Studies on Isocitrate Lyase

in the Unicellular Green Alga *Gloeomonas* sp

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ABSTRACT

Isocitrate lyase (EC 4.1.3.1), the key enzyme of the glyoxylate cycle has been partially purified from the unicellular green alga Gloeomonas sp grown either photoautotrophically (CO_2 and light) or photoheterotrophically (acetate and light). The method used in the purification procedure resulted in an approximately 50-fold increase in specific activity.

Enzyme preparations either from autotrophic or photoheterotrophic cultures were eluted from a DEAE cellulose column at almost the same position. They showed classical Michaelis-Menten kinetics with similar apparent K_m values (1.14 and 1.4 mM) and responded in a similar manner to added metabolites. This provided some evidence that there was only one type of isocitrate lyase in Gloeomonas irrespective of the nature of the carbon source provided in the culture. The enzyme has an optimum pH 7.4. Enzyme activity was completely dependent on Mg^{++} ions and glutathione (reduced form). The optimum temperature for the enzyme reaction was 35 C and the activation energy was 14.1 k_{cal}/mole.

Early products of photosynthetic CO_2 fixation, e.g. glycollate and 3-phosphoglycerate as well as ribulose diphosphate, the CO_2 acceptor in the Calvin cycle, inhibited isocitrate lyase at physiological concentrations (10 mM). Intermediates of the tricarboxylic cycle, namely,

α-ketoglutarate, succinate, malate, fumarate and oxaloacetate are all powerful inhibitors at 10 mM concentration. Of the nucleotides tested, only ADP and 3'-AMP at 10 mM, exerted any inhibitory effect on the enzyme. Succinyl CoA activated the enzyme by 155% at a concentration of 1 mM. α -hydroxy-2-pyridinemethanesulphonate, an inhibitor of glycollate oxidase and dichlorophenyldimethyl urea, a specific inhibitor of photosynthetic 0_2 evolution had no influence on enzyme activity *in vitro*. Cycloheximide, which blocks protein synthesis, inhibited the formation of isocitrate lyase *in vivo* in autotrophic cells.

Determination of isocitrate lyase activity in *Gloeomonas* during the life cycle in synchronous cultures revealed that changes in the level of the enzyme activity may occur due to inactivation, activation and *de novo* synthesis of the enzyme. The presence of isocitrate lyase activity throughout the whole life cycle of the photoautotrophically grown *Gloeomonas* provided strong evidence that this enzyme is constitutive. The question whether algal isocitrate lyase may function in a catabolic (degradative) or anabolic (biosynthetic) pathway is discussed.

ABBREVIATIONS

AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
DCMU	N'(3, 4, dichlorophenyl) N-N dimethyl urea
DEAE	diethylaminoethyl cellulose
EDTA	ethylenediaminetetraacetic acid
HPMS	α-hydroxy-2-pyridine methanesulphonate
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
MES	2-(N-morpholino) ethanesulphonic acid
TRIS HCl	Tris (hydroxy methyl) HCl
TEM BUFFER	Tris HCl buffer (0.014 M) containing 1 mM EDTA and 1 mM mercaptoethanol
DNP	2, 4 dinitrophenylhydrazone
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid

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INTRODUCTION

Isocitrate lyase (EC 4.1.3.1), the key enzyme of the glyoxylate cycle (Kornberg,1965), has been shown to occur in various unicellular algae when forced to grow on acetate as the sole carbon source (Wiessner and Kuhl, 1962; Wiessner, 1963; Haigh and Beevers, 1964; Syrett, 1963 and Wiessner, 1968). The presence of this enzyme in algal cells has been considered to be a good indicator for the operation of the glyoxylate shunt in which malate synthetase (EC 4.1.3.2) mediates the formation of malate from acetyl CoA and glyoxylate produced by isocitrate lyase. Nevertheless, Wiessner (1968) observed substantial activity of the latter enzyme in Chlamydomonas and Chlamydobotrys grown in light with CO_2 as the sole carbon source. Relatively low enzyme activity has been also reported in photoautotrophically grown Chlorella pyrenoidosa (Lord and Merrett, 1970).

Recently, Badour and Waygood (1971) have demonstrated the presence of isocitrate lyase in the unicellular green alga Gloeomonas grown under continuous illumination with 5% CO_2 in air (v/v). These authors showed that the activity of the second enzyme of the glyoxylate cycle, malate synthetase, was very low and not sufficient to account for the operation of the glyoxylate shunt. Instead, consistent and substantial activity of glyoxylate carboligase was evident. This

finding has led to the suggestion that isocitrate lyase in photoautotrophically grown Gloeomonas may function in a catabolic (degradative) pathway in which CO_2 is produced and tartronic acid semialdehyde or its oxidised form mesooxalic (ketomalonate) acid semialdehyde partially or completely excreted (Badour and Waygood, 1971).

In the present work, some kinetic studies were carried out on partially purified isocitrate lyase preparations from photoautotrophically as well as photoheterotrophically grown Gloeomonas, in order to establish some properties of this enzyme in vitro. Data on the response of the latter to various metabolites particularly products of photosynthesis and the glyoxylate cycle as well as some intermediates of the tricarboxylic acid cycle are reported. In addition, changes in the level of isocitrate lyase during the life cycle of Gloeomonas were determined.

LITERATURE REVIEW

The initial observation which led to the discovery of isocitrate lyase was made in 1953 by Campbell, Smith and Eagles while studying terminal respiration in aerobic microorganisms. Many features of the conventional tricarboxylic acid cycle were noted, but when attempts were made to detect α-ketoglutarate by chromatography of the fermentation liquors, only spots corresponding to glyoxylate were found. However, it was reported that isocitrate, unlike citrate and cis-aconitate, was not dissimilated to glyoxylate, and no explanation of this observation was given in subsequent papers. During the next few years several laboratories reported finding a C_2-C_4 cleavage of isocitrate in aerobic and facultative bacteria (Saz, 1954, 1956; Smith, 1954, 1955), yeasts and molds (Olson, 1954, 1957). Isocitrate lyase was purified sufficiently to remove aconitase and the reaction clearly defined as the cleavage of one mole of isocitrate to yield one mole of glyoxylate and succinate (Smith, 1954). Recently, reports of its occurrence include green algae (Haigh and Beevers, 1964; Harrop and Kornberg, 1966), germinating seedings of Gymnosperms (Firenzuoli et al, 1968) and Angiosperms (Tanner and Beevers, 1965; Breidenbach and Beevers, 1967).

Reaction

Isocitrate lyase (EC 4.1.3.1) is a member of the ketoacid lyase (Dixon and Webb, 1967). It catalyses the cleavage of isocitrate to give succinate and glyoxylate according to the following equation.

$$\text{Threo } D_S \text{ Isocitrate} \xrightleftharpoons{Mg^{2+}\ RSH} \text{succinate} + \text{glyoxylate}$$

Trivial and Systematic names

Isocitratase (Kornberg, 1959) has been variously referred to as 'isocitritase' (Smith, 1957), isocitric lyase (Olson, 1954) and isocitrase (Dixon and Webb, 1958). The systematic name of the enzyme is threo D_S-isocitrate glyoxylate-lyase (Dixon and Webb, 1967). However, the term isocitrate lyase is used throughout the present investigation.

Some Properties

(a) Specificity

Threo D_S-isocitrate is the only substrate known to undergo cleavage. Citrate, cis-aconitate, threo L_S-isocitrate and threo D_S-isocitrate lactone are inert (Smith, 1957 and Olson, 1959). The reaction is readily reversible, having an equilibrium constant for the condensation reaction of 34 (Smith, 1957). Also, detailed investigations have revealed marked specificity for glyoxylate and succinate in the aldol condensation reaction (Rao et al , 1965). But

at physiological concentration of reactants and products, isocitrate cleavage is thermodynamically favoured (Smith, 1957).

(b) Cofactors

Enzyme activity was found to be dependent on the presence of both a divalent metal ion (Mg^{++}, Fe^{++}, Co^{++}, Mn^{++}) and a sulfhydryl compound cysteine or glutathione (Smith, 1955, 1957; Olson, 1959).

Intracellular Localization

Isocitrate lyase was reported as particulate in the Basidiomycete, Coprinus lagopus and in the alga Chlorella (Casselton, 1969; Harrop and Kornberg, 1966). In plant seedlings, the enzymes of the glyoxylate cycle are found in glyoxysomes (Breidenbach and Beevers, 1967). On the other hand in bacteria and in Sacchromyces cerevisiae, isocitrate lyase is in the soluble fraction (Duntzie et al, 1969; Wegener et al, 1968).

Role of Isocitrate

(a) In bacteria;

Kornberg et al (1953) have established that the most probable role of isocitrate lyase in conjunction with malate synthetase is the anaplerotic function of the glyoxylate bypass. The latter enzyme catalyses the condensation of glyoxylate and acetyl CoA to give

malate (Ajl, 1957). By the operation of these two enzymes in the glyoxylate bypass, C_4 dicarboxylic acid is formed from acetate and isocitrate, while isocitrate is reformed by the Kreb's cycle. The importance of this sequence is that it permits the replacement from acetate of intermediates of the tricarboxylic acid cycle drained away during growth on acetate and this is achieved by bypassing those steps of the tricarboxylic acid cycle which result in the evolution of carbon dioxide and replacing them with the two key enzymes mentioned above. The net effect leads to an increase in the organic acid carbon. The subsequent metabolism of these acids provide the precursors of most constituents and therefore allows the organisms to grow on acetate as sole carbon source.

(b) In algae ;

Isocitrate lyase has been demonstrated in unicellular green algae growing in light on acetate as the sole carbon source (Wiessner and Kuhl, 1962; Wiessner, 1963; Syrett, 1966). Such photoheterotrophic growth is associated with the operation of the glyoxylate cycle which accounts for the synthesis necessary to maintain growth. The presence of isocitrate lyase has been considered (Wiessner, 1968) to be a fairly good indicator for the operation of the glyoxylate cycle induced by growth on acetate.

(c) In seedlings;

Catalysis by isocitrate lyase is also vital in the conversion of lipid reserves to carbohydrates as found for example in the germination of fatty plant seedlings (Beevers, 1953; Tanner and and Beever, 1965).

Control of Isocitrate lyase Activity

Isocitrate lyase occupies a pivotal position and thus it is of significance that the apportioning of carbon between the Kreb's cycle and the glyoxylate cycle be regulated, so as to avoid unbalance in the utilization of the growth substrate for the supply of energy on the one hand and of the precursors of cellular materials on the other. In many organisms, this intracellular control is exerted by influencing both the activity and the formation of isocitrate lyase.

At the optimum pH, intermediates of the tricarboxylic acid cycle namely succinate, pyruvate and oxaloacetate have been reported to be powerful inhibitors in Chlorella (John and Syrett, 1968) while phosphoenolpyruvate does inhibit at physiological concentration under acidic conditions. By the nature of this inhibition, the danger of overproduction of C_4 acids is avoided, should the concentration of such acids rise beyond optimum levels. The continued functioning of the tricarboxylic acid cycle effectively

utilizing these acids, allows a relief of the inhibition of isocitrate lyase by the metabolites. This fine control system may thus be regarded as providing a unique example of the feedback control of a cyclic system by end product inhibition (Umbarger, 1964).

A second type of control mechanism perhaps less delicate in its response is manifested by regulation of the de novo synthesis of isocitrate lyase. Addition of C_4 intermediates such as succinate to cells growing in acetate causes in most systems the repression of enzyme synthesis (Pardee, 1959; Bell, 1967). Kornberg (1965) believed that the coarse control on the synthesis of isocitrate lyase is mediated by phosphoenolpyruvate.

Levels of Isocitrate lyase in Microorganisms as Influenced by Mode of Nutrition

The formation of isocitrate lyase in microorganisms is adaptive in nature (Smith, 1955; Madsen, 1955). Enzyme levels are high when acetate or fatty acids are employed as sole carbon sources, are low when tricarboxylic acid cycle intermediates or amino acids are used, and are severely repressed in cells grown on glucose or glycerol. Aerobic conditions, if not essential for formation of the enzyme, generally favour its presence (Smith, 1955). The obligate anaerobic Chromatium, however contains the enzyme (Sisler, 1959).

However, the activity of isocitrate lyase was reported to be

very low in most organisms grown on carbon dioxide free air as sole carbon source. Experiments with intact cells of Chlorella grown on acetate have shown that isocitrate lyase synthesis is promoted by light, especially at 705 nm which favours production of ATP by cyclic photophosphorylation (Syrett, 1966). The enzyme is not produced when the alga grows photo synthetically on $C0_2$, even if acetate is present, but it is formed under otherwise similar conditions if the assimilation of $C0_2$ is prevented by addition of DCMU (Goulding and Merrett, 1966).

Moreover, Wiessner (1968) found in various unicellular green alga (viz Chlorella, Chlamydobotrys, Chlamydomonas, Euglena and Scenesdesmus) that the enzymes typical of the Calvin cycle are substantially repressed or even inhibited under culture conditions promoting the activity of the glyoxylate cycle enzymes. On the basis of photosynthetic $C0_2$ fixation experiments, Lord and Merrett (1970) concluded that reductive pentose cycle enzymes are absent in acetate grown cells rather than being inhibited or repressed. Their evidence showed that the de novo synthesis of the $C0_2$ fixing enzymes commenced after transferring the cells from acetate to $C0_2$. Recently Badour and Waygood (1971) reported high isocitrate lyase activity in Gloeomonas at high $C0_2$ tension in autctrophic growth whereas the activity in cells maintained at low $C0_2$ concentration was low.

MATERIALS AND METHODS

Organism and Cultures

The organism used in the present investigation was an unicellular green alga tentatively identified as a Gloeomonas sp (Badour and Waygood, 1970). Stock bacteria-free agar slants of this alga were maintained at room temperature and low light intensity to allow slow growth. Inocula were aseptically introduced from the slants into sterilized culture media of the following mineral composition (moles/L). The pH of the media was consistently around 7.0.

Medium	Molarity
NH_4Cl	10^{-2}
KCl	10^{-2}
$MgSO_4 \cdot 7H_2O$	10^{-3}
$CaCl_2$	10^{-4}
$NaH_2PO_4 \cdot H_2O$	4.5×10^{-3}
K_2HPO_4	10^{-2}
$FeSO_4 \cdot 7H_2O$	2.5×10^{-5}
EDTA* (as indicated below)	
H_3BO_3	10^{-6}
$ZnSO_4 \cdot 2H_2O$	10^{-6}
$MnSO_4 \cdot 4H_2O$	10^{-6}
$CuSO_4$	10^{-8}

$CoCl_2 . 6H_2O$	10^{-8}
$(NH_4)_2Mo$	10^{-8}

*Preparation of the Fe-EDTA complex: 0.695g $FeSO_4 . 7H_2O$ and 0.93 g EDTA (disodium salt of ethylenediamine tetraacetic acid) were dissolved in 80 ml boiling H_2O. The volume was made to 100 ml, from which 1.0 ml contained the amount of $FeSO_4 . 7H_2O$ given above.

Algal cultures were exposed to light (1,000 ft c) provided by fluorescent tubes in a growth chamber at 25 C and aerated with a continuous current of 5% CO_2 in air (v/v). After 3 days of continuous illumination, the cultures were subjected for another 3 days to alternating light and dark periods of 12 hr each to increase productivity. Before harvesting, the algal cells were illuminated for 16 hr in order to increase the isocitrate lyase level as reported for this alga (Badour and Waygood, 1971). By this way, photoautotrophically grown cells were produced. On the other hand, photoorganotrophic growth was achieved by using a sterilized media of the same mineral nutrients as given above, but with added sodium acetate at a concentration of 1.5×10^{-2} M. Acetate served as the sole carbon source in these cultures which were continuously illuminated (1,000 ft c) and aerated with CO_2-free air. Air was freed from CO_2 by passing through 40% KOH solution. Algae from seven day-old acetate cultures were used to prepare the cell free extracts.

For some experiments, synchronization of the algal cells was

conducted by subjecting the cultures to periods of 12:12 hr light-dark change (Kuhl and Lorenzen, 1964). At the beginning of each light period, the algal suspension was diluted to a constant cell number (10^6 ml^{-1}). Under continuous bubbling with 5% CO_2 in air (v/v) and at light intensity of 1,000 ft c, the cultures indicated synchrony and separation of daughter cells took place after 16 hr from the commencement of illumination.

Preparation of Cell-Free Extracts

About 10 litres algal suspension obtained either from photoautotrophic (grown on CO_2) or photoheterotrophic (grown on acetate) cultures were used for preparation of cell-free extracts. In case of synchronized cultures only 500 ml algal suspension was used. The cells were collected by centrifugation and washed twice with cold 0.1 M phosphate buffer pH 7.0. The packed cell volume was then suspended in 100 ml of ice cold 0.014 M TEM buffer and sonicated for 5 minutes at invervals of 30 seconds each with a Biosonik (Bronwill) operated at maximum output. The resulting homogenate was spun at 35,000 g for 30 minutes to sediment unbroken cells, cell debris and starch grains as well as heavy organelles. The greenish yellow supernatant was the crude extract. Suitable volumes of the latter were taken for the determination of isocitrate lyase and NADP-linked isocitrate dehydrogenase. For α-ketoglutarate synthetase assay, a dialyzed crude extract in TEM buffer was used.

Purification of isocitrate lyase

(a) Ammonium sulphate precipitation:

For purification, sonication of algae was achieved in phosphate buffer (0.1 M) at pH 6..0 After centrifugation, the crude extract was brought to 40% ammonium sulphate saturation by addition of solid ammonium sulphate crystals (24.3 g per 100 ml of crude extract). The suspension was stirred slowly for 30 minutes after which the precipitate was removed by centrifugation at 35,000 xg for 30 minutes. The supernatant was brought to 60% ammonium sulphate saturation (18.2 g of solid ammonium sulphate per 100 ml). The precipitate was then collected by centrifugation and dissolved in 10 ml of 0.014 M TEM buffer. This was applied to the DEAE cellulose column for further fractionation.

(b) DEAE cellulose column chromatography:

Before packing the column, the DEAE cellulose was thoroughly washed with N NaOH, neutralized with twice-distilled water pH 6.0. Thereafter, the DEAE cellulose was suspended in a column of 230 mm x 10 mm and equilibrated overnight in 0.014 M TEM buffer. The column was run overnight and 5 ml fractions were collected. The protein eluates were measured at 280 nm in a Zeiss spectrophotometer and the peak fractions were assayed for isocitrate lyase activity. Fractions indicating substantial enzyme activity were stored at -16 C. Before use,

5 ml samples were thawed at room temperature, dialyzed for 2 hr against one litre of 0.014 M TEM buffer and then centrifuged resulting in a yellowish enzyme solution.

Enzyme Assays

Isocitrate lyase (EC 4.1.3.1); Two methods were employed to assay the enzyme:

1. The method of Dixon and Kornberg (1959), in which the enzymically produced glyoxylate was trapped by phenylhydrazine. The resulting glyoxylatephenylhydrazone derivative was spectrophotometrically measured at 324 nm by a Zeiss spectrophotometer. The assay system contained the following in a final volume of 3.0 ml.

$MgCl_2$	;	7.5 µmole
MES buffer	;	50.0 µ mole, pH 6.8
Glutathione	;	2.0 µ mole
Enzyme	;	1-10 µg Protein
Phenyl hydrazine (adjusted to pH 6.8 by NaOH);		10 µmole
Three D_s isocitrate (sodium salt)	;	10 µ mole

The reaction was carried out in a quartz cuvette (1 cm) at 30 C and initiated, by adding isocitrate, whereas the reference cuvette lacked the substrate.

2. The incubation method of Rao et al (1970) was also used. By this method the quantity of glyoxylate formed during 10 minutes incubation

at 30 C was colorimetrically determined as given below. The incubation mixture contained the following in a final volume of 1.0 ml.

MES buffer; pH 7.3; 50 μ mole

$MgCl_2$; 7.5 μ mole

Glutathione(reduced form and adjusted to pH 7.3) ; 2.0 μmole

DL threo isocitrate (sodium salt) ;10.0 μmole

After preincubation of enzyme with glutathione for 10 minutes in the presence of Mg^{2+} the reaction was commenced by isocitrate. After 10 minutes the enzyme reaction was stopped by adding 0.4 ml of M oxalic acid. This was followed by the addition of 0.1 ml 5% phenylhydrazine HCl. The reaction mixture was brought to boiling within 1 minute and quickly cooled in ice. Thereafter, 1.8 ml of concentrated HCl and 0.1 ml 25% (w/v) $K_3Fe(CN)_6$ were added. The reddish violet colour developed was measured at 520 nm against a blank carried out without added substrate.

In studies dealing with the effect of metabolites on enzyme reaction, test compounds were preincubated with enzyme for 10 minutes before addition of substrate.

NADP-linked isocitrate dehydrogenase (E. C. 1.1.1.42); Isocitrate dehydrogenase was measured by the procedure described by Syrett et al (1963). The rate of NADP reduction was followed spectrophotometrically at 340 nm. The reaction mixture contained the

following in a final volume of 3.0 ml:

Potassium phosphate buffer, pH 7.8	;	40 µmole
$NADP^+$	;	0.5 µmole
Sodium threo D_s isocitrate	;	0.5 µmole
$MgCl_2$	;	0.1µmole
Enzyme	;	1-2 mg protein

The specific activity of the enzyme was reported as mµ moles NADPH formed min^{-1} mg^{-1} protein.

α-ketoglutarate synthetase: α-ketoglutarate synthetase was assayed according to Buchanan and Arnon (1969). This enzyme catalyses the formation of α-ketoglutarate-^{14}C (as determined by its 2,4 dinitrophenylhydrazone derivative) from $H^{14}CO_3^-$ and succinate in the presence of reduced ferredoxin, ATP, $MnCl_2$, CoA. The chloroplast fragments required for the reaction were prepared essentially according to Whatley and Arnon (1963) as follows:

Ten grams of fresh commercial spinach leaves were washed with water and dried on a paper towel. The mid-ribs were removed and the leaf blades were sliced into 1 cm squares. These were ground with an 'isotonic' solution consisting of 100 ml of 0.35 M NaCl and 10 ml of 0.2 M Tris buffer (pH 8.0) in about 40 grams of cold sand for less than 2 minutes. The chloroplast suspension was filtered through a double layer of cheesecloth and the filtrate was centrifuged at 0 C for one minute at 200 xg. The supernatant was centrifuged for 7 min at 1000 x g. The sedimented whole chloroplasts were suspended in 2 ml of ice cold 0.35 M NaCl, homogenized by gentle stirring

and diluted to 50 ml with 0.035 M NaCl, then centrifuged again for 7 min at 1000 x g. The green pellet was suspended in 10 ml of 0.035 M NaCl and included in the reaction mixture.

The reaction was carried out in a Warburg flask using a Gilson respirometer. The sidearm contained 1 μC of $NaH^{14}CO_3$ and 0.1 ml of each of the following: HEPES buffer, pH 7.5, 17 μ mole; dichlorophenolindophenol, 17 μ mole; ferredoxin (1 mg/ml); sodium ascorbate, 6.7 μmole, ATP, 1.7 μ mole; potassium succinate, 3.6 μmole; CoA, 0.17 μmole, semicarbazide, 17 μmole (pH 7.0); thiamine pyrophosphate, 2 μmole. The main compartment contained 1 ml of soluble enzyme extract (1 mg protein/ml) and 1 ml of spinach chloroplast fragments. The flask was gassed with nitrogen for 10 min after which the vent was closed and the contents of the side arm were tipped into the main compartment. The reaction mixture was illuminated from below (about 1000 ft c) and maintained at 30 C. After one hour, the reaction was stopped by adding 0.5 ml 12N HCl and the precipitated protein was removed by centrifugation for 5 min at 1000 xg at 0 C. Then 0.04 ml of 0.1% dinitrophenylhydrazine in 2 N HCl was added to the clear supernatant and left for 1 hr at 30 C. The resulting 2, 4-dinitrophenylhydrazones were extracted into ethyl acetate, concentrated by evaporation with air and made to 0.2 ml, 40 μ l were withdrawn and mixed with 10 ml of scintillator cocktail (100 g naphthalene, 7 g 2, 5-diphenyl oxazole, 0.3 g 1, 4 bis-2

5 phenyl oxazolyl benzene dissolved in P-dioxane and made up to one litre) for the measurement of radioactivity by a Picker Nuclear Liquimat, 220 liquid scintillator counter.

Thin-layer Chromatography

Products of isocitrate lyase obtained by either the forward or backward reactions as well as the products of α-ketoglutarate synthetase were identified by chromatography on 0.25 mm layers of MN cellulose with calcium sulphate binder using 20 x 20 cm plates. Ascending chromatography of the 2,4-dinitrophenylhydrazone derivatives (DNP) of glyoxylate and α-ketoglutarate were run in a tertiary amyl alcohol:ethanol:H_2O (5:1:4 by volume) solvent. The spots were treated with a solution of 4 N NaOH in ethanol to visualize the reddish colours characteristic of these DNP derivatives. Radioactivity was detected in these spots by exposing the plates to x-ray film. Authentic 2,4-dinitrophenylhydrazones of glyoxylate and α-ketoglutarate were run simultaneously as markers.

For the identification of succinate and isocitrate the deproteinized reaction mixture was dried in a stream of air and redissolved in 0.2 ml H_2O. About 40 µl were spotted on TLC MN cellulose plate which were run in an ascending fashion using ethyl acetate:formic acid: H_2O (11:5:3) as solvent. The spots were located by spraying the plates with aniline glucose solution and heating at 120 C (Smith, 1960).

Their R_f values were compared with those of authentic acids.

Spectral Studies

The dried 2, 4-dinitrophenylhydrazone derivatives of the enzymically produced substances were dissolved in 2 N NaOH and the absorption spectra of the colour produced was measured in a recording Unicam Spectrophotometer Model SP-800 and compared with alkaline DNP derivatives of authentic reference standards.

Protein Determination

Protein was determined by the folin phenol reagent method of Lowry et al (1951). Crystalline bovine albumin was used as a standard. Determination of the protein of whole cells was achieved by mixing equal volumes of washed cell suspension with 2 N NaOH and incubating at 100 C for 30 min. The soluble protein was estimated as mentioned before.

RESULTS

Purification of isocitrate lyase

The steps carried out for the purification of isocitrate lyase (assayed by Method 2) from the unicellular green alga Gloeomonas are summarized in Table I. The procedure resulted in an approximately 50 fold increase in specific activity. The method of purification was initiated by addition of ammonium sulphate to the crude extract maintained at pH 6.0 (John and Syrett 1967), followed by centrifugation. The collected pellet was applied to a DEAE cellulose column, equilibrated with TEM buffer containing dithiothreitol. The latter maintained the enzyme activity during elution. Fig. 1 shows that the enzyme was recovered in tube numbers 18-24. To the eluate 2 mM of glutathione was added and the enzyme solution was kept at -16 C for ten days without any significant loss in activity. Purification of isocitrate lyase from either photoautotrophically or photoheterotrophically grown Gloeomonas exhibited virtually the same pattern of elution and responded equally to the effect of dithiothreitol. The increased recovery in Stage III must be due to the removal of an inhibitor of the reaction.

Progress of the enzyme reaction

The time course for isocitrate cleavage was measured by the increase in the absorbance at 324 nm due to the formation of

Table 1

Summary of Partial Purification of Isocitrate Lyase from Gloeomonas

	Fraction	Total volume (ml)	Total protein mg/ml	Total enzyme units #	Specific activity*	Recovery (%)
I	Crude extract	100	2200	19,500	8.4	100
II	Ammonium sulphate (40 - 60%)	10	126	8,946	71.0	46
III	DEAE cellulose effluent	20	90	36,000	400	185

one enzyme unit is defined as the amount of enzyme which caused an O.D. increase of 0.10 in 10 min at 520 nm.

* specific activity is expressed in units/mg of protein.

Fig. 1 Chromatography of fraction II (Table I) on DEAE cellulose by the application of a linear gradient of NaCl concentration (0.5 M mixed with 0.05 M in TEM buffer). Protein concentration measured as O.D. 280 nm (●—●—●—●). Isocitrate lyase activity measured as O.D. 324 nm (○—○—○—○) using the continuous spectrophotometric method 1.

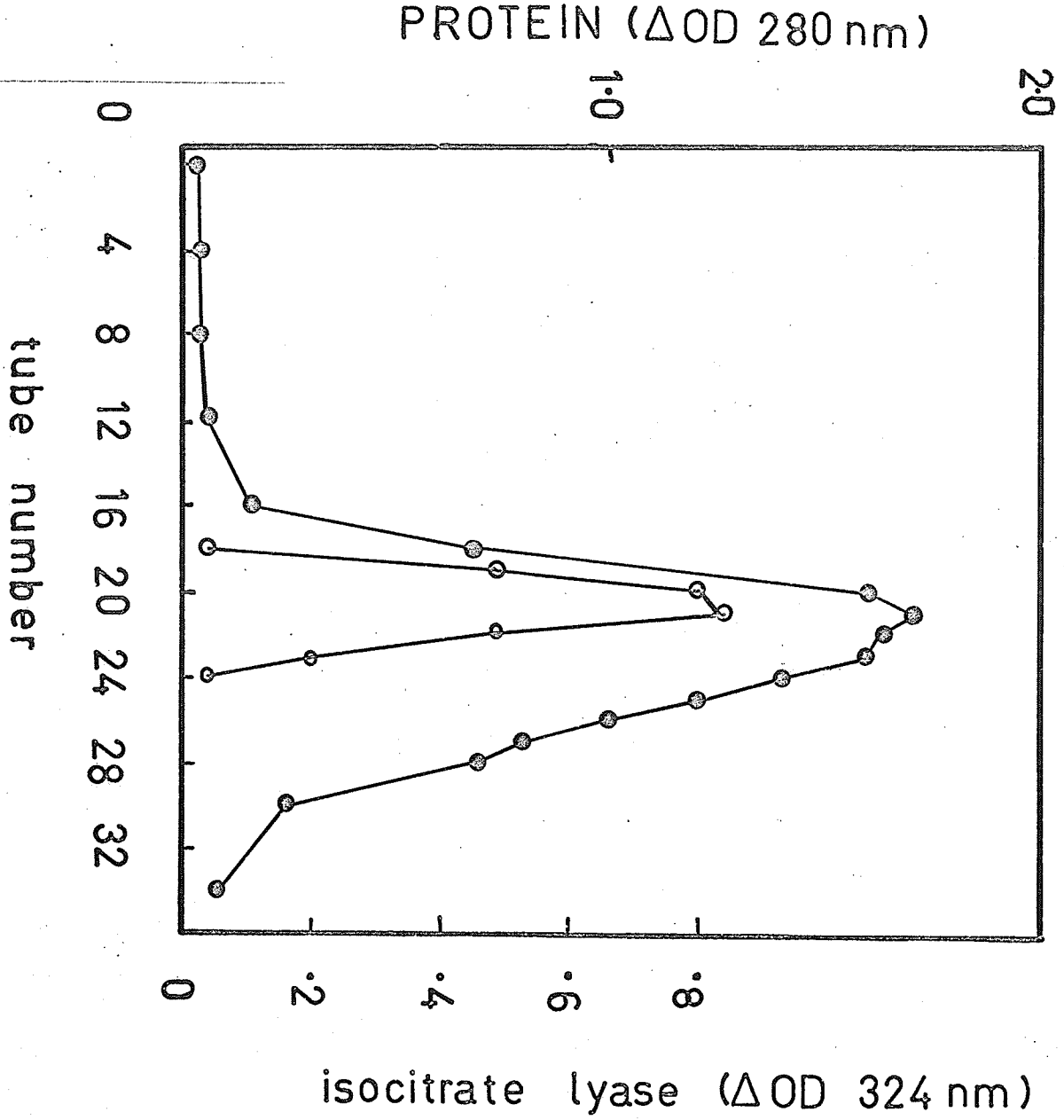
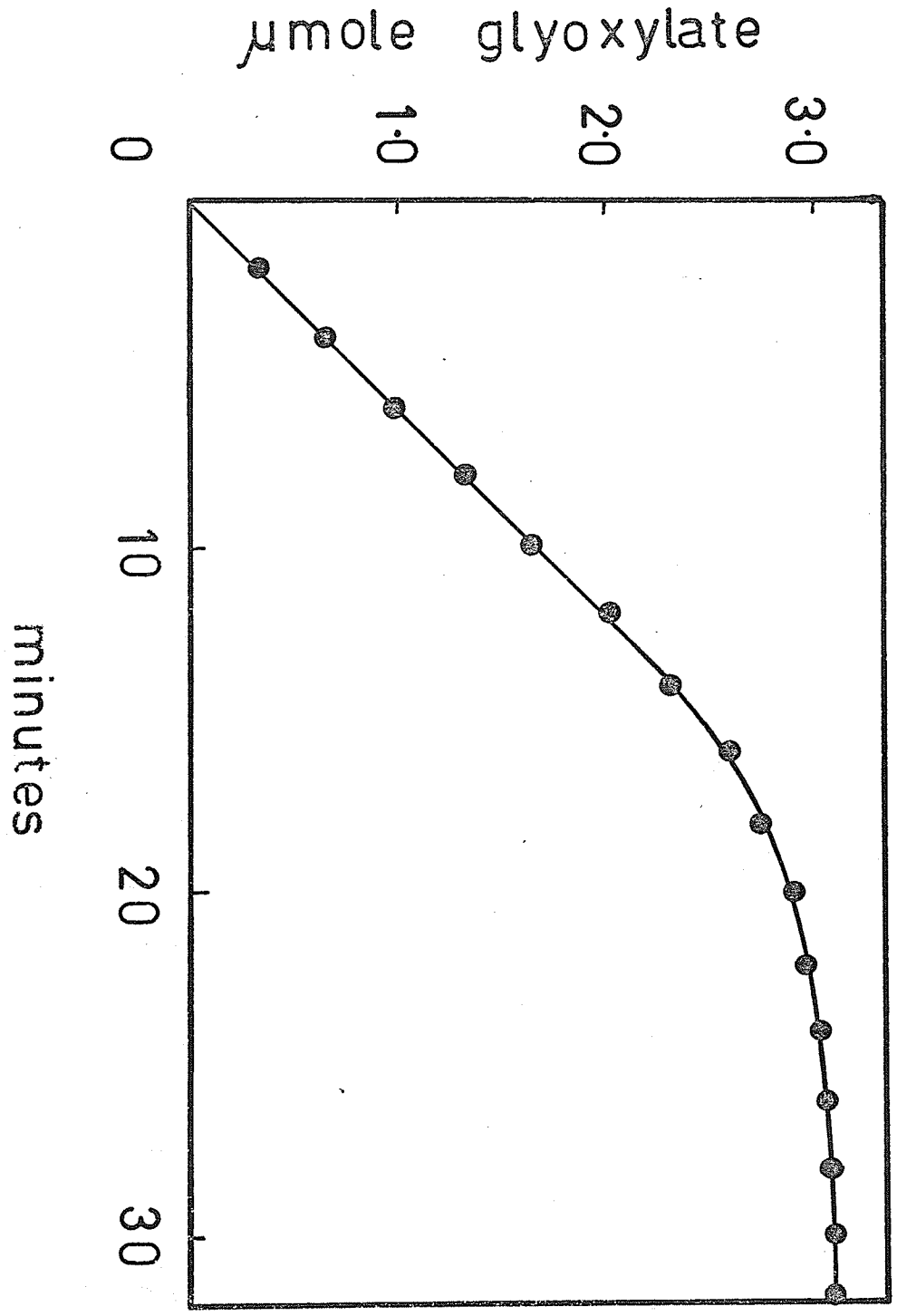

PROTEIN (ΔOD 280 nm)
0
1·0
2·0
0
4
8
12
16
20
24
28
32
tube number
0
·2
·4
·6
·8
isocitrate lyase (ΔOD 324 nm)

Fig. 2 (A) Progress curve of algal isocitrate lyase. Method 1 was used.


µmole glyoxylate
0
1.0
2.0
3.0
minutes
10
20
30

glyoxylatephenylhydrazone. As shown in Fig. 2, the reaction is linear over the period of 14 minutes, beyond which it slows down and ultimately attained an equilibrium.

Identification of glyoxylate as reaction product

Glyoxylate was identified as one of the products of isocitrate cleavage by partially purified algal isocitrate lyase prepared from cells grown on acetate or CO_2 in light. The enzyme system was incubated for one hour at 30 C and stopped by addition of 0.2 ml 15% trichloroacetic acid. After centrifugation, glyoxylate was extracted and identified in the clear supernatant as described in Methods. Fig. 3 shows that the absorption spectrum of the enzymically formed glyoxylatedinitrophenylhydrazone derivative (DNP) dissolved in N NaOH was identical with that of authentic glyoxylate DNP derivative. Furthermore, the enzyme product extracted as DNP derivative cochromatographed with authentic glyoxylate DNP (Fig. 4).

Effect of enzyme concentration on rate of enzyme reaction

The effect of enzyme concentration on the rate of reaction is illustrated in Fig. 2B. Enzyme concentrations varying from 100 µg to 250 µg/ml indicated a linear relationship with reaction velocity.

Cofactors

Divalent ions: As shown in Table II, the enzyme activity was

Fig. 2 (B) Effect of enzyme concentration on reaction velocity. Method 2 was used.

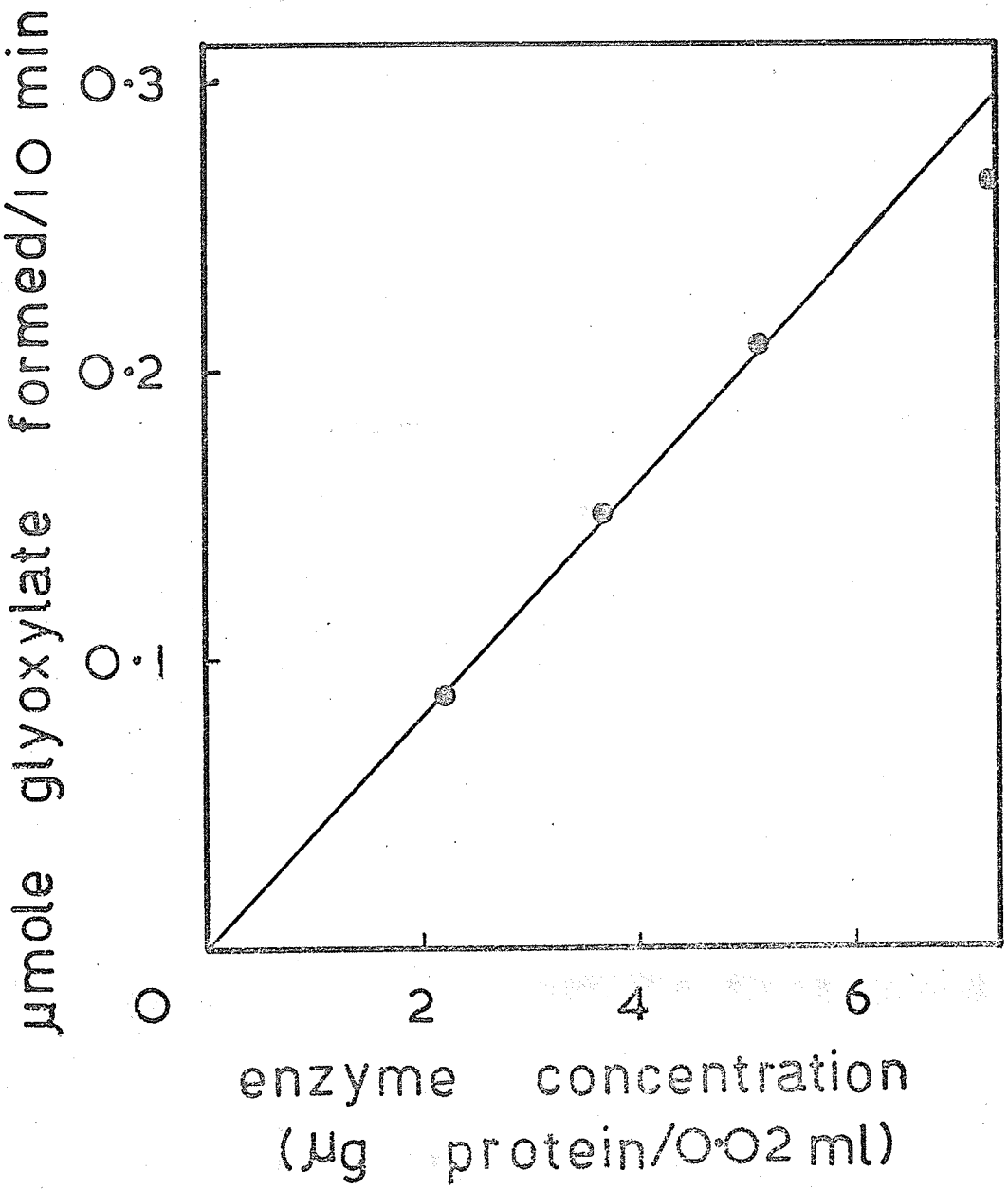

µmole glyoxylate formed/10 min
0·3
0·2
0·1
0
2
4
6
enzyme concentration
(µg protein/0·02 ml)

Fig. 3 Absorption spectra of:

(A) Glyoxylate DNP from isocitrate cleavage by isocitrate lyase

(B) Authentic glyoxylate DNP

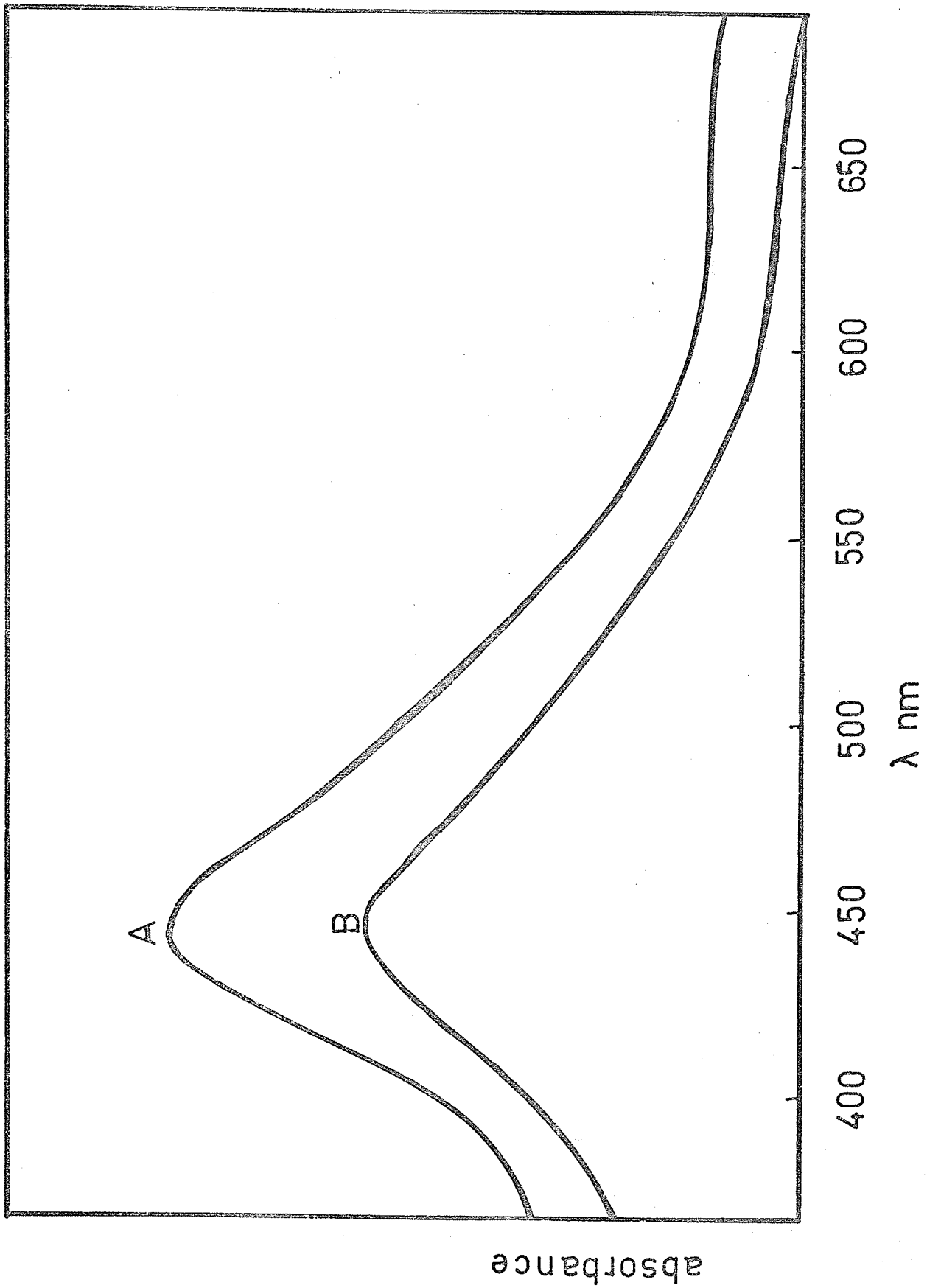

absorbance
A
B
400
450
500
550
600
650
λ nm

Fig. 4 Thin layer chromatogram of the enzymatically formed glyoxylate from isocitrate

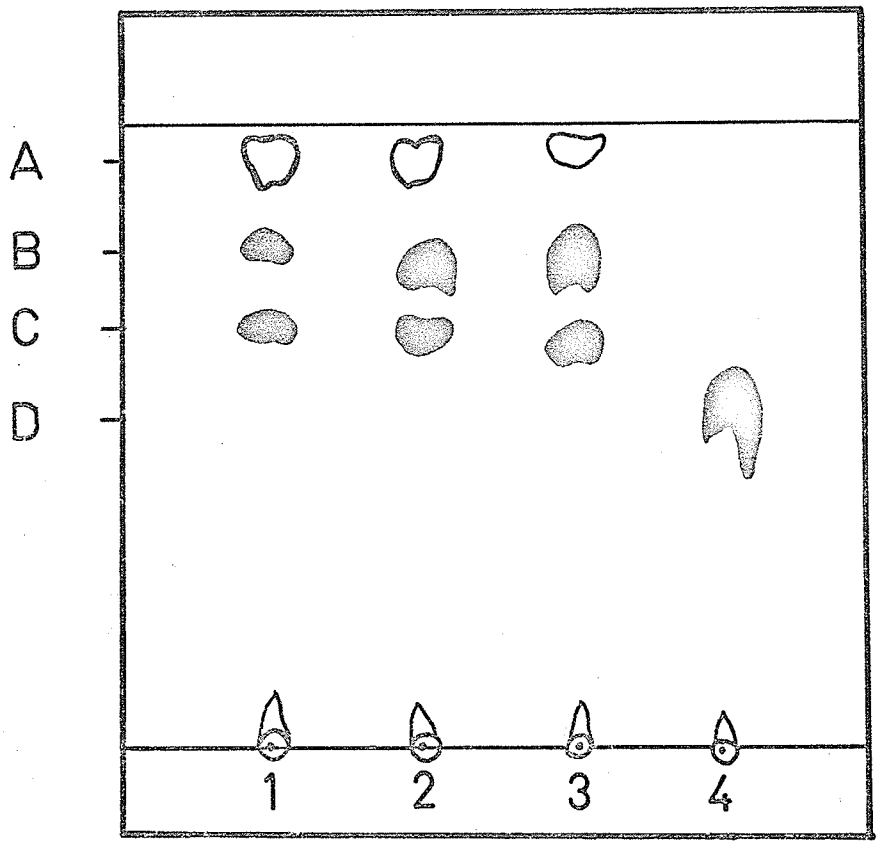
1. Authentic glyoxylate dinitrophenylhydrazone (DNP)

   A: 2, 4 Dinitrophenylhydrazine
   B and C: glyoxylate (DNP)

2. Reaction product as DNP derivative

3. Cochromatography of 1 and 2.

4. Authentic α-ketoglutarate DNP derivative


A
B
C
D
1
2
3
4

completely dependent on Mg^{++}. Its omission or substitution by other divalent metal ions showed no enzyme activity. Varying the magnesium concentration as shown in Fig. 5A showed clearly that 7.5 mM was the optimum concentration for maximum activity. Higher Mg^{++} concentrations were inhibitory.

Sulfhydryl Compounds: The presence of 2 mM reduced glutathione was required for maximum activity (Fig. 5B). Higher concentrations of glutathione led to substantial inhibition as indicated by the rapid decrease in the enzyme activity. Mercaptoethanol and dithiothreitol were as effective as glutathione, however unlike glutathione, they did not inhibit at higher concentrations (Table III).

Accordingly, magnesium and glutathione at the optimum concentrations were used as cofactors in all subsequent assays.

Effect of temperature

The activity of isocitrate lyase was determined over a temperature range of 15 - 60 C using the incubation method. Control samples containing no substrate were run to measure the actual temperature inside the reaction mixture. As illustrated in Fig. 6a, the activity was lowest at 16 C, optimum at 35 C, beyond which the rate steadily declined.

The Arrhenius plot of log velocity versus the reciprocal of the

Table II

Effect of Divalent Ions on Isocitrate Lyase Activity

Ions	Concentration (mM)	Specific Activity (μmole glyoxylate/10 min)
Nil	-	0
$MgCl_2$	7.5	0.40
$MnCl_2$	7.5	0
	5.0	0
$NiCl_2$	7.5	0
	5.0	0
$CaCl_2$	7.5	0
	5.0	0
$CoCl_2$	7.5	0
	5.0	0

Fig. 5 (A) Effect of Mg^{++} concentration on the enzyme activity.

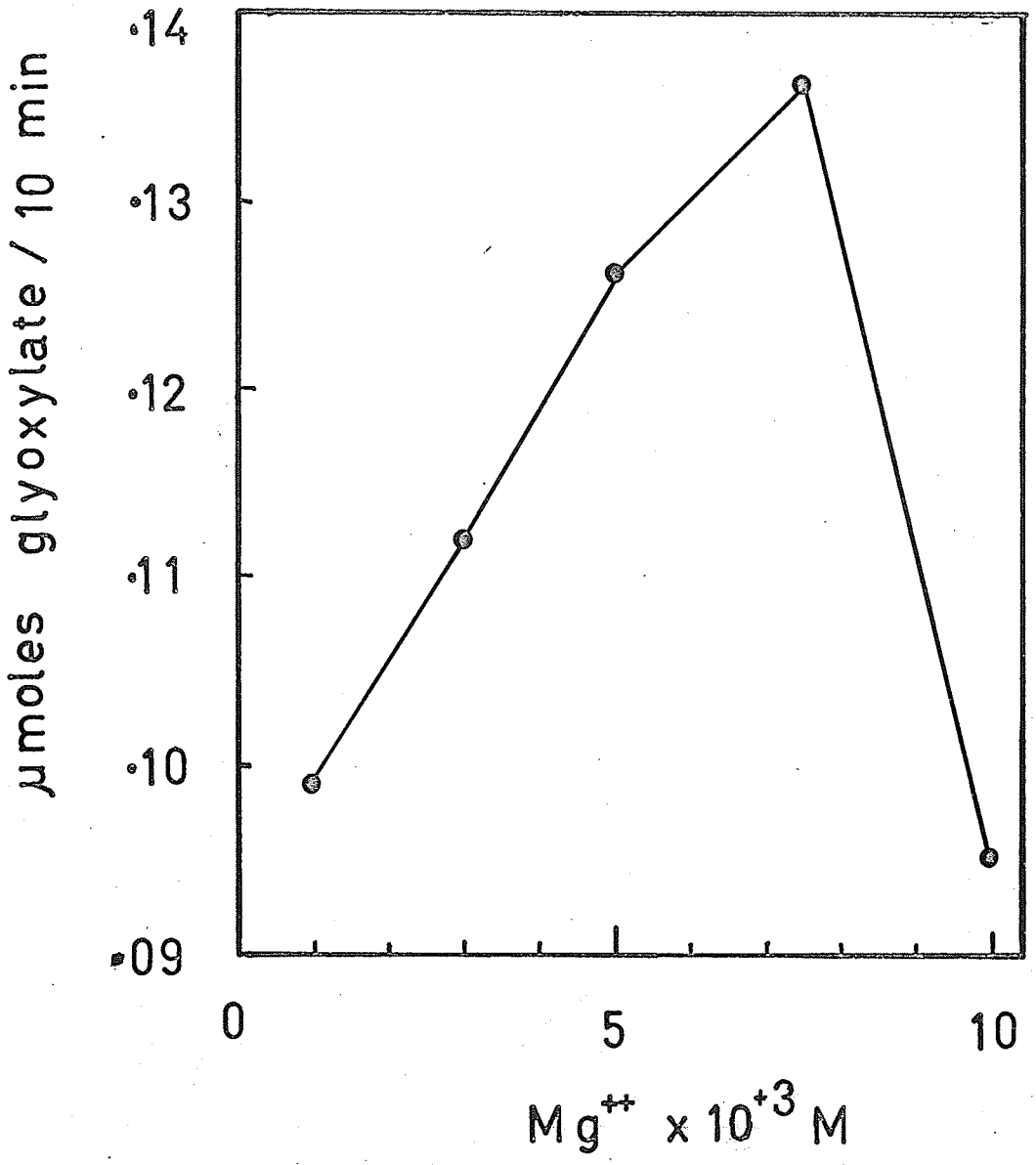

µmoles glyoxylate / 10 min
·14
·13
·12
·11
·10
·09
0
5
10
Mg++ x 10+3 M

Table III

Effect of Sulfhydryl Compounds on Isocitrate Lyase Activity

Compounds	Concentration (mM)	Specific Activity (µmole glyoxylate/10 min)
Nil	-	0
Glutathione	2.0	0.22
Cysteine	2.0	0.03
	10.0	0.03
Dithiothreitol	2.0	0.18
	10.0	0.24
Mercaptoethanol	2.0	0.21
	10.0	0.21

Fig. 5 (B) Enzyme activity as influenced by glutathione concentration

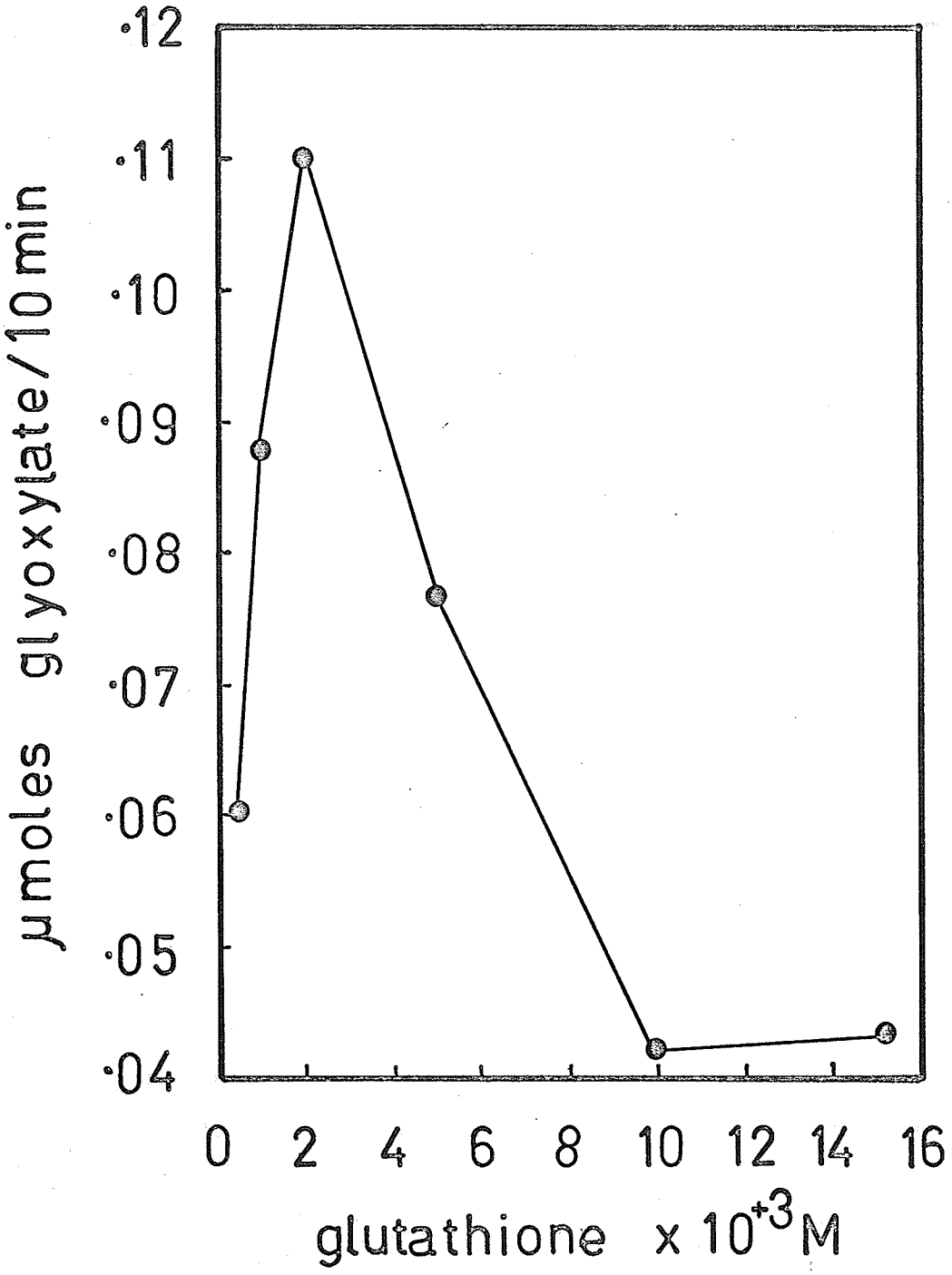
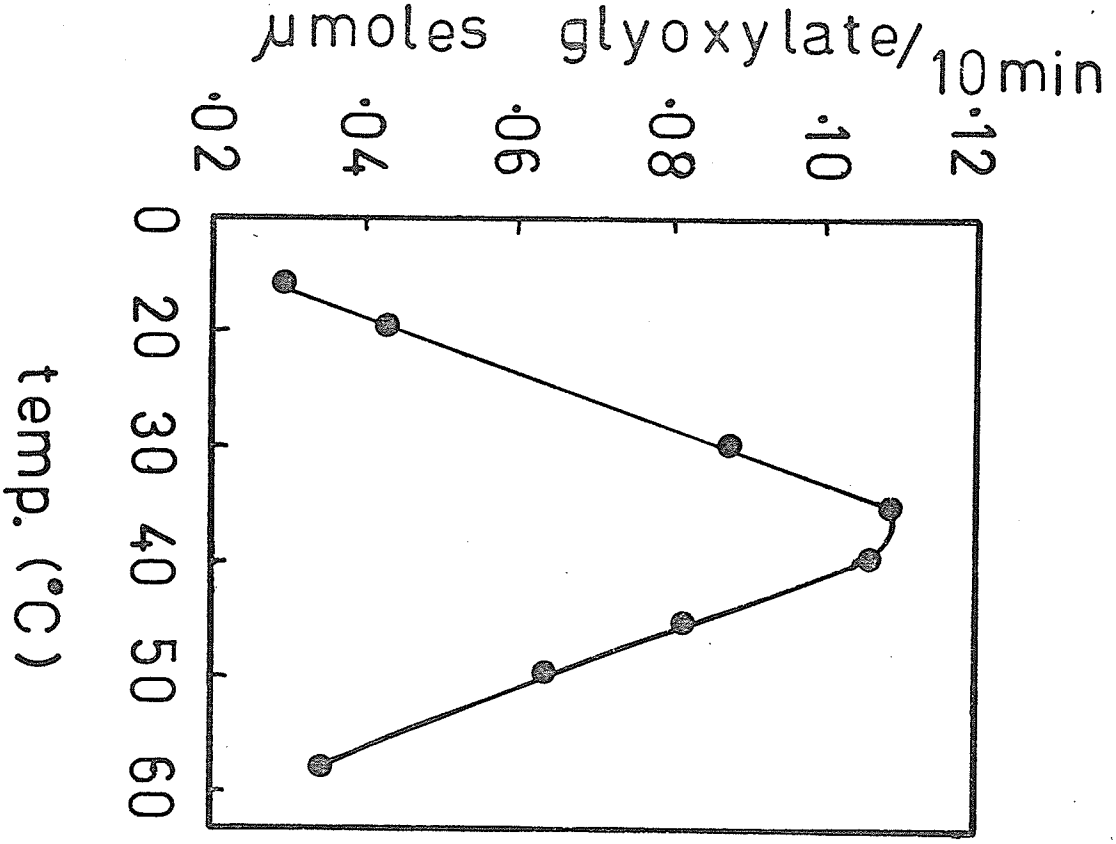
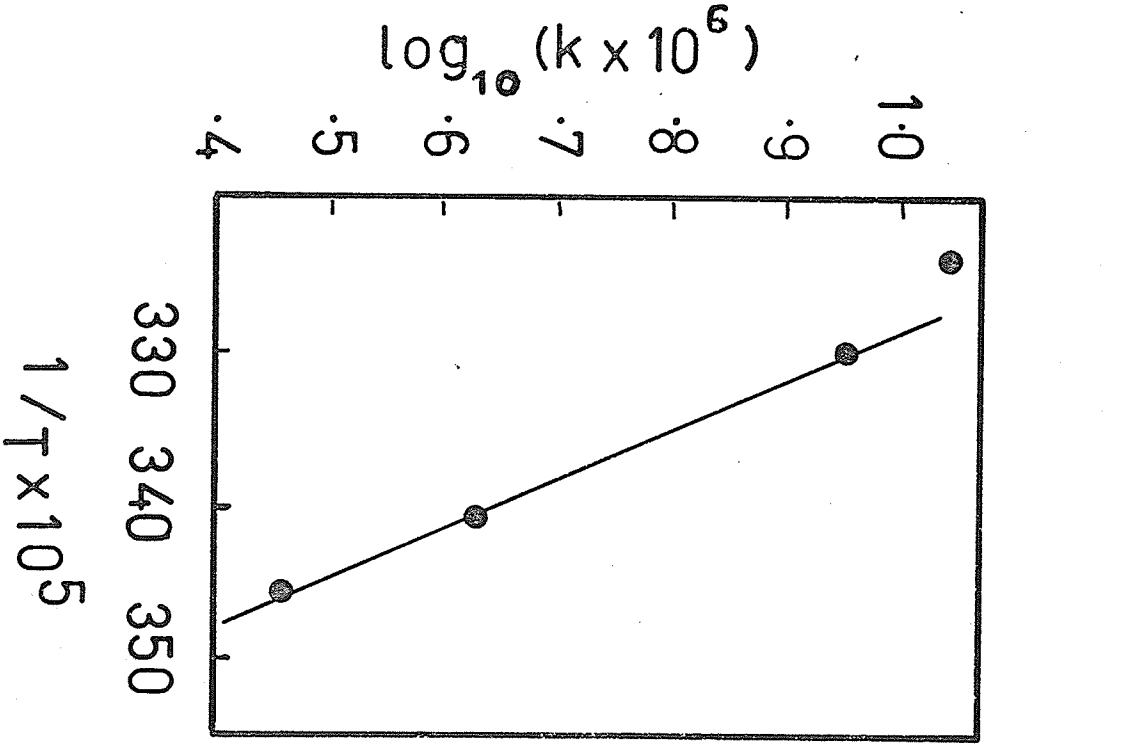

·12
·11
·10
·09
·08
·07
·06
·05
·04
µmoles glyoxylate/10 min
0 2 4 6 8 10 12 14 16
glutathione x 10+3 M

Fig. 6 (a) Effect of temperature on the activity of isocitrate lyase

(b) Arrhenius plot of log velocity against 1/T


µmoles glyoxylate/10min
.02
.04
.06
.08
.10
.12
0
20
30
40
50
60
temp. (°C)

(a)


log10 (k x 10^6)
.4
.5
.6
.7
.8
.9
1.0
330
340
350
1/T x10^5

(b)

absolute temperature shows linearity from 15 - 35 C (Fig. 6b). Velocity k is in mole^{-1} litre^{-1} min^{-1}.

From the Arrhenius equation:

$$-E = \frac{d(\log v) \times 2.303R}{d(1/T)}$$

$$E = - \text{slope} \times 2.303\ R$$

where T is the absolute temperature and R the gas constant, the energy of activation can be calculated from Fig. 5b.

$$E = \frac{-(1.00 - 0.40) \times 2.30 \times 1.99}{(348 - 328.5)/10^5}$$

$$E = 14.1\ k_{cal}/\text{mole}$$

Effect of pH on the activity of isocitrate lyase

The activity of isocitrate lyase was determined using morpholino-ethane sulphonate (MES) buffer over a pH range of 5.0 - 8.5. The incubation method was employed in which the reaction mixture contained 2-5 µg protein of the enzyme solution, cofactors and buffer at the indicated pH values. The pH optimum of the enzyme prepared from either CO_2 or acetate grown cells was 7.3 - 7.5. There was a sharp increase in the activity above pH 6.0, whereas a comparatively slow decrease was observed between pH 7.5 - 9.0.

Effect of substrate concentration on enzyme activity

Fig. 8 A represents initial velocity versus isocitrate concentration

Fig. 7 Effect of pH on the activity of isocitrate lyase

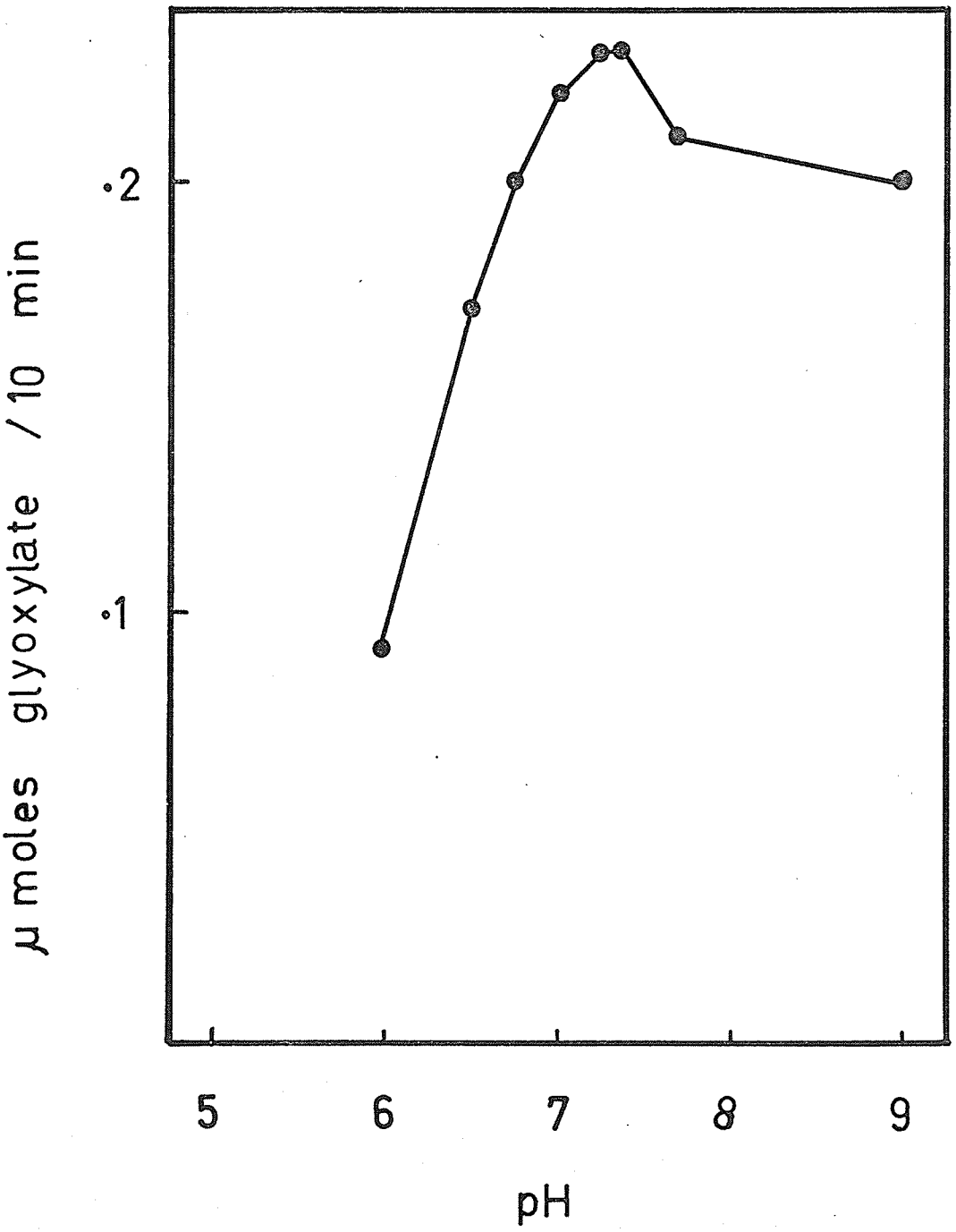

μ moles glyoxylate /10 min
·2
·1
5
6
7
8
9
pH

Fig. 8 (A) Effect of substrate concentration on isocitrate lyase from photoautotrophically grown Gloeomonas. Initial velocity is reported in μmole glyoxylate formed after 10 min incubation (see Methods).

(B) Double reciprocal plot of curve A.

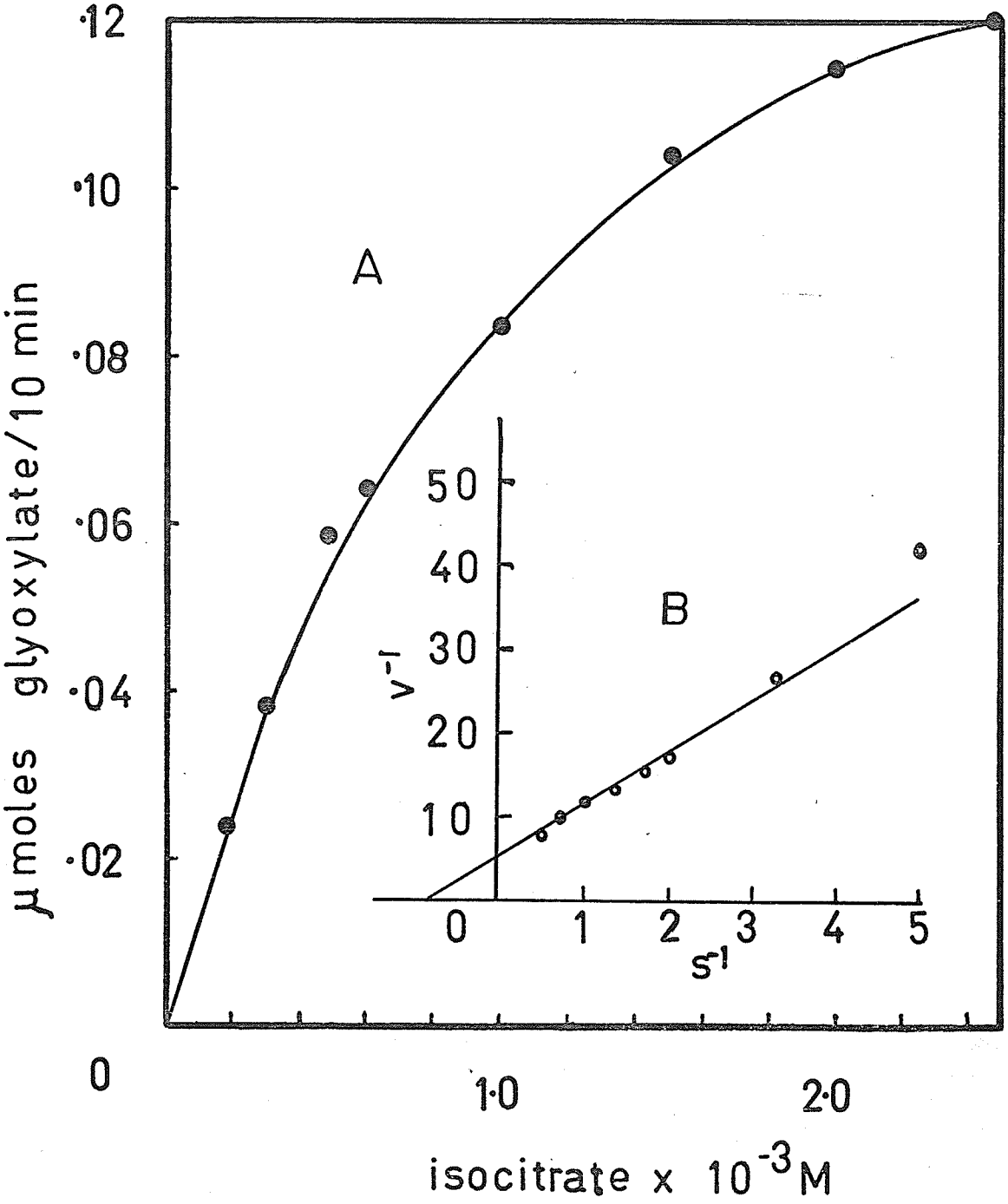
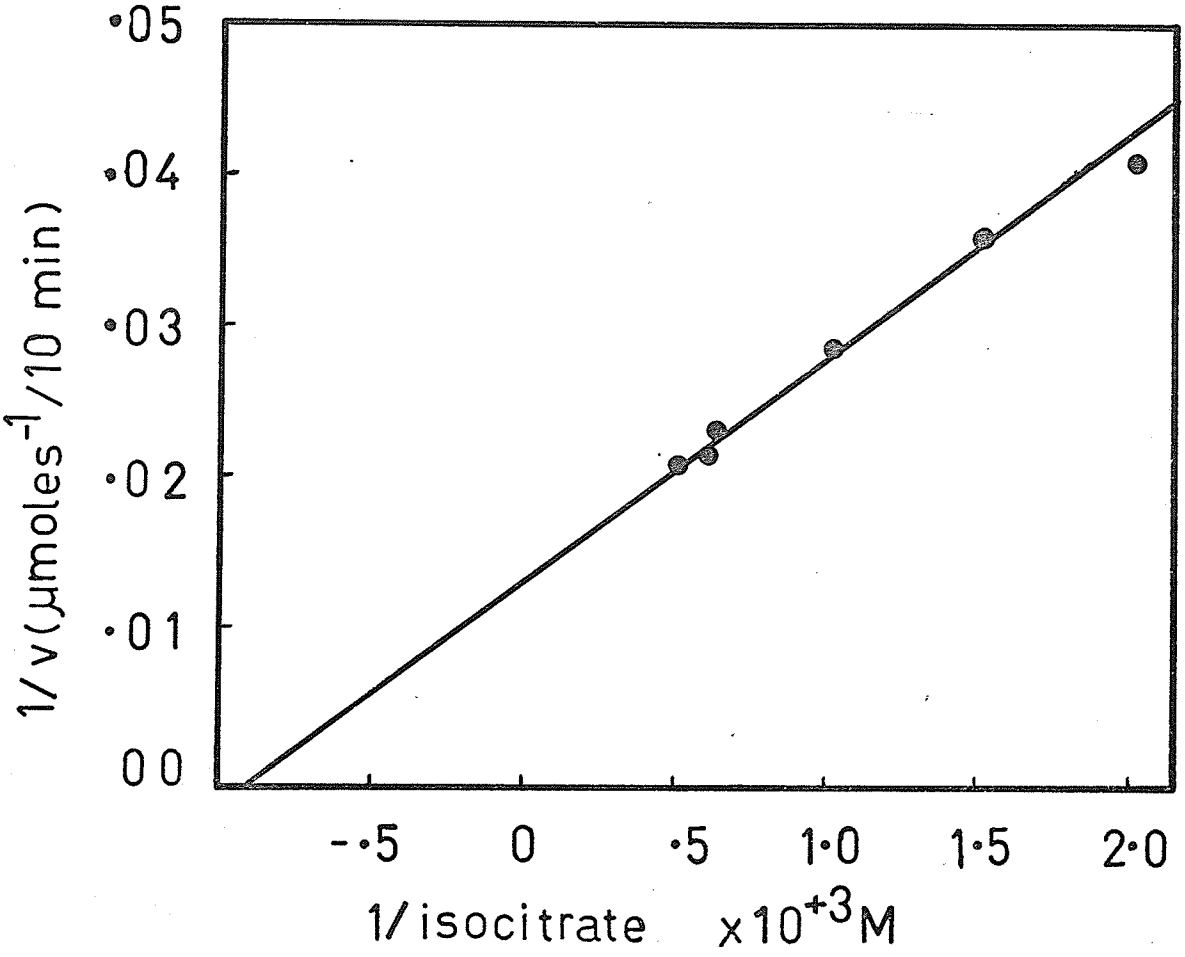

·12
·10
·08
·06
·04
·02
0
µ moles glyoxylate/10 min
A
B
50
40
30
20
10
v^{-1}
0
1
2
3
4
5
s^{-1}
1·0
2·0
isocitrate x 10^{-3}M

Fig. 9 Double reciprocal plot of the effect of substrate concentrations on isocitrate lyase from photo-heterotrophically grown Gloeomonas.


·05
·04
·03
·02
·01
00
1/v(μmoles^{-1}/10 min)
-·5
0
·5
1·0
1·5
2·0
1/isocitrate x10^{+3}M

using an enzyme preparation from photoautotrophically grown algal cells. The curve obtained was a normal hyperbola showing normal Michaelis-Menten kinetics. A Lineweaver-Burk plot (Lineweaver and Burk, 1934) of these data gave a K_{app} of 1.4×10^{-3} M (Fig. 8B). When an enzyme preparation from photoheterotrophic algal cultures were used, the K_{app} was 1.1×10^{-3} M (Fig. 9) which is similar to the value reported for cells grown in CO_2.

Incorporation of glyoxylate-1-^{14}C into isocitrate by isocitrate lyase

In order to demonstrate the aldol condensation reaction between glyoxylate and succinate by isocitrate lyase, glyoxylate-1-^{14}C was used. The latter compound forms a DNP derivative with acidic dinitrophenylhydrazine (0.1% in 2 N HCl) and can be readily extracted in ethyl acetate. Since isocitrate is also a reaction product and does not react with DNP, it therefore can be recovered in the aqueous phase. Thus, the amount of radioactivity incorporated into the aqueous phase would indicate isocitrate formation, which was identified by chromatography.

The decrease of the reactant glyoxylate-1-^{14}C due to condensation with succinate by isocitrate lyase could be determined by measuring the radioactivity in the glyoxylate DNP derivative, soluble in ethyl acetate, initially and after reaction initiated by addition of succinate.

For this reason the assay system contained the following

Table IV

Radioactivity in water soluble and ethyl acetate fractions

	Water Soluble (Isocitrate)	Ethyl Acetate Soluble (Glyoxylate - DNP)
	dpm x 10^{-3} per ml	
Zero time	20.4	670
30 min incubation	250.0	470
Difference (due to enzymic reaction)	209.6	-200

reactants and cofactors with isocitrate lyase (10 µg protein): 2 µmole Na-glyoxylate; 0.16 µ mole glyoxylate-1-^{14}C (3.2 mCi/mM); succinate 2 µ mole; 7.5 µ mole $MgCl_2$; 2 µmole glutathione and 50 µmole of MES buffer pH 7.5 in total volume of 1.0 ml. The reaction was started by addition of succinate and stopped after 30 min incubation at 30 C by 0.2 ml TCA. After deproteinization of the reaction mixture 0.4 ml of acid 2,4-dinitrophenylhydrazine was added and after 30 minutes the DNP derivative was extracted into ethyl acetate, evaporated and made to 0.2 ml with the solvent. The water extract was made to 0.2 ml with twice distilled water as described for that of ethyl acetate. From each of these two extracts 40 µl was used for counting the radioactivity and the remainder for TLC as described in Methods.

As described in Table IV, incorporation of glyoxylate-1-C^{14} into isocitrate in the presence of isocitrate lyase was demonstrated. The decrease of radioactivity in the glyoxylate DNP derivative after 30 minutes reaction period was almost recovered in the water extract which contained radioactive isocitrate.

The effect of some intermediary metabolites on the activity of isocitrate from photoauto- or photoheterotrophically grown Gloeomonas.

In these studies, metabolites were adjusted to pH 7.4 with HCl or NaOH and assay conditions followed were the same as given in Method 2. Reaction mixtures contained 10 mM of each of the tested metabolites (Tables V, VI and VII), as preliminary studies showed that 1 mM and

Table V

Inhibition of Isocitrate Lyase by Some Intermediates of Pentose Reductive Cycle and Glycolysis

Additives (10 mM)	Photoorganotrophic O. D. /10 min	% inhibition	Photoautotrophic O. D. /10 min	% inhibition
Nil	0.79	0	0.78	0
3-phosphoglycerate	0.29	66	0.25	69
3-phosphoglyceraldehyde (1 mM)	0.60	24	0.58	26
dihydroxyacetone-P	0.70	11	0.65	15
fructose-1, 6-di P	0.72	8	0.70	11
fructose-6-P	0.69	11	0.68	13
ribose-5-P	0.78	0	0.61	22
ribulose-1, 5-di P	0.56	30	0.45	44
glycollate	0.38	52	0.30	61
glucose-1 P	0.65	18	0.62	22
glucose-6-P	0.76	3	0.78	0
phosphoenolpyruvate	0.78	0	0.61	22
pyruvate (1 mM)	0.69	11	0.66	15
acetyl phosphate	0.70	11	0.63	20
glycoaldehyde (1 mM)	0.76	3	0.78	0

Table VI

Inhibition of Isocitrate Lyase by Some Intermediates of the Tricarboxylic Acid Cycle and the Glyoxylate Shunt

Additives (10 mM)	Photoorganotrophic O.D./10 min	% inhibition	Photoautotrophic O.D./10 min	% inhibition
Nil	0.79	0	0.78	0
Acetyl CoA (1 mM)	0.78	0	0.77	0
Citrate	0.72	9	0.71	9
Cis-aconitate	0.48	39	0.52	33
α-ketoglutarate	0.43	45	0.42	46
Succinate	0.36	53	0.37	53
Fumarate	0.44	44	0.48	39
Malate	0.43	45	0.39	50
Oxaloacetate (1 mM)	0.52	34	0.50	36
Oxalate	0.35	56	0.32	60
Tartronic Semialdehyde	0.70	11	0.68	13
Glycerate	0.64	19	0.60	23
Tartrate	0.58	27	0.61	22

Table VII

Effect of Some Amino Acids, Nucleotides and Related Compounds on Isocitrate Lyase Activity

Additives (10 mM)	Photoorganotrophic O.D./10 min	% inhibition	Photoautotrophic O.D./10 min	% inhibition
Nil	0.79	0	0.78	0
NAD	0.77	3	0.69	11
NADP	0.63	20	0.67	14
NADH	0.70	11	0.72	8
NADPH	0.69	13	0.66	15
ATP	0.65	18	0.68	13
ADP	0.43	45	0.44	45
AMP 3'	0.38	52	0.39	50
AMP 5'	0.72	8	0.77	3
Serine	0.63	20	0.64	18
Glycine	0.71	8	0.77	3
β -alanine	0.65	18	0.68	13
Aspartate	0.60	24	0.62	22
Glutamate	0.70	11	0.68	11

5 mM concentrations generally had no sigificant effect on the enzyme activity.

Table V shows clearly that isocitrate lyase was inhibited by 3-phosphoglycerate and ribulose diphosphate, two intermediates of the pentose reductive cycle. Furthermore, glycollate, an early product of photosynthetic CO_2 fixation inhibited the enzyme activity.

Among the organic acids from the TCA cycle and the glyoxylate shunt, α-ketoglutarate, succinate, malate, oxaloacetate (1 mM) and oxalate inhibited isocitrate lyase (Table VI).

The effect of some nucleotides and amino acids (Table VII) on the enzyme activity was also tested. All compounds failed to alter the enzymatic rate with the exception of ADP and 3'AMP. The latter two compounds caused substantial inhibition at high concentrations (5 - 10 mM). In addition, there was no significant difference in response to these metabolites between enzyme preparations from algal cells grown in light on CO_2 or acetate as the carbon source.

Mode of inhibition

To further define the mechanism of action of various inhibitors, the mode of inhibition of malate, succinate, glycollate and 3-phosphoglycerate was examined. Data for all are presented as double reciprocal plots (1/v vs 1/(S)) in Figures 10 - 17. Succinate and glycollate were found to be competitive inhibitors (Fig. 10 and 12) and their K_i were

Fig. 10 Double reciprocal plot of the inhibition of succinate on isocitrate cleavage.

For enzyme assay, Method (1) was used (see Methods).

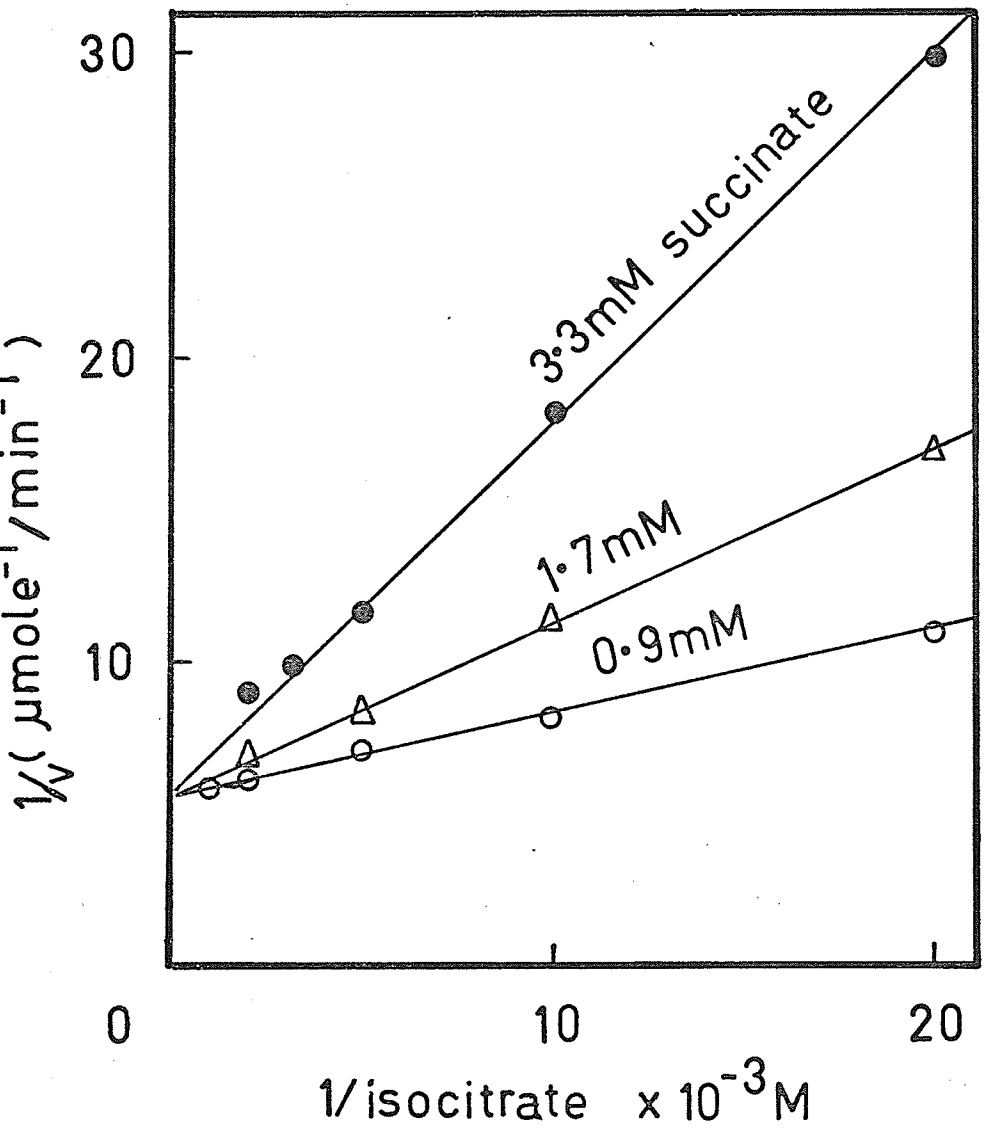

30
20
10
0
10
20
3·3mM succinate
1·7mM
0·9mM
1/v(µmole^{-1}/min^{-1})
1/isocitrate x 10^{-3}M

Fig. 11 Determination of inhibitor constant (K_i) for succinate (Dixon, 1953).

For enzyme assay, Method (2) was used (See Methods).

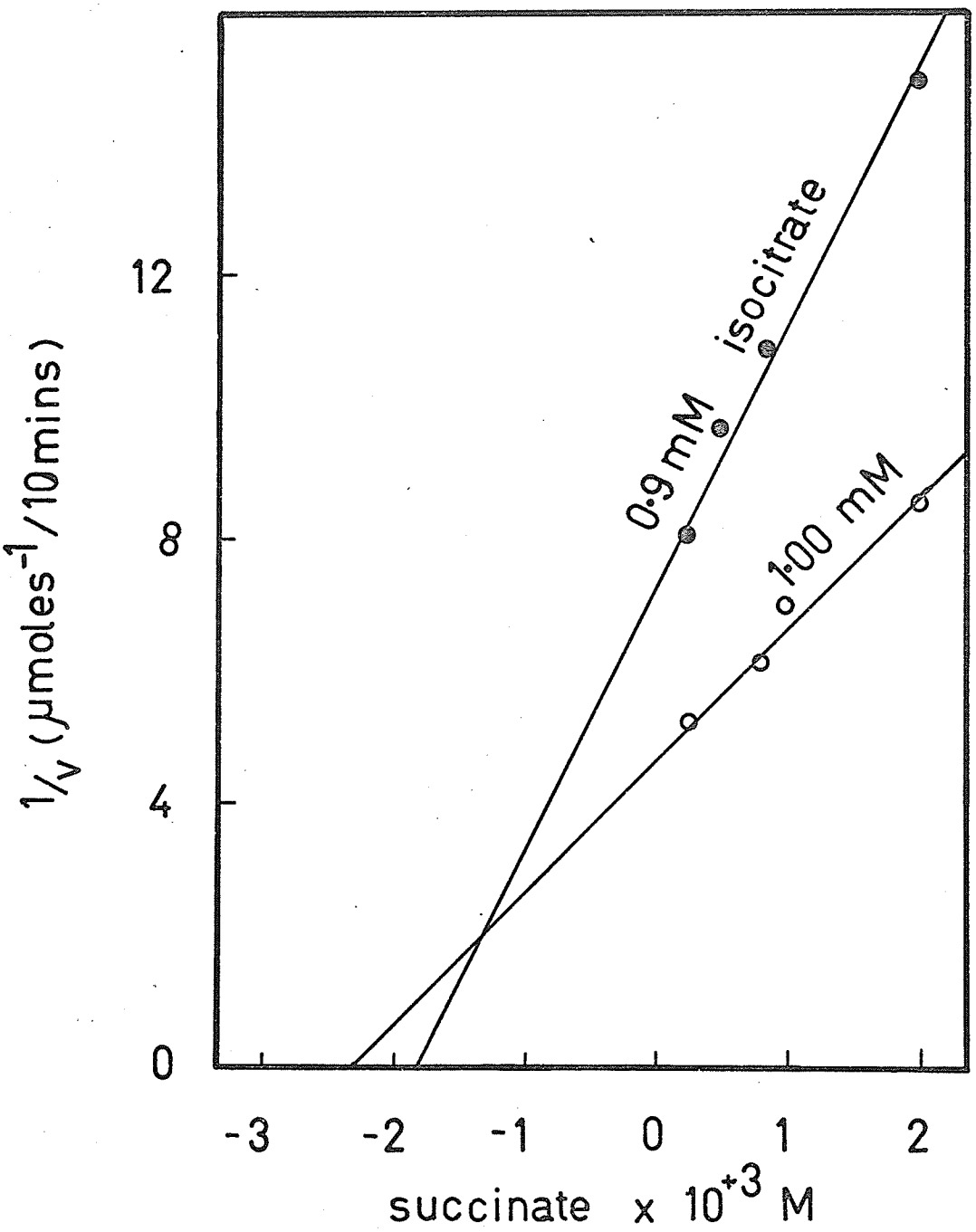

12
8
4
0
$1/_V$ (μmoles^{-1}/10mins)
-3
-2
-1
0
1
2
succinate x 10^{+3} M
0·9 mM isocitrate
o 1·00 mM

Fig. 12 Double reciprocal plot of the inhibition of glycollate on isocitrate cleavage.

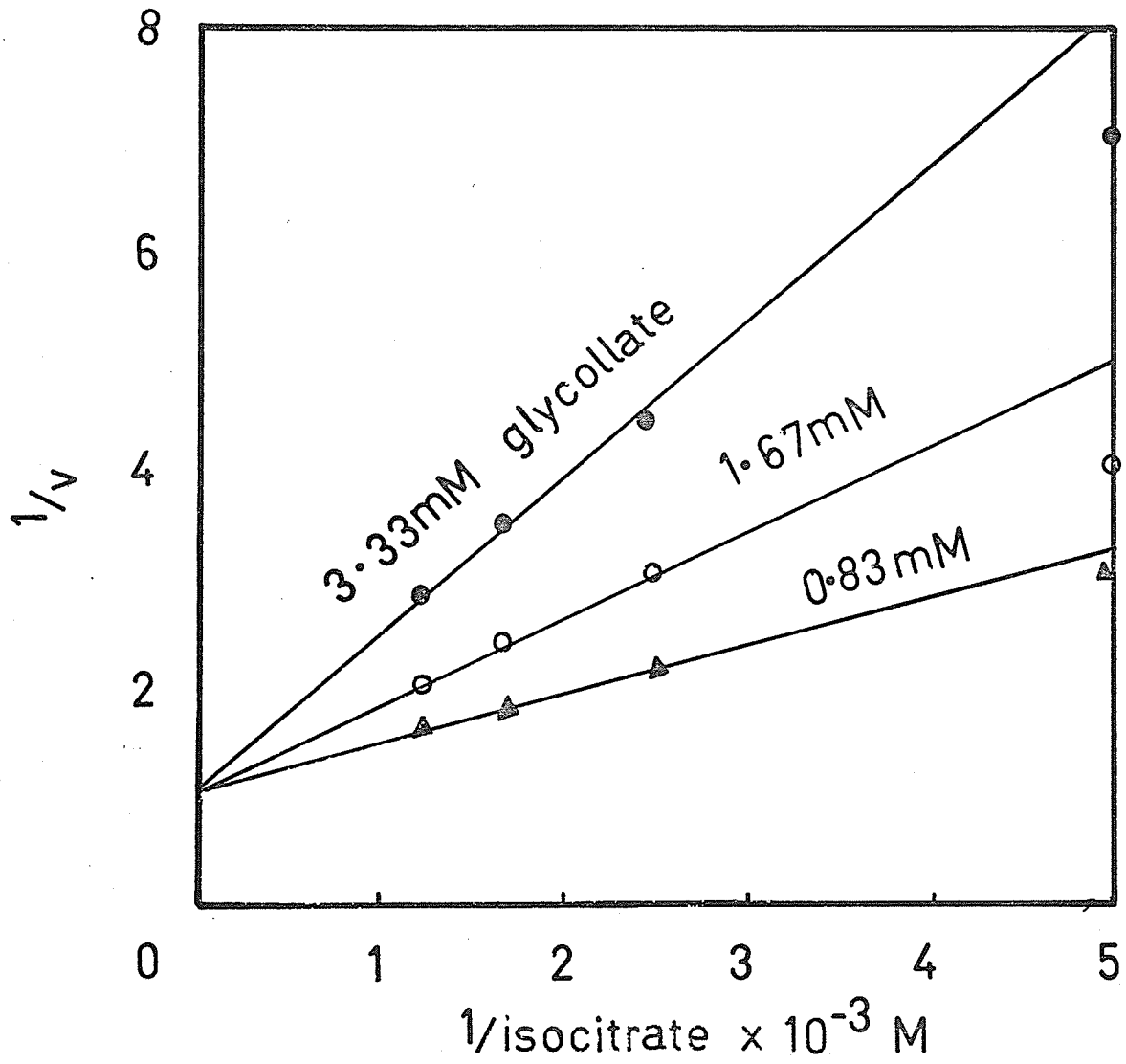

8
6
4
2
0
1/v
3·33mM glycollate
1·67mM
0·83mM
1
2
3
4
5
1/isocitrate x 10⁻³ M

Fig. 13 Determination of inhibitor constant (K_i) for glycollate.

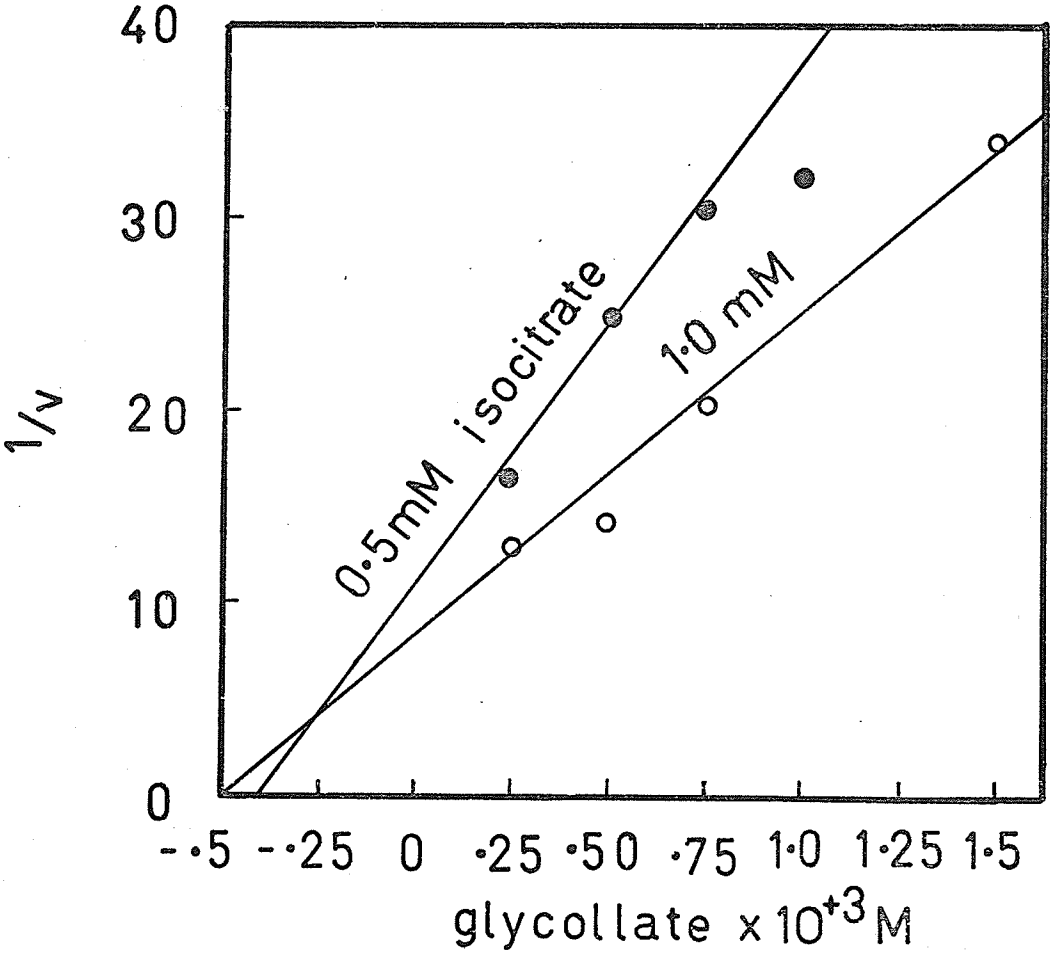

40
30
20
10
0
1/v
0·5mM isocitrate
1·0 mM
-·5 -·25 0 ·25 ·50 ·75 1·0 1·25 1·5
glycollate x 10+3 M

Fig. 14 Double reciprocal plot of the inhibition of 3-phosphoglycerate on isocitrate cleavage.

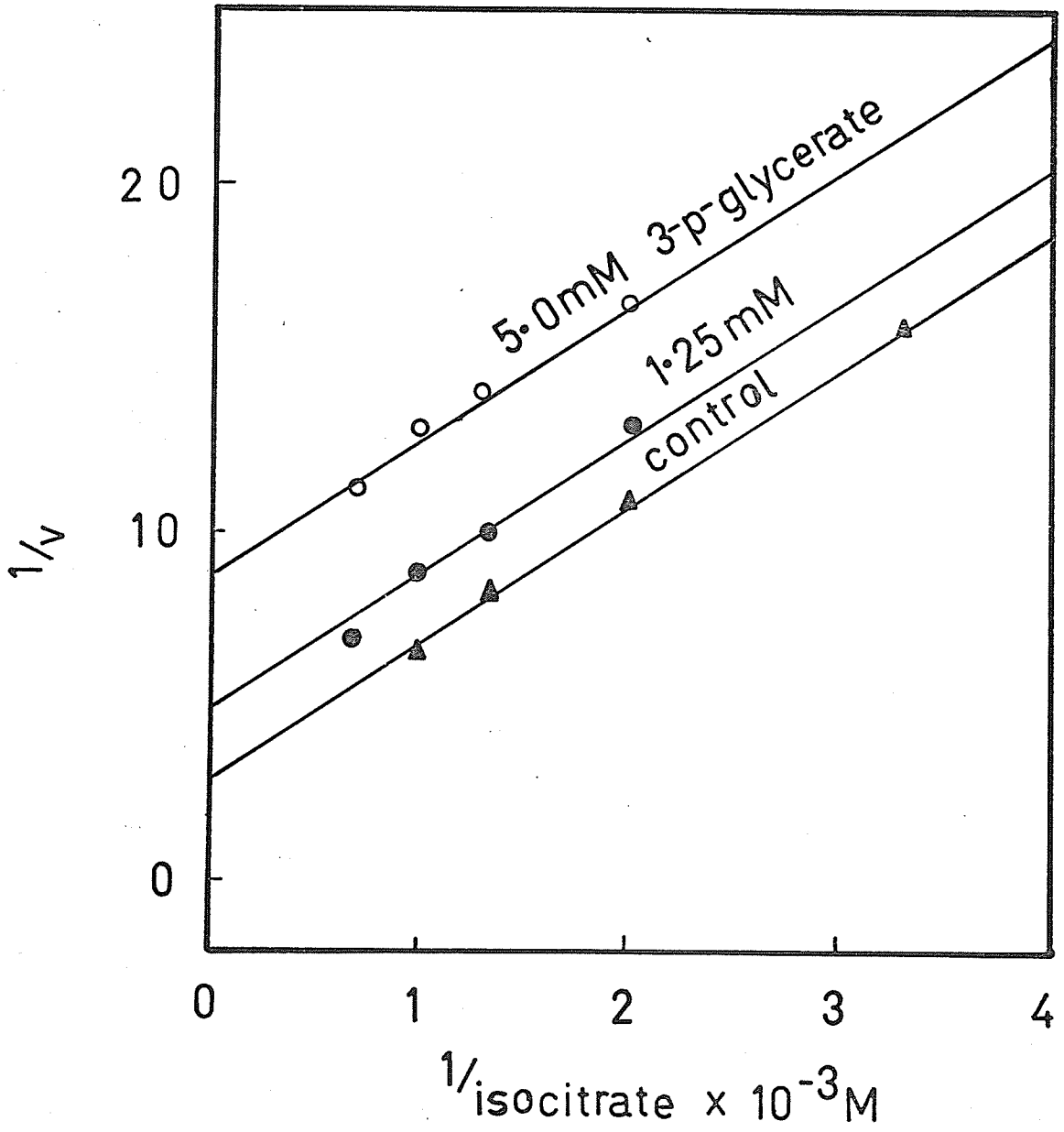

5·0mM 3-p-glycerate
1·25mM
control
20
10
0
1/v
0
1
2
3
4
1/isocitrate x 10-3M

Fig. 15 Replot of Figure 14.

Intercept vs (3-phosphoglycerate).

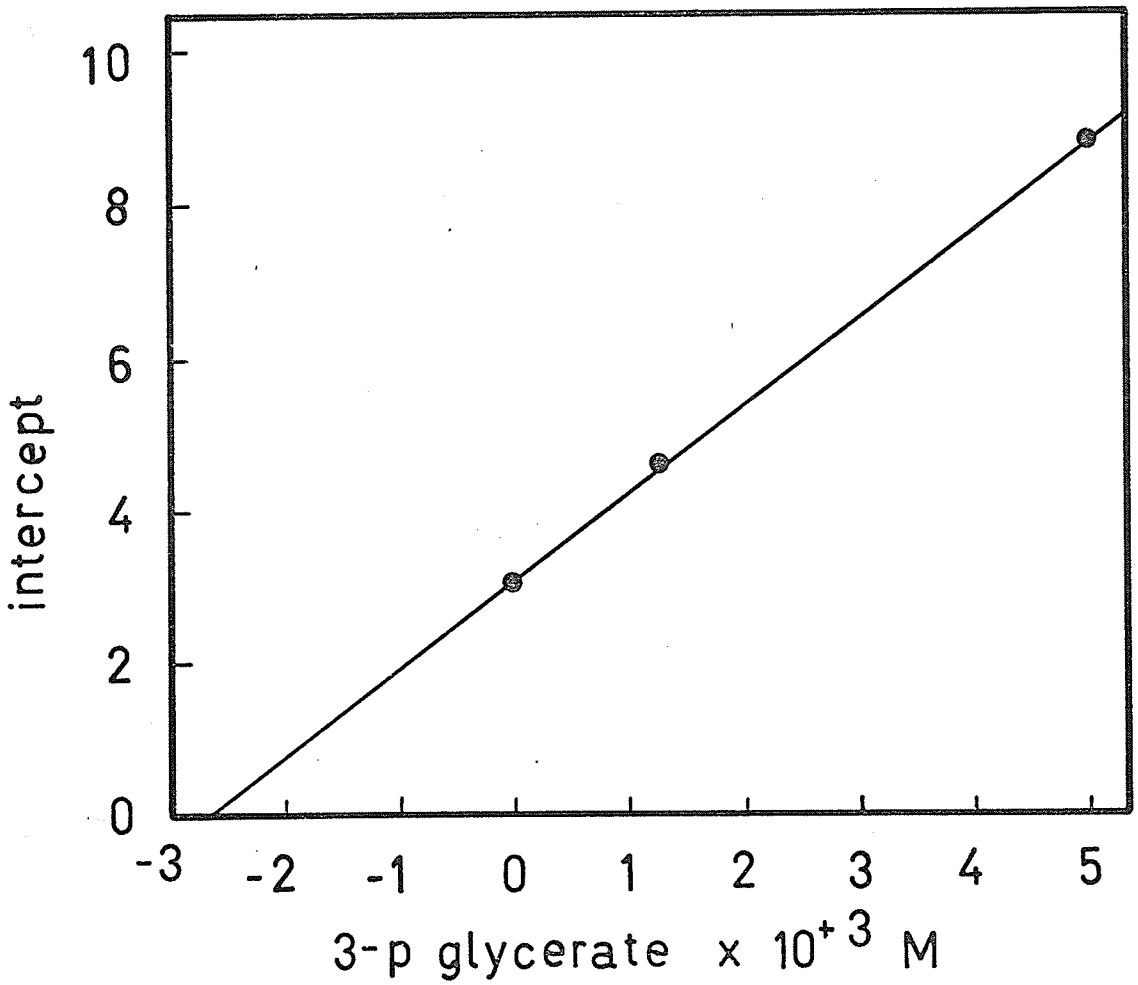

10
8
6
4
2
0
intercept
-3
-2
-1
0
1
2
3
4
5
3-p glycerate x 10+3 M

Fig. 16 Double reciprocal plot of the inhibition of malate on isocitrate cleavage.

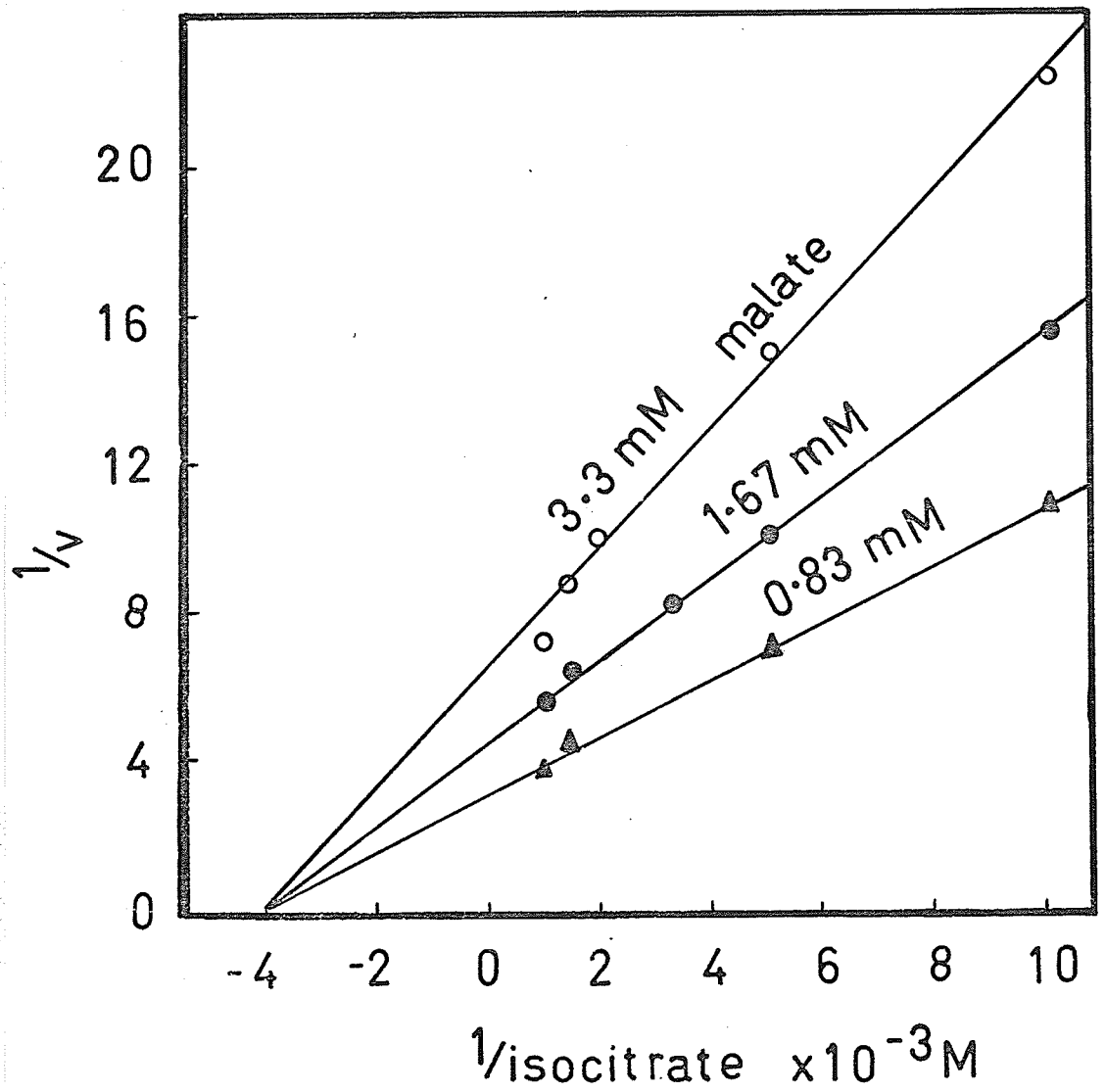
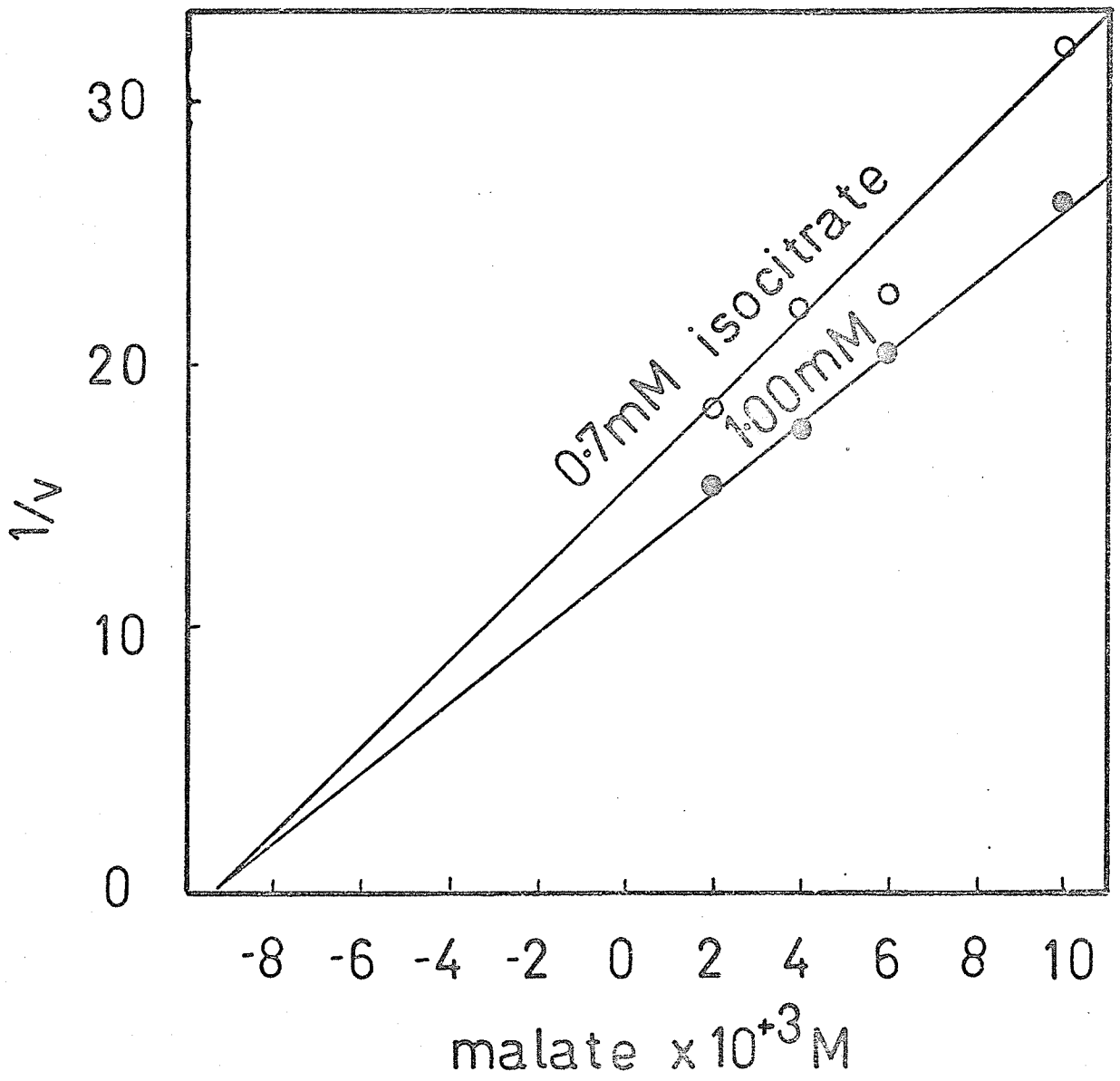

20
16
12
8
4
0
1/v
3·3 mM malate
1·67 mM
0·83 mM
-4
-2
0
2
4
6
8
10
1/isocitrate x10⁻³M

Fig. 17 Determination of inhibitor constant (K_i) for malate.


30
20
10
0
1/v
0·7mM isocitrate
1·00mM
-8 -6 -4 -2 0 2 4 6 8 10
malate x 10+3 M

1.27 mM and 0.25 mM respectively (Figs. 11 and 13) while 3-phosphoglycerate was an uncompetitive inhibitor (Fig. 14) with a K_i of 2.7 mM(Fig. 15). Malate appeared to give non-competitive inhibition (Fig. 16) with a K_i of 8.6 mM (Fig. 17).

Activation of isocitrate lyase by succinyl CoA

As seen in Table VIII, 1 mM of succinyl CoA increased isocitrate lyase activity to 155% as compared to control, but higher concentration of this compound (4 mM) had a pronounced inhibitory effect on enzyme activity (Table VIII). It is evident from Tables V and IX that neither acetyl CoA (Table V) or CoA (Table IX) were effective in activating the enzyme reaction. Acetyl CoA had no effect, whereas CoA apparently inhibited isocitrate lyase activity at 4 mM (Table IX). The stimulatory effect of succinyl CoA is clearly demonstrated in Fig. 18 whereas succinate is inhibitory (Fig. 10).

Effect of cycloheximide (actidione) on the formation of isocitrate lyase

Cycloheximide, an antibiotic produced by Steptomyces grieseus, inhibits the growth of fungi (Whiffen et al, 1946, Whiffen, 1948), algae (Palmer and Maloney, 1955), and protozoa (Loefer and Matney, 1952), but does not affect bacteria (Whiffen et al, 1946). Work with intact yeast and mammalian cells (Kerridge 1958, Bennett et al, 1964; Siegel and Sisler, 1964) showed that cycloheximide inhibited protein, RNA and DNA synthesis.

Table VIII

Effect of Succinyl CoA on Isocitrate Lyase Activity

Succinyl CoA Added (mM)	Activity μ mole/10 min	Activity % of control
Nil	0.060	100
0.01	0.054	90
0.10	0.050	90
1.00	0.093	155
4.00	0.010	33

Fig. 12 Double reciprocal plot of the effect of succinyl, CoA on isocitrate lyase activity.

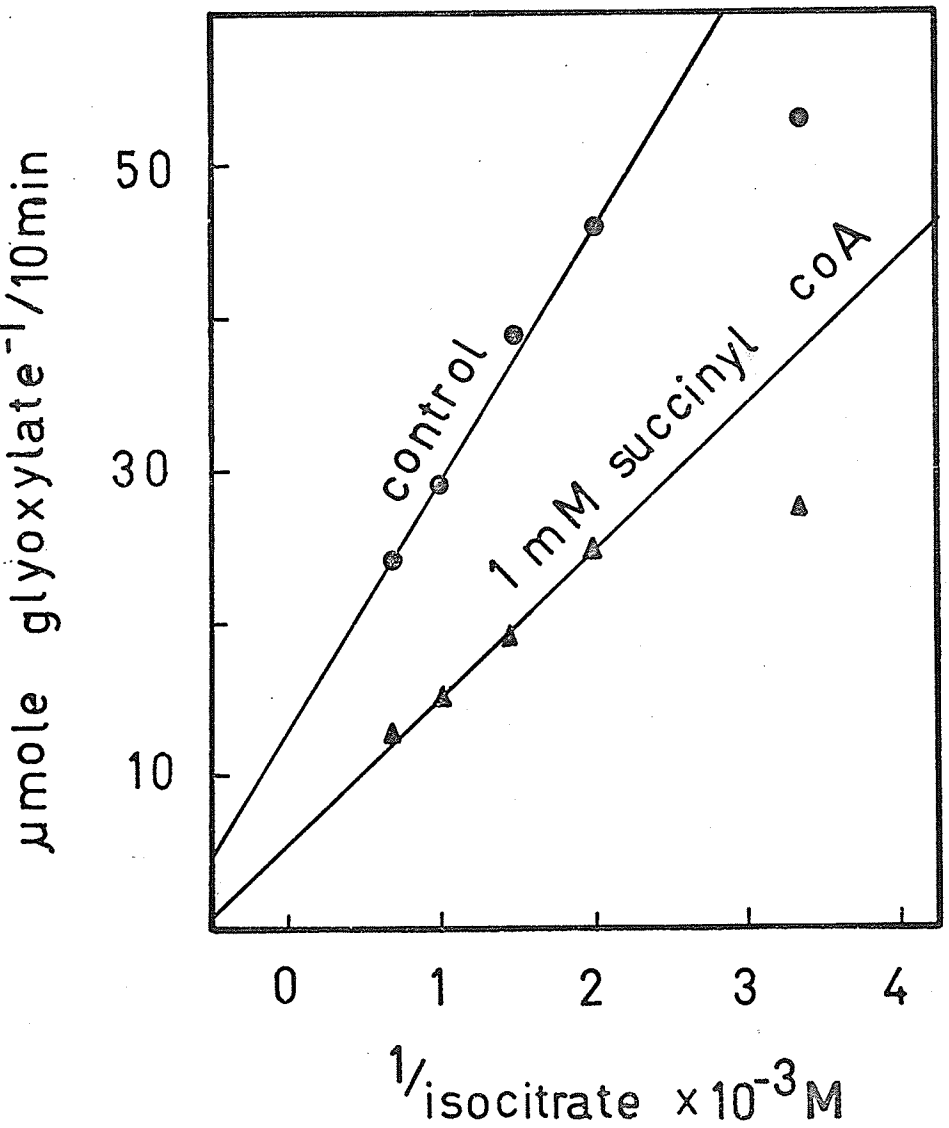

μmole glyoxylate^{-1}/10min
50
30
10
control
1 mM succinyl coA
0
1
2
3
4
1/isocitrate x10^{-3}M

Table IX

Effect of Coenzyme A on Isocitrate Lyase Activity

Coenzyme A added (mM)	Activity mole/10 min	Activity expressed as a % of the control
Nil	0.085	100
0.01	0.073	86
0.10	0.068	80
1.00	0.60	70
4.00	0.060	70

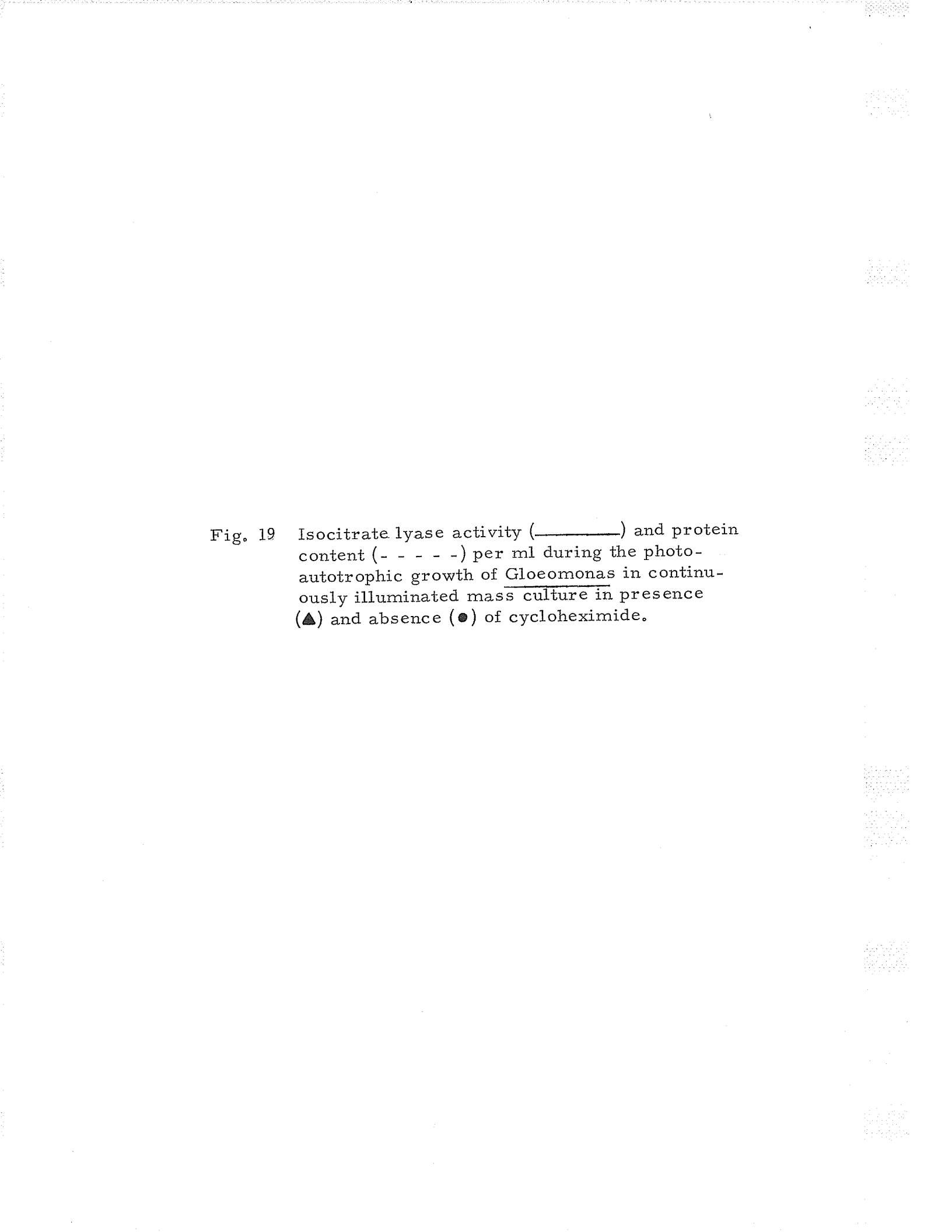
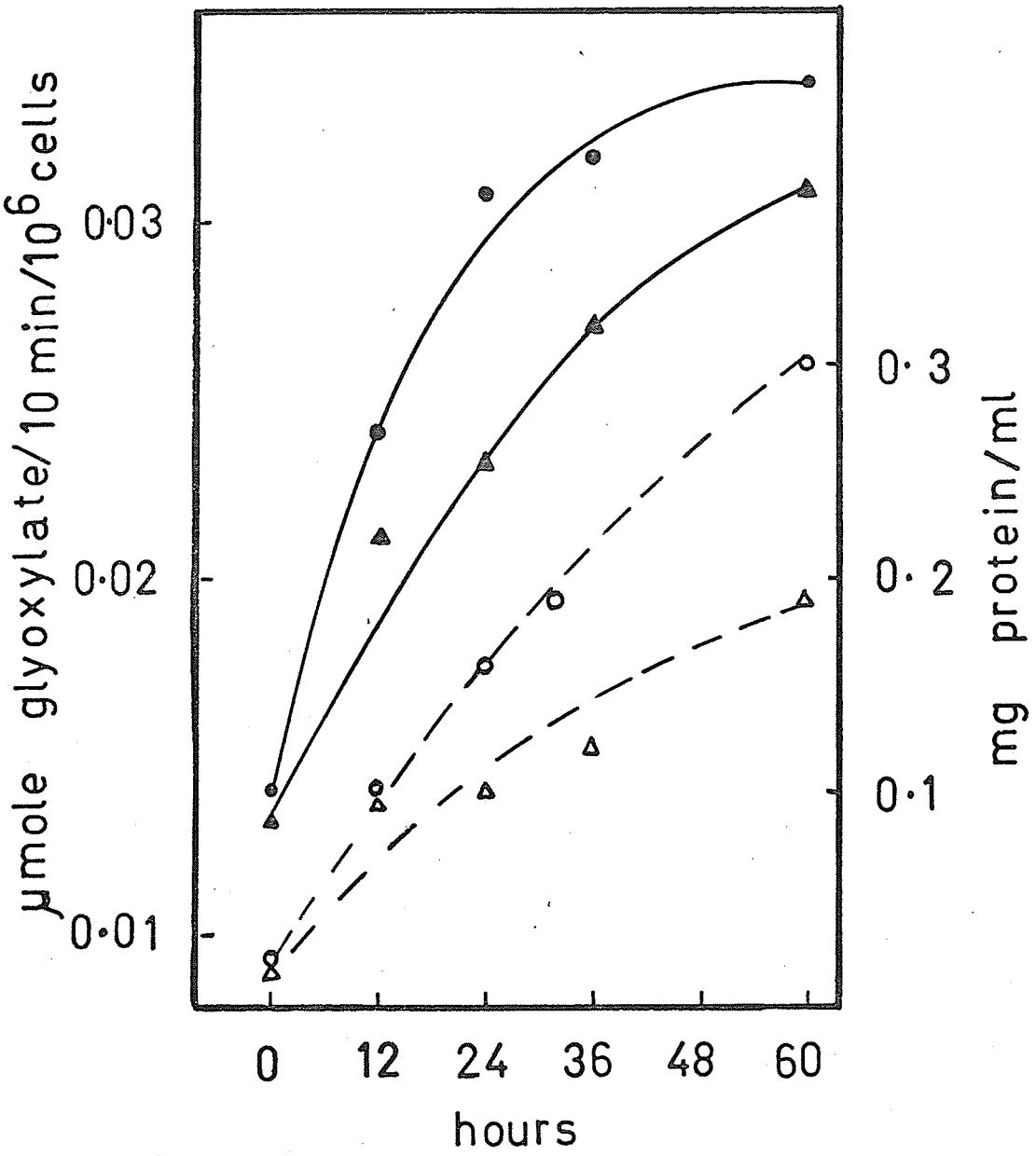


Fig. 19 Isocitrate lyase activity (————) and protein content (- - - - -) per ml during the photoautotrophic growth of Gloeomonas in continuously illuminated mass culture in presence (▲) and absence (●) of cycloheximide.


µmole glyoxylate/10 min/10^6 cells
0·03
0·02
0·01
0·3
0·2
0·1
mg protein/ml
0
12
24
36
48
60
hours

Isocitrate lyase is formed rapidly when algal cells are supplied with acetate under non-photosynthetic conditions (Syrett et al, 1963) and the increase in enzyme activity is accompanied by the synthesis of a new protein identifiable after purification with acrylamide gel (Syrett, 1966). However the development of enzyme-activity is inhibited by cycloheximide (Morris, 1966). This antibiotic has since been used by many investigators to differentiate between the activation or inactivation of enzyme activity and the de novo synthesis of the enzyme (Morris, 1966; Nelson and Tolbert, 1970). It was therefore considered important to determine whether the synthesis of isocitrate lyase in Gloeomonas was inhibited by cycloheximide.

In this experiment, algal cells growing in mass cultures were secured at the exponential phase of growth and resuspended in nutritive media with or without added cycloheximide (5 µg/ml). Protein synthesis and isocitrate lyase activity in both cultures were followed over a period of 60 hours. 500 ml of the algal suspension were withdrawn at intervals for protein determination and enzyme assay. Fig. 19 showed that during the first 36 hours the increase in enzyme activity was closely associated with protein synthesis. The latter was relatively lower in cycloheximide treated cultures. Later on, however, levels of enzyme activity indicated slight increases though protein synthesis continued.

The results showed that cycloheximide at a concentration of 5 µg/ml

inhibited isocitrate lyase formation by about 30%.

Effect of α-hydroxy-2-pyridinemethanesulphonate and Dichlorophenyldimethyl urea on isocitrate lyase

α-hydroxy-2-pyridinemethanesulphonate (HPMS) has been used as a specific inhibitor for plant glycollate oxidase (EC 1.1.3.1) in vitro (Zelitch, 1957). However, its non-specificity has been reported in studies dealing with its effect in vivo (Tolbert and Hess, 1966 and Lord and Merrett, 1970). Addition of this inhibitor at varying concentrations to isocitrate lyase reaction system (Table X) had virtually no influence on enzyme activity.

Dichlorophenyldimethylurea (DCMU), a specific inhibitor for photosynthetic 0_2 evolution (Bishop, 1958), was also tested within the physiological concentration (Table XI) usually used in vivo experiments. The results obtained showed no significant effect on the activity of isocitrate lyase.

Levels of isocitrate lyase and isocitrate dehydrogenase (NADP-linked enzyme) activities during the life cycle of Gloeomonas

The starting material for these experiments was the daughter cells collected at the end of the dark period from well synchronized (12 hr light : 12 hr dark) autotrophic cultures of Gloeomonas. These cells, referred to as "nascent dark cells" (Tamiya, 1966; Pirson and Lorenzen, 1966) are characterized by active photosynthetic $C0_2$ fixation, 0_2 evolution and carbohydrate synthesis during the growth period; 8 - 10

Table X

Effect of α-Hydroxy-2-Pyridinemethanesulphonate on Isocitrate Lyase Activity

HPMS (mM)	Activity μ mole/10 min	Activity expressed as a % of the control
0	0.125	100
0.01	0.122	100
0.1	0.122	100
1.0	0.120	98
10.0	0.122	100

Table XI

Effect of Dichlorophenyldimethylurea (DCMU) on Isocitrate Lyase Activity

DCMU (μ M)	Activity μ mole/10 min	Activity expressed as a % of the control
0	0.085	100
0.25	0.080	94
0.5	0.080	94
1.0	0.080	94

Table XII

Effect of crude extracts from cells at an early stage of growth (8 hr) on isocitrate lyase activity of the mature cells (12 hr)

Time (hr)	Specific Activity μ mole	Specific Activity glyoxylate/10 min/mg protein
	Experiment I	Experiment II
8	3.4	3.4
12	6.3	5.70
8 + 12	4.2	4.10

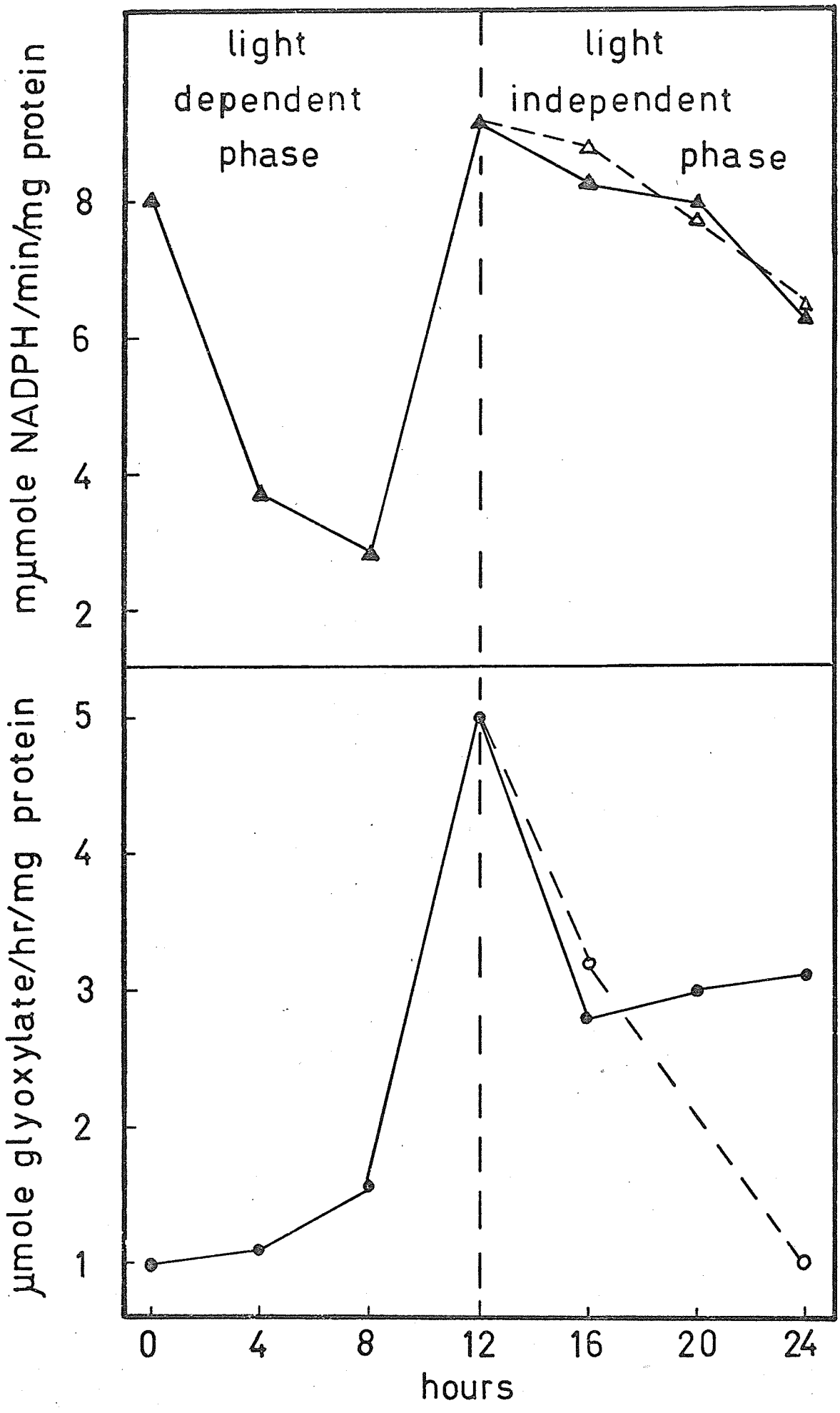
Fig. 20 Activity levels of isocitrate lyase and isocitrate NADP linked dehydrogenase during the life cycle of Gloeomonas in synchronized cell culture.

●-●-●-●- activity of isocitrate lyase in the light

o-o-o-o- activity of isocitrate lyase in the dark

▲-▲-▲-▲- activity of isocitrate NADP-linked dehydrogenase in the light

△-△-△-△- activity of isocitrate NADP-linked dehydrogenase in the dark


light
dependent
phase
light
independent
phase
mμmole NADPH/min/mg protein
2
4
6
8
μmole glyoxylate/hr/mg protein
1
2
3
4
5
0
4
8
12
16
20
24
hours

hr from the commencement of illumination (Tan, Badour and Waygood, 1970). During this state of development isocitrate lyase activity in the crude algal extracts was relatively low (Fig. 20). This was associated with a decreasing activity of isocitrate dehydrogenase. During the phase of maturity or ripening (8 - 12 hr light) abrupt increase in the activity of the two enzymes was clearly observed.

Since the further development of the mature algal cells (cellular division, and separation of daughter cells) is a light independent process it was possible, therefore, to examine the effect of light on the observed high levels of both enzymes. This is of interest because during cellular division light energy has been shown to be trapped mainly through cyclic phosphorylation (Senger, 1970) since photosystem II is repressed (Senger and Bishop, 1967). Thus one set of the algal mature cells were kept in the light whereas the other set was darkened. As illustrated in Fig. 20 isocitrate dehydrogenase activity declined progressively and attained the initial level of the nascent dark cells (time = 0) without being influenced by light. On the other hand, while isocitrate lyase showed comparatively strong decrease in its activity which continued in the dark after the separation of daughter cells (20 - 24 hr), the activity level at the same time was maintained in those cells subjected to illumination.

From these results, the following may be inferred:

(a) Isocitrate lyase and isocitrate dehydrogenase were detectable throughout the entire life cycle of Gloeomonas, but the level of enzyme activity changed as the alga was developing in the light dependent phase.

(b) Decreasing activity of isocitrate dehydrogenase associated with comparatively low isocitrate lyase activity were found during the phase of active photosynthesis performed by the young actively growing cells. Maximal activities were attained during the phase of maturity.

(c) Light maintained the level of isocitrate lyase activity in the daughter cells if produced under illumination, presumably due to activation of synthesis of the enzyme protein mediated by cyclic ATP formation (Syrett, 1966).

(d) Darkness led to declining enzyme activity.

In order to show whether the low isocitrate lyase activity during the phase of growth was attributable to repression of enzyme synthesis or to inactivation by some intracellular metabolites at these developmental stages, crude extracts from 8 hr old cells were mixed with extracts from mature cells. As shown in Table XII, extracts from growing cells (8 hr light) exerted an inhibitory effect on the enzyme activity of the mature cells. Therefore, the observed low activity of isocitrate lyase during the phase of growth and active photosynthesis may be due to enzyme inhibition by endogenous substance(s).

α -ketoglutarate synthetase

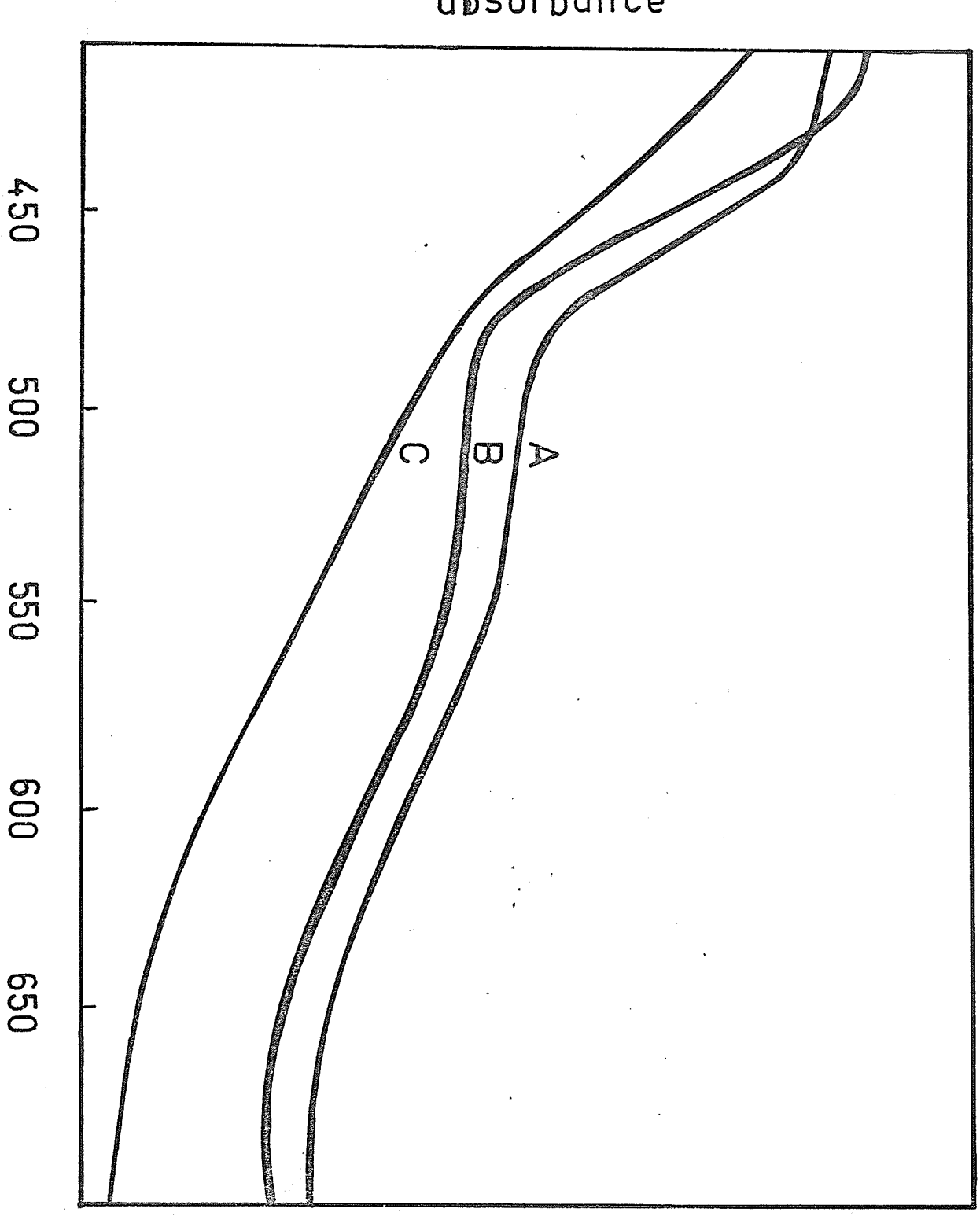
The presence of isocitrate lyase as well as isocitrate dehydrogenase (NADP-linked) during the entire life cycle of Gloeomonas and the activation of the former enzyme by succinyl CoA (Table VIII and Fig. 18) have led to the suggestion that these enzymes might be implicated in CO_2 fixation via the reductive carboxylic cycle operating in some photosynthetic bacteria (Evans, Buchanan and Arnon, 1966). However, when algal extracts were tested for the presence of α -ketoglutarate synthetase using $NaHC^{14}O_3$ as described in Methods, no significant radioactivity was detected at the end of the incubation period. Nevertheless, it was possible to demonstrate the presence of the reaction product α -ketoglutarate by comparing the absorption spectrum of its alkaline 2, 4-dinitriphenylhydrazone derivative as illustrated in Fig. 21. However, further studies are required to clearly demonstrate the presence of this system.

Fig. 21 Absorption spectrum of the reaction product of;

α-ketoglutarate synthetase.

Alkaline DNP derivative of:

A - reaction mixture
B - authentic α-ketoglutarate
C - control


absorbance
450
500
550
600
650
A
B
C

DISCUSSION

As shown in the present work, isocitrate lyase has been purified 50-fold from autotrophic and photoheterotrophic cultures of the unicellular green alga Gloeomonas. The enzyme reaction was found to be dependent on Mg^{++} and glutathione for maximum activity. The optimum pH was about 7.4 which agrees with that of the enzymes from other sources (McFadden, 1963; John and Syrett, 1967). MES buffer was used in the reaction mixture since Rao et al (1970) has pointed out that tris may induce a subtle change in the structure of the enzyme and phosphate buffers (Olson, 1959; McFadden, 1962) may inhibit the enzyme activity. The enzyme attained its maximum activity at 35 C and the Arrhenius activation energy for isocitrate cleavage was 14.1 k_{cal} $mole^{-1}$ l similar to that in Pseudomonas indigofera (Rao et al, 1965).

It has been shown that preparations of isocitrate lyase either from autotrophic or photoheterotrophic cultures of Gloeomonas were eluted from the DEAE cellulose column virtually at the same position. Both enzyme preparations gave classical Michaelis-Menten kinetics and had almost the same K_m values (1.14 and 1.4 mM). They responded in a similar manner to added metabolites (Tables V, VI, VII). Therefore, it may be inferred that irrespective of the nature of the carbon source provided in the culture, there was only one type of isocitrate lyase

in Gloeomonas. The presence of acetate did not induce another isocitrate lyase as in Neurospora crassa where two isocitrate lyase enzymes have been reported (Sjogren and Ramono, 1967).

The presence of isocitrate lyase in a variety of photoautotrophically unicellular green alga as Chlorella (Lord and Merrett, 1970), Chlorogonium, Chlamydomonas and Chlamydobotrys (Wiesnner, 1968) is evident though the measured activity is low as compared to that when growth occurred in acetate as the sole carbon source.

In bacteria, Kornberg (1963) proposed that acetate does not directly induce isocitrate lyase, but rather acts to derepress enzyme synthesis by removal of a repressor close to oxaloacetic acid. On the other hand, Baechtel et al (1970) showed that during the photoautotrophic growth (4% $C0_2$ and light)of a thermophilic strain of Chlorella pyrenoidosa in synchronous cultures, isocitrate lyase synthesis could be accomplished solely by placing the cells in the dark. Addition of acetate (0.2% w/v) in the dark slightly increased the enzyme synthesis whereas higher concentration actually repressed the dark induction of the enzyme. This observation provided evidence that repressors or inhibitors of isocitrate lyase originally present in this Chlorella may arise mainly from photosynthesis. These repressors in Chlorella could be eliminated by darkening rather than by acetate as in bacteria.

Glycollate and 3-phosphoglyceric acid are early products of the photosynthetic CO_2 fixation (Wilson and Calvin, 1955) and ribulose diphosphate is the CO_2 acceptor in the Calvin cycle. The three compounds inhibited isocitrate lyase in vitro (Table V). Such inhibitory effect may occur undoubtedly in vivo in the eucaryotic cells of Gloeomonas as well as other algae with rigid compartmentational controls. This is because of the free exchange of certain Calvin cycle intermediates between chloroplast and cytoplasmic pools, at least for equilibration (Hatch and Slack, 1970). Photosynthetically formed glycollate is excreted into to the surrounding media by algae (Cheng and Tolbert, 1970). Furthermore, 3-phosphoglycerate and pentosephosphate are provided by the chloroplasts for the synthesis of amino acids and for nucleic acid formation respectively (Bassham et al, 1968).

The inhibition of isocitrate lyase in vitro by ADP (Table VII) and in inorganic phosphate (McFadden, 1962) may indicate a relationship between the energy state of the cell and the activity of the initiating enzyme of the glyoxylate cycle. Thus low ATP/ADP ratio during active photosynthetic CO_2 fixation and further carbohydrate synthesis may lead to inactivation of isocitrate lyase. The activity of the latter may be partially restored as ATP/ADP ratio increases with declining CO_2 assimilation. The abrupt increase in isocitrate lyase activity (Fig. 20) at the end of the growth phase (active photo-

synthesis) which has been observed in Chlorella also (Baechtel, 1970) may sustain this view.

Since isocitrate lyase and isocitrate (NADP-linked) dehydrogenase compete for the same substrate; control mechanisms should be present to ensure their efficient operation. High activities of the latter enzyme may indicate enhanced activities of the tricarboxylic acid cycle enzymes, thus promoting biosynthetic processes and production of NADH (Pleut and Aogaichi, 1968; Stein and Stein, 1967; Cook and Carver, 1966). Isocitrate lyase produces glyoxylate which inhibits the TCA cycle (Davies and Ribereau-Gayon, 1969(whereas intermediates of the TCA cycle namely, α -ketoglutarate, succinate, malate, fumarate, oxaloacetate (Table VI) are powerful inhibitors of isocitrate lyase in Gloeomonas similar to that reported in Chlorella (John and Syrett, 1968). As pointed out by Umbarger (1964) this inhibitory effect may thus represent a feedback control of a cyclic system by end product inhibition. Such a control, however, may not necessarily occur during active photosynthesis as the levels of isocitrate lyase increase very slowly while isocitrate dehydrogenase activities were decreasing during the first eight hours of illumination (Fig. 20). It may rather exist as C_4 acids formed by β-carboxylation via phosphoenolpyruvate carboxylase (EC 4.1.1.31) or the acid PEP carboxylase. Both of the latter enzymes have been detected in Gloeomonas grown under the culture conditions described (C.K. Tan, unpublished results; Pan and Waygood, 1971). This does not rule out

the possibility of inhibition due to TCA intermediates and low ATP/ADP ratio in the dark as concommitant with the abrupt decrease of the enzyme activity in the dark nascent daughter cells.

The activation of algal isocitrate lyase by succinyl CoA at a physiological concentration (1 mM) is of interest since this compound is directly implicated in chlorophyll biosynthesis in plants (Kirk, 1970) through the formation of δ-aminolaevulinate. For the cell it is more rational to derive succinate via isocitrate lyase rather than from α-ketoglutarate drained for protein synthesis. It appears therefore, that the alga has to control succinate production and succinyl CoA may be the signal of this process. Bleaching of Chlorella by glucose (Matsuka and Hase, 1965) which has been reported recently (John et al, 1970) to cause the degeneration of isocitrate lyase protein may support this opinion. Furthermore, Pirson et al, 1959 showed that at the phase of maturity, chlorophyll synthesis is inhibited in Chlorella at low temperature (4 C) and the cells become bleached. At this stage of development, isocitrate lyase activity in Gloeomonas was maximal at 25 C (Fig. 20). The enzyme in vitro has been shown to be inactive (Fig. 6) at low temperature (16 C) whereas its optimum activity was attained at 35 C. It is therefore conceivable to suggest the involvement of isocitrate lyase in such bleaching processes.

DCMU at physiological concentrations had no effect on isocitrate lyase activity *in vitro* (Table XI), though it has been found to stimulate the enzyme activity in intact cells of *Chlorella pyrenoidosa* grown on acetate in light and aerated with air containing 5% CO_2 (Goulding and Merrett, 1966). It is, therefore, obvious that the stimulatory effect of DCMU *in vivo* under such mixotrophic mode of nutrition is due to inhibition of photosynthesis. The site of inhibition of the latter is the photosynthetic O_2 production which has been shown by Gibbs (1969) to be implicated in glycollate formation. The latter process and the subsequent excretion of glycollate in algae are inhibited by DCMU (Chang and Tolbert, 1970). In other words, it is possible that addition of DCMU would eliminate the inhibitory effect exerted by glycollate and other photosynthates (e.g. 3 PGA) on isocitrate lyase.

Cycloheximide inhibits the synthesis of isocitrate lyase protein in *Gloeomonas* (Fig. 19) as previously reported by (Morris, 1966) in *Chlorella*. However, inhibition of the enzyme activity *in vitro* by various metabolites (Tables V, VI, VII) and its activation by succinyl CoA indicated that the control of enzyme activity is not solely by repression.

The presence of isocitrate lyase activity throughout the whole life cycle of photoautotrophically grown *Gloeomonas* (Fig. 20) may provide strong evidence that this enzyme is constitutive. The failure

to detect the enzyme in crude extract of photoautotrophic unicellular green algae appears to be more likely due to inactivation rather than by repression.

Finally, it is worthwhile to discuss the role of the glyoxylate bypass in Gloeomonas since Badour and Waygood (1971) have suggested a possible function of isocitrate lyase in a 'degradative pathway' rather than the well known anabolic pathway (Kornberg, 1959). The former authors detected very low activity of malate synthetase associated with substantial activity of isocitrate lyase. Instead, a high glyoxylate carboligase activity has been demonstrated. The absence of malate synthetase activity in Euglena and the decreased activity of this enzyme in visible light (Cook and Casser, 1966) confirm this observation in Gloeomonas. Thus replenishment of C_4 compounds in such photosynthetic organisms may be achieved most likely by β-carboxylation rather than by glyoxylate bypass. The latter, however, may provide succinate for chlorophyll synthesis. On the other hand, glyoxylate may undergo decarboxylation yielding tartronic semialdehyde and hence bypass condensative decarboxylation with α-ketoglutarate (Davies and Ribereau-Gayon, 1969) required for protein synthesis. Furthermore, the results of Cook and Carver suggested very strongly that increased oxygen consumption is induced with the glyoxylate bypass in some system which may not yield consumable energy. The decarboxylation of glyoxylate by glyoxylate

carboligase may elucidate this result since the enzyme is a flavo-protein, restored by oxygen.

Thus, in the light of this available data and present discussion of isocitrate lyase, the initiating enzyme of glyoxylate bypass, may serve a catabolic as well as anabolic function. However, it is premature to consider the glyoxylate shunt to be an amphibolic pathway (Sanwal, 1970) in the sense used in bacterial metabolism.

BIBLIOGRAPHY

Ajl, S.J., and Wong, D.T.O., 1957. Significance of the Malate Synthetase Reaction in Bacteria. Science 126: 1013

Badour, S.S., and Waygood, E.R. 1971. Excretion of an acid semialdehyde by Gloeomonas. Phytochem. In press.

Badour, S.S., and Waygood, E.R. 1971. Glyoxylate carboligase activity in the unicellular green alga Gloeomonas sp. Submitted to Biochim. Biophys. Acta.

Bassham, J.A., Kirk, M., and Jensen, R.G. 1968. Photosynthesis by isolated chloroplasts I. Diffusion of labelled photosynthetic intermediates between isolated chloroplasts and suspending medium. Biochim. Biophys. Acta 153: 211-218.

Beevers, H. 1953. 2,4 Dinitrophenol and Plant Respiration. Am. J. Bot. 40: 91-96.

Bell, E.J., Herman, N.J. 1967. Effect of succinate on isocitrate lyase synthesis in Mima polymorpha. J. Bacteriol. 93: 2020-2021.

Bennett, L.L., Jr., Smithers, D., and Ward, C.T. 1964. Inhibition of DNA synthesis in mammalian cells by actidione. Biochim. Biophys. Acta. 87: 60-69

Bishop, N. 1958. The influence of the herbicide DCMU on the O_2 evolving system of photosynthesis. Biochim. Biophys. Acta (Amst.) 27: 205-206.

Braechtel, F.S., Hopkins, H.A. and Schmidt, R.R. 1970. Continuous inducibility of isocitrate lyase during the cell cycle of the eucaryote Chlorella. Biochim. Biophys. Acta, 217: 216-219

Breidenbach, R.W. and Beevers, H. 1967. Association of the glyoxylate cycle enzymes in a novel sub-cellular particle from castor bean endosperm. Biochim. Biophys. Res. Commun. 27: 462-469.

Buchanan, B.B. and Arnon, D.I. 1969. 'Isocitrate Lyase'., Vol. XIII, p. 170. In Methods of Enzymology. Ed. Colowick, S.P. and Kaplan. Academic Press, New York..

Campbell, J.J., Smith, R.A. and Eagles, B.A. 1953. A deviation from the conventional tricarboxylic acid cycle in Pseudomonas aeruginosa. Biochim. Biophys. Acta 11: 594.

Casselton, P.J., Faivole, M.O. and Casselton, L.L. 1969. Isocitrate Lyase in Coprinus lagopus. Can. J. Microbiol. 15: 637-640.

Cheng, W.H., and Tolbert, 1970. Excretion of glycolate, mesotartrate and isocitrate lactone by Scenedesmus braunii. Plant Physiol. 46: 377-85.

Cook, J.R. and Carver, M. 1966. Partial photorespiration of the glyoxylate bypass in Euglena. Plant and Cell Physiol. 7: 377-383

Davies, D. and Ribereau-Gayon, G. 1969. Inhibition of the Kreb's cycle by glyoxylic acid. Phytochem. 8: 1101-1108.

Dixon, G.H. and H.L. Kornberg, 1959. Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. 72: 3P

Dixon, M. and Webb, E.C. 1958. In 'Enzymes', p. 226, Academic Press, N.Y.

Dixon, M. and Webb. E.C. 1967. in 'Enzymes' p. 766, Longman

Duntzie, W.D. Newman, 1969. Studies on the regulation and localization of the glyoxylate cycle enzymes in Sacchromyces cerevisiae. Eur. J. Biochem. 10: 83-89.

Evans, M.C.W., Buchanan, B.B. and Arnon, D.I. 1966. A new ferredoxin-dependent carbon reduction cycle in photosynthetic bacteria. Proc. Natl. Acad. Sci. 55: 928-934.

Firenzuoli, A.M., Vanni, P., Mastronuzzi E., Zanobini A., and Baccari V. 1968. Participation of the glyoxylate cycle in the metabolism of germinating seeds of Pinus pinea. Life Sciences 7: 1251-1258.

Gibbs, M. 1969. Photorespiration, Warburg effect and glycollate. Ann. N.Y. Acad. Sci. 168: 356-368.

Goulding, K.H. and Merett, M.J. 1966. The photometabolism of acetate by Chlorella pyrenoidosa. J. Expt. Bot. 17: 678-689.

Haigh, W.G. and Beevers, H. 1964. The occurrence and assay of isocitrate lyase in algae. Arch. Biochem. Biophys. 107: 147-151

Harrop, L., and Kornberg H.L. 1966. Role of isocitrate lyase in metabolism of algae. Proc. Royal Soc. 166, 1002.

Hatch, C.R. and Slack, M.D. 1970. Photosynthetic CO_2 fixation pathways. Ann. Rev. Pl. Physiol. 21: 141-162.

John, P.C.L. and Syrett, P.J. 1967. The purification and properties of isocitrate lyase from Chlorella. Biochem. J. 105: 409-416.

John, P.C.L. and Syrett P.J. 1968. The inhibition by intermediary metabolites of isocitrate lyase from Chlorella pyrenoidosa. Biochem. J. 110: 481-484.

John, P.C.L., Thurston, C.F., Syrett, P.J. 1970. Disappearance of isocitrate lyase enzymes from cells of Chlorella pyrenoidosa. Biochem. J. 119: 913-919.

Kerridge, D. 1958. The effect of actidione and other antifungal agents on nucleic acid and protein synthesis in Sacchromyces carlsbergensis J. Gen. Microbiol. 19: 497-506.

Kirk, J.T.O. 1970. Biochemical aspects of chloroplast development. Ann. Rev. Plant Physiol. 21: 11-42.

Kornberg, H.L. 1955. 'Isocitric dehydrogenase of yeast (TPN)' in "Methods of Enzymology", vol. I. Ed. Colowick, S.P. and Kaplan, N.O. p. 705. Academic Press, N.Y.

Kornberg, H.L. 1959. Aspects of terminal respiration in microorganisms, Ann. Rev. Microbiol. 13: 49-78.

Kornberg, H.L. 1963. The role of acetate in isocitrate lyase induction Biochim. Biophys. Acta 73: 517-519.

Kornberg, H.L. 1965. The role and control of the glyoxylate cycle in Escherichia coli. Biochem. J. 99; 1-11.

Kornberg, H.L. 1965. Control of biosynthesis from C_2 Compounds France: Center Nationale de la Recherche Scientifique Colloques Internationaux Paris, 124; 193-207.

Kuhl, A. and Lorenzen, H. 1964. 'Handling and culturing of Chlorella methods', in 'Cell Physiology, p. 159-187. Ed. D.M. Prescott, N.Y. Acad. Press.

Lord, J.M. and Merrett, M.J. 1970. Growth inhibition of free-living protozoa by actidione. Physiol. Zool. 25: 272-6.

Lord, J.M. and Merrett, M.J. 1970. The regulation of glycollate oxidoreductase with photosynthetic capacity in Chlamydomonas mondana. Biochem. J. 117: 125-127.

Lowry, O.H., Rosebrough, N.J. Farr, A.L. and Randall, R.J. 1951. Protein measurement with the folinphenol reagent. J. Biol. Chem. 193: 265-275.

Madsen, N.B. and Hochester, R.M. 1959. The tricarboxylic acid and glyoxylate cycles in Xanthomonas phaseoli (XP8). Can. J. Microbiol. 5: 1

Matsuka, M. and Hase, E. 1965. The role of respiration and photosynthesis in the chloroplast regeneration in the 'glucose bleached' cells of Chlorella protothecoides. Plant and Cell Physiol. 7: 149-161.

McFadden, B.A. and Howes, W.V. 1962. Isocitrate lyase and malate synthetase in Hydrogenomonas facilis. J. Biol. Chem. 237: 1410

McFadden, B.A. and Howes, W.V. 1963. Crystallization of some properties of isocitrate lyase from Pseudomonas indigofera. J. Biol. Chem. 238, 1737-1742.

Morris, J. 1966. The effect of cycloheximide on protein and nucleic acid synthesis by Chlorella. J. Expt. Bot. 18: 54-64.

Nelson, E.B., Tolbert, N.E. 1969. The regulation of glycollate metabolism in Chlamydomonas reinhardtii. Biochim. Biophys. Acta 184: 263-270

Olson, J.A. 1954. The d-isocitric lyase system: the formation of glyoxylic and succinic acids from d-isocitric acid. Nature 194: 695-697

Olson, J.A. 1957. Fed. Proc. 16: 229; Cited in Daron, H.H. Thesis, University of Illinois 1961.

Olson, J.A. 1959. The purification and properties of yeast isocitrate lyase J. Biol. Chem. 234; 5-10

Palmer, C. and Maloney, T.E. 1955. Preliminary screening for potential algicides. Ohio J. Sci. 55: 1-8.

Pan, D. and Waygood, E.R. 1971. A fundamental, thermostabile, cyanide-sensitive photosynthetic enzyme in corn leaves and other organisms. Can. J. Bot. In press

Pardee, A.B. 1959. "The control of enzyme activity"; In 'The Enzymes', Vol. 1, p. 681. Ed. Boyer, P.D., Lardy, H. and Myrback, K. Academic Press, N.Y.

Pirson, A., Lorenzen, H. and Kopper, A. 1959. A sensitive stage in synchronized cultures of Chlorella. Plant Physiol. 34: 353-5

Pirson, A. and Lorenzen, H. 1966. Synchronized dividing algae. Ann. Rev. Plant Physiol. 17: 439-451.

Pleut, G.W.E. and Aogaichi, T. 1968. Purification and properties of diphosphopyridine nucleotide-linked isocitrate dehydrogenase of mammalian liver. J. Biol. Chem. 243: 5572.

Rao, G.R. and McFadden, B.A. 1965. Isocitrate lyase from Pseudomonas indigofera. IV. Specificity and inhibition. Arch. Biochem. Biophys. 112: 294-303.

Roche, T.E., Williams, J.O. and McFadden, B.A. 1970. Effect of pH and buffer upon K_m and inhibition by phosphoenolpyruvate of isocitrate lyase from Pseudomonas indigofera. Biochim. Biophys. Acta 206, 193-195.

Sanwal, B.D. 1970. Allosteric controls of amphibolic pathways in bacteria. Bacteriol. Rev. 34: 20-39.

Saz, H.J. 1954. The enzymatic formation of glyoxylic and succinic acids from the tricarboxylic acids. Biochem. J. 58: 20-21.

Saz, H.J. and Hillary, E.P. 1956. The formation of glyoxylic and succinic acids from the tricarboxylic acid cycle from Pseudomonas aeruginosae Biochem. J. 62: 563.

Senger, H. 1970. Charakterisierung einer synchrononkultur vun Scenesdesmus obnliqus, iher potentiellen photosyntheseleistung potentiellen photoynthese-Quotienten isahrend des Entwick lungscyclus. Planta (Berl.) 90: 243-266.

Senger, H. and Bishop, N.I. 1967. Quantum yield of photosynthesis in syn chronous Scenesdesmus cultures. Nature 214: 140-142

Siegel, M.R. and Sisler, H.D. 1964a. Site of action of cycloheximide in cells of Saccbromyces pastorianus. I. The effect of the antibiotic on cellular metabolism. Biochim. Biophys. Acta 87: 70-82.

Sisler, E.C. and Fuller, R.C. 1959. Cited in: The Enzymes, vol. V, p. 388, Ed. Boyer, P.D., Lardy, H. and Myrback, K. Academic Press, N.Y.

Sjogren, R.E. and Romano, A.H. 1967. Evidence for multiple forms of isocitrate lyase in Neurosporra crassa. J. Bacteriol. 93, 1638-1643.

Smith, I. 1960. "Chromatographic and electrophoretic techniques", vol. I, p. 279. Interscience Publ. Inc., N.Y.

Smith, R.A. and Gunsalus, I.C. 1954. Isocitritase: a new tricarboxylic acid cleavage system. J. Amer. Chem. Soc. 76: 5002-5003.

Smith, R.A. and Gunsalus, I.C. 1955. Distribution formation of isocitritase Nature 175: 774.

Smith, R.A. and Gunsalus, I.C. 1957. Isocitratase. J. Biol. Chem. 229: 305-319

Stein, A., Stein, J.H. and Kirkman, S.K. 1967. Diphosphopyridine nucleotide specific isocitrate dehydrogenase of mammalian mitochondria. Biochemistry 6: 1370.

Syrett, P.J. 1963. Enzymes of the glyoxylate cycle in Chlorella vulgaris J. Expt. Bot. 14: 249-64.

Syrett, P.J. 1966. The kinetics of isocitrate lyase formation in Chlorella: Evidence for the formation of enzyme synthesis by photophosphorylation. J. Expt. Bot. 17: 641-654.

Tamiya. 1966. Synchronous cultures of algae. Ann. Rev. Plant. Phys. 17: 1-26

Tan, C.K., S.S. Badour and E.R. Waygood. 1970. Photosynthesis of the unicellular green alga Gloeomonas. In Advances in C_4 Photosynthesis and Photorespiration in Plants. Symposium Extract; Canberra, Australia.

Tanner, W. and Beevers, H. 1965. The competition between the glyoxylate cycle and the oxidative breakdown of acetate in Ricinus endosperm II. The effect of NH_4^+ and an uncoupler on the metabolism of endosperm slices. Z. pflanzenphysiol. 53: 126-139.

Tolbert, N.E. and Hess, J.L. 1966. The effect of hydroxymethanesulfonate on $^{14}CO_2$ photosynthesis by algae. J. Biol. Chem. 24: 5712-5715.

Wegener, Reeves, W.S.H. and Ajl, S.J. 1968. Alternate pathways of metabolism of short chain fatty acids. Bacteriol. Rev. 32: 1-26.

Whatley, F.R. and Arnon, D.I. 1963. "Isolation of Chloroplasts" in Methods of Enzymology, vol. VI, p. 308. Ed. Colowick, S.P. and Kaplan, N.O. Academic Press, N.Y.

Whiffen, A.J. 1946. The production of an antifungal antibiotic from Streptomyces griseus. J. Bact. 52: 610-611.

Whiffen, A.J. 1948. The production assay and antibiotic activity of actidione an antibiotic from Streptomyces griseus. J. Bacteriol. 56: 283-291.

Wiessner, W. and Kuhl, A. 1962. Die bedeutung des glyoxyl saure-Zyklus fur die photo-assimilation von acetate bei phototrophen. Algen: In vortrage aus dem Gesamtgebiet der Botanik, N.F., Nr. 1, S. 102-108. Stuggart: Gustav Fischer.

Wiessner, W. 1963. Stoff wechselleistung und enzym aktivitat bei Chlamydobotrys (vol. vocales). Arch. Mikrobiol. 45: 33-45.

Wiessner, W. 1968. Enzymaktivitat and Kohlenstoff-assimilation bei grunalgen unterschiedlichen erncihrungsphysiologischen Typs. (Beol.) 79: 92-98.

Wilson, A.T. and Calvin M. 1955. The photosynthetic CO_2 dependent transients J. Am. Chem. Soc. 77: 5948-5957.

Umbarger, H.E. 1964. Intracellular regulatory mechanisms. Science. 145: 674.

Zelitch, I. 1857. α-Hydroxysulfonates as inhibitors of the enzymatic oxidation of glycollic and lactic acids. J. Biol. Chem. 224: 251.