



UNIVERSITÉ D'OTTAWA
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α -KETOGLUTARATE-DEPENDENT METABOLISM OF
GLYOXYLATE IN TETRAHYMENA PYRIFORMIS

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TO MY PARENTS, HUSBAND AND DAUGHTER.

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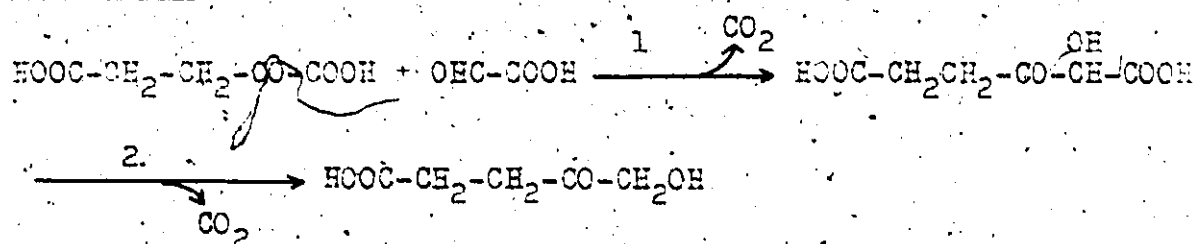
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ABBREVIATIONS.

ALA	: δ -Aminolevulinic acid.
ALA dehydrase	: δ -Aminolevulinic acid dehydrase
AAP	: Aminoantipyrine.
CO ₂	: Carbon dioxide.
2,4-DNPH	: 2,4-dinitrophenylhydrazine.
DNPH	: 2,4-dinitrophenylhydrazone.
<u>E. coli</u>	: <u>Escherichia coli</u> .
EDTA	: Ethylene diamine tetraacetic acid.
HKA	: α -Hydroxy- β -ketoadipic acid.
HLA	: δ -Hydroxylevulinic acid.
HKPA	: α , β -Dihydroxy- γ -ketopimelic acid.
LDH	: Lactate dehydrogenase.
Mg ⁺²	: Magnesium ions.
BME	: β -Mercaptoethanol.
NAD	: Nicotinamide-adenine dinucleotide.
NADP	: Nicotinamide-adenine dinucleotide phosphate.
OAA	: Oxaloacetate.
PEP	: Phosphoenolpyruvate.
PCA	: Perchloric acid.
PPO	: 2,5-diphenyloxazole.
S.M.G.	: Synthetic medium supplemented with glucose.
S.M.A.	: Synthetic medium supplemented with acetate.
TSA	: Tartronic semialdehyde.
TPP	: Thiamine pyrophosphate.
TCA	: Trichloroacetic acid.
TCA cycle	: Tricarboxylic acid cycle.
DCA cycle	: Dicarboxylic acid cycle.

SUMMARY

The present studies were concerned with the α -keto-glutarate-dependent metabolism of glyoxylate in Tetrahymena pyriformis. The following reaction (1)



was found to occur in the presence of mitochondrial preparations of Tetrahymena and consists of the condensation of glyoxylate and α -ketoglutarate to form α -hydroxy- β -keto-adipic acid (HKA) and carbon dioxide. The product of the spontaneous decarboxylation of HKA, β -hydroxylevulinic acid (HLA) (reaction 2) was identified as a derivative of 2,4-dinitrophenylhydrazine by paper chromatography and comparison to synthesized authentic HLA. The enzyme known as α -ketoglutarate:glyoxylate carboligase (in fact a lyase; recommended name: α -hydroxy- β -keto adipate synthase) required thiamine pyrophosphate and magnesium for maximal activity (the latter requirement could be demonstrated only in the presence of EDTA).

Kinetic experiments with mitochondrial preparations showed that the carboligase has greater affinity for α -ketoglutarate ($K_m = 0.1 \text{ mM}$) than for glyoxylate ($K_m = 5 \text{ mM}$).

Attempts to purify the enzyme failed due to large losses in preliminary steps. It was possible, however, to obtain evidence that the carbolligase may be part of the α -ketoglutarate dehydrogenase system as has been reported for beef heart mitochondria.

The activity of the enzyme is not affected by growth of the cells in defined or undefined media supplemented with acetate or glucose, nutrients which affect the activity of a number of enzymes in Tetrahymena.

α -(1- ^{14}C) ketoglutarate was detected in the assay mixtures and evidence is presented that its formation is not due to malate synthase and the tricarboxylic and dicarboxylic acid cycles. Rather, its formation may be due to the operation of a cycle initiated by the carbolligase of mitochondria, since both enzyme activity and α -(1- ^{14}C) ketoglutarate production were decreased in assay mixtures supplied by mitochondria from thiamine-deficient cells.

A metabolic cycle is proposed to account for the observed regeneration of α -ketoglutarate and is discussed in connection with the oxidation of glyoxylate in Tetrahymena and other organisms.

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PURPOSE OF THE RESEARCH.

The principal purpose of the research described in this thesis was to study the α -ketoglutarate-dependent metabolism of glyoxylate in Tetrahymena which hinged upon the demonstration of α -ketoglutarate: glyoxylate carboligase in this organism, its subcellular distribution, regulation, and physiological role.

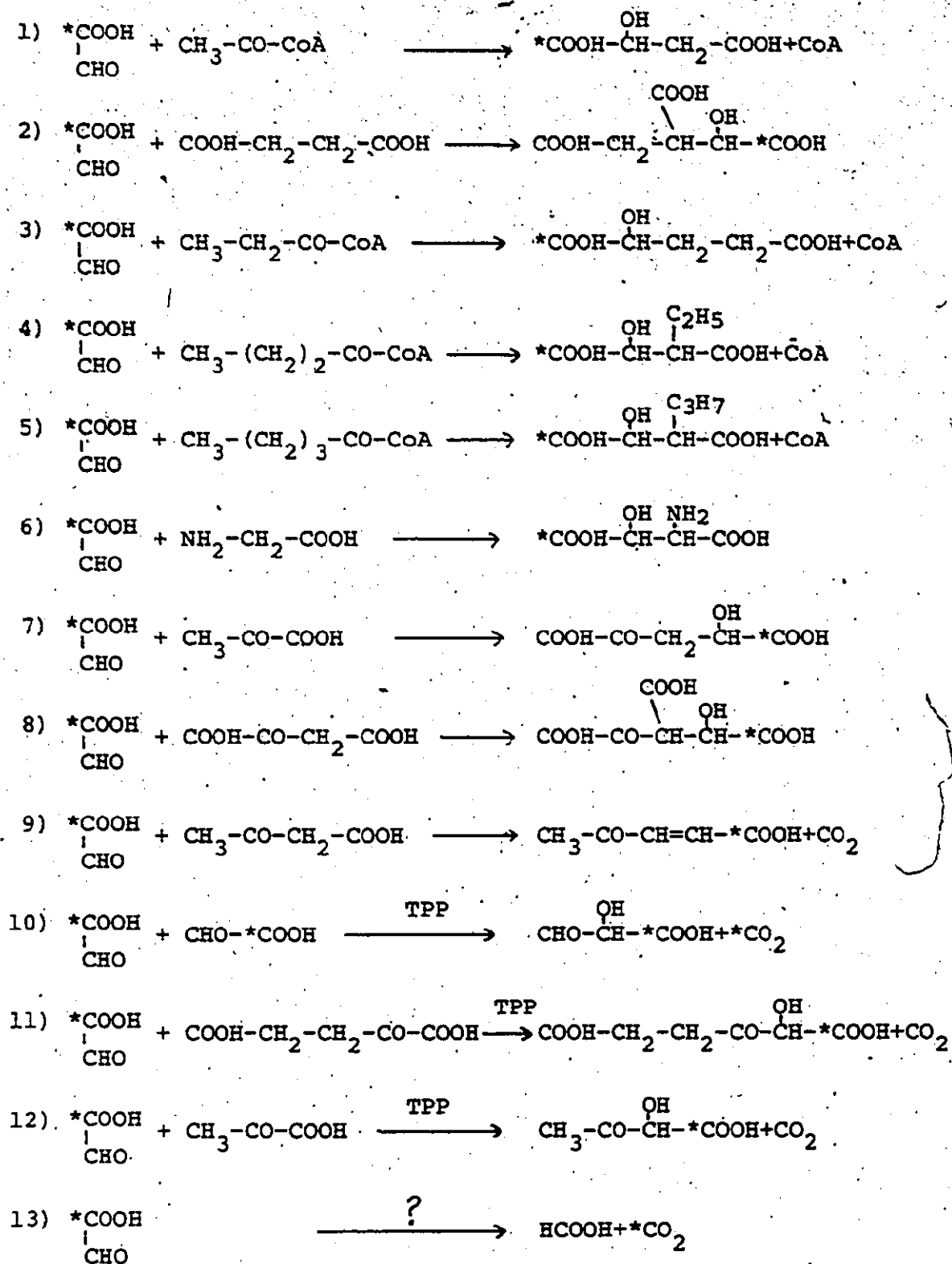
A subsidiary aim of the research was to provide evidences that the condensation of α -ketoglutarate and glyoxylate is an activity of the α -ketoglutarate dehydrogenase complex.

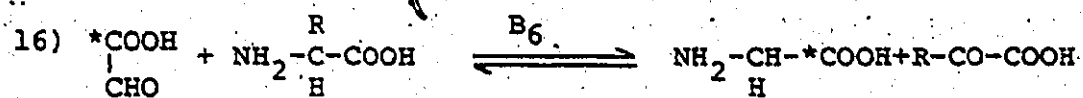
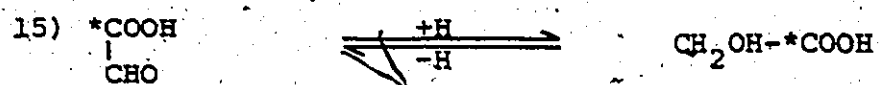
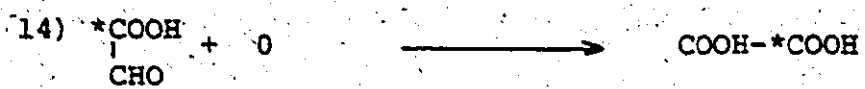
CHAPTER 1 : INTRODUCTION

Section 1.1. PATHWAYS OF GLYOXYLATE METABOLISM

Glyoxylate is known to be very active biochemically. Several enzymatic reactions for its formation and for its further metabolism have been reported as it is shown in Table 1.1. Glyoxylate participates in condensation reactions (reactions 1-12); oxidations (reactions 13 and 14); reduction (reaction 15) and transamination (reaction 16). Among the condensation reactions are the condensation with acetyl-CoA to malate (1), with succinate to isocitrate (2), with propionyl-CoA to α -hydroxyglutarate (3), with butyryl-CoA to β -ethylmalate (4), with valeryl-CoA to n-propylmalate (5), with glycine to β -hydroxyaspartate (6), and with pyruvate to form γ -hydroxy- α -ketoglutarate which has shown to be reversible (7). In some cases the condensation is accompanied by a concomitant decarboxylation of one of the reactants. The condensation of two glyoxylate molecules yields tartronic semialdehyde (TSA) and CO_2 (10), the condensation of glyoxylate and α -ketoglutarate yields α -hydroxy- β -ketoadipate and CO_2 (11) and with pyruvate α -hydroxy- β -ketobutyrate and CO_2 (12). Non-enzymatic condensation with oxaloacetate to oxalomalate (8), and with acetoacetate to β -acetylacrylate (9) also has been reported.

Table 1.1. Enzymatic reactions in glyoxylate metabolism.





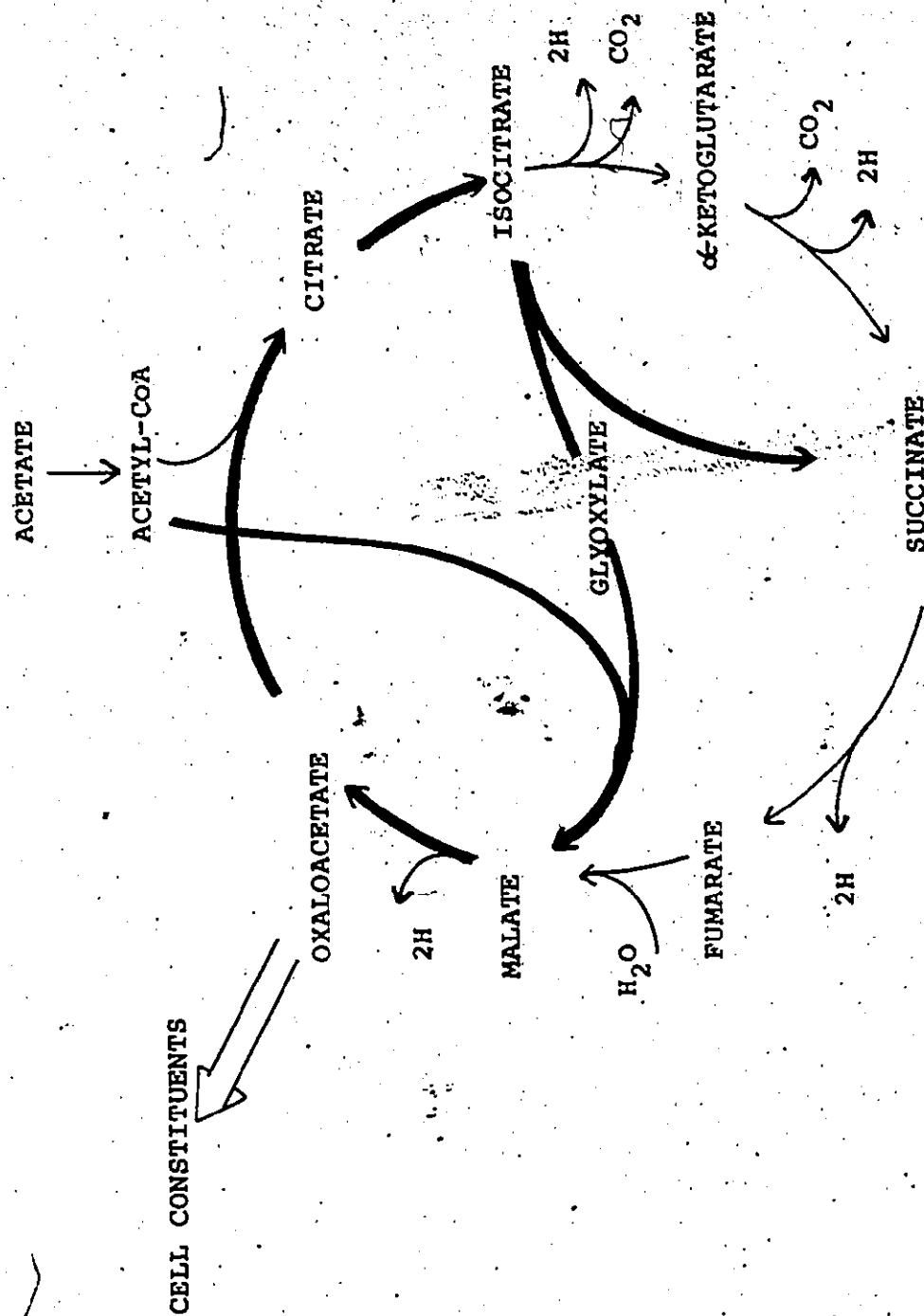


Figure 1.1: Routes for the provision of energy and of cell constituents during microbial growth on acetate. The catabolic route (TCA cycle) is shown by light arrows; The anaplerotic pathway (glyoxylate cycle) by heavy arrows. (From Kornberg, 1966 a).

The purpose of this section is to summarize these reactions, the metabolic pathways initiated by some of these reactions and the proposed significance of these pathways.

1.1.1. Condensation reactions.

1.1.1.A. Condensation with short fatty acids.

The central role of glyoxylate during growth of microorganisms on acetate or in the presence of more reduced C_2 -compounds such as ethanol is now well documented. During the growth of microorganisms intermediates of the tricarboxylic acid cycle (TCA) are utilized to provide the carbon skeletons of many cell constituents (Krebs et al., 1952). When C_2 -compounds are the sole source of carbon means must exist whereby such intermediates, drained from the cycle, are replenished by ancillary reactions (Kornberg and Gotto, 1959). When the C_2 -substrate for growth is acetate, this is accomplished via the glyoxylate cycle (Kornberg and Madsen, 1958; Kornberg and Krebs, 1957) (figure 1.1). The net effect of the glyoxylate cycle is the synthesis of one molecule of C_4 -dicarboxylic acid from two molecules of acetate and it therefore serves to replenish C_4 -acids drained from the TCA cycle. The two reactions which bypass the decarboxylative steps of the TCA cycle have been referred to as the "glyoxylate by-pass" (Kornberg and Madsen, 1957). The enzymes that catalyze

these reactions are isocitrate lyase and malate synthase. Isocitrate lyase catalyses the reversible aldol cleavage of Ds(+)-isocitrate to succinate and glyoxylate (For review see Kornberg, 1966a). Malate synthase catalyses the condensation of glyoxylate with acetyl-CoA to form malate (Wong and Ajl, 1956; Kornberg, 1966).

The genes governing the synthesis of malate synthase and of isocitrate lyase in Escherichia coli appear to be members of a single operon (Vanderwinkel and De'Vlieghere, 1968) and thus subject to identical control. Kornberg (1966,b) has shown that this control is largely a repression exerted by a metabolite closely related to pyruvate and phosphoenolpyruvate (PEP). In addition, PEP inhibits the activity of isocitrate lyase (Ashworth and Kornberg, 1963). Thus isocitrate lyase and malate synthase are governed by end-product repression and inhibition, mechanisms commonly employed to regulate biosynthetic sequences.

Two malate synthases have been described in Escherichia coli (Falmagne et al., 1965) and independent genetic loci govern their synthesis. One form (malate synthase A) is formed during growth on acetate but not on glycolate, the other form (malate synthase G) is formed during growth on glycolate (Vanderwinkel and De'Vlieghere, 1968). Malate synthase G contributes about 40% of the activity observed in extracts of acetate-grown cells and over 90% of the

activity observed on glycolate-grown cells. In Rhizopus (Wegener et al., 1967) and in E.coli (Falmagne et al., 1965) the two malate synthase forms observed during growth on glycolate and acetate can be differentiated by their rates of thermal inactivation.

The glyoxylate by-pass has been demonstrated in a variety of microorganism such as bacteria, fungi and algae (For review see Wegener et al., 1968).

In protozoa and especially in Tetrahymena pyriformis the evidences for the presence of the glyoxylate by-pass enzymes was obtained by Hogg (1959). These organisms have a great capability to convert fats into glycogen (Hogg and Warner, 1956). Strong evidence supports the conclusion that cellular lipids are converted into glycogen via the glyoxylate cycle (Hogg and Kornberg, 1963; Hogg, 1969; Levy and Scherbaum, 1965a). It is also known that malate synthase and isocitrate lyase are located in peroxisomes (Müller et al., 1968).

The regulation of the glyoxylate by-pass enzymes in Tetrahymena is not completely elucidated. The compartmentation of the enzymes in a special structure is probably a means of regulation but the process by which it is accomplished is unknown (Hogg, 1969). As in bacteria the formation of these enzymes in Tetrahymena has been found to be influenced by the composition of the nutrient medium.

The by-pass could be induced by growth in the presence of acetate and repressed by growth in the presence of glucose (Hogg and Kornberg, 1963; Whitlow et al., 1972). However, it was found that when this organism was grown aerobically in peptone media, the level of isocitrate lyase was low and it was only slightly increased by subsequent aerobic incubation in acetate media. In contrast, incubation under static conditions resulted in high enzyme activity and the addition of acetate did not further promote enzyme formation (Levy, 1967). It was suggested that in Tetrahymena the levels of intracellular metabolite repressors of isocitrate lyase are controlled more by changes in growth conditions, particularly the oxygen content, than by the presence of acetate.

The glyoxylate by-pass activity measured by radioactivity incorporation from ^{14}C -acetate into CO_2 and glycogen reaches a maximum in late log phase or early stationary phase in Tetrahymena (Connet and Blum, 1971). Similar results for isocitrate lyase activity have been found by Whitlow et al., (1972). However, Levy and Scherbaum (1965a, 1965b) had previously reported that maximal glyoxylate by-pass activity occurred in stationary phase cells. They induced stationary phase by transferring log cultures to conditions of restricted aeration. Lowered oxygen tension resulted in cessation of cell multiplication.

and increased isocitrate lyase and malate synthase activity within three hours. Recently, Kemper et al. (1973) showed that the glyoxylate by-pass reached a maximum at the end of log phase, declined until the middle of stationary phase and then increased again to a maximum near the end of stationary phase. They suggested that the first peak could be caused by acetate induction and the second by oxygen depletion in the culture medium.

The glyoxylate by-pass also operates in plant tissues. Studies with germinating fatty seeds have provided considerable evidence for the operation of a glyoxylate cycle during the conversion of fat into carbohydrate (Bradbeer and Stumpf, 1959; Carpenter and Beevers, 1959; Marcus and Velasco, 1960; Calvin and Beeves, 1961). The glyoxylate cycle enzymes occur exclusively in the glyoxysomes (Breidenbach and Beevers, 1967). Isocitrate lyase is lost during growth and maturation of fatty seeding (Beevers, 1969; Carpenter and Beevers, 1959). In spite of an early report to the contrary (Carpenter and Beevers, 1959) isocitrate lyase has been demonstrated in crude dialyzed extracts of spinach leaves. The reason for the failure of earlier investigations to detect the enzyme may be the presence of endogenous inhibitors recently reported by Godavari et al., (1973).

The operation of the glyoxylate cycle has not been conclusively demonstrated in animal tissues although the presence of malate synthase has been reported in rat liver (Ganguli and Chakraverty, 1961). Production of glyoxylate from fatty acids has been observed in vitro in mitochondria from both rat and pigeon liver following addition of ADP (Kondrashova and Rodionova, 1971).

The discovery of malate synthase in E. coli by Wong and Aji (1956) and its significance in acetate metabolism initiated a series of investigations on the enzymatic condensation of glyoxylate with other monocarboxylic acids. As a result, the condensation of glyoxylate with propionyl-CoA to form α -hydroxyglutarate was demonstrated. This reaction is catalyzed by α -hydroxyglutarate synthase obtained from aerobically grown propionate-adapted E. coli (Reeves and Aji, 1962). Condensation with butyryl-CoA to form β -ethylmalate was demonstrated in extracts of Pseudomonas aeruginosa grown in butyrate-mineral salts media (Rabin et al., 1963). Condensation of glyoxylate with n-valeryl-CoA to form β -n-propylmalate was demonstrated in extracts of valerate-grown E. coli (Imai et al., 1963).

The condensation of glyoxylate with propionyl-, butyryl-, and valeryl-CoA are analogous to that catalyzed by malate synthase, but several important differences should be noted. First, the activities of α -hydroxyglutarate and β -ethylmalate synthase formed during growth on propionate

and butyrate, respectively, are considerably lower than the activity of malate synthase found in acetate-grown cells. Moreover, growth on propionate and butyrate also results in high activity of malate synthase suggesting that these substrates may be metabolized principally via acetate (Wegener et al., 1967).

1.1.1.B. Condensation with glycine.

This condensation reaction has been described in Micrococcus denitrificans grown on glyoxylate as sole carbon source. Glyoxylate is transaminated to glycine which condenses with another molecule of glyoxylate to form β -hydroxyaspartate. This substance then undergoes a dehydratase reaction to yield oxaloacetate and ammonia. The operation of this pathway effects the net formation of an intermediate of the TCA cycle required both for energy and for biosynthesis (Kornberg and Morris, 1963).

1.1.1.C. Condensation with pyruvate.

γ -Hydroxy- α -ketoglutarate aldolase catalyzes the reversible cleavage of γ -hydroxy- α -ketoglutarate to pyruvate and glyoxylate (Kuratomi and Fukunaga, 1963). The enzyme has been purified from rat liver (Kuratomi and Fukunaga, 1963; Maitra and Dekker, 1964); from bovine liver (Kobes and Dekker, 1969); from a soil bacterium grown on α -ketoglutarate (Aronson et al., 1967). The activity also has been demonstrated in extracts of plant tissues (Payes and Laties, 1963a). This reaction represents a terminal step in hydroxyproline degradation by mammals (Adams and Goldstone, 1960a, 1960b; Goldstone and Adams, 1962; Kuratomi and Fukunaga, 1963; Kuratomi et al., 1963). The enzymatic conversion of γ -hydroxy- α -ketoglutarate to malate by a mitochondrial dehydrogenase and by a peroxidase has been described (Payes and Laties, 1963a). These reactions may be physiologically significant as a pyruvate-catalyzed

mechanism for the oxidation of glyoxylate (Páyes and Laties, 1963 a).

1.1.1.D. Condensation with oxaloacetate.

Conflicting results have appeared in the literature with regard to the condensation of glyoxylate and oxaloacetate concerning the primary product of the condensation. A non-enzymatic condensation of glyoxylate with oxaloacetate was described (D'Abramo et al., 1957, 1958; Ruffo et al., 1959; Ruffo, 1961). The product of this reaction was identified as α -hydroxy- β -oxalosuccinate (oxalomalate) (Ruffo et al., 1962 a). This reaction did not occur in an acid medium, was rather slow at pH 7.4, but increased significantly either by the addition of magnesium ions or by increasing the temperature to 40°. The free oxalomalate is very unstable and loses CO₂ even at room temperature to yield β -hydroxy- α -ketoglutarate.

The enzymatic condensation has been reported in Acetobacter suboxidans and can be distinguished from the non-enzymatic reaction. The former occurs at pH 6 in the absence of magnesium, whereas the non-enzymatic condensation has an optimal pH at 7.5 and requires magnesium ions (Sekizawa et al., 1966).

Inhibition of respiration in animal tissue by glyoxylate was enhanced by oxaloacetate and was accompanied by the accumulation of citrate (D'Abramo et al., 1958; Ruffo et al., 1962b; 1959). This is in accord with the finding that oxalomalate competitively inhibits aconitase (Ruffo et al., 1959; 1962a) and isocitrate dehydrogenase (Ruffo and Adinolfi, 1963).

However it has been claimed that condensation of glyoxylate and oxaloacetate takes place with concomitant decarboxylation and at pH 7.4. Oxalomalate in fact represents a mixture of γ -hydroxy- α -ketoglutarate and bicarbonate (Payes and Laties, 1963b). γ -Hydroxy- α -ketoglutarate proved to be an effective competitive inhibitor of aconitase, isocitrate dehydrogenase and α -ketoglutarate dehydrogenase. A possible physiological role of glyoxylate for the control of the TCA cycle through its condensation with oxaloacetate was suggested (Ruffo et al., 1962b). Subsequent studies (Ruffo et al., 1967) suggested that oxalomalate is 10-fold more effective as an inhibitor of aconitase and isocitrate dehydrogenase than is γ -hydroxy- α -ketoglutarate.

1.1.1.E. Condensation with acetoacetate.

The non-enzymatic condensation of glyoxylate with acetoacetate to form β -acetylacrylate has been described

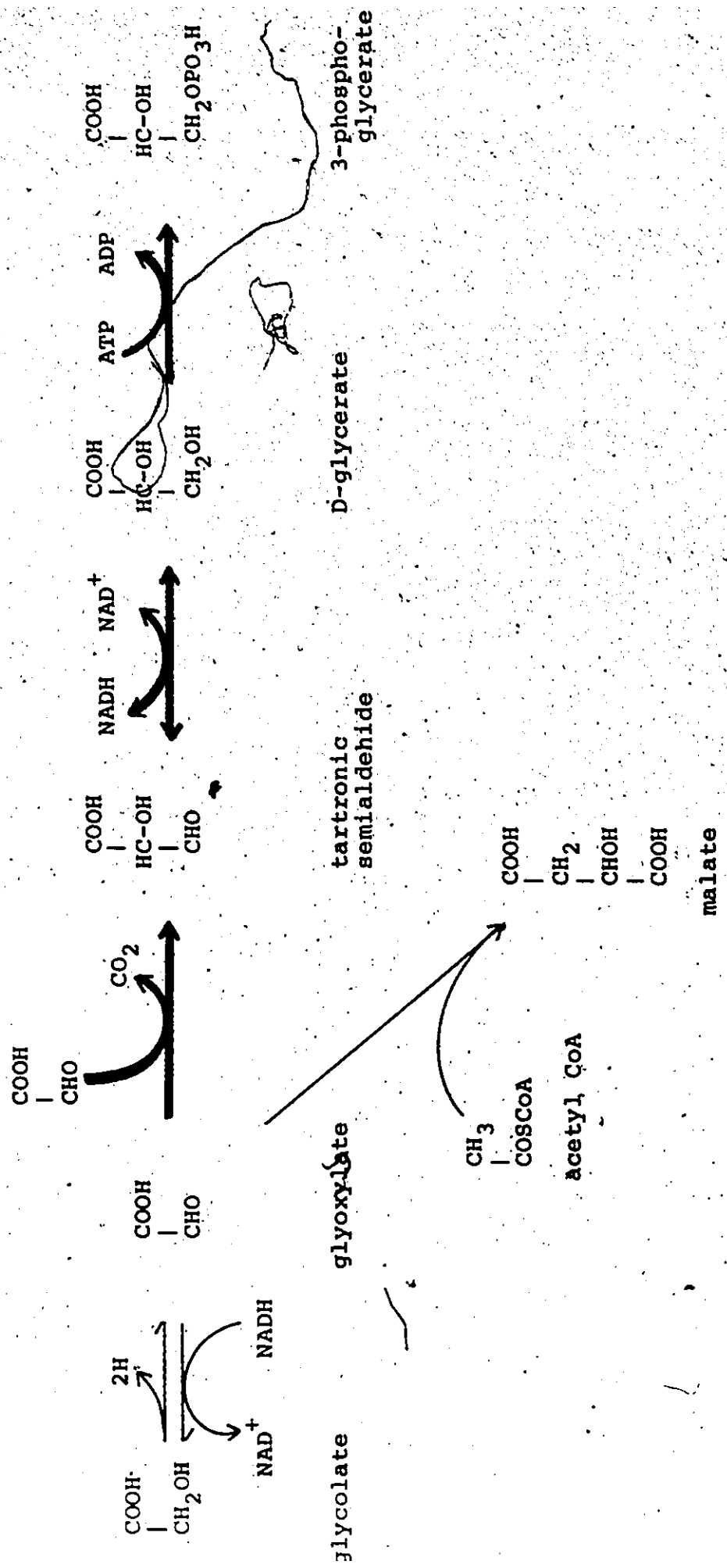


Figure 1.2: Reactions responsible for the conversion of glyoxylate to common intermediary metabolites. The glycerate pathway is shown by heavy arrows. (From Ornston and Ornston, 1969).

(Ellington et al., 1964). It proceeds at neutral pH and is accelerated by magnesium ions. Although the physiological significance of this reaction is obscure (the normal concentration of glyoxylate occurring in vivo is very low, but appears in considerable amounts in the organs and blood of rats given a thiamine-deficient diet, (Liang, 1960, 1962)), it may account for the observed antiketogenic properties of glyoxylate in rat liver slices.

1.1.2. Condensation with concomitant decarboxylation.

1.1.2.A. Condensation with glyoxylate.

Glyoxylate or glycolate can serve as a sole carbon source for some microorganisms. When one of these substances is the growth substrate a series of specialized reactions convert it to common intermediary metabolites (Fig. 1.2). The interconversion of glycolate and glyoxylate can be catalyzed by two distinct enzymes: a flavin mononucleotide-dependent glycolate oxidase (Zelith and Ochoa, 1953) and a NADH or NADPH glyoxylate reductase (section 1.1.6). As shown in Figure 1.2 a point of metabolic divergence occurs at the level of glyoxylate, which can participate in either of two condensing reactions: malate synthase (Wong and Aji, 1956) joins glyoxylate and acetyl-CoA to give rise to L-malate; glyoxylate carboligase (actually a lyase; recommended name: tartronic semialdehyde synthase 4.1.1.47) catalyzes the conversion of two molecules of glyoxylate to tartronic

semialdehyde (TSA) and CO_2 (Krakow and Barkulis, 1956). The latter reaction is the first step in the "glycerate pathway" a series of reactions that results in the synthesis of 3-phosphoglycerate from glyoxylate (Kornberg and Elsdon, 1961). Glyoxylate carboligase requires magnesium ions and thiamine pyrophosphate (TPP) for maximal activity (Krakow and Barkulis, 1956). The enzyme has been purified from E. coli grown in glycolate (Krakow et al., 1961; Gupta and Vennesland, 1964) and identified in Pseudomonas grown on glycolate (Kornberg and Gotto, 1961).

The next enzyme in the glycerate pathway is the tartronic semialdehyde reductase which catalyzes the reduction of TSA to D-glycerate with concomitant oxidation of reduced NAD or NADP (Gotto and Kornberg, 1961a, 1961b). D-Glycerate thus formed is phosphorylated via a glycerate kinase to yield either 2- or 3-phosphoglycerate (Kornberg and Gotto, 1961).

The divergent condensation reactions have fostered a divergence of views regarding their role in the oxidation of glyoxylate. According to one proposal (Hansen and Hayashi, 1962; Ornston and Ornston, 1969) (Fig. 1.3 a) glyoxylate is converted to acetyl-CoA via 3-phosphoglycerate (3-PGA) and is oxidized via the TCA cycle. Malate synthase replenishes those intermediates of the TCA cycle that are used for

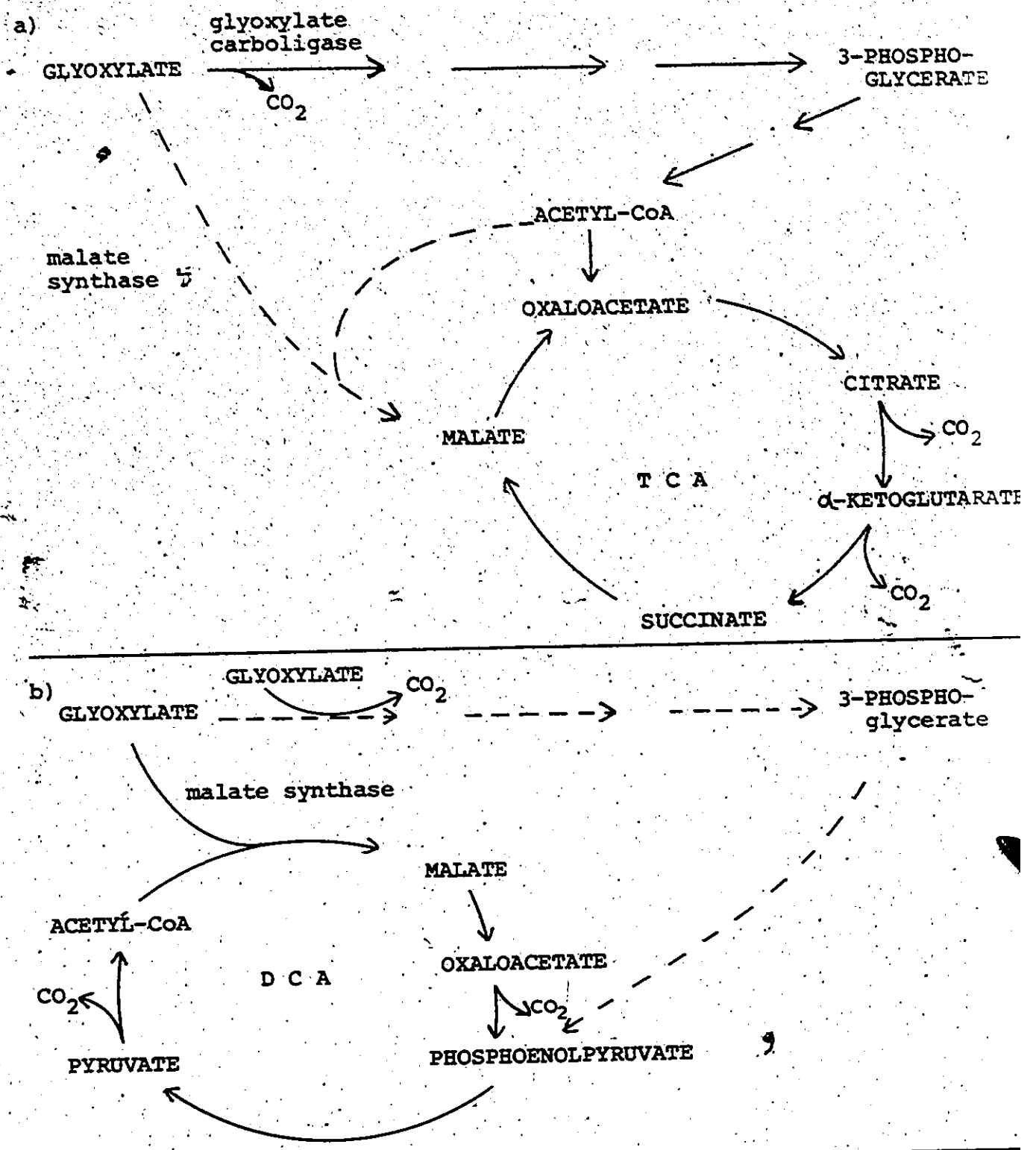


Figure 1.3: Role of the tricarboxylic acid cycle (a) and the dicarboxylic acid cycle (b) in the metabolism of glyoxylate. (From Ornston and Ornston, 1969).

biosynthesis and hence may be termed an anaplerotic enzyme.

Another viewpoint (Kornberg and Sadler, 1961) is that the TCA cycle is not essential for the oxidation of glycolate; instead the dicarboxylic acid cycle performs the oxidation and the glycerate pathway performs an anaplerotic function (Fig. 1.3 b).

The first enzyme of the glycerate pathway, glyoxylate carboligase, is also formed by E. coli and other organisms when glyoxylate is added to cultures growing on other carbon sources, and in Acetobacter the formation of this enzyme may even be induced through overproduction of glyoxylate inside the cell if isocitrate lyase is very active (Kornberg, 1966).

The synthesis of malate synthase G (See section 1.1.1.A) appears to be induced directly by glycolate which may be formed by a constitutive glyoxylate reductase in glyoxylate or acetate-grown cells (Ornston and Ornston, 1969).

The glycerate pathway has been found to operate in extracts of Pseudomonas (A) grown with glycine as sole carbon source (Callery and Dagley, 1959; Dagley, et al., 1961); in extracts of Pseudomonas oxalaticus grown in oxalate (Quayle and Keech, 1959, 1960, 1961); and in

extracts of Streptococcus allanticus grown in allantoin medium (Valentine et al., 1962, 1964). In all of these organism glyoxylate is formed inside the cell from the corresponding carbon source present in the grown medium.

The presence of TSA reductase has been reported in several enterobacteria grown in D-glucarate (Blumental and Fish, 1963; Trudgill and Widons, 1966).

The operation of the glycerate pathway has not been demonstrated in eukaryotic cells, although glyoxylate carboligase was reported in the green algae (Gloeomonas sp. (Badour and Waygood, 1971).

1.1.2.B. Condensation with α -ketoglutarate.

Preliminary studies on glyoxylate metabolism on mammalian systems (Weinhouse and Friedmann, 1951; 1952; Nakada and Weinhouse, 1953; Nakada et al., 1955) suggested that glyoxylate when present in physiological amounts was metabolized principally to formate and CO₂. Later it was reported (Nakada and Sund, 1958) that the addition of L-glutamate enhanced the oxidative decarboxylation of glyoxylate by rat liver homogenates and extracts of mitochondria and N-formylglutamate was the intermediate formed. Crawhall and Watts (1962) studied the metabolism of

glyoxylate by rat and human liver mitochondria in the presence of L-glutamate. They failed to demonstrate N-formylglutamate as an intermediate and were able to account for all the glyoxylate metabolized by decarboxylation oxidation to oxalate and amination to glycine. Furthermore, L-glutamate could be replaced by α -ketoglutarate. The mechanism of this synergistic decarboxylation of glyoxylate and α -ketoglutarate was not established in these studies.

Earlier, Franke and Jilge (1961) tentatively identified α -keto- β -hydroxyadipate as an intermediate in the condensation between glyoxylate and α -ketoglutarate in Aspergillus niger. Using similar techniques, Okuyama et al., (1965) demonstrated an analogous reaction with extracts of Rhodopseudomonas spheroides. Later several investigators (Koch and Stokstad, 1965; Stewart and Quayle, 1967; Schlossberg et al., 1968) demonstrated the occurrence of a similar system in rat liver mitochondria, pig liver mitochondria and a particulate fraction from beef heart. These investigators demonstrated that glyoxylate and α -ketoglutarate undergo an initial condensation to yield α -hydroxy- β -keto adipate (HKA) rather than α -keto- β -hydroxyadipate as claimed earlier by Franke and Jilge, (1961) and Okuyama et al., (1965). HKA then decarboxylates to δ -hydroxylevulinic acid (HLA) in the presence of acid (Fig.1.4).

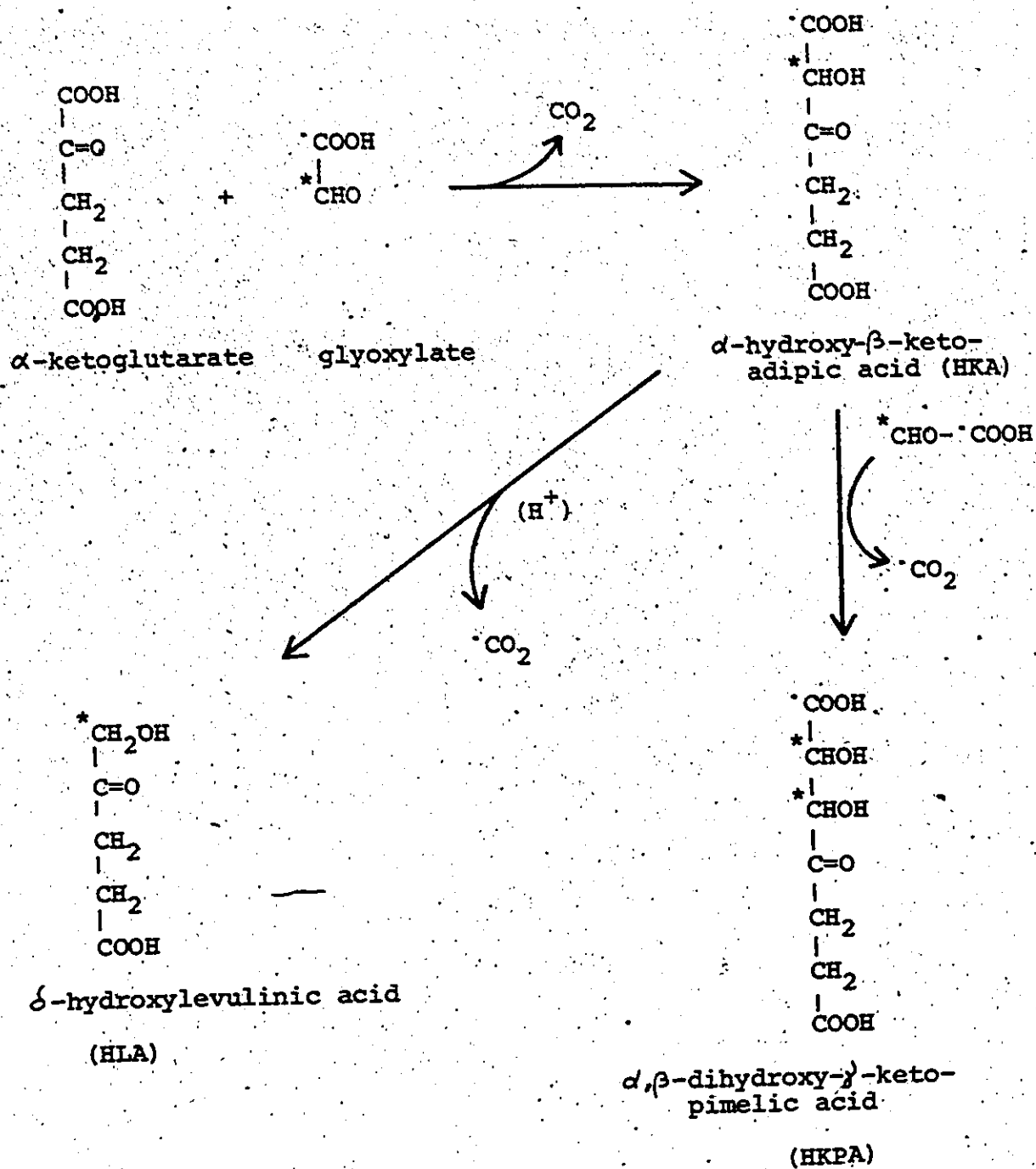


Figure 1.4: Products of d-ketoglutarate:glyoxylate carboligase reaction. (From Schlossberg et al., 1970)

In addition to HLA a second minor product has been identified as α,β -dihydroxy- γ -ketopimelic acid (HKPA) (Schlossberg et al., 1970).

The enzyme α -ketoglutarate: glyoxylate carboligase (actually a lyase: recommended name: α -hydroxy- β -ketoadipate synthase) has been purified from rat liver mitochondria and requires magnesium and TPP for maximal activity (Koch and Stockstad, 1966), from beef-heart mitochondria (Schlossberg et al., 1970), pig-liver mitochondria (Stewart and Quayle, 1967), Mycobacterium takeo (Moriyama and Yui, 1966), E. coli (Yamasaki and Moriyama, 1970a), and Mycobacterium phlei (Yamasaki and Moriyama, 1971).

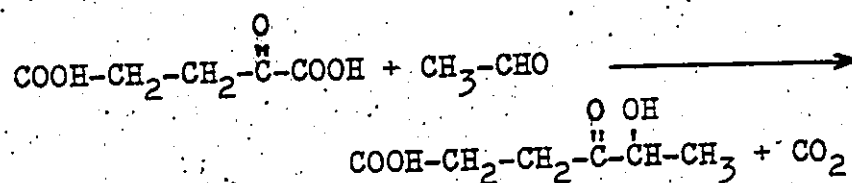
α -Ketoglutarate: glyoxylate carboligase was found not only in the mitochondria but also in the soluble fraction of human liver cells (Koch et al., 1967).

Although the nature of the reaction was recognized to be a carboligase reaction between α -ketoglutarate and glyoxylate it was not clear whether it was catalyzed by an independent enzyme specific for this reaction. Experiments with purified enzymes from beef heart (Schlossberg et al., 1970), rat liver and R. spheroides (Saito et al., 1971), and E. coli (Kubasik et al., 1972) strongly support the view that the enzyme with carboligase activity is

identical with the α -ketoglutarate dehydrogenase complex. In plant mitochondria the carbolicase activity may be an inherent property of pyruvate or α -ketoglutarate oxidase (Davies and Kenworthy, 1970).

It is not clear whether the enzyme in the soluble liver fraction (Koch *et al.*, 1967) is a specific carbolicase or it is identical with α -ketoglutarate decarboxylase (section 1.3) involved in the α -ketoglutarate dehydrogenase complex.

The α -ketoglutarate dehydrogenase complex prepared from pig heart also catalyzed the carbolicase reaction between α -ketoglutarate and some other aldehydes (Table 1.2). Aldehydes, in turn, inhibited the α -ketoglutarate dehydrogenase activity as measured by the reduction of NAD^+ (Saito *et al.*, 1971). The condensation of α -ketoglutarate and acetaldehyde, a metabolite of ethanol, yields δ -hydroxy- γ -ketohexanoic acid and has been reported by Bloom and Westerfeld, (1966):



Reaction system	$^{14}\text{CO}_2$ formed (μmoles)
Standard	0.145
+ Ferricyanide (20 μmoles)	0.975
+ Glyoxylate (10 μmoles)	3.20
+ Formaldehyde (10 μmoles)	0.660
+ Acetaldehyde (10 μmoles)	1.52
+ Succinic semialdehyde (10 μmoles)	0.207

Table 1.2: $^{14}\text{CO}_2$ formation from α -(1- ^{14}C) ketoglutarate by α -ketoglutarate dehydrogenase complex. The standard reaction mixture contained in a final volume of 3 ml: 100 μmoles of phosphate buffer (pH 7.0), 10 μmoles of α -(1- ^{14}C) ketoglutarate, 5 μmoles of Mg Cl_2 , 0.5 μmoles of TPP, 30 mg of bovine serum albumin, and 0.67 mg protein of the α -ketoglutarate dehydrogenase complex. Incubation were carried out for 30 min. This reaction system measures the α -ketoglutarate decarboxylase activity of the α -ketoglutarate dehydrogenase complex. (From Saito et al., 1971).

The biological significance of the carbolligase is unknown. A cyclic mechanism was proposed (Okuyama et al., 1965); Kawasaki et al., 1966) involving α -keto- β -hydroxyadipate and α -ketoglutarate. The effect of one turn of the cycle is the complete oxidation of one mole of glyoxylate to two moles of CO_2 and one of water. The cycle could account for the catabolism of glyoxylate in rat liver mitochondria (Kawasaki et al., 1966). In Mycobacterium takeo where isocitrate lyase but not malate synthase was detected the α -ketoglutarate: glyoxylate condensation may serve a biosynthetic function (Moriyama and Yui, 1966).

The effect of α -ketoglutarate: glyoxylate carbolligase activity on porphyrin synthesis has been studied (Yamasaki and Moriyama, 1970b). It was found that when extracts of Mycobacterium phlei were incubated with the substrate and cofactors for the carbolligase activity a remarkable inhibition of porphyrin synthesis occurred. It was suggested that the carbolligase activity might be playing an important role for the regulation of porphyrin synthesis from δ -aminolevulinic acid (ALA) by producing HLA which may inhibit ALA dehydratase competitively as does levulinic acid. On the other hand, formation of aspartate, glutamate, and ALA was observed when HLA was incubated with sonicated rat liver mitochondria (Wang et al., 1970). Formation of

these products suggests that HLA may be converted to α -ketoglutarate and ALA via γ,δ -dioxovalerate. In vivo oxidation of ^{14}C -HLA to $^{14}\text{CO}_2$ has also been shown in the rat (Wang et al., (1970).

α -Ketoglutarate:glyoxylate carboligase activity was studied in tissues of patients with primary hyperoxaluria (Koch et al., 1967) a genetic disorder of glyoxylate metabolism characterized by increased urinary excretion of oxalate, glycolate and glyoxylate (section 1.1.4). Cytoplasmic extracts showed a significantly lower activity of this enzyme than did control tissues suggesting that this condensation may be of physiological significance in the metabolism of glyoxylate by animal tissues.

1.1.2.C. Condensation with pyruvate.

A condensation reaction between pyruvate and glyoxylate yields two products. One of these was identified as acetol and was presumably formed by a nonenzymatic decarboxylation of the initial product of the reaction, α -hydroxy- β -keto butyric acid. The second was identified as α,β -dihydroxy-ketovaleric acid. The reaction was reported to be catalyzed by the pyruvate decarboxylase component of the pyruvate dehydrogenase complex of E. coli (Kubasik et al., 1972) (Fig. 1.5).

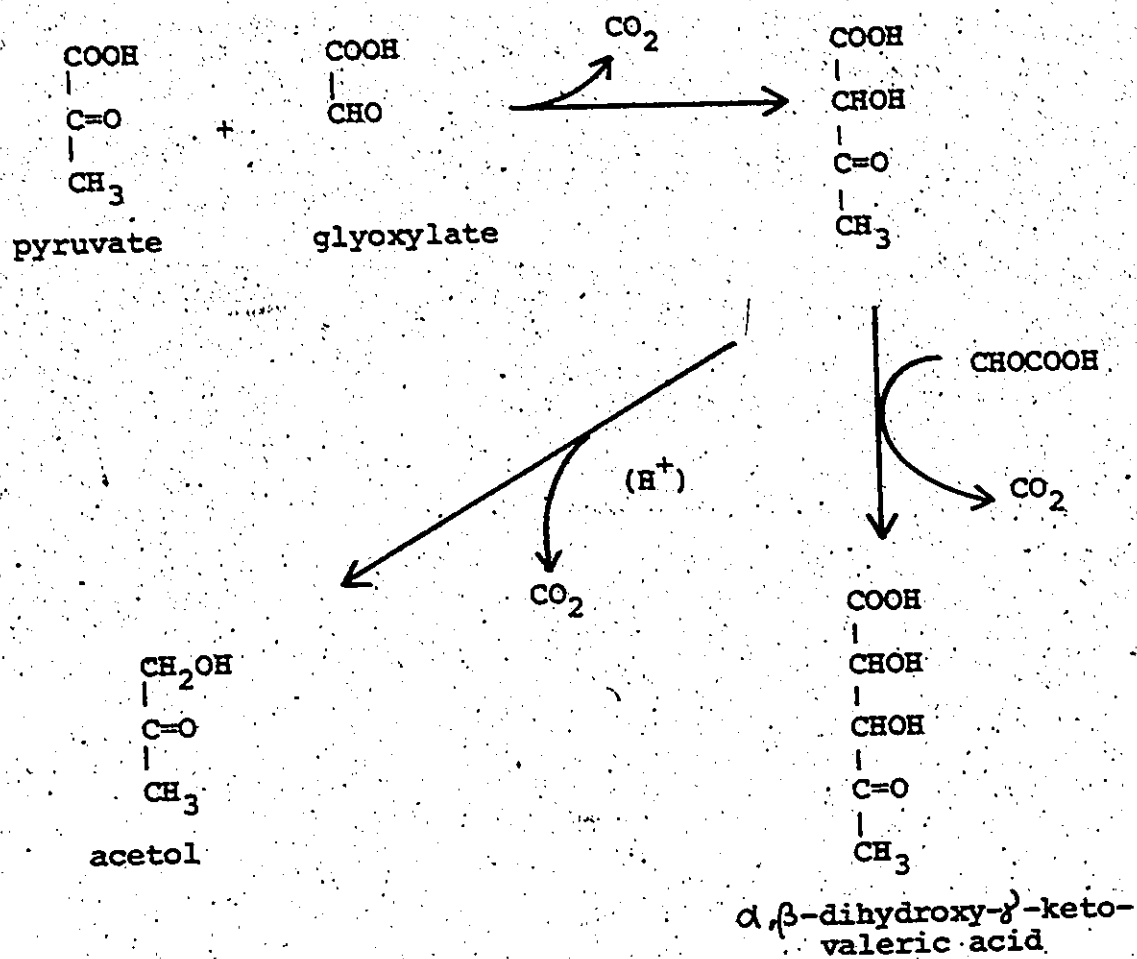


Figure 1.5: Products of pyruvate:glyoxylate condensation reaction. (From Kubasik et al., 1972).

Both the α -ketoglutarate dehydrogenase and the pyruvate dehydrogenase complexes are thiamine-containing enzymes capable of reacting with glyoxylate and both reactions represent potential pathways for the normal metabolism of glyoxylate. The ease with which TPP is removed from the pyruvate as compared with the α -ketoglutarate dehydrogenase complex and the concomitant defect in glyoxylate metabolism during thiamine deficiency (Liang, 1962) suggest a major role for the pyruvate reaction. However, in mammalian tissues the reaction of glyoxylate with pyruvate is much slower than its reaction with α -ketoglutarate and it is not yet clear which reaction is more important in preventing hyperoxaluria (Kubasik *et al.*, 1972).

1.1.3. Oxidation to formate and CO₂.

Intact rats oxidized glyoxylate to CO₂ (Weinhouse and Friedmann, 1951) and formate production was observed from the C₂ of glyoxylate (Weinhouse and Friedmann, 1952). In vitro experiments with various liver preparations also have shown formation of formate from the C₂ of glyoxylate (Nadada and Weinhouse, 1953). Later on studies of glycine catabolism in rat liver Nakada *et al.*, (1955) suggested that glyoxylate was in intermediary of glycine

catabolism to yield formate and CO_2 .

Trying to characterize the enzyme system Nakada and Sund (1958) showed that a partially purified preparation isolated from rat liver mitochondria catalyzed the oxidative decarboxylation of glyoxylate. The system required NAD, TPR, L-glutamate and MnCl_2 to achieve maximal rates of decarboxylation. The product of these oxidative decarboxylation were CO_2 and N-formylglutamate. It was suggested that N-formylglutamate was hydrolyzed to formate and glutamate. Despite similarities to the previous reaction using rat and human liver mitochondria Crawhall and Watts (1962) were unable to detect N-formylglutamate as an intermediate or as a product of the reaction and further found that α -ketoglutarate was more effective in promoting the reaction than L-glutamate. The nature of this synergistic decarboxylation of glyoxylate was not known. (Presumably it was identical with that of α -ketoglutarate:glyoxylate carboxylase, section 1.1.2.B). Further studies on formate production in mammalian tissues have not been described.

In plant tissues the oxidation of glyoxylate by hydrogen peroxide yields formate and CO_2 . Hydrogen peroxide is generated mainly by the oxidation of glycolate to glyoxylate in the peroxisomes (Zelitch and Ochoa, 1953;

Halliwell and Butt, 1974); or it may be generated in the chloroplasts in the presence of light and manganous ions in an atmosphere containing oxygen (Zelitch, 1973). It is not yet clear which series of reactions may function in photorespiration. In this last process 50% of the net CO_2 assimilated during photosynthesis by leaves of many species may be released in a light-dependent oxidation (Zelitch, 1966). This CO_2 arises mainly from the carboxyl group of glyoxylate, little is released from the α -carbon atom. Against the peroxisomal series of reactions is that the hydrogen peroxide may be decomposed by the great excess of catalase actively present in these organelles (for review see Zelitch, 1972) or the finding that the formate, derived from C-2 of glyoxylate, can be further oxidized to CO_2 (Leeks and Butt, 1972) by the peroxidatic action of catalase (Halliwell, 1974). The release of CO_2 from both carbon atoms of glyoxylate argues against a significant participation of this oxidation in photorespiration. However, although most of the hydrogen peroxide was destroyed by catalase, enough escaped decomposition to allow significant rates of nonenzymatic glyoxylate decarboxylation, and also catalase has been found to be converted into an inactive form under conditions of continuous hydrogen peroxide generation (Halliwell and Butt, 1974). The activity of

catalase in leaf peroxisomes in vitro and in vivo may thus be insufficient to prevent the attack by peroxide on glyoxylate.

1.1.4. Oxidation to oxalate.

The conversion of glyoxylate to oxalate may be catalyzed by three enzymes: glycolic acid oxidase, xanthine oxidase and lactic acid dehydrogenase (LDH).

Glycolic acid oxidase (glycolate:oxygen oxidoreductase) catalyzes both the conversion of glycolate to glyoxylate and of glyoxylate to oxalate, the latter being a functionally irreversible step. This enzyme is inhibited by oxalate thus possibly providing a regulatory mechanism through product inhibition (Richardson and Tolbert, 1961). Xanthine oxidase (Ratner et al., (1944); Nakada and Weinhouse, 1953) plays only a minor role in the overall production of oxalate. This is supported by the observation that patients with xanthinuria and those treated with xanthine oxidase inhibitors (allopurinol) excrete normal amounts of oxalate (Gibbs and Watts, 1966). Lactate dehydrogenase (LDE) can bring about the reversible conversion of glyoxylate to glycolate although the equilibrium lies far in favour of glycolate. LDH can also catalyze the formation of oxalate from glyoxylate (Sawaki et al., 1966). Evidence has been

presented that LDB has a major role in catalyzing the reaction in both liver and heart tissues. The NAD-independent oxidations, catalyzed by xanthine oxidase and glycolate oxidase in the liver are relatively unimportant and they were not detected in the heart (Gibbs and Watts, 1973).

Oxalate, an end-product of metabolism, is normally excreted in human urine. Its main immediate precursors in man and other mammals are L-ascorbate and glyoxylate each accounting for slightly less than one-half the total endogenous production (for review see Hagler and Herman, 1973).

The association of oxalate with human disease is of considerable importance. Two-thirds of all kidney stones (Nordin and Hodgkinson, 1967) and over three-fourths of endemic bladder stones contain calcium oxalate (Gershoff et al., 1963). Oxalate excretion is increased in four conditions in man: Primary hyperoxaluria type I (glycolic aciduria), primary hyperoxaluria type II (L-glyceric aciduria), pyridoxine deficiency and certain intestinal disorders with diminished reabsorption of glycine-conjugated bile salts (William and Smith, 1972).

Enteric hyperoxaluria responds to taurine. Pyridoxine deficiency hyperoxaluria (presumably secondary to diminished transamination of glyoxylate to glycine) responds to vitamin replacement. At the present time there is no method for control of excess oxalate synthesis and excretion in the two forms of genetic hyperoxaluria, glycolic aciduria and L-glyceric aciduria (O'Keefe et al., 1973).

Several lines of evidence have suggested that excess oxalate synthesis in primary hyperoxaluria results from a block in the metabolism of glyoxylate: (1) increased urinary excretion of oxalate, glycolate and glyoxylate occurs (Hockaday et al., 1965). (2) ^{14}C -glyoxylate administered intravenously exhibits an increased conversion to glycolate and oxalate. A similar increased conversion of ^{14}C -glycolate to oxalate occurs (presumably via glyoxylate) (Hockaday et al., 1964). (3) The rate of metabolism of ^{14}C -glyoxylate and ^{14}C -glycolate to ^{14}C - CO_2 is reduced (Hockaday et al., 1964). (4) The metabolism of ascorbic acid, another oxalate precursor, is normal in hyperoxaluria (Atkins et al., 1965). From in vivo incorporation studies it was concluded that the defect probably was in the enzymatic conversion of glyoxylate to glycine (Hockaday et al., 1965). In recent studies, however, no such defect of either glutamate or alanine: glyoxylate aminotransferase in liver or kidney could be found (Williams and Smith, 1972), in contrast to a reported defect in glycine synthesis from glyoxylate in autopsy kidney specimens in primary hyperoxaluria reported by others (Deen et al., 1966).

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More recently, low activity of α -ketoglutarate:glyoxylate carboligase has been found in cytoplasmic preparations of liver, spleen and kidney of five patients with primary hyperoxaluria. Mitochondrial activities of the enzyme were not uniformly reduced (Koch et al., 1967). Crawhal and Watts (1962) also found the synergistic decarboxylation of glyoxylate and α -ketoglutarate to be normal in hepatic mitochondria from three patients with primary hyperoxaluria. These investigators did not study cytoplasmic fractions. It was suggested that cytoplasmic α -ketoglutarate:glyoxylate carboligase may be an activity selectively reduced in primary hyperoxaluria (Koch et al., 1967).

1.1.5. Transamination reactions.

Glyoxylate may undergo amination or transamination to glycine (Weinhouse and Friedman, 1951; Nakada and Weinhouse, 1953). The activities with ornithine, glutamine and asparagine have all been purified (Meister, 1954; Meister and Fraser, 1954; Meister et al., 1952). The glutamine transaminase is functionally irreversible but the asparagine enzyme has been shown to be reversible and

includes glycine among the amino group donors. The glutamate:glyoxylate aminotransferase from rat liver (Nakada, 1964) irreversibly transfers the amino group from L-glutamate to glyoxylate, and it has been reported to be localized in liver peroxisomes (Vader and Tolbert, 1970). The alanine:glyoxylate aminotransferase and ornithine: α -oxoglutarate aminotransferase which also has activity toward glyoxylate, have also been studied (Thompson and Richardson, 1967). The alanine:glyoxylate aminotransferase was completely irreversible under all experimental conditions in favor of glycine formation. About 80% of the activity was recovered from the mitochondrial fraction of rat liver and the remaining activity with the cytosol fraction (Rowell et al., 1972).

Glyoxylate transaminase activity has also been reported in plant tissues (Sinha and Cossin, 1965; Wilson et al., 1954), in algae (Lord and Marrett, 1970), and in bacteria (Tsuiki and Kikuchi, 1962). Reversibility studies in higher plant tissues have shown that the glyoxylate transaminase systems favors glycine synthesis in vitro (Cossin and Sinha, 1965). Several amino group donors were studied and among them alanine and glutamic acid were the more effective. Although the glyoxylate transaminase activity has been reported to be localized in the cytoplasmic

fraction of plant tissues (Cossin and Sinha, 1965), the glutamate:glyoxylate transaminase has been found located in leaf peroxisomes (Kisaki and Tolbert, 1969).

Non-enzymatic transamination between glycine and glyoxylate occurs at pH 7.0 and 37° (Fleming and Crosbie, 1960; Lord and Merret, 1970). The equilibrium of the reaction was completely dependent on coordination-complex formation as indicated by the Cu^{+2} catalysis and the inhibition by ethylenediaminetetraacetate (EDTA) (Fleming and Crosbie, 1960).

1.1.6. Reduction to glycolate.

Three enzymes have been reported to catalyze the reduction of glyoxylate to glycolate and hydroxypyruvate to glycerate, namely, D-glycerate dehydrogenase, glyoxylate reductase, and lactate dehydrogenase.

The mammalian D-glycerate dehydrogenase catalyzes the reduction of hydroxypyruvate to glycerate with either NADH or NADPH as cofactors and has no activity towards pyruvate (Willis and Sallach, 1962; Dawkins and Dickens, 1965). A similar plant dehydrogenase exhibits a high degree of specificity toward NADH and was at least 4-fold more active with hydroxypyruvate than with glyoxylate and

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inactive with pyruvate (Stafford et al., 1954).

The NADH-glyoxylate reductase found in higher plants (Zelitch, 1955; 1955) was at least 2.8-fold more active with glyoxylate than with hydroxypyruvate and inactive with pyruvate. A similar enzyme isolated from rat liver peroxisomes catalyzed the reduction of glyoxylate to glycolate. This glyoxylate reductase activity utilized NADH and also reduced hydroxypyruvate to glycerate and pyruvate to lactate (Vandor and Tolbert, 1970). NADPH-glyoxylate reductase from plants also has been reported (Zelitch and Gotto, 1962). This enzyme reacted only slowly with hydroxypyruvate and no reaction was observed with pyruvate.

Lactate dehydrogenase catalyzes the reversible inter-conversion of glyoxylate and glycolate, although the equilibrium lies far in favour of glycolate (Sawaki et al., 1966). It is interesting to note that lactate dehydrogenase acts on hydroxypyruvate as effectively as on pyruvate (Dawkins and Dickens, 1965) the product of the reaction being L-glycerate.

Section 1.2. SOURCES OF GLYOXYLATE IN MAMMALIAN TISSUES.

The sources of glyoxylate in mammalian tissues are not completely known, but glycine is probably the major

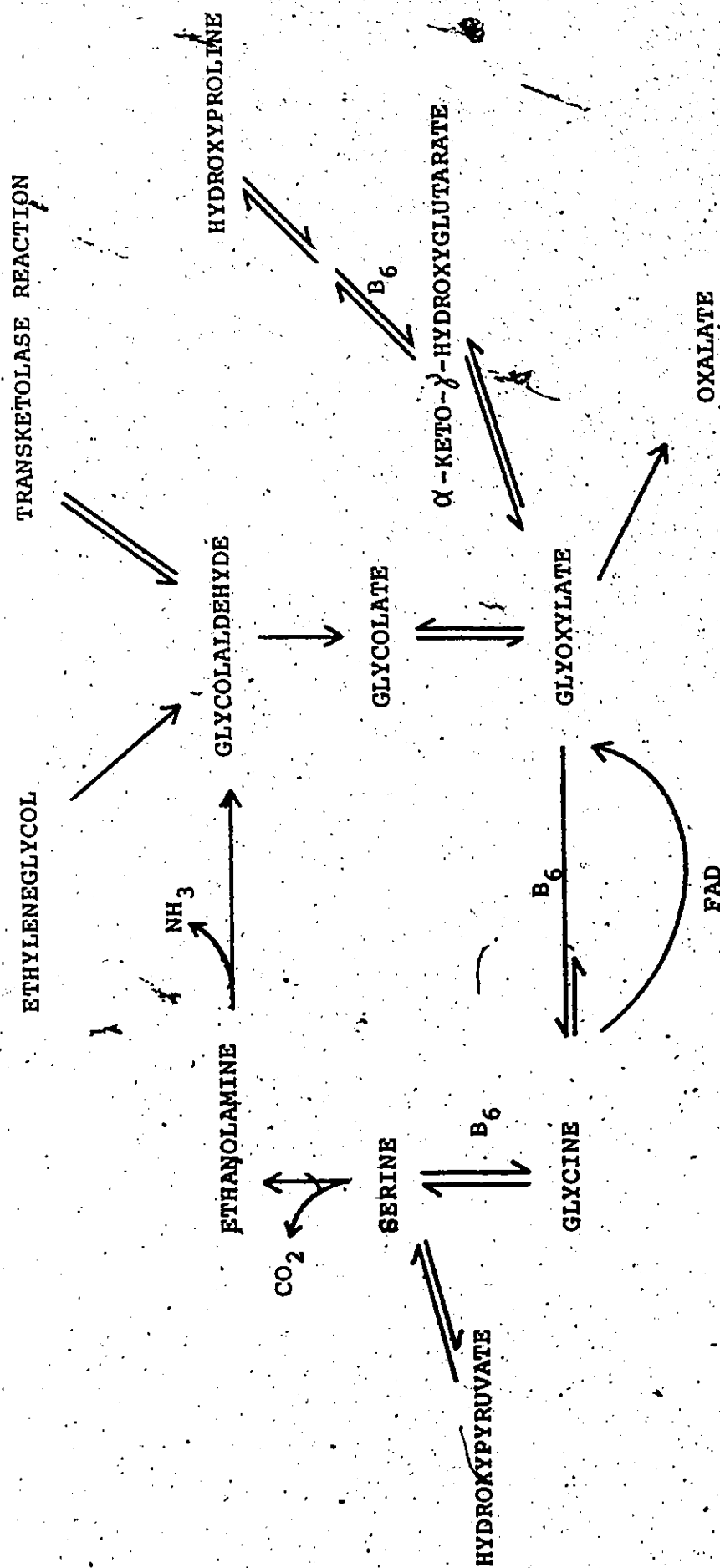
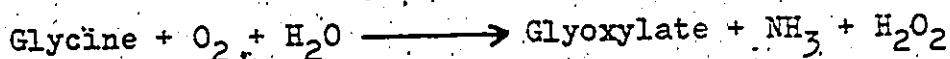


Figure 1.6: Precursors of glyoxylate in mammalian tissues. (From Hagler and

Herman, 1973).

source (Fig. 1.6). Studies on the conversion of glycine to oxalate in normal and hyperoxaluric subjects have been done with glycine labeled in the C₁ position (Crawhall *et al.*, 1959) and conversion of glycine to oxalate via glyoxylate was assumed. The operation of the alternative metabolic pathway via serine and ethanolamine (Fig. 1.6) was also demonstrated by comparing the ¹³C-enrichment of the urinary oxalate when (1-¹³C) glycine and (2-¹³C) glycine were given to a patient with primary hyperoxaluria (Dean *et al.*, 1968).

Glycine can be converted directly to glyoxylate by oxidative deamination catalyzed by glycine oxidase originally described in mammalian liver and kidney preparations by Ratner *et al.*, (1944). Glycine oxidase is probably identical with D-amino acid oxidase.



Although in theory glycine might also be converted to glyoxylate by transamination in most of the cases so far described the equilibrium of the reaction lies far in the direction of glycine synthesis (section 1.1.5).

Glycolate is both a precursor and a product of glyoxylate. The only metabolic fate established for glycolate is its oxidation to glyoxylate. This oxidation

is catalyzed by glycolate oxidase, a flavoprotein present in plants, animals and microorganisms (Zelitch and Ochoa, 1953; Kun et al., 1954). In mammals the only known precursor of glycolate other than glyoxylate (section 1.1.6) is glycolaldehyde (Kun et al., 1954) which may be derived from ethanolamine via the glycine-serine pathway (Dean et al., 1968). Glycolaldehyde also exists as an enzyme-bound intermediate in the transketolase reaction of the pentose-phosphate pathway. The oxidation of glycolaldehyde to glycolate is catalyzed by both aldehyde oxidase and dehydrogenases. The oxidation of ethylene glycol to oxalate probably occurs by way of glycolaldehyde and glycolate (Williams and Smith, 1972).

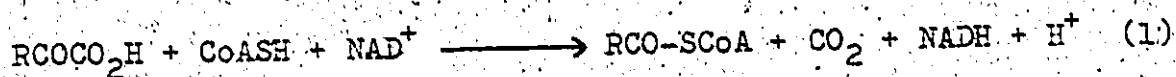
In addition to glycine and glycolate, α -keto- γ -hydroxy glutarate is an immediate precursor of glyoxylate (section 1.1.1.C). The former is formed by transamination between γ -hydroxyglutamate and α -ketoglutarate (Kuratomi et al., 1963). Current evidence does not suggest that hydroxyproline is an important precursor of glyoxylate and therefore of oxalate (Williams and Smith, 1972) but it seems that the contribution of hydroxyproline to urinary oxalate may be both age and sex-dependent and requires further study before its precise contribution can be

appreciated (Hagler and Herman, 1973).

Section 1.3. α -KETOGLUTARATE DEHYDROGENASE COMPLEX.

GENERAL INFORMATION.

Enzyme systems that catalyze a coenzyme A- and diphosphopyridine nucleotide-linked oxidative decarboxylation of pyruvate and α -ketoglutarate (reaction 1)



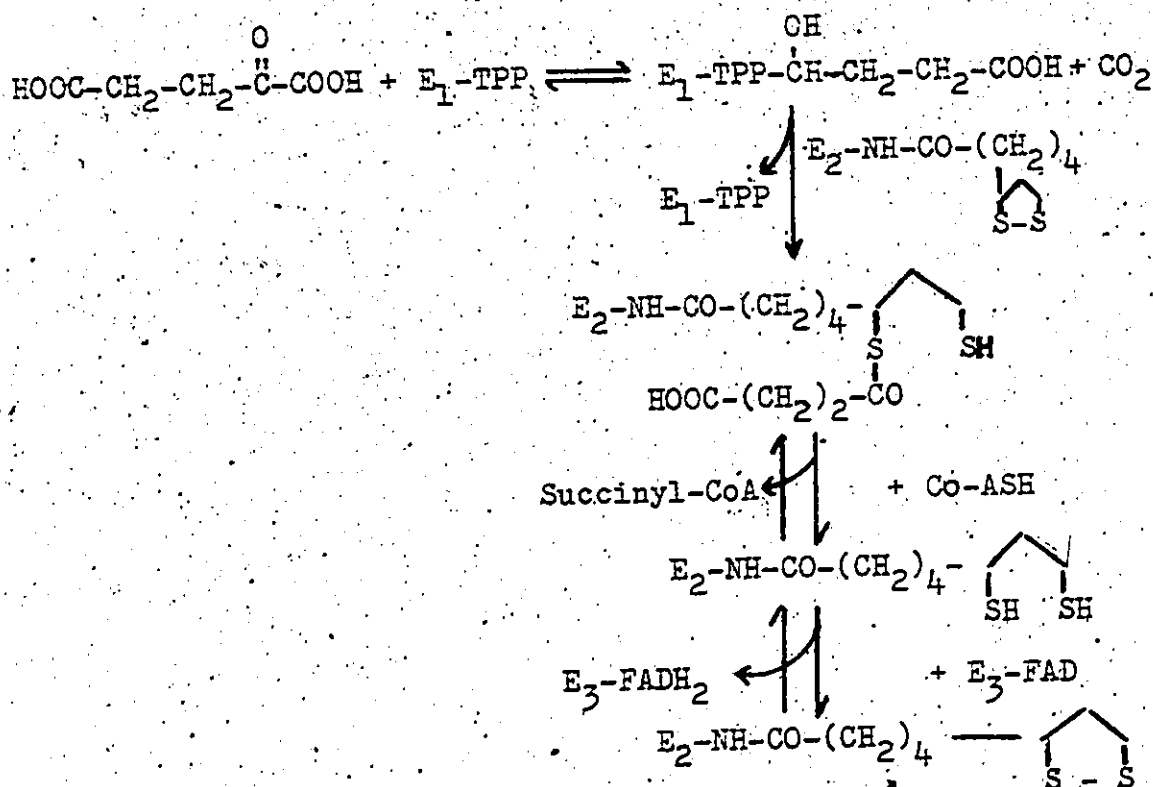
have been isolated as multienzyme systems with molecular weights of several million from pigeon breast muscle (Jagannathan and Schweet, 1952; Schweet et al., 1952), pig heart muscle (Sanadi et al., 1952; Hayakawa et al., 1964), and E. coli (Koike et al., 1960).

Two classes of complexes have been obtained, one specific for pyruvate the other for α -ketoglutarate. α -Ketoglutarate dehydrogenase complex has also been purified from bovine kidney (Linn et al., 1972), from Acinetobacter lwoffii (Parker and Weitzman, 1973) and from cauliflower flowerets (Wedding and Black, 1971).

The E. coli α -ketoglutarate dehydrogenase complex

has been separated into three enzymes (Mukherjee et al., 1965): α -ketoglutarate decarboxylase, dihydrolipoyl transsuccinylase and a flavoprotein dihydrolipoyl dehydrogenase. The complex has been reconstituted from the isolated enzymes. The E. coli pyruvate dehydrogenase complex also has been separated into three enzymes, analogous to those obtained from the α -ketoglutarate dehydrogenase complex and it too has been reassembled from the isolated enzymes (Koike et al., 1963).

The reactions catalyzed by the various components are:



E_1 , the α -ketoglutarate decarboxylase also catalyzes the transfer of the succinaldehyde group to the second protein of the complex, transsuccinylase, with a resultant oxidation to form a succinyl group. The decarboxylase is occasionally being referred to as a dehydrogenase. E_2 is the dehydrolipoate transsuccinylase and E_3 the dihydrolipoate dehydrogenase.

section 1.4. TETRAHYMENA AS AN EXPERIMENTAL ORGANISM

1.4.1. General considerations.

The ciliate Tetrahymena pyriformis is certainly not representative of all protozoa but it may be considered representative of the 6,000 species of ciliates that have been described (Hill, 1972). Tetrahymena has been studied far more than any other ciliate and their contribution to scientific knowledge is significant. The ciliate has been used in studies ranging from basic biology to cancer research (Hill, 1972). A primary reason for the success of Tetrahymena as an experimental subject is that it is a eukaryote which can be grown in large quantities on bacteria-free media including defined media.

From the information known about Tetrahymena a disputed point is whether these organisms are plants or animals (Holz, 1966). There are strong arguments in favor of calling them animals. Under the microscope they are seen to be motile, to ingest food and to have no chloroplasts. They have the same amino acid requirements as man and rat (Kidder and Dewey, 1951) and the vitamin requirements for all three are quite similar. Tetrahymena contains, as do higher animals, glycogen as a storage form of carbohydrate (Ryley, 1952) which it breaks down anaerobically to lactate (Warneck and van Eys, 1962). The biosynthesis of phosphatidyl serine proceeds in a manner similar to that in animals but not to that in bacteria (Dennis and Kennedy, 1970) and the ribonucleic acid polymerase is similar to that of animal but not to that of bacteria. (Byfield et al., 1970). The organism also possesses the catecholamines norepinephrine and epinephrine (Janakidevi et al., 1966a), serotonin (Janakidevi et al., 1966b), and 3'5'-cAMP phosphodiesterase activity (Blum, 1970). However the physiological function of these substances has not been determined. Tetrahymena also contains hemoglobin (Keilin and Ryley, 1953) and

N-phosphorylarginine as a phosphagen (Robin and Viala, 1966) and neither substance is found in plants. However, Tetrahymena, like plants has an operative glyoxylate cycle (Hogg and Kornberg, 1963) and contains a pentacyclic triterpenoid (Mallory et al., 1963) both of which have been found in plants. Tetrahymena lacks the urea cycle enzymes (Dewey et al., 1957) and it required preformed pyrimidines (Kidder and Dewey, 1951). The organism can synthesize ubiquinones from shikimate (Miller, 1965) but cannot synthesize phenylalanine.

1.4.2. Glyoxylate metabolism in Tetrahymena

The glyoxylate metabolism in Tetrahymena has been studied fundamentally in relation to its relation to the glyoxylate cycle (section 1.1.1.A). The presence of glyoxylate oxidase activity in the peroxisomes provides an alternative pathway for the glyoxylate formed by isocitrate lyase (Müller et al., 1968). It was suggested that if the reaction leads to formate and CO_2 , then the particles have ability to oxidize the 2-carbon fragment formed from isocitrate, since formate is a good substrate for the subsequent catalytic peroxidation (section 1.1.3).

However, in vivo studies have shown that the ratio of $^{14}\text{CO}_2$ produced from (1- ^{14}C) glyoxylate to that produced from (2- ^{14}C) glyoxylate was 2.1 (Blum, 1972). This suggested that glyoxylate was metabolized via glyoxylate oxidase. On the other hand, it was found that the nucleotide adenosine monophosphate (AMP) caused a marked inhibition of the oxidation of labelled glyoxylate but it had little effect on $^{14}\text{CO}_2$ production from labelled glucose, acetate or pyruvate. Furthermore, acetate, and to a lesser degree pyruvate, also inhibited the oxidation of labelled glyoxylate. It was suggested that AMP and the presence of substrate which produced acetyl CoA interferes with malate synthase activity or malate transfer from the peroxisomes to mitochondria (Blum, 1972). The above studies suggest that glyoxylate may be metabolized in Tetrahymena also by a route other than that involving malate synthase.

CHAPTER 2 : MATERIALS AND METHODS.

SECTION 2.1: MATERIALS.

Tetrahymena pyriformis, strain W, was obtained from the Culture Collection of Algae and Protozoa, Cambridge, England. Proteose-peptone, yeast extract and thioglycolate were all from Difco laboratories. Potassium-permanganate, chromotropic acid, sodium sulphate (anhydrous), ethyl acetate, potassium phosphate, ethylenediamine tetraacetic acid, disodium salt (EDTA) and other inorganic reagents were purchased from Fisher Scientific.

Diethyl ether, thiourea, sucrose, Tris buffer, and mannitol were purchased from J.T. Baker. 2,4-Dinitrophenylhydrazine was from Eastman Kodak Co. Amino acids, δ -aminolevulinic acid, thiamine pyrophosphate, glyoxylic acid, as the sodium salt, 4-aminoantipyrine, adenine nucleotides, and guanine nucleotides were from Sigma Chemical Co.

(U- ^{14}C) glyoxylic acid as the sodium salt, (2- ^{14}C) glyoxylate acid as the sodium salt, (1- ^{14}C) glyoxylic acid, sodium salt, Hyamine hydroxide (1 Molar solution in methanol) were purchased from Amersham/Searle. (2- ^{14}C) glycine, 2,4-diphenyloxazole (PPO), α -(1- ^{14}C) ketoglutarate from New England Nuclear.

Section 2.2. METHODS.

2.2.1. General methods for the growth of Tetrahymena.

T. pyriformis was isolated by Lwoff in 1923 and thanks to the work of Dewey et al. (1950) can be grown in a defined medium. The organism also can be grown in undefined but axenic medium (Lwoff, 1923) or even in a medium that contains bacteria as a food source. The culture media used throughout this work are either a proteose-peptone medium or a defined medium.

(A) Media.

(i) Proteose-peptone medium.

The medium contained per 100 ml of aqueous solution: proteose-peptone, 2 g; yeast extract, 0.1 g. The solution was neutralized with dilute ammonium hydroxide, filtered through Whatman N° 41 filter and paper under vacuum and then autoclaved at 121° for 20-30 minutes in an AMSCO autoclave.

(ii) Synthetic media.

The synthetic medium described by Dewey et al. (1950) but without Tween 85 was used. Stock solutions of the

components of the medium were prepared and stored under a layer of toluene at 4°. These components were labelled A, B, C, D, E, F, G, H, I. The medium contained 3% A, 2% C to H, and 0.1% I and was neutralized with dilute ammonium hydroxide. It was autoclaved at 121° for 20-30 minutes. The composition of the stock solutions is given on Table 2.1. When required glucose was sterilized separately and added to a final concentration of 0.25%. In this Thesis "synthetic medium" refers to the medium lacking acetate. The latter and glucose were added as supplements.

(B) Maintenance of stock cultures.

Stock cultures were grown in 5 ml proteose-peptone medium contained in 15 ml screw-cap culture tubes. Triplicate transfers were made each week. All cultures were grown at 25 ± 1° in an incubator.

(C) Parent cultures.

Parent cultures were started with a 2% inoculum (v/v) from the stock cultures in 100 ml of proteose-peptone medium contained in 300 ml nepheloflasks. These were grown for 72 hours and then used to inoculate the experimental cultures. This insured that the history of the inoculum was similar for each experiment.

A		g/l	D		g/l
L-Arginine		4.0	MgSO ₄ ·7H ₂ O		10.0
L-Histidine		1.5	Fe(NH ₄) ₂ (SO ₄) ₂ ·6H ₂ O		2.5
DL-Isoleucine		5.0	MnCl ₂ ·4H ₂ O		0.05
L-Leucine		5.0	ZnCl ₂		0.005
L-Lysine		4.0			
DL-Methionine		6.0	E		
L-Phenylalanine		2.0	CaCl ₂ ·2H ₂ O		5.0
DL-Serine		8.0	CuCl ₂ ·2H ₂ O		0.5
L-Tryptophan		1.6	FeCl ₃ ·6H ₂ O		0.125
DL-Valine		1.0			
B			F		
DL-Alanine		5.5	K ₂ HPO ₄		100.0
L-Arginine		4.3	KH ₂ PO ₄		100.0
L-Aspartic acid		6.1			
Glycine		0.5	G		
L-Glutamic acid		11.65	Sodium acetate		100.0
L-Histidine		2.1			
DL-Isoleucine		6.3	H		g/l
L-Leucine		9.7	Guanylic acid		3.0
L-Lysine		7.6	Adenylic acid		2.0
DL-Methionine		3.4	Cytidylic acid		2.5
L-Phenylalanine		5.0	Uracil		1.0
L-Proline		8.75			
DL-Serine		7.7	I		mg/l
DL-Threonine		8.8	Thioctic acid		4.0
L-Tryptophan		1.2			
DL-Valine		6.6			
C		mg/l			
Ca pantothenate		10.0			
Nicotinamide		10.0			
Pyridoxine.HCl		100.0			
Pyridoxamine.HCl		10.0			
Pyridoxal.HCl		10.0			
Riboflavin		10.0			
Pteroylglutamic acid		10.0			
Thiamine.HCl		100.0			
Biotin (free acid)		0.05			
Choline chloride		100.0			

Table 2.1: Stock solutions for preparation of the synthetic medium.

(D) Experimental cultures.

Experimental cultures were started with a 2% inoculum (v/v) from 72 hours old parent cultures.

(E) Sterility.

Periodically, the sterility of the Tetrahymena cultures was checked by inoculating 10 ml thioglycolate medium contained in 30 ml screw-cap culture tubes. These were incubated at 37°. Tetrahymena cells do not survive at this temperature but contaminating bacteria would thrive. Bacterial growth would be detected visually after 2-3 days by the appearance of turbidity.

2.2.2. Procedure for harvesting cultures of T. pyriformis.

The cells were harvested by centrifugation at 270xg for 2 minutes in a refrigerated Servall centrifuge set at 0°, after growth for about 50 hours at which time the cells are in the transitional phase of growth.

2.2.3. Preparation of cell extract.

Cells were washed twice with ice-cold 0.9% NaCl and resuspended in 0.25 M sucrose. Cell disruption was achieved either by sonicating for about 30 seconds in a

Biosonic 111 ultrasonic disintegrator or by freezing at -45° for 15 min and thawing in the presence of 0.25 M sucrose. The completeness of disruption was routinely checked by viewing under the microscope.

2.2.4. Fractionation methods.

The methods utilized for the fractionation of Tetrahymena homogenates were those described by Mager and Lipmann (1958) with some modifications (method A) and by Kobayashi (1965) (method B).

Method A.

The cells were collected (section 2.2.2.) and washed twice with ice-cold distilled water. The washed cells (about 5 g wet weight) were suspended in 50 ml of 0.4 M sucrose solution containing 0.1 M Tris.HCl buffer, pH 7.5, and 0.005 M potassium chloride and the suspension was left in ice for 10 min to lyse partially. This preparation separated on centrifugation for 15 min at 18,000xg into a well packed bottom layer, a fluffy intermediate layer and a clear supernatant fluid. The supernatant fluid and the intermediate layer were discarded and the pellet was homogenized in a glass homogenizer with Teflon pestle of 15 ml capacity in 0.25 M sucrose, 0.01 M phosphate, pH 7.0 and

1 mM β -mercaptoethanol (BME) to complete cellular disintegration. During homogenization the temperature was kept near 0° by holding the homogenizer in an ice-water mixture. The homogenate was diluted to about 45 ml with sucrose-phosphate-BME solution. Cell debris and nuclei were removed by centrifugation at $500\times g$ for 5 min. The supernatant fluid will be referred to as the "homogenate". For further fractionation of the homogenate the sucrose concentration was raised to 0.35 M by adding 0.8 M sucrose. On centrifugation at $12,000\times g$ for 15 min the mitochondrial fraction was sedimented. The turbid supernatant fluid will be referred to as the "supernatant fraction".

The mitochondrial fraction was washed twice with 0.25 M sucrose and finally suspended in about 3 ml of the sucrose-phosphate-BME solution. All the fractions were kept at -20° until assayed.

Method B.

The cells were washed twice with a medium containing 0.35 M mannitol, 0.1 mM EDTA and 1 mM Tris. HCl, pH 7.2. The washed cells were suspended in the same medium and homogenized at 0° in a glass homogenizer with a tightly fitting Teflon pestle. During this procedure the medium was kept at pH 7.0 to 7.4 by adding 1 M KOH. The homogenate was made up to 45 ml with the same medium and was immediately centrifuged at $10,000\times g$ for 5 min. The resulting sediment consisted of two layers. The upper

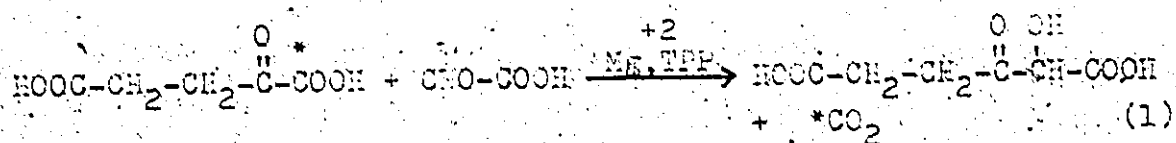
layer was whitish-gray and of a jelly-like consistency, while the bottom layer was yellowish-brown and tightly packed. The mannitol medium was carefully poured into the tube and stirred gently to remove the loosely packed upper layer. This procedure was repeated three to four times and the suspensions were combined. The final suspension was centrifuged at 12,000xg for 15 min and the supernatant fluid so obtained will be referred to as the "supernatant fraction". The sediment was discarded. The bottom layer which contained mitochondria, unbroken cells and some cilia was suspended in 50 ml of medium with a loosely fitting homogenizer and centrifuged at 200xg for 2 min. The supernatant fluid was then centrifuged at 10,000xg for 5 min to sediment the mitochondrial particles. The latter were washed once with 50 ml of the preparation medium and then suspended in about 1 ml of 0.02 M potassium phosphate, pH 7.0, containing 1 mM BME. All the fractions were kept at -20° until assayed.

A detailed discussion on the merits and drawbacks of the two methods will be presented in Chapter 3.

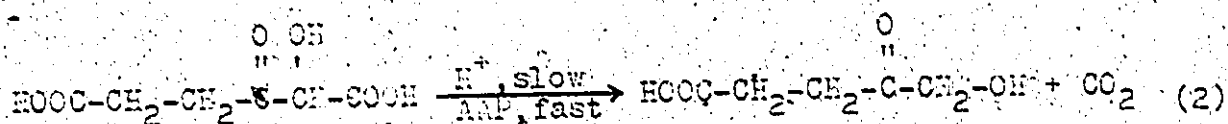
2.2.5. Enzyme assays.

2.2.5.A. α -Ketoglutarate:glyoxylate carbolipase.

The enzyme catalyzes the following reaction:



The product α -hydroxy- β -ketoadipate (HKA) decarboxylates spontaneously in the presence of acid or aminoantipyrine (AAP) to yield δ -hydroxylevulinic acid (HLA).



When (U- ^{14}C) glyoxylate was the labelled substrate the enzyme was assayed by following either the slow decarboxylation in acid medium or the fast decarboxylation in the presence of AAP (Koch and Stokstad, 1966) according to reaction 2. AAP is a well-known catalyst for the decarboxylation of β -ketoacids (section 3.5).

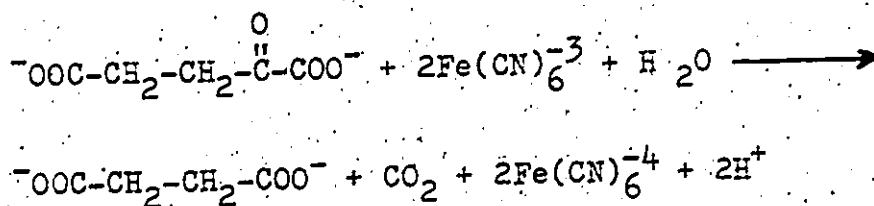
When α -(1- ^{14}C) ketoglutarate was the labelled substrate the reaction was followed by measuring the $^{14}\text{CO}_2$ according to reaction 1.

Incubations were carried out for 60 min at 30° in a nitrogen atmosphere in Warburg flasks in a final volume of 2.0 ml. The main compartment contained: 100 μmoles potassium phosphate, pH 7.0, 5 μmoles MgCl_2 , 0.2 μmoles thiamine pyrophosphate (TPP), 2 μmoles α -ketoglutarate and the enzyme source. The solutions were previously neutralized. The reaction was started after 10 min of gassing by tipping from one arm 2 μmoles of labelled glyoxylate or unlabelled glyoxylate when α -(1- ^{14}C) ketoglutarate

was the labelled substrate. These incubation conditions will be referred to as "standard assay conditions". The reaction was stopped by adding 0.1 ml of 70% perchloric acid (PCA) or a mixture of 0.05 ml of 25% trichloroacetic acid (TCA) and 0.1 ml of 0.2 N AAP from the other side arm. $^{14}\text{CO}_2$ was trapped in Hyamine hydroxide (0.06 ml or 0.2 ml when higher substrate concentrations were used, in methanol on a filter paper) in the center well during an additional 60 min when PCA was used, or during an additional 30 min when TCA-AAP mixture was used. The content of the center well was counted in 10 ml of scintillation solution (ethanol:toluene, 3:7, 0.4% in 2,5-diphenyloxazole (PPO) in a liquid scintillation counter with channels ratio standardization (section 2.2.7.).

2.2.5.B. α -Ketoglutarate decarboxylase:

α -Ketoglutarate decarboxylase is a component (E_1) of the α -ketoglutarate dehydrogenase complex (section 1.3). In presence of potassium ferricyanide, the decarboxylase component catalyzes the following reaction:



The reaction can be followed spectrophotometrically

by measuring the decrease in absorbance at 410 nm (Massey, 1960). 2,6-Dichlorophenol-indophenol may be used as the electron acceptor instead of ferricyanide, in which case the absorbance decrease at 600 nm is measured. These spectrophotometric methods are satisfactory after the active mitochondrial fraction is separated from the homogenate. The reaction rate with the artificial acceptors decreases with time, and meaningful assays are obtained only during the first 30 seconds of the reaction. This may explain the previously unsuccessful attempts to demonstrate α -ketoglutarate dehydrogenase activity in homogenates of Tetrahymena (Müller et al., 1968). This difficulty has been largely overcome in the manometric assay by using bovine serum albumin as a protective agent. Furthermore this procedure is applicable at all states of purification including the homogenate fraction (Sanadi and Littlefield, 1952). This assay as modified by Schlossberg et al., (1970) was chosen and is described below.

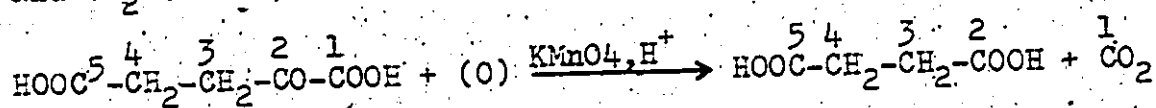
Incubations were carried out for 30 min at 30° in an air atmosphere in Warburg flasks in a final volume of 2 ml. The main compartment contained: 100 μ moles potassium phosphate, pH 7.0, 5 μ moles $MgCl_2$, 0.5 μ moles neutralized TPP, 2 μ moles neutralized α -(1- ^{14}C) ketoglutarate

(about 140,000 d.p.m.); 20 mg albumin, pH 6.9, and the enzyme source. After a 10 min equilibration the reaction was started by tipping from one arm 4 μ moles of potassium ferricyanide and was stopped by adding 0.1 ml of 70% PCA from the other arm. The incubation was continued for an additional 30 min. $^{14}\text{CO}_2$ was trapped and counted as in the carbolicase assay.

Units of enzyme activities were defined as μ moles of $^{14}\text{CO}_2$ liberated per hour at 30° .

2.2.6. Permanganate oxidation of 2,4-dinitro-phenylhydrazine of α - ^{14}C ketoglutarate.

In the presence of excess potassium permanganate in acid medium DNPH- α -ketoglutarate is oxidized to succinate and CO_2 (Krebs, 1938).



This technique originally used for quantification of α -ketoglutarate from biological sources may be utilized for the determination of the radioactivity in the C_1 carboxylic carbon of the DNPH- α -ketoglutarate (Okuyama et al., 1965).

Procedure: DNPH derivatives of α -(1- ^{14}C) or α -(5- ^{14}C)

ketoglutarate were prepared from the corresponding acid, chromatographed on paper, extracted and dried as described in section 2.2.9.

To the dry ^{14}C -DNPH derivatives (about 0.5 mg) 1 mg of cold α -ketoglutarate derivative was added and they were dissolved in 0.6 ml of 10 mM Na_2CO_3 . From this solution 0.05 ml was counted in Bio-Solv-containing scintillation solution (section 2.2.7) and 0.5 ml was transferred to Warburg flasks and 0.4 ml 50% H_2SO_4 was added to the main compartment. The flasks were incubated at 30° for 5 min and the reaction was started by tipping 1.0 ml of 3% KMnO_4 from the two side-arms of the flasks (0.5 ml from each arm). If the purple color of the permanganate disappeared before 10 min fresh oxidizing agent was added carefully through the venting plug. An additional incubation of 60 min was allowed for complete trapping of the $^{14}\text{CO}_2$ liberated during the reaction. Measurements of the $^{14}\text{CO}_2$ was conducted as described for the enzyme assays (section 2.2.5). The mixture was filtered through Whatman No 1 filter paper to remove MnO_2 formed in the reaction and the filtrate was extracted with diethyl ether for 48 hours in a Kutscher-Steudel extractor. The ethereal extract which contained succinate was transferred to tubes and

evaporated over 2.0 ml of water under a stream of nitrogen. The aqueous solution was evaporated to 0.5 ml and applied to chromatography paper Whatman No. 1. The solvent system consisted of 95% ethyl alcohol:water:ammonia (8:1:1 v/v) (Jones et al., 1953). The chromatograms were developed by the ascending method for 6 hours. The acid was visualized by spraying with 0.04% bromophenol blue. The chromatograms were cut in 1 cm segments and counted in 0.4% PPO in toluene (section 2.2.7).

Results: In Table 2.2. the recovery of radioactivity from the D-¹⁴FH- α -ketoglutarate as CO_2 is shown. When the α -ketoglutarate was labelled in the C_1 95.7% in one assay and 83% in the other assay were recovered as $^{14}\text{CO}_2$ and no radioactivity was detected as succinate. When the label was in position C_5 only 2% was obtained in the $^{14}\text{CO}_2$ and about 203 d.p.m. (45%) were recovered as succinate.

2.2.7. Scintillation solutions. Instrument.

The following counting solutions were used:

- (a) 0.4% (w/v) 2,5-diphenyloxazole (PPO) in toluene.
- (b) 0.4% PPO (w/v) in ethanol:toluene (3:7 v/v).
- (c) 0.4% PPO (w/v) in toluene containing 10% Bio-Solv BBS-3 (v/v) (Beckman Instruments).

DNPH- α - 14 C- ketoglutarate	d.p.m.		
	Initial in the flasks	As 14 CO ₂	As 14 C- succinate
1- 14 C	5,600 46,563	5,356 38,590	0 0
5- 14 C	451	9	203

Table 2.2: Oxidation of the 2,4-dinitrophenylhydrazone of α -(1- 14 C) and (5- 14 C) ketoglutarate. Main compartement contained: 1.5 mg of labelled DNPH- α -ketoglutarate dissolved in 0.5 ml of 10 mM Na₂CO₃ and 0.4 ml of 50% H₂SO₄. From the side arms 1 ml of 3% KMnO₄ was added. After 60 min of incubation the trapped 14 CO₂ was counted and the succinate formed was extracted and chromatographed as described in the text.

Counting was performed in a Nuclear Chicago, model Isocap 300, liquid scintillation apparatus, refrigerated at 4°. In some experiments the Beckman LS-133 counter was utilized. Commercial quenched standards (Amersham/Searle) were used to establish ^{14}C quench correction curve.

2.2.3. Measurement of $^{14}\text{CO}_2$

The reported methods for the estimation of $^{14}\text{CO}_2$ obtained in the assay of the carbolligase are as follows:

(i) precipitation of the $^{14}\text{CO}_2$ as $\text{Ba}^{14}\text{CO}_3$ and counting in a conventional Geiger-Mueller counter or proportional counter (Crawford and Watts, 1962; Saito et al., 1971; Davies and Kenworthy, 1970; Schlossberg et al., 1968; Okuyama et al., 1965). (ii) measuring of the trapped radioactive CO_2 by liquid scintillation counting (Koch and Stokstad, 1966; Yamasaki and Moriyama, 1970; Steward and Quayle, 1967; Schlossberg et al., 1970). The first procedure is rather tedious and not convenient for experiments involving measurements of a great number of samples. In the second method alkali-soaked filter paper is generally utilized to trap the $^{14}\text{CO}_2$ and counting is performed in a dioxane counting solution. However, no information is given by the users on measures to avoid chemiluminescence in the counting solutions.

It is well known that alkaline medium is essential for the chemiluminescence reaction in standard scintillation solutions (Kalbhen, 1967, 1970). This results in spurious counts unrelated to the radioactivity of the sample, which can be eliminated by dark adaptation of the samples or by the addition of acid to neutralize the basicity of the counting solution. Chemiluminescence is believed to be due to organic peroxides that may react in an alkaline medium to produce emission of light (Gundermann, 1968) and is more pronounced at higher temperatures (Kalbhen, 1967). In contrast to dioxane which is notorious for the formation of peroxides on contact with air, toluene-based scintillation solutions generally contain much less peroxides and should be preferred with basic solubilizers such as KOH, Hyamine and NCS (trade mark for a solution of a quaternary ammonium base dissolved in toluene, Amersham/Searle) (Kalbhen, 1970).

For the foregoing reasons the method of Kobayashi and Maudsley (1969) which utilizes toluene-based scintillation solution was chosen, with some modifications, and is described below.

Description of the method.

The $^{14}\text{CO}_2$ produced in a Warburg flask was trapped

in 0.06 ml of Hyamine hydroxide (1.0 M solution in methanol) on Whatman No 3 filter paper (7 x 15 mm) placed in the center well. The contents of the center well were transferred to a polyethylene vial and maximum transfer was ensured by washing the well with small portions of scintillation solution using a Pasteur pipette (total ~~0.5~~ ml). The final volume in the vials was 10 ml. The counting solution contained ethanol (section 2.2.7).

Reliability of $^{14}\text{CO}_2$ -trapping and counting techniques.

To test the reliability of the trapping and counting techniques portions of a 2 mM $\text{NaH}^{14}\text{CO}_3$ were pipetted into the main compartment of Warburg flasks. Water was added to bring the total volume to 2.0 ml. Sulphuric acid (0.2 ml, 6 N) was added from the side-arm and the evolved $^{14}\text{CO}_2$ was trapped as described above. After shaking for 1 hour at 30° the contents of the center well were transferred to counting vials and counted in a liquid scintillation apparatus at 4° (section 2.2.7).

Hyamine hydroxide (trade name for 1 M p-(diisobutyl-cresoxyethoxyethyl)-dimethyl-benzyl-ammonium hydroxide in methanol) is a high molecular weight quaternary amine.

Passman et al., (1956) were the first to use this substance in collecting $^{14}\text{CO}_2$ for liquid scintillation counting.

because the carbonate formed is soluble in toluene. However, because the counting solution described by Kobayashi contains ethanol the influence of the latter on counting efficiency was also studied. Thus in one series of experiments ethanol:toluene (3:7 v/v) scintillation mixtures were prepared. All the assays were done in duplicate. The vials with the samples were allowed to stand at room temperature in the dark for 1 hour and were counted after cooling for at least 45 minutes. Then the vials were again allowed to stand for 15 hours at room temperature in the dark and after cooling were counted again. Both the external standard and channels ratio methods were used to calculate the counting efficiency.

Results.

The most reliable counting was observed when ethanol was present in the counting solution as shown in Figure 2.1. A straight-line relationship between the quantity of $^{14}\text{CO}_2$ liberated and the observed counting rate was obtained which closely corresponded to the theoretically expected counts. As is evident in Figure 2.1 the channels ratio method yielded a straight line much closer to the theoretical line than the external standard method did. Therefore, the former method was considered to be more reliable. In

Figure 2.1

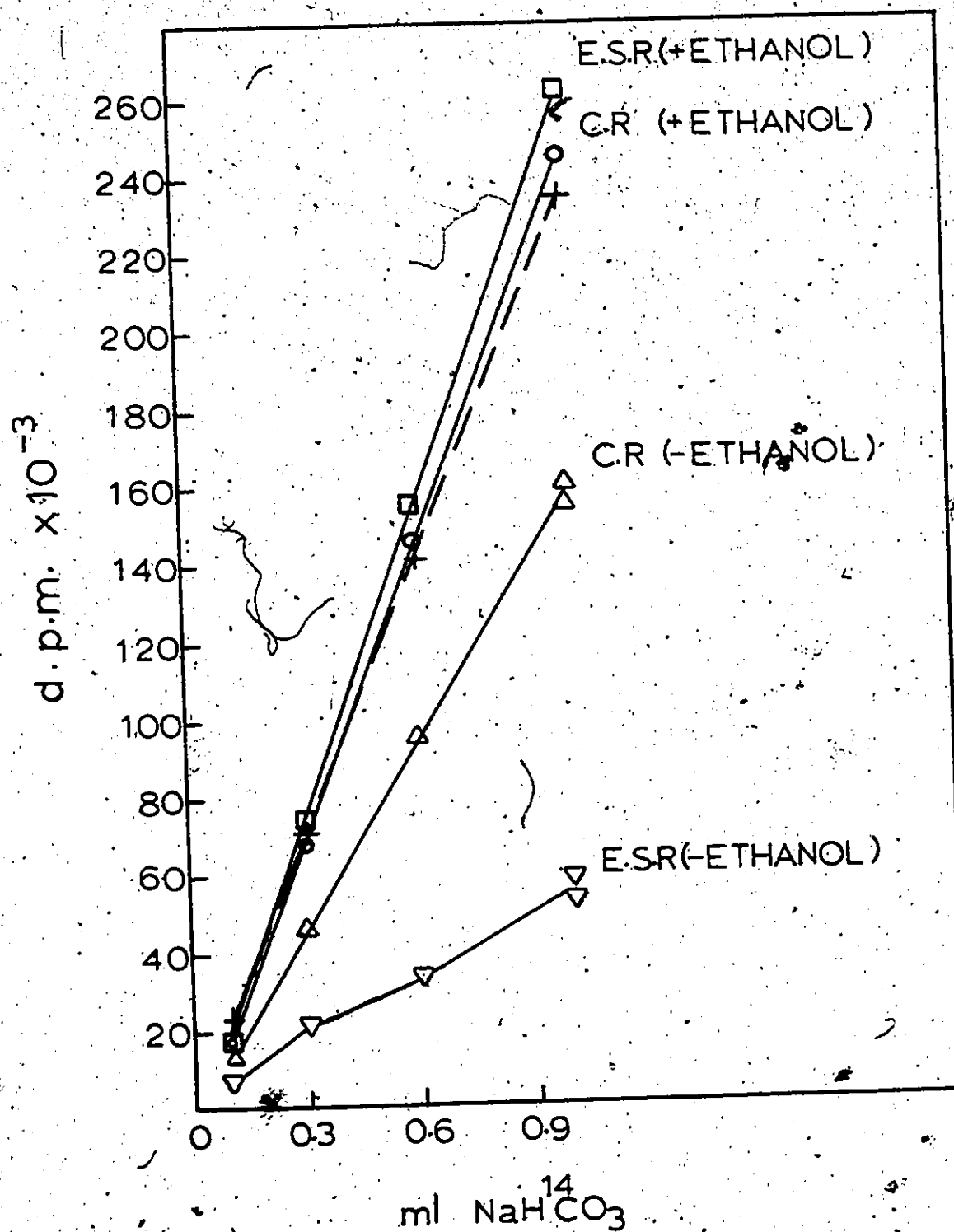


Figure 2.1: Effect of counting solution and of the method of standardization on the estimation of the trapped $^{14}\text{CO}_2$. The main compartment contained the indicated volumes of 2 mM $\text{NaH}^{14}\text{CO}_3$ (234,810 d.p.m./ml) in a total of 2 ml; Sulphuric acid (0.2 ml, 6N) was added from the side arm. Incubations were carried out for 60 min. The central well contained filter paper impregnated with 0.06 ml of Hyamine hydroxide. The contents of the central well were transferred into counting solutions containing ethanol (+ ethanol) or without ethanol (- ethanol), and counted after 15 hours of standing in the dark at room temperature, with the channels ratio method (C.R.) or the external standard ratio method (E.S.R.). The calculated theoretical d.p.m. from the amount of $\text{NaH}^{14}\text{CO}_3$ are represented by the dashed line.

Counting conditions

- Ethanol

+ Ethanol

ml	Channels Ratio		Ext.St. Ratio		Channels Ratio		Ext.Est. Ratio	
	c.p.m.	Eff. (%)	c.p.m.	Eff. (%)	c.p.m.	Eff. (%)	c.p.m.	Eff. (%)
0.3	17,991	38.6	18,217	83.7	51,593	75.6	51,813	70.6
	17,365	37.6	17,862	83.7	52,315	75.9	52,551	70.5
0.6	27,057	28.9	27,482	83.4	110,521	75.7	111,720	71.4
	27,750	28.7	28,407	83.7	110,929	75.7	110,829	71.4

Table 2.3: Counting efficiencies obtained in the presence and absence of ethanol in the counting solutions using the channels ratio and external standard ratio methods. Data were taken from Fig. 2.1.

the absence of ethanol the efficiency calculated by the external standard method was abnormally high thus resulting in abnormally low corrected d.p.m. (Fig.2.1, Table 2.3).

The low registered d.p.m. generally obtained in the absence of ethanol was due to the low solubility of the Hyamine carbonate in toluene. As shown in Table 2.4, 95.5% of the radioactivity was in solution when ethanol was present and only 1% in its absence, after the paper was removed from the vial.

The efficiencies obtained in the presence and absence of ethanol are shown in Table 2.5. In the presence of ethanol about 75% efficiency was obtained when the channels ratio method was used and about 70.5% when the external standard ratio method was used. In the absence of ethanol a variable and less than 50% efficiency was obtained with the channels ratio method.

No chemiluminescence could be detected after 1 hour of standing in the dark at room temperature, since a longer period (15 hours) did not result in a decrease in the counting rate.

In summary, under our experimental conditions Hyamine hydroxide and filter paper were effective in

Counting solution	d.p.m.		
	with the filter paper	without the filter paper	released from the filter paper. %
+ Ethanol	68,585	65,397	95.5
- Ethanol	46,609	463	1.0

Table 2.4: Solubility of Hyamine carbonate in the counting solutions. The filter papers were taken out from the counting vials and the solutions counted again with the channels ratio method. The counting vials used were those containing 0.3 ml of $\text{NaH}^{14}\text{CO}_3$ from Fig. 2.1.

trapping the CO_2 liberated from the reaction. The presence of ethanol in the counting solution was essential for optimal solubility of the carbonate from the paper, and the channels ratio method for the correction of quenching proved to be more reliable. Standing of the vials in the dark at room temperature for 1 hour and counting at low temperature were adopted as routine to exclude the possibility of chemiluminescence interfering with the counting. In later experiments 0.2 ml of Hyamine was used. This increase in Hyamine did not result in chemiluminescence and the efficiency was 73-74%.

2.2.9. Preparation of δ -hydroxylevulinic acid (HLA).

HLA is not available from commercial sources. Deamination of δ -aminovaleric acid by nitrous acid with the formation of δ -hydroxylevulinic acid as described by Schlossberg et al. (1968), with some modifications, was used for its preparation.

NaNO_2 (50%, 1.0 ml) was added dropwise over a period of 10 min to 100 mg of δ -aminovaleric acid (128 mg of the hydrochloride) dissolved in 5 ml of 1 N H_2SO_4 . The solution was allowed to stand at room temperature for 25 min and was then immersed in boiling water for 5 min.

Excess nitrous acid was removed by transferring the solution to a round-bottomed flask, adding 20 ml of 0.25 N H_2SO_4 and flash-evaporating at 40° to about 12 ml. This solution, diluted and neutralized, was passed through a column (20 x 60 mm) packed with Dowex 50W-X8 (H^+ form, 100-200 mesh). The column was washed with distilled water and the first 90 ml were collected, concentrated in a flask evaporator to about 6 ml and passed through a column (10 x 160 mm) packed with Dowex AG 1-X10, (acetate form, 100-200 mesh). The column was first washed with 25 ml water and then eluted with a concentration gradient of acetic acid from 0 to 0.5 N at a flow rate of 15.6 ml/hour. Fractions of 4.2 ml were collected and 0.2 ml aliquots were tested with the periodate-chromotropic acid method. A peak was obtained in tubes 35 through 44.

Periodate-chromotropic acid method for the
determination of HLA

This procedure was described by Frisell and McKenzie (1958) for the determination of serine and it was used for the determination of HLA by Schlossberg et al., (1968). The standardization procedure using serine as standard is described below.

(i) Oxidation of serine to formaldehyde.

Different aliquots of a solution of serine (10 μ moles/ml) were transferred to 10 ml volumetric flasks and 1.5 ml of 0.2 M phosphate buffer, pH 7.0, and water were added to a final volume of 3 ml. The final amount of serine ranged between 1 and 5 μ moles. After adding 1 drop of methyl red to the solution, 10% of TCA was pipetted dropwise to make the solution slightly basic to the indicator (yellow color). One ml of 0.075 M NaIO_4 was then added and after 5 min TCA was introduced dropwise to the exact acid end-point of the indicator. One ml of 10% NaHSO_3 was next added and the volume was brought to 10 ml with distilled water.

(ii) Determination of formaldehyde.

One ml of the above solution was transferred to test tubes, 10 ml of chromotropic acid reagent was added and the color was developed by heating in a boiling water-bath for 30 min. After the solutions were cooled to room temperature, 1.0 ml of 5% thiourea solution was added to each tube to reduce to a minimum a red color contributed by the reagents. The absorbancy was measured at 570 nm in a Coleman spectrophotometer. Known formaldehyde solutions (1.22 μ moles/ml) also were analyzed with the chromotropic acid method. Figure 2.2 shows the standard curves obtained

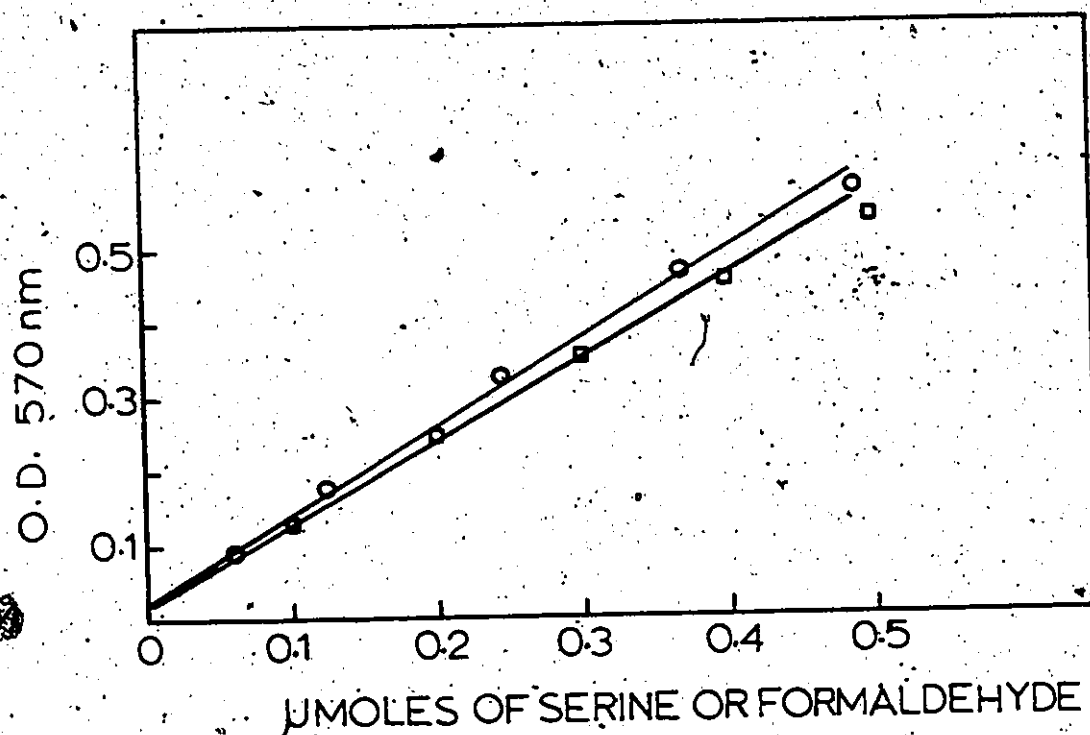


Figure 2.2: Standard curves for the estimation of formaldehyde by the chromotropic acid method. Different amounts of a formaldehyde solution ($1.22 \mu\text{moles/ml}$) were assayed with chromotropic acid reagent (o-o-o-); different amounts of a serine solution were oxidized with NaIO_4 , and then the formaldehyde determined (-o-o-o-) as described in the text.

with serine and formaldehyde. The two straight lines practically coincided.

2.2.10. Preparation of dinitrophenylhydrazone derivative.

The reaction product of α -ketoglutarate:glyoxylate carboligase was identified as the dinitrophenylhydrazone derivative (DNPH derivative).

The usual methods (for review see Kun and Garcia Hernandez, 1957) for the preparation and further identification of DNPH derivatives are not adequate in the case of HLA, the product of the carboligase reaction and of subsequent acidification (section 2.2.5). These methods utilize extraction of the DNPH derivatives from the organic phase with 1.0 M Na_2CO_3 . However, the recovery of some derivatives is incomplete, especially in the case of the less polar keto acids as those corresponding to valine, leucine and isoleucine (Kawano *et al.*, 1962). Dagly *et al.* (1961) have reported the lack of extraction in Na_2CO_3 of the DNPH-tartronic semialdehyde, which is a bis derivative (osazone). Preliminary experiments have shown that the DNPH-HLA was poorly extracted when this procedure was applied.

Isolation of the DNPH-HLA for further identification

by paper-chromatography from the reaction mixture has been accomplished by ether extraction in a Kutscher-Steudel extractor (Koch and Stokstad; 1966; Koch et al., 1967) which is not practical when several samples must be processed. Extraction of the derivatives from the reaction mixture with ethyl acetate and subsequent application on paper for chromatography without reextraction in an aqueous phase has also been reported (Davies and Kenworthy, 1970). This procedure results in derivatives with a high degree of contamination with excess reagent (2,4-DNPH) and its decomposition products. In preliminary experiments such extraction resulted in poor chromatograms. For these reasons the above procedures were discarded.

The replacement of the oxygen atom of a carbonyl group by the residue $=NNHC_6H_5$ is attended with an increase in molecular weight and consequent decrease in solubility. Hence, an aldehyde or a ketone often can be precipitated from a dilute solution as the phenylhydrazone (Fieser and Fieser, 1961). Derivatives of still decreased solubility are obtained with 2,4-dinitrophenylhydrazines. Therefore a method based on these properties was chosen. The method utilized by Dagely et al. (1961); with some modifications, was used in our experiments and is described below.

2.2.10.A. Preparation of DNPH derivatives.

After the enzymatic reaction had been carried out, 2.5 ml of 0.2% 2,4-DNPH reagent was added to the reaction mixtures directly or after deproteinization with PCA. The mixtures were kept at room temperature overnight. The crystals were collected, after cooling in ice for about 20 min, by centrifugation in the presence of 0.5 g kaolin (to aid the precipitation of crystals), washed once with cold 2 N HCl and twice with water, and drained by inversion. The precipitate was extracted from the kaolin paste three times with 5 ml ethyl acetate. The ethyl acetate solution was dried with anhydrous Na_2SO_4 and evaporated to dryness under N_2 at about 35° . This procedure was quantitative for the bis derivatives but not for the mono derivatives, and will be referred to as "crystal extraction". In some experiments where quantitative determination of the hydrazone of α -ketoglutarate was required the remaining aqueous solution and the HCl washings were extracted once more, and the procedure will be referred to as "total extraction".

The DNPH derivatives can be identified by paper chromatography, a procedure that has been widely used for the derivatives of oxaloacetate, α -ketoglutarate and

pyruvate (Cavallini et al.; 1949a; 1949b; El Hawary and Thompson, 1953).

2.2.10.B. Chromatography of the DNPH derivatives.

The dry residue containing the DNPH derivatives was dissolved in 0.5 ml of ethyl acetate and applied to Whatman No 1 filter paper. In some cases it was necessary to apply 0.1-0.2 ml. For development two solvent systems were employed. The first system was ethanol:water (83:17 v/v) on paper which had been previously impregnated with 0.1 M potassium phosphate buffer, pH 7.3, and subsequently dried for 12 hours at room temperature (Kun and Garcia-Hernandez, 1957). Development was carried out for 8 hours at room temperature by the descending method. The second system was n-butanol:ethanol:2N NH_3 (7:1:2 v/v) (Koch and Stockstad, 1966) developed for 24 hours unless otherwise indicated at room temperature by the ascending method.

2.2.10.C. Identification of DNPH derivatives.

This was accomplished by their migration on paper chromatograms and the color developed after spraying with a saturated solution of NaOH in ethanol. The DNPH derivatives yield the following colors after such treatment: α -ketoglutarate, dark green; hydroxylevulinic acid (HLA),

blue, one isomer of glyoxylate, brown-orange, and the other isomer, yellow.

2.2.10.D. Extraction of the derivatives from the paper.

The papers were cut in small pieces, moistened with 2 N HCl and extracted three times with 5 ml ethyl acetate in a 50 ml Erlenmeyer flask. The ethyl acetate extract was dried with Na_2SO_4 and evaporated to dryness under a stream of nitrogen. In some cases, when only a qualitative identification of the HLA derivative was needed, extraction was accomplished directly with 1.0 N NaOH.

Absorption spectra were taken, after dissolving the dry DNPH derivative in 1.0 N NaOH, in the visible region of the spectrum.

2.2.10.E. Counting of DNPH derivatives.

The dry DNPH derivatives were dissolved in 10 mM Na_2CO_3 and counted in 10 ml of counting solution containing Biq-Solv (section 2.2.7) or, when the radioactive pattern was required, the paper strips (3 cm wide) were cut into 1 cm-long segments and counted in toluene (section 2.2.7).

2.2.11. Protein determination.

Protein was determined using the method of Lowry et al. (1951) and bovine serum albumin was used as standard.

2.2.12. Column chromatography of amino acids.

Analysis of amino acids was carried out with a Beckman automatic amino acid analyzer, model 120B, in a 50x0.9 cm column.

The standard amino acids were prepared in a mixture containing 0.5 μ moles of the ^{14}C -amino acid, 5 mg albumin and water to a final volume of 2 ml. The mixture was deproteinized by TCA at a final concentration of 6.6%. Samples from enzymatic assay mixtures containing 5 mg mitochondrial fraction protein were also deproteinized with TCA.

Amino acids were eluted from the column with 0.2 M Na-citrate, pH 3.28, at 59°. Samples of 0.5 ml each were collected and counted using Bio-Solv in a Beckman instrument (section 2.2.7).

2.2.13. Growth of thiamine- deficient cells.

Cells deficient in thiamine were obtained as follows:

Figure 2.3

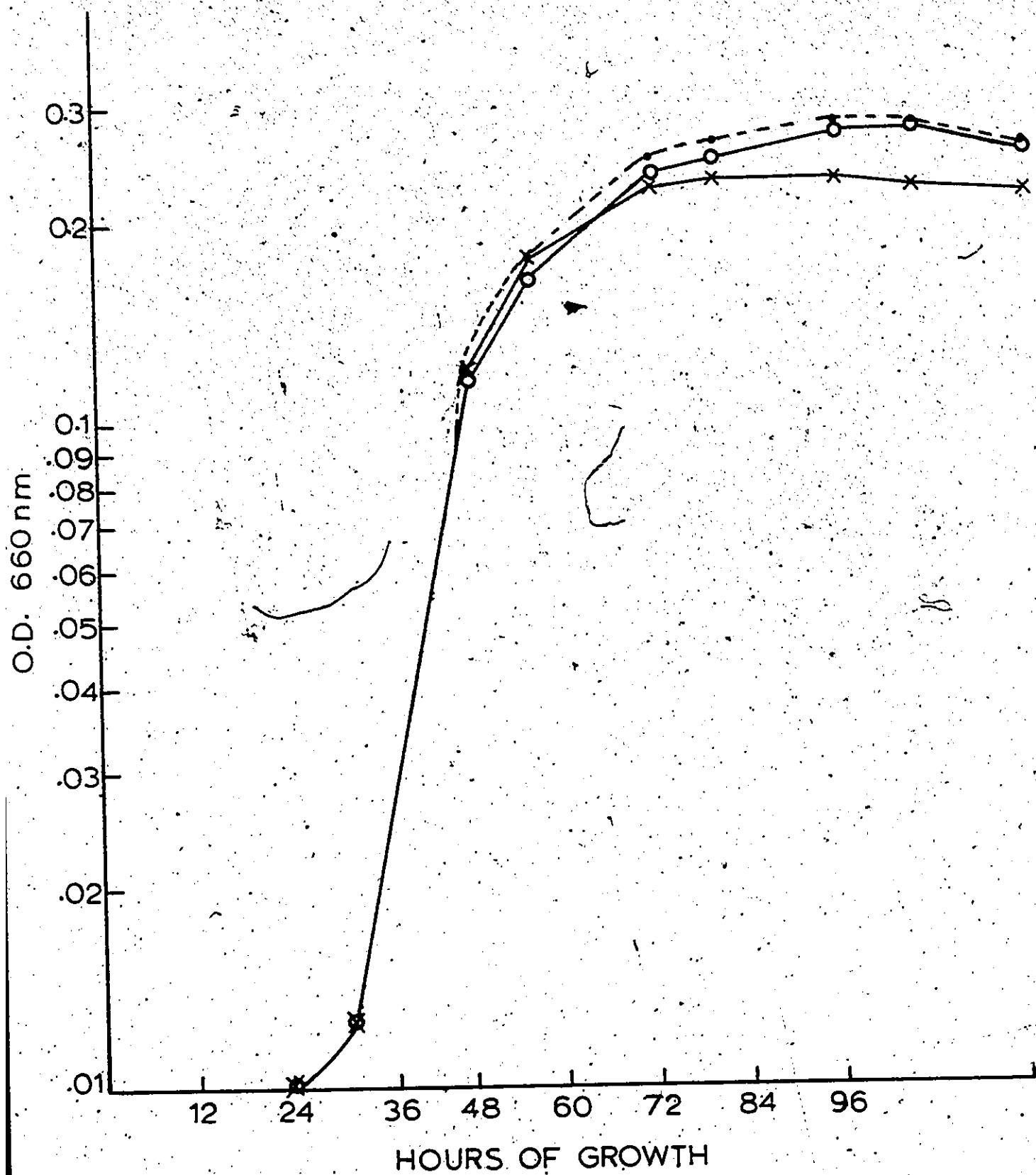


Figure 2.3: Effect of thiamine on the growth of Tetrahymena. 100 ml synthetic medium plus acetate and varying amounts of thiamine were inoculated with 1% inoculum from cultures grown for 3 days in proteose-peptone medium. The normal (control) medium contained 100ug of thiamine (-o-o-). The deficient media contained either 10 ug (-.-.-) or no thiamine (x-x-x-).

1% of cells from parent cultures (section 2.2.10) were inoculated into 100 ml synthetic medium supplemented with acetate but no thiamine (thiamine- deficient parent cultures). These were grown for 4 days and then used to inoculate the experimental cultures. The latter were grown in synthetic medium supplemented with acetate and different amounts of thiamine and were started with a 2% inoculum (v/v) from the thiamine- deficient parent cultures.

Figure 2.3 shows that when organisms previously grown in proteose-peptone medium were transferred to thiamine- deficient medium no significant effect on the growth was observed compared to organisms grown in the presence of the normal (100 μ g) or 10 μ g of thiamine. A marked inhibition of growth was observed after a second transfer of the cells grown in the absence of thiamine to synthetic thiamine- deficient medium (plus acetate). (Fig. 2.4.). Growth in the presence of 1 μ g or 5 μ g of thiamine was normal but it was reduced to about 80% of the control value on the presence of 0.2 μ g of thiamine. The latter concentration was chosen for the growth of partially thiamine- deficient cells.

Figure 2.4

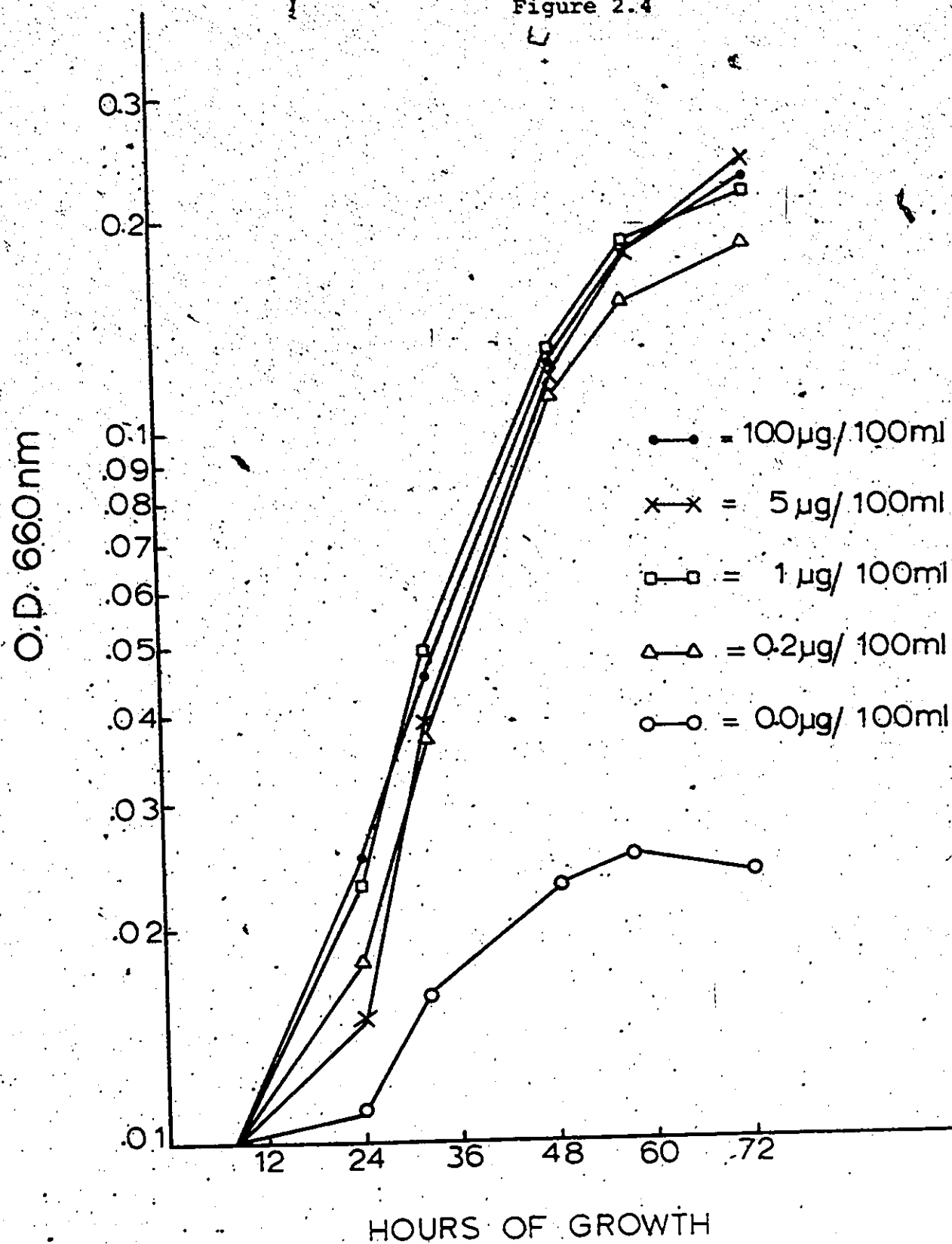


Figure 2.4: Effects of thiamine on the growth of Tetrahymena. 100 ml of synthetic medium plus acetate containing the indicated amounts of thiamine, were inoculated with a 2% inoculum from 4-day old cells grown in thiamine-deficient medium plus acetate, this last culture was obtained as described in Figure 2.3.

CHAPTER 3 : RESULTS

Section 3.1: INTRACELLULAR DISTRIBUTION OF α -KETOGLUTARATE: GLYOXYLATE CARBOXYLASE AND α -KETOGLUTARATE DECARBOXYLASE.

During the studies on the distribution of the α -ketoglutarate:glyoxylate carboxylase activity a major problem was the cell fractionation procedure. The fractionation described by Mager and Lipmann(1956) does not yield subcellular fractions analogous to those obtained for rat liver homogenates and the usual methods for the preparation of mitochondria are not adequate for the ciliate. However, suitable procedures involving gentle homogenization and differential centrifugation have been developed for Tetrahymena (Byfield et al., 1962; Kobayashi, 1965).

The procedures for the subcellular fractionation used for the work in this Thesis are methods A and B described in Methods (section 2.2.4). In this section data are presented on the subcellular distribution of the α -ketoglutarate:glyoxylate carboxylase activity when the original methods were utilized and when some modifications were introduced to them (methods A and B).

Experimental procedures:

i) Method of Mager and Lipmann: Cells were grown for 50 hours in proteose-peptone medium and fractionated as described in method A (section 2.2.4) except that the nuclear fraction was collected as follows: After homogenization with the Teflon-pestle homogenizer had been completed, cell debris and nuclei were sedimented by centrifugation at 1,500xg for 5 min. Two layers were obtained of which the lower was small, brown-gray and well packed and the upper bulky, whitish and loosely packed. When the two layers were resuspended in 0.25 M sucrose buffered with 0.01 M phosphate buffer, pH 7.0, supplemented with 0.001 M BME (medium A) and centrifuged at 500' x g for 5 min the same two layer were obtained. The upper one was collected, washed once and finally suspended in medium A (it will be referred to as "nuclear fraction"). The lower layer, presumably containing intact cells was discarded. The post-nuclear supernatant fluid (homogenate) was processed as described in method A (section 2.2.4) for obtaining the mitochondrial and supernatant fractions.

ii) Method of Kobayashi: Cells were grown for 48 hours in proteose-peptone or for 60 hours in synthetic medium and fractionated as described in method B (section

2.2.4) except that the supernatant fraction was that obtained after the centrifugation at 10,000xg for 5 min without further centrifugation.

Results and discussion: Table 3.1 shows the subcellular distribution of α -ketoglutarate:glyoxylate carboligase activity when the method of Mager and Lipmann was used as described above. It was found that 57% of the units (considering the total units equal to the sum of the nuclear fraction and the homogenate fraction) were recovered in the nuclear fraction with a specific activity of 0.348. The remaining 43% of the homogenate fraction were distributed as follows: 69.0% in the mitochondrial fraction with a specific activity of 0.232 and 31% in the supernatant fraction with a specific activity of 0.039. Somewhat similar distribution was obtained when the Kobayashi method was employed (Table 3.2). About 50% of the units present in the crude homogenate were recovered in the mitochondrial fraction with a specific activity of 1.08 which represented a 4-fold increase in specific activity over that of the crude homogenate. The remaining units were found in the supernatant fraction with a specific activity of 0.16. However, it should be noted

Subcellular fractions	Protein mg/ml	Units ml	Total units	Units mg.prot.	Percent of:	
					nuclear f. +homogenate	homo-genate
Nuclear	12.7	4.41	35.3	0.348	57.4	-
Homogenate	4.1	0.34	26.2	0.083	42.6	100
Mitochondrial	7.8	1.81	18.1	0.232	29.4	69
Supernatant	2.9	0.11	8.3	0.039	13.4	31.4

Table 3.1: Subcellular distribution of α -ketoglutarate: glyoxylate carboligase activity in cells fractionated by the method of Mager and Lipman (1956). The fractions were obtained as described in the text. The enzyme was assayed in the standard conditions with (U-¹⁴C) glyoxylate (285,925 d.p.m.) as the labelled substrate. The reaction was stopped with TCA-AAP. The cells were grown in proteose-peptone medium.

Subcellular fraction	Protein mg/ml	Units ml	Total units	Units mg.prot.	Percent
Crude homogenate	30.8	8.8	105	0.270	100
Mitochondrial	28.1	30.5	51.8	1.086	49
Supernatant	8.3	1.4	54.2	0.162	52

Table 3.2: Subcellular distribution of α -ketoglutarate: glyoxylate carboligase activity in cells fractionated by the method of Kobayashi (1965). Cells were grown in proteose-peptone medium. Crude homogenate represents the initial preparation obtained after disruption of the cells. The enzyme was assayed as described in Methods (section 2.2.5.A), with 10 μ moles of (1- 14 C) glyoxylate (222,727 d.p.m.) and 4 μ moles of α -ketoglutarate for 30 min. The reaction was stopped with TCA-AAP.

that the starting preparations for the mitochondrial preparations were very different in the two methods. In Table 3.2 the crude homogenate corresponded to the homogenate obtained after complete disruption of the cells and no subsequent centrifugation and contained 100% of the enzyme. On the other hand, the homogenate fraction in Table 3.1 was already deficient by about 57% of the units present in the original preparation, which had been lost to the nuclear fraction. Kobayashi had considered that the nuclei were destroyed during homogenization by released proteases (known to be present in high amounts in Tetrahymena, Viswanatha and Liener, 1955; 1956). As a result, the mitochondria which can be sedimented at as little as 600xg would be the heaviest subcellular particles. For this reason the intact cells are removed by centrifugation at 200xg for 2 min. These mitochondria were shown by Kobayashi to have high respiration and phosphorylative efficiency. From this consideration, then, it appears that the carbolligase activity found in the nuclear fraction (Table 3.1) is due to mitochondria that have been sedimented at 1,500xg. As a result of this, a different pattern of activity distribution was observed when the homogenate fraction, in the Mager and Lipmann method, was obtained by

Subcellular fractions	Protein mg/ml	Units ml	Total units	Units mg.prot.	Per cent
Homogenate	22.1	2.68	69.6	0.120	100
Mitochondrial	15.7	2.63	48.9	0.168	70
Supernatant	10.4	0.04	9.1	0.004	13

Table 3.3: Subcellular distribution of α -ketoglutarate: glyoxylate carboligase activity in cells fractionated by method A. Cells were grown in proteose-peptone. Fractionation was carried out by the modified method of Mager and Lipman (1956). The homogenate was obtained by centrifugation at 500xg for 5 min. Assay conditions as described in Table 3.1.

method A after centrifugation at 500xg for 5 min (Table 3.3). Of the total units 70% were recovered in the mitochondrial fraction and only 13% in the supernatant fraction. With this modification the specific activity of the mitochondrial fraction was about 0.16. This modified procedure is that described as method A and was used in some experiments in this Thesis.

In relation to the high percentage of units found in the supernatant fraction with the Kobayashi method (Table 3.2), it seems clear that 10,000xg for 5 min was not enough for sedimenting most of the mitochondria. However, in some experiments this method was preferred because it yielded a better preparation in which the specific activity increased with respect to the homogenate fraction by a factor of 4.

It was later found that when the method B was used for the preparation of mitochondria from cells grown in the synthetic medium containing acetate or glucose the yield of enzyme activity in the mitochondrial fraction was very low. In a systematic study it was found that this procedure yielded a recovery of only about 10% of the mitochondrial fraction as compared to the 50% yield from cells grown in proteose-peptone medium (Table 3.4 a).

Subcellular fractions	Protein mg/ml	Total protein	Units ml	Total units	Units mg prot.	Per cent
a)						
Homogenate	6.8	279	0.797 (0.858)	32.7 (35.2)	0.117 (0.126)	100 (100)
Mitochondrial	12.8	13.5	2.990 (3.788)	3.1 (4.0)	0.233 (0.296)	9 (11)
Supernatant	3.3	104	0.160 (0.156)	5.0 (4.9)	0.048 (0.048)	15 (14)
b)						
Homogenate	6.8	277	0.787 (0.858)	32.3 (35.2)	0.116 (0.126)	100 (100)
Mitochondrial	9.8	137	1.620 (1.718)	22.7 (24.1)	0.165 (0.175)	70 (68)
Supernatant	3.5	114	0.132 (0.127)	4.2 (4.1)	0.037 (0.036)	13 (12)

Table 3.4: Subcellular distribution of α -ketoglutarate:glyoxylate carbolligase activity in cells fractionated by method B.

continued...

Table 3.4: Subcellular distribution of α -ketoglutarate: glyoxylate carboligase activity in cells fractionated by method B. Cells were grown in synthetic medium supplemented with acetate.

- a) cells were fractionated by method B;
- b) cells were fractionated by method B but the mitochondrial fraction was obtained after centrifugation at 12,000xg for 15 min.

Assay conditions were those described in Table 3.1, with (1-¹⁴C) glyoxylate (178,482 d.p.m.)

Figures in parentheses represent the α -ketoglutarate decarboxylase activity assayed as described in Methods (section 2.2.5.B).

That this was not due to inactivation of the enzyme during the preparation of the mitochondrial fraction is shown in Table 3.4,b, where the mitochondrial fraction obtained after centrifugation at 12,000xg for 15 min contained 70% of the units present in the crude homogenate. Parallel assays of the α -ketoglutarate decarboxylase activity in these fractions were also performed. The same distribution was found for this enzyme also, suggesting either that the mitochondria from cells grown in synthetic medium were more fragile yielding particles upon homogenization which co-sedimented with the components of the fluffy layer which overlays the mitochondrial pellet, or that lighter mitochondria were obtained from cells grown in synthetic medium as compared with those obtained from cells grown in proteose-peptone medium. The possibility that qualitatively different mitochondria are obtained from cells grown in nutritionally different media is supported by the different sedimentation values reported for mitochondria. Byfield et al., (1962) reported that they were able to obtain mitochondria from cells grown in proteose-peptone medium under vigorous aeration by centrifuging between 5,000 and 100,000xg. On the other hand, Kobayashi has reported sedimentation of mitochondria by centrifugation between 200

and 10,000xg from cells grown in proteose-peptone but without aeration. It would then appear that under the latter conditions larger mitochondria were present. We have also observed (section 3.9.2.) that cells grown in proteose-peptone were much larger from static cultures than from shaken cultures. It would not then be surprising that cells grown in our synthetic or proteose-peptone media would yield mitochondria of different size.

In summary, most of the α -ketoglutarate:glyoxylate carboligase activity in Tetrahymena is in the mitochondrial fraction as obtained by the methods used in this work and only 15% of the total activity was found in the supernatant fraction obtained after centrifugation at 12,000xg. α -Ketoglutarate decarboxylase gave the same distribution pattern to that of the carboligase.

(Koch et al, (1967) reported that the α -ketoglutarate:glyoxylate carboligase was present not only in the mitochondria but also in the soluble fractions of human liver cells (about 30%) and human spleen and kidney cells (about 50%). The cells were fractionated by the procedure of Hogeboom (1955) and the supernatant fraction was obtained at 27,000xg. On the other hand, distribution studies with different rat tissues showed negligible activity in the

supernatant fraction after centrifugation at 105,000xg (Schlossberg et al., 1970).

Section 3.2: STUDIES ON THE CO_2 FORMED DURING THE REACTION.

3.2.1. Origin of the $^{14}\text{CO}_2$.

Experimental procedures. Cells were grown for about 55 hours in proteose-peptone medium. The mitochondrial fraction was obtained by method A (section 2.2.4): Assay conditions were as described in Methods (section 2.2.5.A). The labelled substrates were α -(1- ^{14}C) ketoglutarate, α -(5- ^{14}C) ketoglutarate, (U- ^{14}C) glyoxylate, (1- ^{14}C) glyoxylate and (2- ^{14}C) glyoxylate.

Results. It is clear from Table 3.5 that neither the C_2 from glyoxylate nor the C_5 from α -ketoglutarate contributed to the label of CO_2 evolved after acidification of the reaction mixture. Clearly the label originated at the C_1 of glyoxylate (Exper. 1). The labelled CO_2 also originated from the C_1 of α -ketoglutarate as shown by experiment 2 (Table 3.5). There was no significant decarboxylation when either α -ketoglutarate or glyoxylate

Labelled substrate	Assay conditions	$\mu\text{moles}^{14}\text{CO}_2/\text{hr}$
Experiment 1		
(1- ^{14}C) glyoxylate (2.2×10^5 d.p.m.)	Complete	1.305
	Enzyme omitted	0.009
(2- ^{14}C) glyoxylate (2.0×10^5 d.p.m.)	Complete	0.002
	Enzyme omitted	0.001
α -(5- ^{14}C) ketogluta- rate (1.0×10^5 d.p.m.)	Complete	0.060
	Enzyme omitted	0.006
Experiment 2		
(U- ^{14}C) glyoxylate (3.0×10^5 d.p.m.)	Complete	0.665
	α -ketoglutarate omitted	0.019
	Enzyme omitted	0.005
α -(1- ^{14}C) ketogluta- rate (1.2×10^5 d.p.m.)	Complete	0.541
	Glyoxylate omitted	0.054
	Enzyme omitted	0.017

Table 3.5: Origin of the $^{14}\text{CO}_2$ from the labelled substrates.

continued...

Table 3.5: Origin of the $^{14}\text{CO}_2$ from the labelled substrates. Incubations were carried out in the standard conditions of α -ketoglutarate:glyoxylate carboligase (section 2.2.5.A) with the indicated omissions. In experiment 1, mitochondrial fraction (8 mg) were incubated with 4 μ moles of glyoxylate and 4 μ moles of α -ketoglutarate, the labelled substrate being that indicated in the Table. The reaction was carried out for 60 min and stopped with PCA. In experiment 2, the mitochondrial fraction was incubated with 2 μ moles of glyoxylate and 2 μ mples of α -ketoglutarate. When glyoxylate was the labelled substrate the reaction (60 min) was stopped with TCA-AAP. When α -ketoglutarate was the labelled substrate, with PCA.

were omitted (exper. 2) or in absence of mitochondria. In conclusion, the labelled CO_2 was a mixture of C_1 from both glyoxylate and α -ketoglutarate and the reaction required the mitochondrial fraction.

3.2.2. Kinetics of the $^{14}\text{CO}_2$ -release in the different assay systems.

Experimental procedures. Cells were grown for about 55 hours in proteose-peptone medium. The mitochondrial fraction was obtained by method A (section 2.2.4). Three series of flasks were prepared (A, B, and C) for the assay of the enzyme (section 2.2.5.A). In series C, 2 μmoles of α -(1- ^{14}C) ketoglutarate (134,4000 d.p.m.) was the labelled substrate. In series A and B 2 μmoles of (U- ^{14}C) glyoxylate (296,655 d.p.m.) was the labelled substrate. After 1 hour of incubation one flask from each series was removed without further addition, for $^{14}\text{CO}_2$ estimation. In these flasks the nonenzymatic reaction (2) (section 2.2.5) was very low and the flasks would serve as zero time controls for the nonenzymatic reaction (after addition of acid) in the remaining flasks. The flasks of series A and C received PCA and those of series B received TCA-AAP mixture and incubation was continued for another

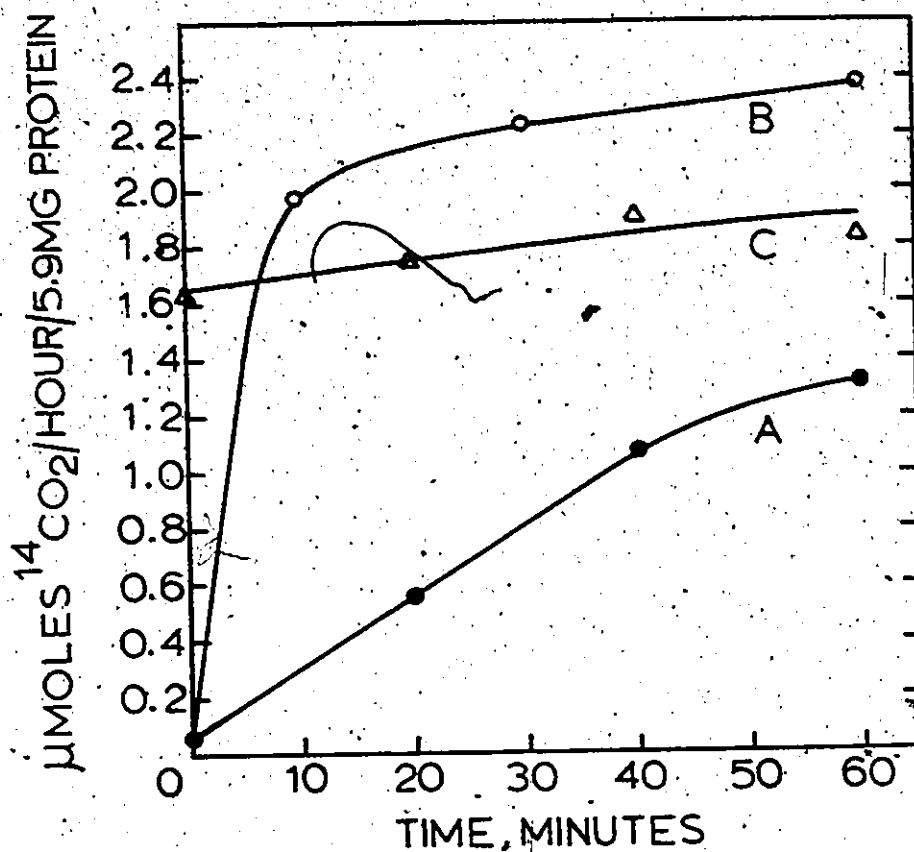


Figure 3.1: $^{14}\text{CO}_2$ production as a function of time after stopping the reaction in the carbamoylase assay: A, (U- ^{14}C) glyoxylate, stopped with PCA; B, (U- ^{14}C) glyoxylate, stopped with TCA plus AAP; C, (L- ^{14}C) - ketoglutarate, stopped with PCA. Mitochondrial fraction protein (1.18mg) was present in each flask.

60 min. The rate of $^{14}\text{CO}_2$ evolution was followed by removing flasks at various time intervals.

Results and discussion. As can be seen in Figure 3.1 little $^{14}\text{CO}_2$ was detected after 60 min of incubation of the complete assay mixture when the labelled substrate was (U- ^{14}C) glyoxylate (zero time for curves A and B). Rapid production of $^{14}\text{CO}_2$ occurred upon addition of TCA-AAP (curve B) and a slower one upon addition of PCA (curve A). When the labelled substrate was (1- ^{14}C) ketoglutarate, addition of PCA resulted in little further increase in $^{14}\text{CO}_2$ production (curve C). The results support the presence of reaction (1) during the assay and of reaction (2) after addition of PCA and AAP (section 2.2.5.A) and are in agreement with the data of Koch and Stokstad (1966) for the rat liver mitochondria.

Section 3.3. COFACTOR REQUIREMENTS OF α -KETOGLUTARATE: GLYOXYLATE CARBOXYLASE.

Experimental procedures. Cells were grown in proteose-peptone medium. The enzyme sources were: (i) Cell extracts: the washed cells were suspended in 0.25 M sucrose and kept frozen at -20° . After one day they were thawed and sonicated for 1 min (section 2.2.3). (ii) the homogenate and the

mitochondrial fractions were obtained by method A (section 2.2.4). α -Ketoglutarate:glyoxylate carboligase was assayed in the standard assay conditions with the omissions or additions described in the legends of the appropriate tables. (U- 14 C) glyoxylate was the labelled substrate. Incubations were carried out for 1 hour and the reactions were stopped by addition of PCA.

Results and discussion. In the presence of the total cell extract it was found (Table 3.6) that TPP was required for maximal carboligase activity. In the absence of added TPP the activity dropped to about 50% of the control after a 20 hour dialysis and to 10% with the longer dialysis. α -Ketoglutarate was also required for maximal activity, however, an absolute requirement could not be demonstrated even after 72 hours of dialysis. No requirement at all could be demonstrated for magnesium.

With mitochondrial preparations α -ketoglutarate was an absolute requirement (exp. 1, Table 3.7 and exp. 2, Table 3.5). TPP was required for maximal activity but 50% of the activity was still found in its absence, and the complete requirement was demonstrated after 43 hours of dialysis. As with the total cell extract no magnesium

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Assay conditions	m units/mg protein	
	Dialyzed 20 hrs	Dialyzed 72 hrs
Complete	26	9
- Mg^{+2}	29	12
- TPP	12	0.9
- Mg^{+2} , - TPP	11	0.7
- α -ketoglutarate	8	5

Table 3.6: Requirements of α -ketoglutarate:glyoxylate carboligase in the total cell extract. The enzyme was assayed in the standard conditions (section 2.2.5.A) but with ($U-^{14}C$ -glyoxylate (300,000d.p.m.) as the labelled substrate. The reaction was stopped with PCA. The cell extract was dialyzed for 20 hours against 150 volumes of 10 mM K-phosphate, pH 7.0, supplemented with 1 mM BME.

Assay conditions	m units/mg protein	
	Undialyzed	Dialyzed
Experiment 1		
Complete	89	74
- Mg^{+2}	109	75
- Mg^{+2} , + EDTA (1 mM)	-	2
- TPP	45	8
- TPP, - Mg^{+2}	43	6
- α -ketoglutarate	8	2
Boiled mitochondrial fraction (10 min)	0.4	0.2
Experiment 2		
- Mg^{+2}	128	
- Mg^{+2} , + EDTA (1 mM)	19	
+ Mg^{+2} (0.25 mM), + EDTA (1 mM)	43	
+ Mg^{+2} (0.50 mM), + EDTA (1 mM)	72	
+ Mg^{+2} (2.50 mM), + EDTA (1 mM)	120	
+ Mn^{+2} (0.25 mM), + EDTA (1 mM)	28	
+ Mn^{+2} (0.50 mM), + EDTA (1 mM)	67	
+ Mn^{+2} (2.50 mM), + EDTA (1 mM)	125	

Table 3.7: Requirements of α -ketoglutarate:glyoxylate carboligase of undialyzed and dialyzed mitochondrial fractions. The enzyme was assayed as indicated in Table 3.6. In experiment 1 dialysis was against two changes of 200 volumes of 10 mM K-phosphate, pH 7.0, supplemented with 1 mM BME, for 43 hours.

requirement could be demonstrated. However, EDTA at a final concentration of 1 mM totally inhibited the reaction. The requirement for glyoxylate was demonstrated using α -(1- ^{14}C) ketoglutarate as the labelled substrate as shown in experiment 2, Table 3.5. Boiled mitochondrial fraction was unable to produce labelled CO_2 when present in the complete reaction mixtures (exp. 1, Table 3.7), indicating that the reaction is enzyme-catalyzed.

Additions of Mg^{+2} or Mn^{+2} to assay mixtures in which EDTA was present completely restored the activity (exp. 2, Table 3.7). Dialysis against the usual buffer supplemented with 1 mM EDTA irreversibly inactivated the enzyme (Table 3.8). The effect of EDTA and the requirements for the carbolligase reaction reported in the literature are summarized in Table 3.9. In all the cases α -ketoglutarate was necessary for the reaction as measured by the decarboxylation of glyoxylate. TPP was required in different degrees. An absolute requirement was found for the enzyme from Mycobacterium phlei (Yamasaki and Moriyama, 1971) and a slight one with a cytoplasmic preparation from human kidney (Koch et al., 1967). The highest requirement for Mg^{+2} has been reported with a partially purified rat-liver



Subcellular fraction	m units/mg protein		Inactivation %
	Undialyzed	Dialyzed	
Crude homogenate	28.2	0.7	97.6
Mitochondrial	186.3	30.1	83.5
Supernatant	5.9	1.2	80

Table 3.8: Effect of dialysis against EDTA on α -keto-glutarate:glyoxylate carboligase.

The enzyme was assayed as indicated in Table 3.6. Dialysis was carried out for 20 hours against 10 mM K-phosphate buffer, pH 7.0, containing 1 mM BME and 1 mM EDTA.

Preparation	+EDTA	- Mg ⁺²	- TPP	- α-keto-glutarate
<u>Mycobacterium phlei</u>				
purified enzyme (1).....	7%	71%	0%	0%
<u>Rhodopseudomonas</u>				
<u>speroides</u> , partially				
purified enzyme (2).....		53%	34%	7%
Rat liver mitochondria, partially purified				
enzyme (3).....		57%	48%	5%
Pig-liver mitochondria, purified enzyme (4).....	0%	55%	83%	1%
Rat liver mitochondria(5).. Rat liver mitochondria, partially purified (6).....		100%		less than 10%
Human-kidney mitochondrial enzyme (7)...		52%	58%	5%
cytoplasmic enzyme (7).....		74%	78%	3%
		87	89%	3%
<u>Tetrahymena pyriformis</u>				
mitochondrial fraction present work.....	2%	100%	11%	3%

Table 3.9: Effect of EDTA and cofactor and substrate requirements for α-ketoglutarate:glyoxylate carboligase reported in the literature. The figures represent the percentage of the activity left after the indicated addition or omission.

- (1) Yamasaki and Moriyama (1971); (2) Okuyama et al., (1965);
 (3) Nakada and Sund (1958); (4) Steward and Quayle (1967);
 (5) Chrawhall and Watts (1962); (6) Koch and Stockstad (1966)
 (7) Koch et al., (1967).

mitochondrial enzyme (Koch and Stockstad, 1966) where the activity of the enzyme dropped to about 50% compared to that obtained in the presence of added Mg^{+2} . In one case no magnesium requirement at all was observed (Crawhall and Watts, 1962) and when tested (Table 3.9) EDTA strongly inhibited the reaction.

These data from the literature and our own suggest that magnesium (or manganese) is strongly bound to the enzyme and may be effectively removed by EDTA. This is supported by the reversal of EDTA inhibition upon addition of the metals (Table 3.7). However, magnesium did not reactivate the enzyme dialyzed in the presence of EDTA. It is possible that the long exposure time to EDTA during dialysis resulted in irreversible structural change with a concomitant loss of catalytic activity.

Section 3.4: CO_2 EVOLUTION IN THE ABSENCE OF α -KETOGlutARATE.

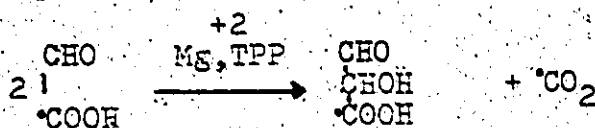
The limited $^{14}CO_2$ evolution before addition of PCA or the TCA-AAP mixture was inhibited by 59% rather than stimulated by α -ketoglutarate (Table 3.10). This inhibitory effect of α -ketoglutarate on the decarboxylation

Substrate	$\mu\text{moles } ^{14}\text{CO}_2/\text{hr}$ after the following additions		
	None	PCA	TCA-AAP
Glyoxylate	0.044	0.090	0.182
Glyoxylate <u>plus</u> α -ketoglutarate	0.018	0.218	0.492
% inhibition or enhancement by α -ketoglutarate	inhibition 59%	enhancement 59%	enhancement 63%

Table 3.10: Effect of α -ketoglutarate on the decarboxylation of glyoxylate in the absence and presence of the nonenzymatic decarboxylation of the reaction product. Homogenate (3.3 mg) obtained by method A (Section 2.2.4) was incubated with or without α -ketoglutarate under the assay conditions for α -ketoglutarate:glyoxylate carboxylase (section 2.2.5.A) with (U- ^{14}C) glyoxylate (295,365 d.p.m.) as the labelled substrate. The reaction was carried out for 30 min and the $^{14}\text{CO}_2$ estimated without further additions, or after an additional 30 min incubation with either PCA or TCA-AAP.

of glyoxylate was observed on several occasions with cells homogenates but not with mitochondrial fractions.

The evolution of $^{14}\text{CO}_2$, in the absence of α -ketoglutarate and its inhibition by the latter is suggestive of the presence of glyoxylate carboligase in Tetrahymena. The latter enzyme catalyzes the condensation of two molecules of glyoxylate to yield tartronic semialdehyde and CO_2 as shown below:



The reaction also required magnesium and TPP for maximal activities (Krakow et al., 1961). The assay conditions for α -ketoglutarate:glyoxylate carboligase are therefore favorable for measurement of the glyoxylate carboligase when α -ketoglutarate is omitted from the reaction mixtures and the observed inhibition may reflect the competition on the part of α -ketoglutarate:glyoxylate carboligase for the common substrate glyoxylate. It is likely that the residual activity observed with the dialyzed cell extracts in the absence of α -ketoglutarate (Table 3.6) is due to the presence of the glyoxylate carboligase, at least in part.

Assay system	mmoles CO ₂ /hr
Complete - α -ketoglutarate	100
Mg ⁺² omitted	42
TPP omitted	15
Mg ⁺² , TPP omitted	8
Enzyme omitted	2

Table 3.11: Cofactor requirements of glyoxylate decarboxylation in the absence of added α -ketoglutarate. Assay conditions are those described in Table 3.6, except that α -ketoglutarate was omitted. The homogenate obtained by method A (section 2.2.4) (3.1 mg) was used as the enzyme source without prior dialysis. After 30 min the reaction was stopped with PCA and incubation was extended for another 30 min. 290,595 d.p.m. were present as (U-¹⁴C)glyoxylate.

Table 3.11 shows that the cofactor requirements for $^{14}\text{CO}_2$ evolution in the absence of α -ketoglutarate were quantitatively distinct from those obtained in its presence (Table 3.6). The difference is particularly prominent in the case of magnesium whose absence cause a drop of 58% in decarboxylation whereas it did not affect the activity in the presence of α -ketoglutarate (Table 3.7).

Attempts, not described in this Thesis, to identify tartronic semialdehyde from the appropriate reaction mixtures failed, however, to produce more definitive evidence for the presence of glyoxylate carboligase in Tetrahymena.

Section 3.5: PRODUCT IDENTIFICATION

Experimental procedures. Cells were grown in proteose-peptone medium for about 55 hours. The enzyme source was either whole cell extracts obtained by sonication (section 2.2.3) or mitochondrial fraction obtained by method A (section 2.2.4). Incubations were carried out for 60 min with the omissions or additions indicated in the legends of the figures. DNPH derivatives were formed by adding 2,4-DNPH reagent to the acidified,

deproteinized assay mixture or directly to the assay mixture, and, when indicated, PCA was added 30 min later. The crystals of DNPH derivatives formed were collected and chromatographed as described in Methods (section 2.2.10). The solvent system n-butanol:ethanol:2 N NH_3 (7:1:2 v/v) is referred to as solvent system 1 and the solvent system ethanol:water (83:17 v/v) as solvent system 2. Identification of the DNPH derivatives was accomplished by their migration on the chromatograms. The mono derivatives are of yellow color and the bis derivatives (osazones) are of red-brown or orange color. Further identification was accomplished by spraying the chromatograms with a saturated solution of NaOH in ethanol. After this treatment DNPH derivatives yield the following colors: α -ketoglutarate, dark green; hydroxylevulinic acid (HLA), blue, (characteristic of bis derivatives); one isomer of glyoxylate (slow-moving) brown-orange, and the other isomer, yellow. Absorption spectra were taken after extracting the derivatives from the chromatograms with 1N NaOH. The radioactive pattern of the chromatograms was obtained by cutting the paper strips in 1 cm-long segments and counting in Toluene-PPO counting solution (section 2.2.10).

Results and discussion. Paper chromatography with solvent system 1 of authentic DNPH-HLA and DNPH derivatives obtained from the acidified mixtures, with the cell extract as the enzyme source, is shown in Figure 3.2. When solvent system 1 was used (Fig. 3.2 a) three yellow compounds were obtained from the control assay mixture (enzyme omitted) corresponding to the DNPH derivatives of α -ketoglutarate (R_f 0.057), and the two glyoxylate isomers (R_f 0.407 and 0.634) (chromatogram A). An additional red-brown DNPH derivative was obtained when the complete assay was used, which turned blue after spraying with ethanolic NaOH (chromatogram C). Authentic DNPH-HLA also yielded a red-brown color which turned blue after spraying with ethanolic NaOH (chromatogram B). Authentic DNPH-HLA and the slowest-moving of the two glyoxylate derivatives overlapped in this system.

Complete separation was obtained when solvent system 2 was used (Fig. 3.2b). In this system the R_f values for the DNPH derivatives of HLA and α -ketoglutarate were 0.15 and 0.05, respectively, and the R_f values for the two glyoxylate isomers were 0.26 and 0.35. The DNPH derivatives of both the enzymatic product and authentic HLA displayed

Figure 3.2: Paper chromatography of authentic DNPH-HLA and DNPH derivatives from the acidified assay mixtures. Dialyzed cell extracts (21 mg) were incubated in the standard conditions as described in Table 3.6. DNPH derivatives were formed in the deproteinized assay mixtures. The crystals were collected and chromatographed on paper, a) in solvent system 1 for 18 hours by the ascending method, b) in solvent system 2 for 8 hours by the descending method. A and D, from the control assay mixtures (no enzyme); B and E, authentic DNPH-HLA; C and G, from the complete assay mixtures; F from a control assay with added authentic HLA. 1 corresponds to DNPH-d-ketoglutarate; 2 and 4 to DNPH-glyoxylate; 3 to DNPH-HLA.

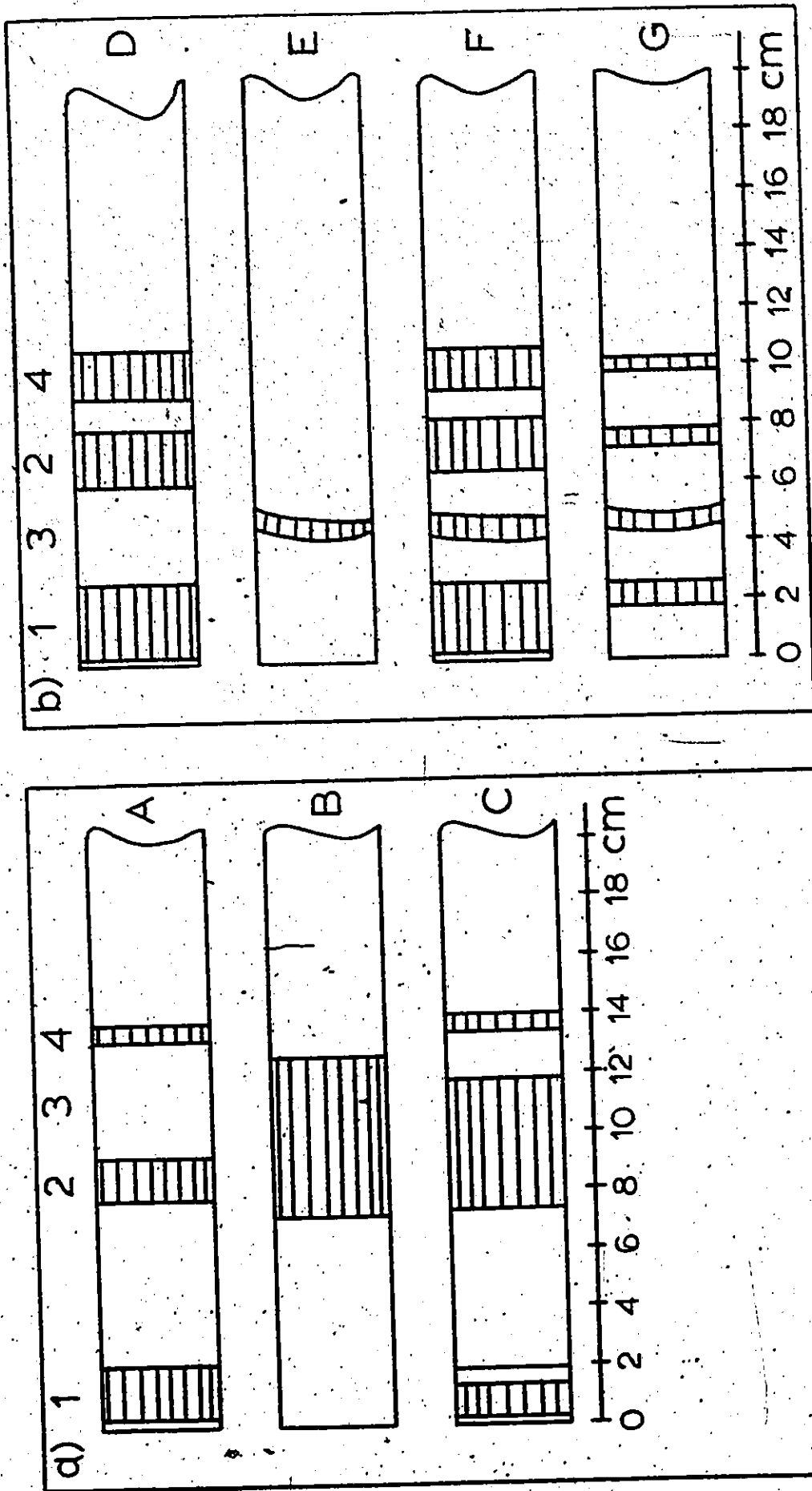


Figure 3.2

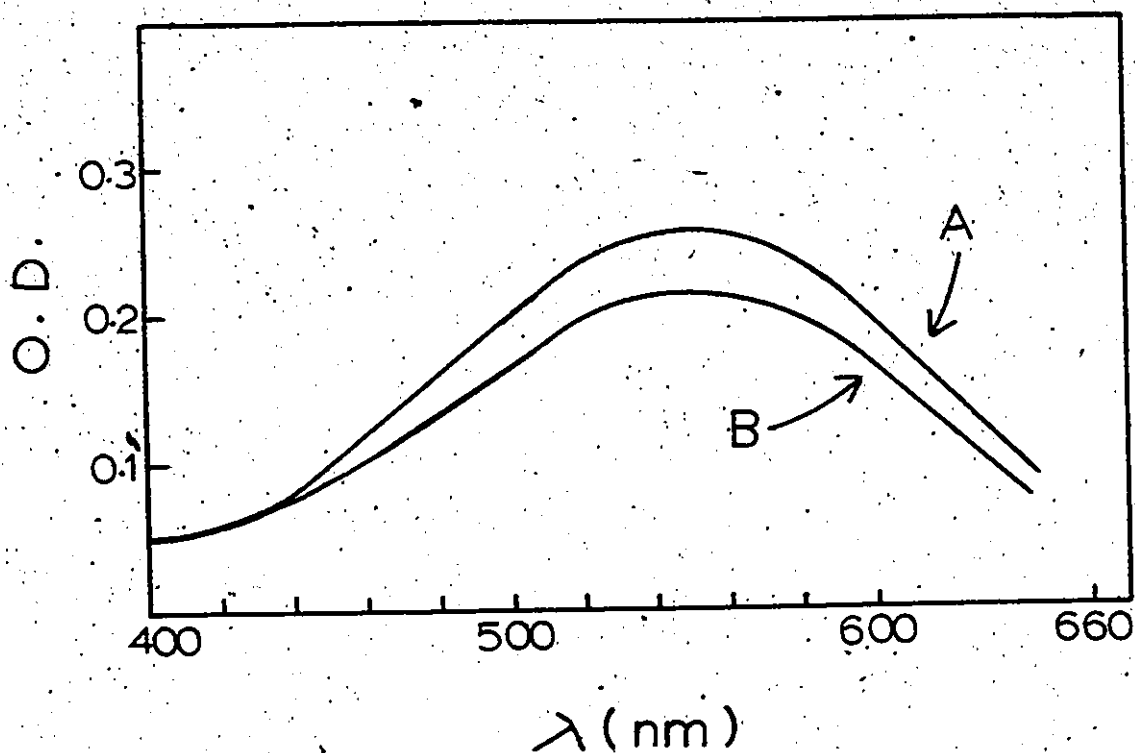


Figure 3.3: Absorption spectrum of authentic and enzymatic DNPH-HLA. The DNPH derivative was extracted with 1 N NaOH from paper chromatograms developed with solvent system 2, and the absorption spectrum determined. A) from the complete assay mixture; B) from authentic DNPH-HLA.

the same broad absorption spectrum with a peak at 550 nm, after extraction with 1N NaOH from the paper chromatograms (Fig 3.5).

When the cell extract was incubated in the standard assay mixture with (U- 14 C) glyoxylate as the labelled substrate and the DNPH derivatives were chromatographed with solvent system 1, a radioactive peak was obtained at the position of DNPH-HLA (Fig. 3.4a). This peak overlaps with the slower-moving glyoxylate derivative as a comparison with the control chromatogram (boiled extract) shows.

In some chromatograms from complete assay mixtures a weakly labelled, faint blue-colored compound was observed near the origin and slightly ahead of the α -ketoglutarate derivative after spraying with ethanolic NaOH (Fig. 3.4a). This compound will be referred to as DNPH-X and its origin was more thoroughly investigated later (section 3.6).

As is shown in Figure 3.4b, when TPP was omitted from the reaction mixture containing a dialyzed cell extract, much less blue color intensity was observed and less radioactivity was counted in the chromatogram of DNPH derivatives as compared to the chromatogram obtained from the complete assay mixture (Fig. 3.4a). When

Figure 3.4: Radioactivity distribution of DNPH derivatives on paper chromatography. The cell extract was dialyzed and assayed under the conditions described in Table 3.6. The crystal of DNPH derivatives formed in the deproteinized assay mixtures were chromatographed in solvent system 1 for about 18 hours. After spraying with saturated ethanolic NaOH the paper strips were cut and counted as described in the Experimental Procedures. The numbers correspond to DNPH derivatives as indicated in Figure 3.2. In a, solid line, complete reaction mixture; dashed line, control (boiled extract). In b, solid line, complete minus magnesium; dashed line, complete minus TPP.

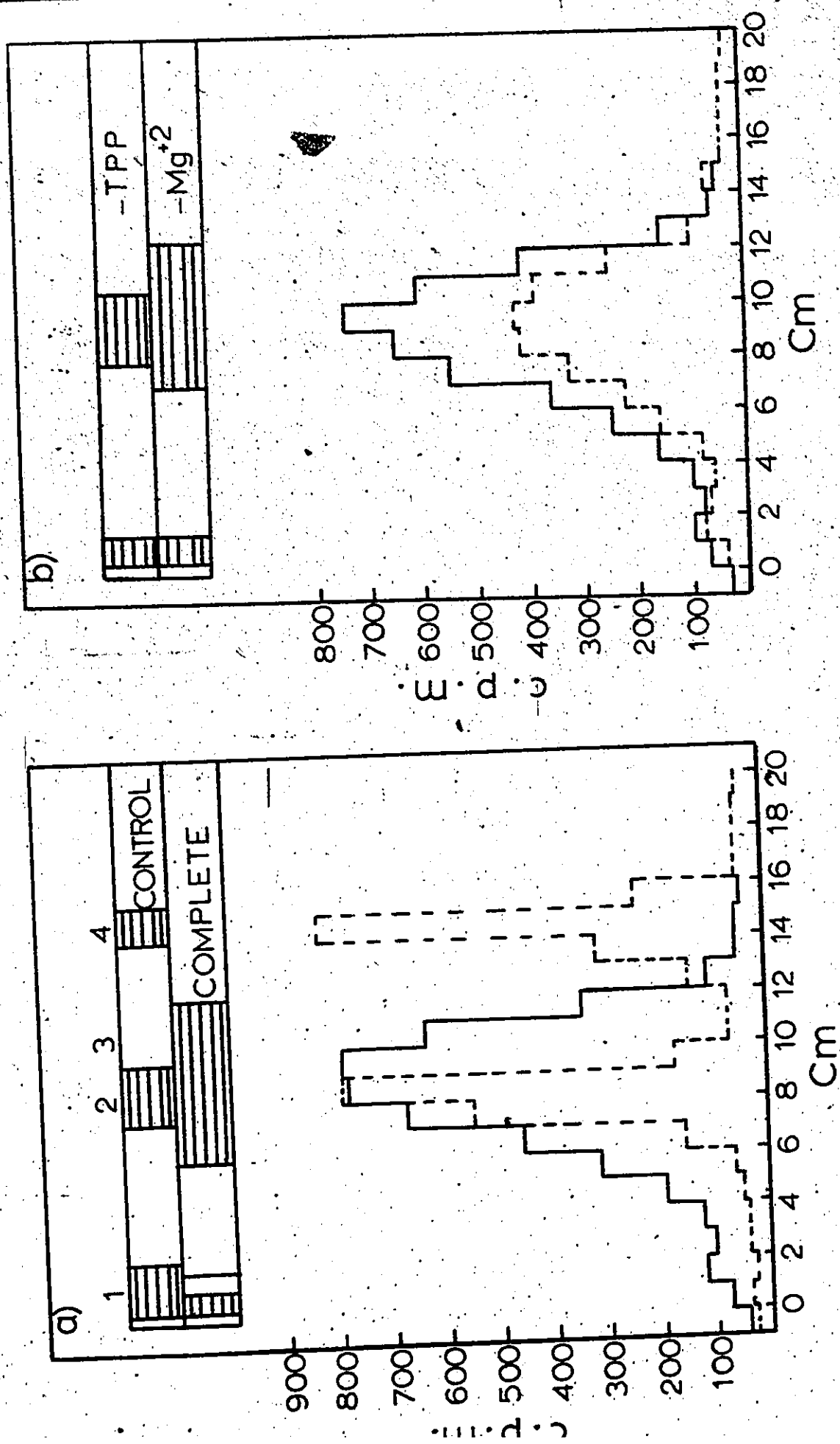


Figure 3.4

magnesium was omitted, both color intensity and radioactivity of the DNPH-HLA were about the same as those obtained when magnesium was present (Fig. 3.4a) and 3.4b). Similar response to these cofactors had been observed when the $^{14}\text{CO}_2$ was measured (section 3.3, Table 3.6). There was then a correlation between $^{14}\text{CO}_2$ evolved and HLA formation which indicated that both products were formed in parallel under the assay conditions for the α -ketoglutarate:glyoxylate carboligase reaction. Furthermore, as in the case of carbon dioxide evolved (section 3.3, Table 3.7), the requirement of magnesium for HLA formation could be demonstrated only when EDTA at a final concentration of 1 mM was added to the assay mixture from which magnesium was omitted (Fig. 3.5a) as judged by both color intensity and radioactivity. The formation of HLA was not affected by the omission of magnesium or the simultaneous presence of 1 mM EDTA and 2.5 mM magnesium, as a comparison of Figs. 3.5a and 3.5b shows.

The labelling pattern of CO_2 and HLA in conjunction was next studied by assaying mitochondrial fractions with either (1- ^{14}C) glyoxylate or (2- ^{14}C) glyoxylate in an atmosphere of either air or nitrogen. The following were studied: a) $^{14}\text{CO}_2$ formation after one hour of enzymatic

Figure 3.5: Cofactor requirements of DNP-HLA formation. Undialyzed mitochondrial fractions were incubated with the indicated additions or omissions, as described in Table 3.7. DNP derivatives were formed in the deproteinized assay mixtures, and the crystal chromatographed in solvent system 2 for 8 hours, by the descending method. The papers after spraying with saturated ethanolic NaOH were cut and counted as described in the Experimental Procedures.

a) complete assay mixtures with added EDTA ($\text{EDTA} + \text{Mg}^{+2}$) is shown in solid line; complete assay without magnesium and supplemented with EDTA ($\text{EDTA} - \text{Mg}^{+2}$) with broken line. b) complete assay mixture (or without magnesium) is shown in solid line; control assay mixture (no enzyme), with broken line.

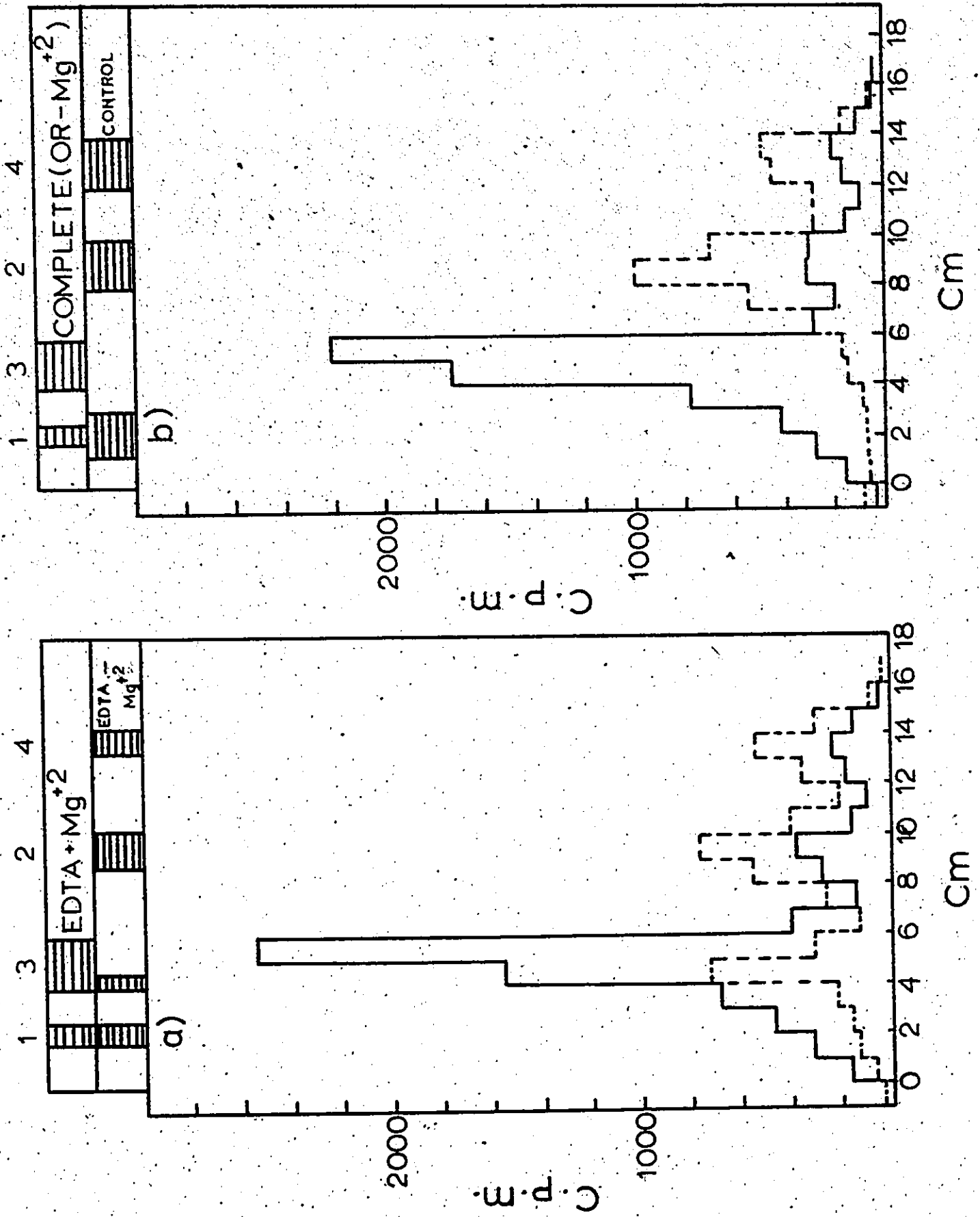


Figure 3.5
 PROFILE OF AGRICULTURE

reaction. b) $^{14}\text{CO}_2$ formation after two additional hours in the presence of PCA. c) Formation of DNPH derivatives in the assay mixtures immediately after one hour of reaction. These mixtures will be referred to as "zero time in acid". d) Formation of DNPH derivatives after an additional two hours in the presence of PCA. In case (c) PCA was also added 30 min after the addition of the 2,4-DNPH reagent so that the conditions of crystal formation be similar with those in case (d). The radioactive distribution after paper chromatography of the DNPH derivatives from the "zero time in acid" mixtures are shown in Figure 3.6 and 3.7. HLA was labelled when (2- ^{14}C) glyoxylate was the labelled substrate indicated by a broad peak of radioactivity at the position of DNPH-HLA when the complete assay was used. A sharp peak corresponding to the slow-moving glyoxylate isomer was obtained in the control assay. That DNPH-HLA was formed was also indicated by the blue area on the chromatogram, typical of this substance, after spraying with NaOH in ethanol (Fig. 3.6). On the other hand, when (1- ^{14}C) glyoxylate was the labelled substrate (Fig. 3.7) the DNPH-HLA formed was not labelled since only two sharp radioactivity peaks corresponding to the glyoxylate isomers were observed. The

Figure 3.6: Radioactive distribution of DNPH derivatives from the "zero time in acid" mixtures incubated under N_2 with $(2-^{14}C)$ glyoxylate as the labelled substrate. 2.4 mg of mitochondrial fraction (from cells grown in proteose-peptone) were incubated in the standard conditions, except that the reaction was stopped by adding 2,4-DNPH reagent, as indicated in Experimental Procedures. $(2-^{14}C)$ glyoxylate (4μ moles; 215,440 d.p.m.) was the labelled substrate. The crystals of DNPH derivatives were collected and an aliquot was chromatographed with solvent system 1 for 26 hours. After spraying with saturated ethanolic NaOH the papers were cut and counted. The numbers indicate the position of DNPH derivatives as described in Fig. 3.2 and X represents an unknown compound. The complete assay is shown in solid line; the control assay, (no enzyme) in broken line.

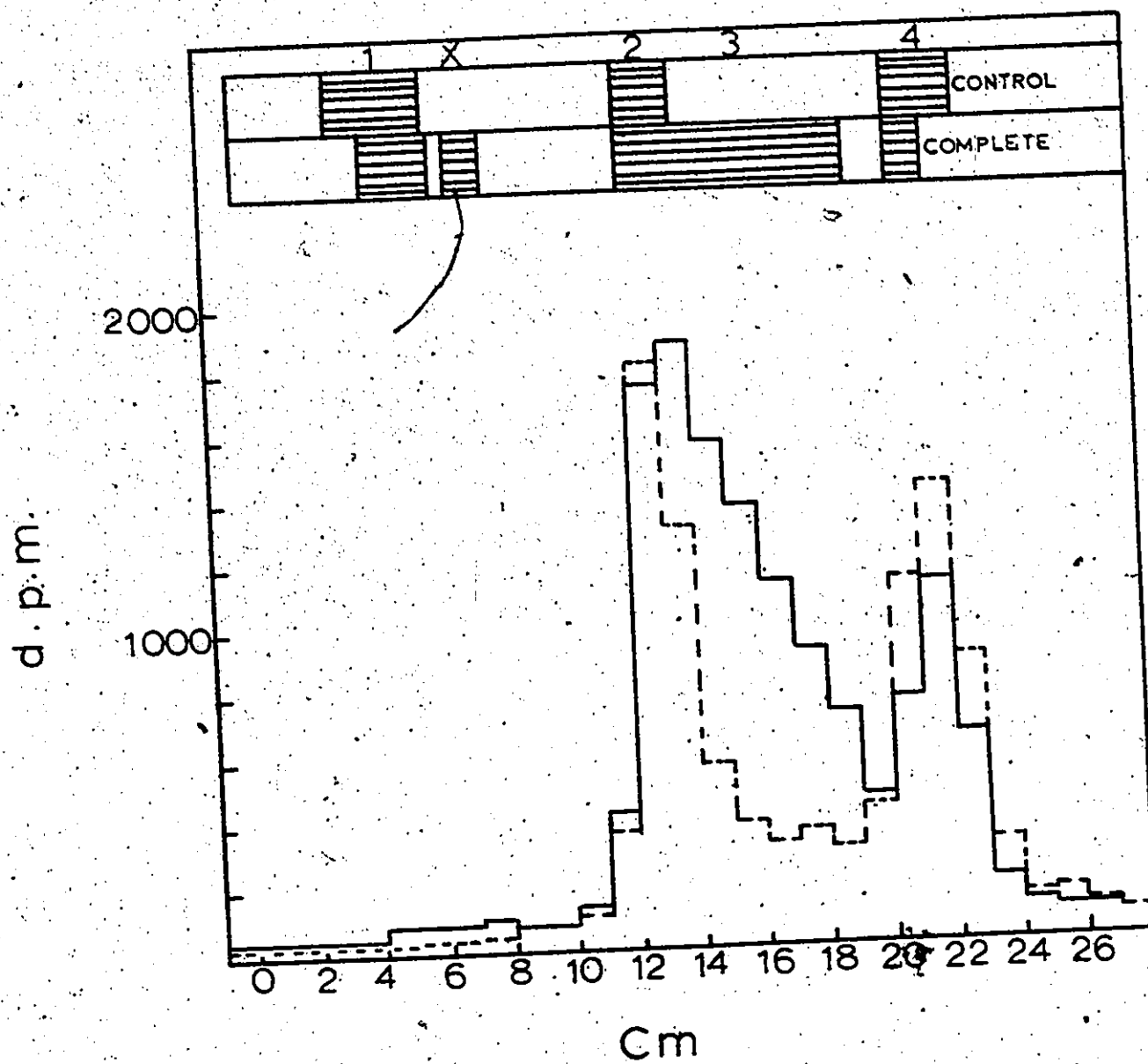


Figure 3.6

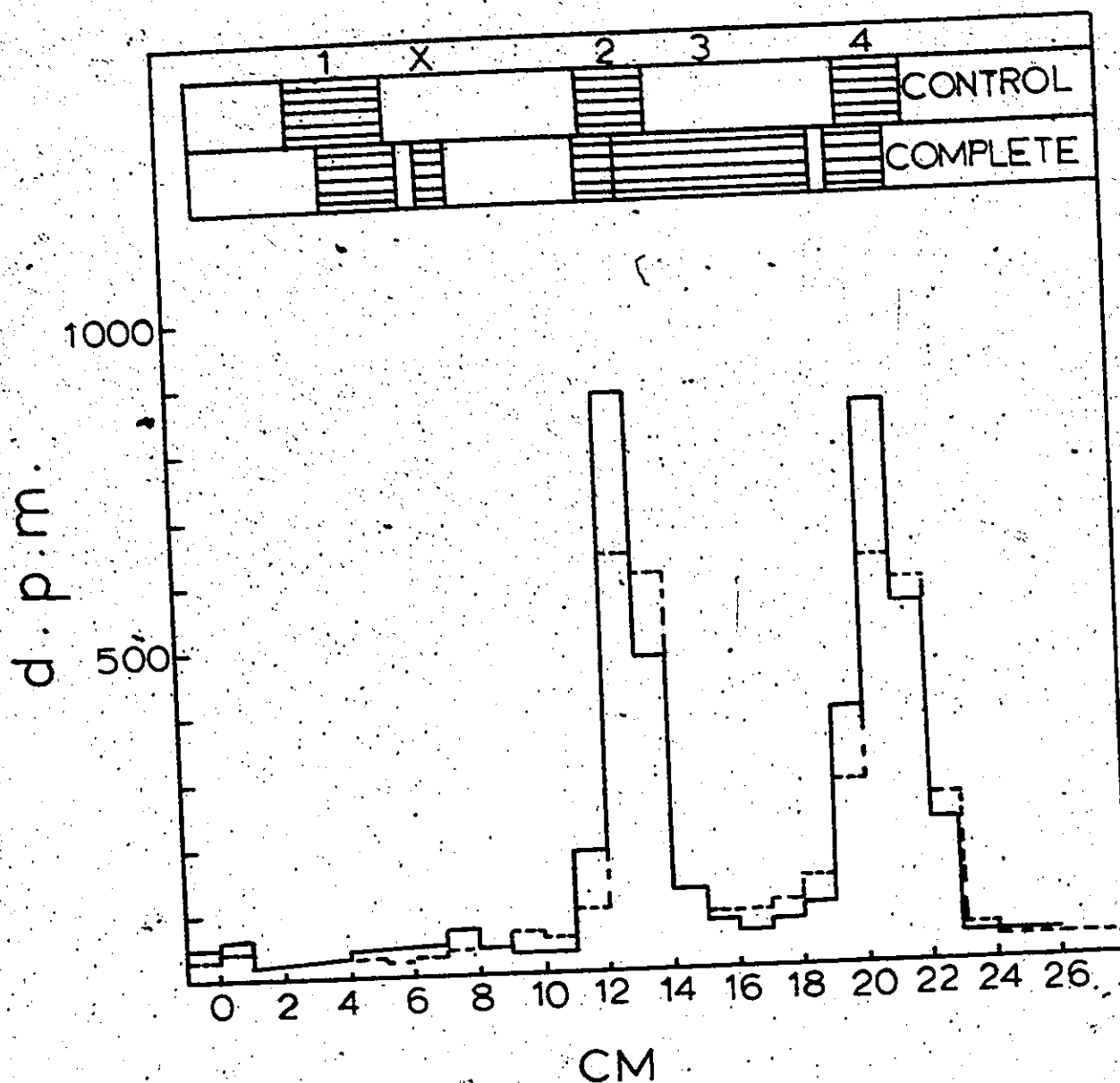


Figure 3.7: Radioactive distribution of DNPH derivatives from the "zero time" in acid" mixtures which were incubated under N_2 with $(1-^{14}C)$ glyoxylate as the labelled substrate. Incubations and chromatography were conducted as described in Fig. 3.6, except that the labelled substrate was $(1-^{14}C)$ glyoxylate (189,200 d.p.m.). The complete assay is shown in solid line; the control assay (no enzyme) in broken line.

carbon dioxide evolved after acidification of the reaction mixtures was labelled when (1- ^{14}C) glyoxylate was the labelled substrate, but was not labelled when (2- ^{14}C) glyoxylate was used (Table 3.12). The $^{14}\text{CO}_2$ evolved and the radioactive pattern of DNPH derivatives were the same whether air or N_2 were present during the reaction (Table 3.12 and Fig. 3.8 compared to Fig 3.6).

The results are in accord with reaction 2 (section 2.2.5), in which α -hydroxy- β -ketoadipate (HKA), the product of the enzymatic reaction which contains the two carbon atoms of glyoxylate, produces upon acidification carbon dioxide from the carboxylic carbon of glyoxylate and HLA which retains the carbonyl carbon of glyoxylate. Therefore, HLA should be labelled only if (2- ^{14}C) glyoxylate is the labelled substrate and the $^{14}\text{CO}_2$ should be evolved only when (1- ^{14}C) glyoxylate is the labelled substrate.

Acidification for 2 hours of the assay mixtures prior to the addition of 2,4-DNPH reagent, resulted in a diminution in both the color intensity and the label of the HLA derivative. Under these conditions the formation of the glyoxylate derivative was also diminished as can be seen by comparing Figures 3.6 and 3.9. It is possible that 2 hours of incubation in acid at 30° may result in losses

Assay conditions	$\mu\text{moles } ^{14}\text{CO}_2/\text{hr}$	
	Additions	
	None	PCA
(1- ^{14}C)glyoxylate, air	0.045	0.713
(1- ^{14}C)glyoxylate, nitrogen	0.020	0.733
(2- ^{14}C)glyoxylate, air	0.000	0.005
(2- ^{14}C)glyoxylate, nitrogen	0.000	0.000

Table 3.12: α -ketoglutarate:glyoxylate carboligase activity under air or nitrogen. Mitochondrial fraction (2.4 mg), from cells grown in proteose-peptone, fractionated by method A, were incubated as described in Experimental Procedures with 4 μ moles of labelled glyoxylate as indicated and 4 μ moles α -ketoglutarate. The reaction was carried out for 60 min under air or nitrogen. The $^{14}\text{CO}_2$ evolved at the end of the enzymatic reaction with no acid added and after further incubations for 120 min after the addition of PCA.

Figure 3.8: Radioactivity distribution of DNPH derivatives from "zero time in acid" assay mixtures under air with (1-¹⁴C) or (2-¹⁴C) glyoxylate as the substrate labelled. Assay condition and chromatography are those described in Figures 3.6 and 3.7, except that air was the gas phase during the enzymatic reaction. Assay mixture containing (2-¹⁴C) glyoxylate is shown in broken line; from assay mixtures containing (1-¹⁴C) glyoxylate in solid line.

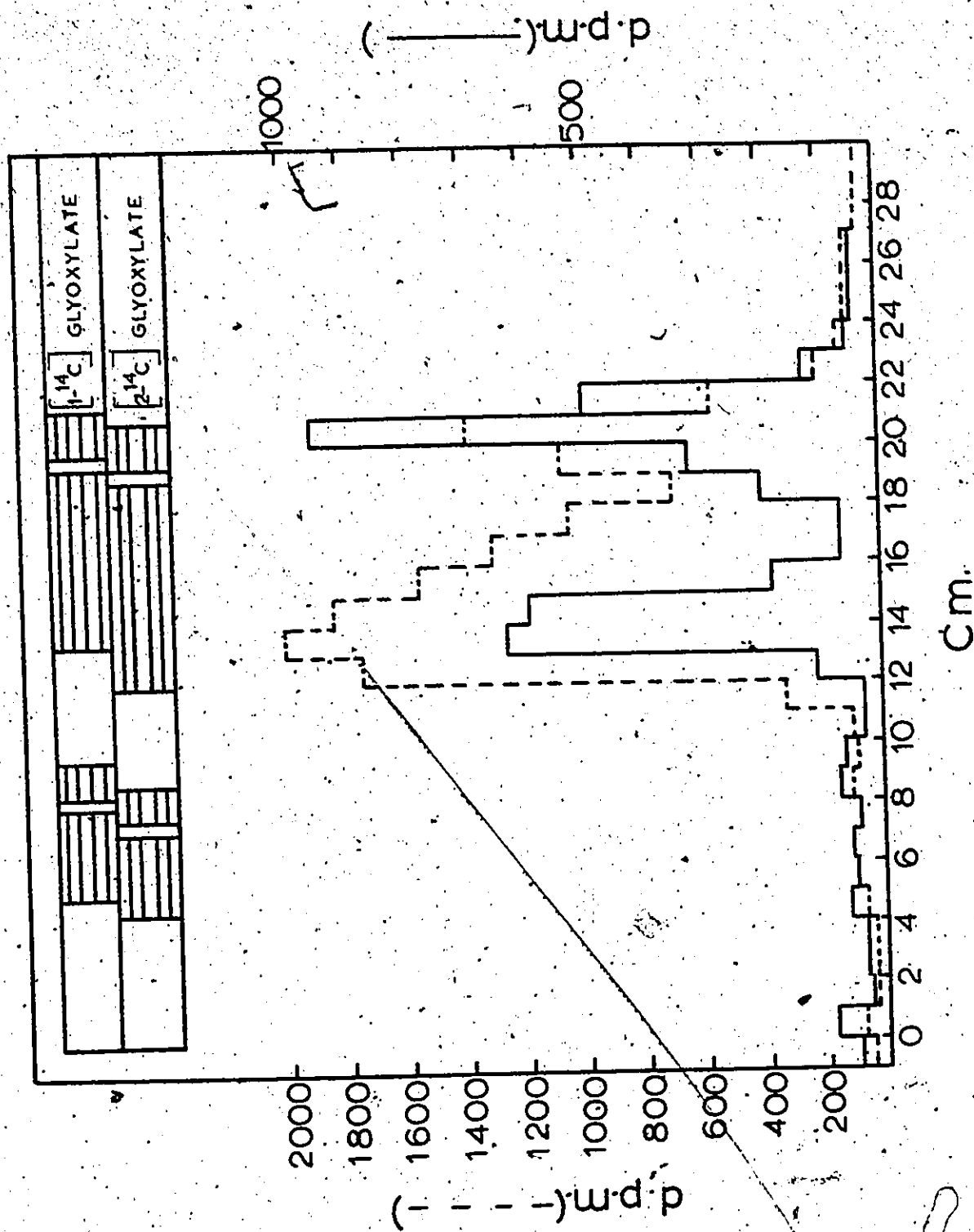


Figure 3.8

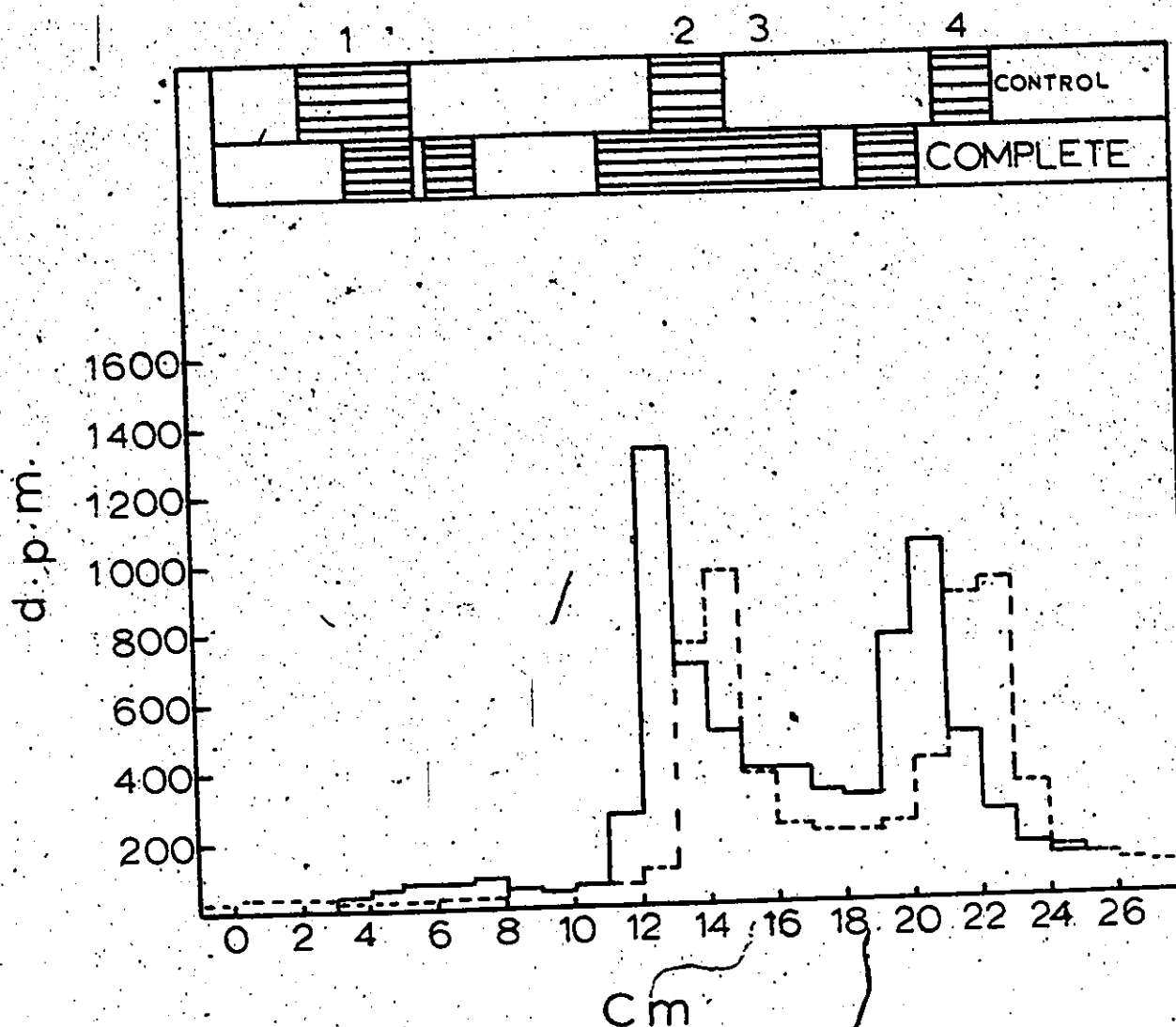


Figure 3.9: Radio-active distribution of DNPH derivatives from the "2 hours in acid" assay mixtures containing $(2-^{14}\text{C})$ glyoxylate as the labelled substrate. Assay conditions and chromatography were conducted as described in Fig. 3.6, except that the mixtures were further incubated for 2 hours in PCA before 2,4-DNPH reagent was added. DNPH derivatives from the complete assay is shown in complete line; those from the control assay (no enzyme) in broken line.

for HLA and glyoxylate. However, visual inspection of the chromatograms did not indicate a similar reduction in color intensity of the areas representing the DNPH derivatives of α -ketoglutarate and the X compound after two hours in acid.

When the mitochondrial fraction was the enzyme source somewhat more of the DNPH-X compound was obtained in comparison with that obtained when the total cell extract was used, but this slight increase in the intensity of the blue color was not accompanied by an increase in radioactivity.

The distribution of the radioactivity of the DNPH derivatives on paper chromatography with solvent system 1 obtained when either mitochondrial or cytoplasmic fraction of rat liver were the enzyme sources for the α -ketoglutarate: glyoxylate carboligase reaction and (U-¹⁴C) glyoxylate the labelled substrate, has been reported by Koch et al., (1967). Two bis derivatives (osazones) were obtained. The slow-moving compound was identified as the DNPH derivative of HKA by assuming the same properties with the synthesized DNPH derivative of γ -hydroxy- β -ketopropionate (tartronic semialdehyde). The faster-moving bis derivative

was suggested to be the DNPH-HLA. Both compounds were red-brown and turned blue after spraying the papers with ethanolic KOH. The same two bis derivatives have been reported when mitochondria from plants were the enzyme source and chromatography accomplished in a similar solvent system 1 (Davies and Kenworthy, 1970). Physical and chemical characterization of the products was accomplished by Schossberg et al., (1970) after isolation by column chromatography from mixtures containing about 100-fold purified carboligase. The products were identified as HLA and 2,3-dihydroxy-4-ketopimelic acid (HKPA) and it was suggested that the DNPH-HKA detected by Koch et al., (1967) might in fact be DNPH-HKPA.

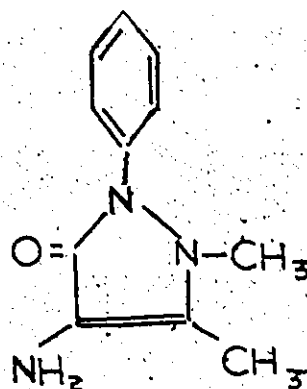
From the experiments reported here it can be concluded that the bis derivative, obtained as the major compound in our chromatograms using solvent system 1, and which overlaps with the slowest-moving of the two glyoxylate derivatives corresponds to the DNPH-HLA, in agreement with Koch et al., (1967). However identification was obtained also with solvent system 2 and the use of synthesized authentic HLA. The slow-moving bis derivative (DNPE-X) which may correspond to the slow-moving bis derivative reported by those workers may not

however be DNPH-HKA, as suggested by Koch et al., (1967) by reason of the following considerations:

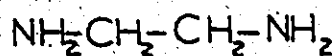
a) The DNPH-X compound (presumably the osazone of HKA) did not diminish after prolonged standing in acid medium as would be expected from its instability in acid as stated before (Fig. 3.9).

b) An osazone of HKA itself cannot, in fact, exist. It is well known that β -keto acids are unstable compounds, and the extensive evolution of CO_2 upon acidification (or addition of AAP) of the complete reaction mixtures support the presence of a β -ketoacid as the primary enzymatic product. AAP which catalyzes the decarboxylation of HKA was first studied by Kaneko (1938) in connection with the catalytic decarboxylation of acetoacetate. A series of other heterocyclic diamino compounds have been demonstrated to be active in catalyzing the β -decarboxylation of acetoacetate. The most active were derivatives of antipyrine such as 1-phenyl-3-hydroxy-4-amino-pyrazolone (5)-imide and AAP. Beniya in 1934 had already observed that ethylene diamine and o-phenylenediamine decarboxylated β -ketoacids more readily than aniline (Table 3.13), but m or p-phenylenediamine were scarcely active as aniline.

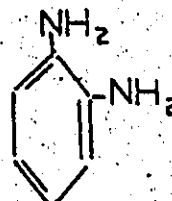
Aminoantipyrine



Ethylenediamine



O-Phenylenediamine



Aniline



2,4-Dinitrophenylhydrazine

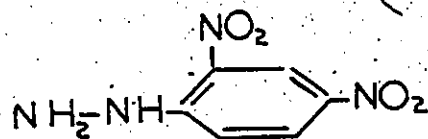


Table 3.13: Amino compounds capable of catalyzing the decarboxylation of β -ketoacid.

(for review see Aka Matsu 1950). Hence a second amino group in neighbouring position enhances the decarboxylating activity. As shown in Table 3.14 the CO_2 formation from acetoacetate by some amino compounds indicates that the heterocyclic compounds such as AAP were the most active catalysts. Aniline at the same concentration was only slightly effective and the catalytic power was increased when a second amino group was present in the molecule as in o-phenylenediamine. Aniline at higher concentration was as active as o-phenylenediamine.

It has also been shown that amines (Byerrum and Rothschild, 1952) as well as lysine and polylysine (Rohlfing, 1967) catalyse the decarboxylation of oxaloacetate to pyruvate.

Furthermore, the presence of an amino group at the active site of decarboxylases seems to be an essential feature of these enzymes as has been shown with acetoacetate decarboxylase. The decarboxylation product, acetone, was found to be bound to the α -amino group of a lysine residue (by a Schiff base) (Westheimer, 1963; Warren et al., 1966).

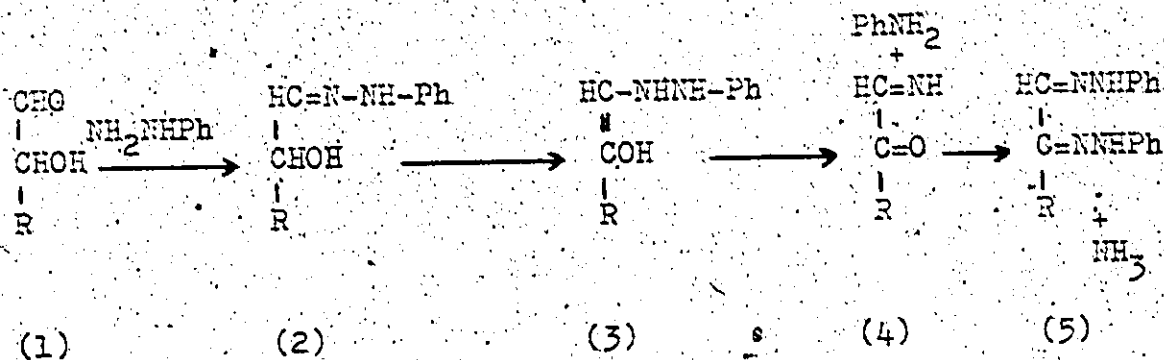
From the foregoing it seems probable that the 2,4-DNPH reagent itself used for the formation of the hydrazone and osazone derivatives in our reaction mixtures, may also

Amino compound	Time in min.			
	5	10	20	30
M/10 Amino-antipyrine	17.15	20.85	23.7	24.05
M/10 1-Phenyl-3-hydroxy- 4-amino-pyrazolone- (5)-imide	20.25	22.25	23.05	23.30
M/10 o-Phenylen diamine	3.75	6.05	8.80	10.80
M/10 Aniline	0.45	0.90	1.45	1.90
M Aniline	3.45	5.65	9.15	11.20

Table 3.14: CO_2 formation in ml from acetoacetic acid by amino compounds.

0.5 ml of 0.1 M catalyst solution plus 10 ml of 0.5 M acetate buffer (pH 4.2) were mixed with 1 ml of 1 M sodium acetoacetate solution. CO_2 evolution was measured at the times indicated at 37 °C. 100% decomposition of acetoacetate should form 25 ml of CO_2 .
(.from Akamatsu, 1950).

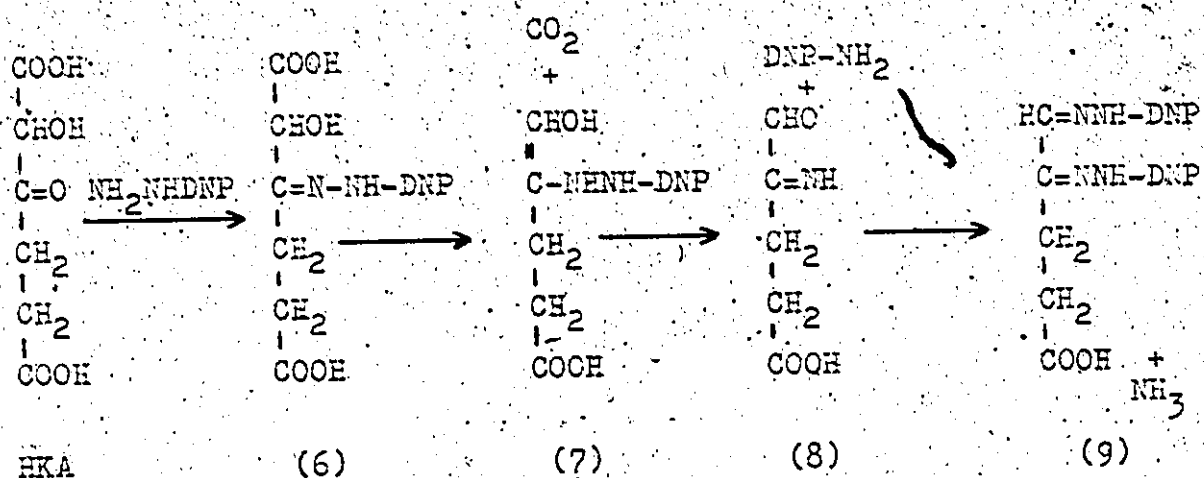
catalyze the decarboxylation of HKA by virtue of the presence of the hydrazino group. Osazone formation would then follow. The mechanism for the formation of osazones is not positively elucidated but one scheme proposed by Weygand in 1940 (for review see El Khadem, 1965) which involves a series of Amadori rearrangements is presented below:



One molecule of phenylhydrazone (2) rearranges to the hydrazino enol (3) which loses aniline to give a keto imine (4) and the latter reacts with two molecules of phenylhydrazine to yield the osazone (5) and ammonia.

By analogy, in the case of HKA below, enolization of the phenylhydrazone (6) would be accompanied by decarboxylation to yield compound (7) and CO_2 , and this would be followed by ketoimine formation (8) and finally formation of the osazone (9). Hence, osazone formation from HKA must be

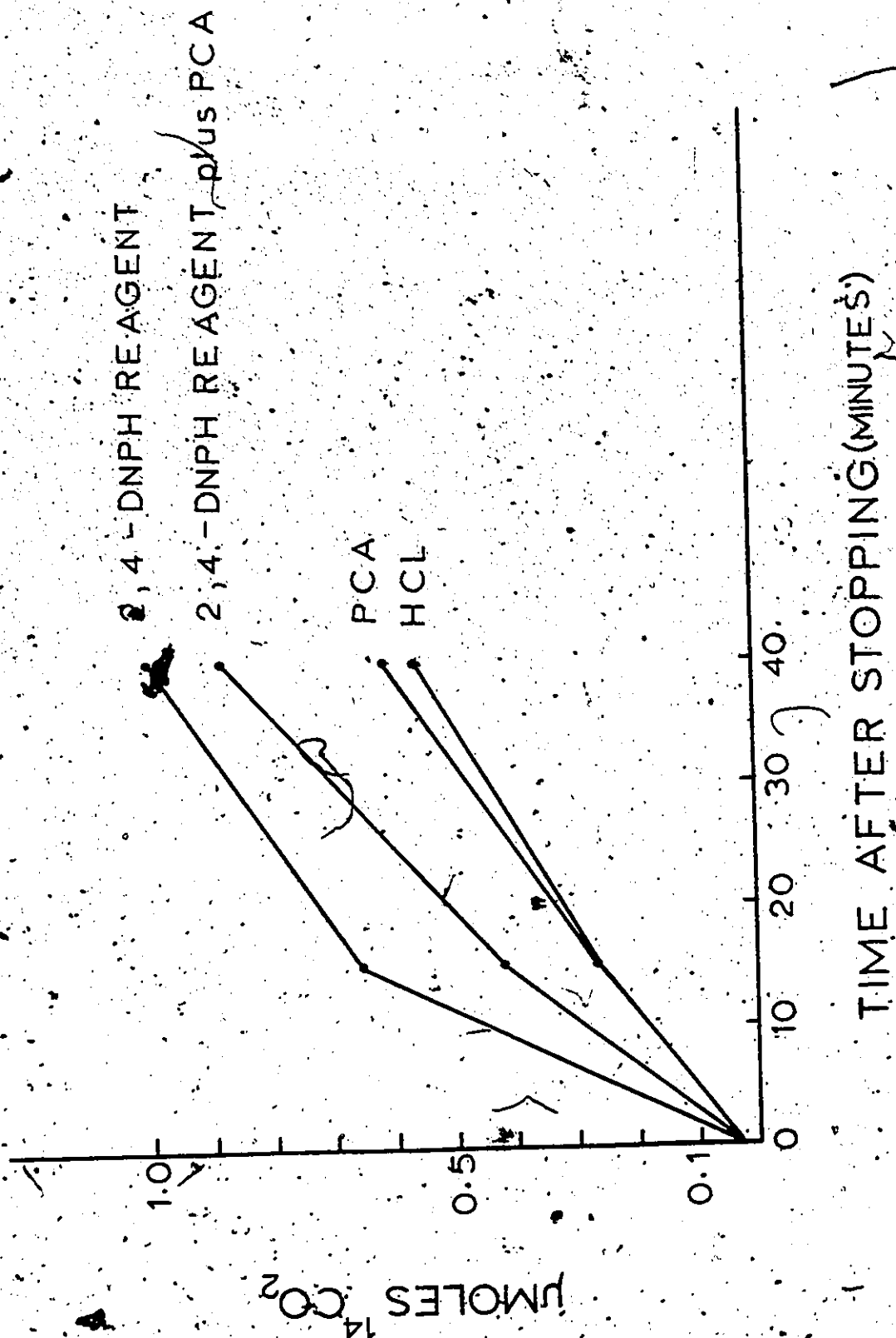
accompanied by decarboxylation and an osazone of HKA itself cannot exist. One would expect therefore that the decarboxylation of HKA by PCA in reaction (2) (section 2.2.5) is further enhanced after the addition of the 2,4-DNPH reagent.



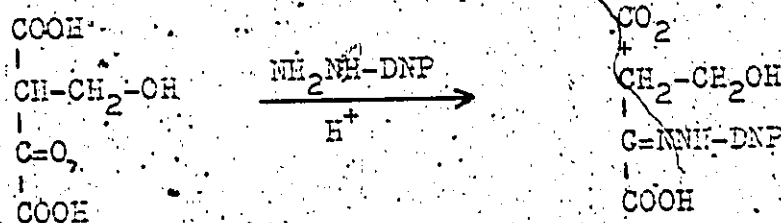
In fact, it was found and shown in Figure 3.10, that when (1-¹⁴C) HKA, enzymatically formed in the complete assay mixture with (1-¹⁴C) glyoxylate as the labelled substrate, was treated with an acid solution of 2,4-DNPH (0.5% in 2N HCl), the ¹⁴CO₂ evolved at 15 min was 142% higher than that evolved in the presence of HCl alone or PCA alone. At that time formation of osazone (orange crystals) were also observed. Some reduction in the rate

Figure 3.10: Evolution of $^{14}\text{CO}_2$ from reaction mixtures after addition of 2,4-DNPH reagent or acid (PCA or HCl). Mitochondrial fraction (0.24 mg) were incubated in the standard conditions (section 2.2.5.A) with (1- ^{14}C) glyoxylate as the labelled substrate except that the side arm contained either 0.1 ml 70% PCA or 0.5 ml 2,4-DNPH reagent (0.5% in 2N HCl) or 0.5 ml 2N HCl or 0.5 ml 2,4-DNPH reagent plus 0.1 ml 70% PCA. After 1 hour incubation the contents of the side arm were tipped into the main compartment and the $^{14}\text{CO}_2$ evolved was estimated at the indicated time.

Figure 3.10



of CO_2 evolution was noted when 2,4-DNPH reagent and PCA were added together. Our results are in agreement with experiments described by Ruffo *et al.*, (1962a), in which both oxalomalate and β -hydroxymethyl oxaloacetate reacted with 2,4-DNPH with liberation of CO_2 and the formation of the corresponding phenylhydrazones:



β -hydroxymethyl-oxaloacetate acid.

Section 3.6: FURTHER STUDIES ON THE ORIGIN OF THE DNPH-X.

Experimental procedures. Cells grown in synthetic and proteose-peptone media were used. Mitochondrial fractions were obtained by method B (section 2.2.4). The assay conditions were as described in Methods (section 2.2.5) with 4 μ moles of glyoxylate and 4 μ moles of α -ketoglutarate in an atmosphere of nitrogen or air. Omissions are indicated in the legends of the Figures. After 60 min of

incubation, 2,4-DNPH reagent was added to the reaction mixtures. PCA was also added after about 30 min in order to reproduce the same acid conditions as in the previous experiments. The crystals of DNPH derivatives were collected as described in section 3.5. The dried DNPH derivatives were dissolved in 0.5 ml of ethyl acetate and two 0.2 ml aliquots were applied on duplicate series of paper chromatograms in solvent system 1. The paper strips of one series were sprayed with saturated ethanolic NaOH and cut for counting in the liquid scintillation counter (section 2.2.10). The DNPH-X was extracted with ethyl acetate (section 2.2.10) from the second series of chromatograms and rechromatographed in solvent system 1. The DNPH-X was finally extracted from the paper and dried under nitrogen and the dry residue was dissolved in 0.5 ml ethyl acetate from which 0.2 ml were dried and dissolved in 0.1 ml 10 mM Na_2CO_3 for counting in Bio-Solv (section 2.2.7), and 0.2 ml were dried and dissolved in 1 ml in NaOH and the absorption measured at 550 nm.

At the same time the DNPH- α -ketoglutarate was processed in the same way and results on this will be presented in section 3.10.

Results and discussion. With fractionation procedure B used to obtain Mitochondria from cells grown in proteose-peptone and synthetic media, a significant difference in the amount of the unknown compound formed was noted, as is clear from Figure 3.11. This difference was reflected both in the intensity of blue color formation and radioactivity on the chromatograms and was in favour of the preparation obtained from cells grown in the synthetic medium by about 220% in terms of radioactivity. Similar results were obtained when incubations were carried out in an atmosphere of nitrogen (not shown). The formation of the compound was dependent on TPP and α -ketoglutarate as demonstrated in Figures 3.12a and 3.12b, respectively. The carboligase activity itself was somewhat higher in mitochondria from cells grown in proteose-peptone (Table 3.15). Although some carboligase activity could always be assayed in supernatant fractions the unknown compound was always absent.

As shown in section 3.5, 2 hours incubation in acid prior to the addition of 2,4-DNPH reagent did not result in a diminution of the counts of DNPH-X obtained from cells grown in synthetic medium.

When the DNPH-X was extracted after the second chromatography and its absorption at 550nm and radioactivity

Figure 3.11: Radiobactivity distribution (solvent system 1) of DNPH derivatives from assay mixtures containing mitochondrial fractions from cells grown in different growth medium. Mitochondrial fraction (2.4 mg) from cells grown in proteose-peptone and from synthetic medium supplemented with acetate (2.2 mg) were incubated in the standard conditions under air with 4 μ moles of (2- 14 C)glyoxylate (215,440 d.p.m.) as the labelled substrate. After 1-hour incubations, 2,4-DNPH reagent was added and the crystals were chromatographed in solvent system 1. The papers were sprayed with ethanolic NaOH and the cut and counted . a) From mitochondrial fraction obtained from cells grown in proteose-peptone b) From mitochondrial fraction obtained from cells grown in synthetic medium supplemented with acetate.

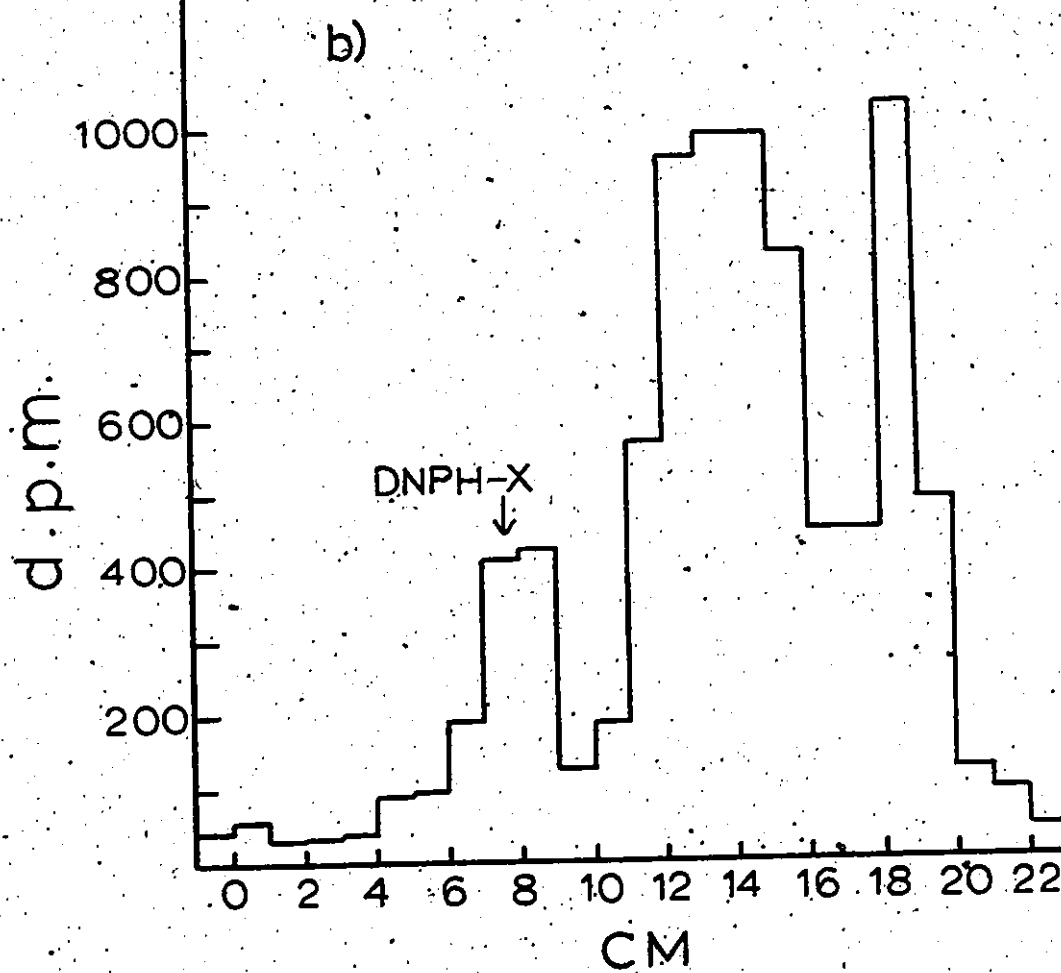
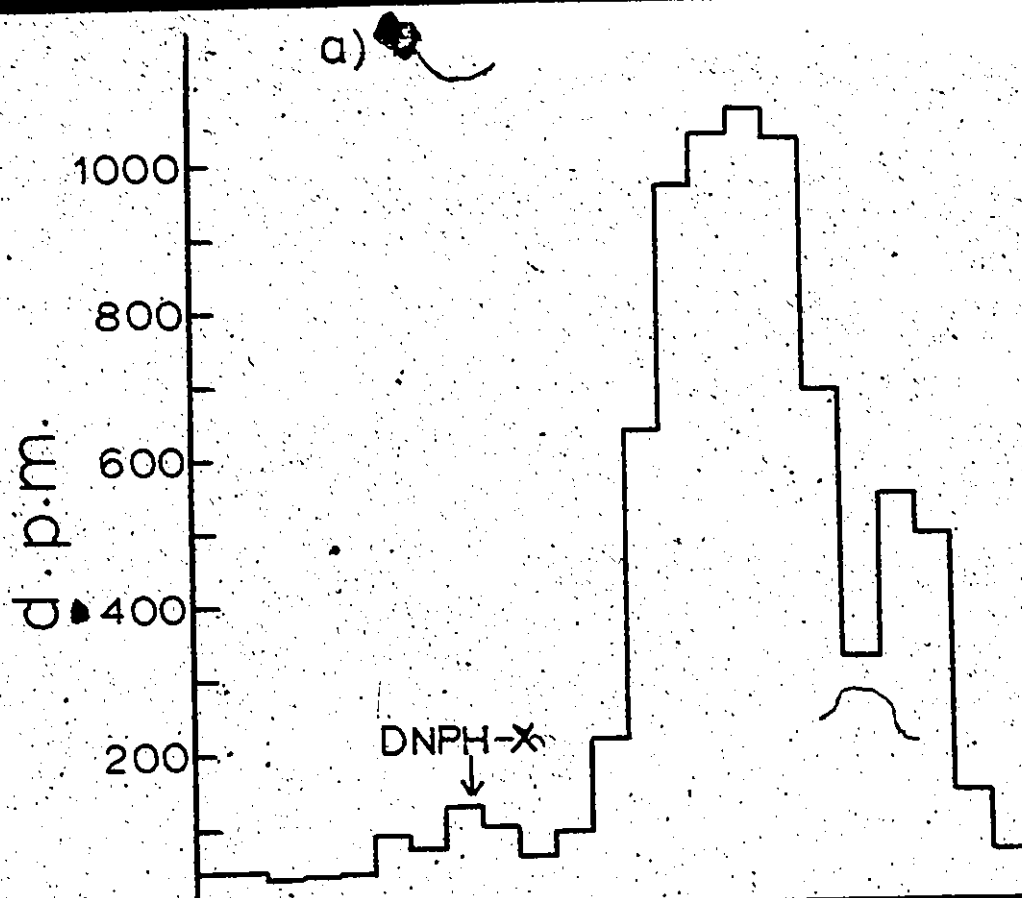


Figure 3.12: Radioactivity distribution (solvent system 1) of DNPH derivatives from assay mixtures in which TPP or α -ketoglutarate was omitted. Assays conditions and chromatography are those described in Fig. 3.11, except that TPP was omitted (a) or α -ketoglutarate was omitted (b), from the reaction mixtures. (This figure is to be compared with Fig. 3.11.b).

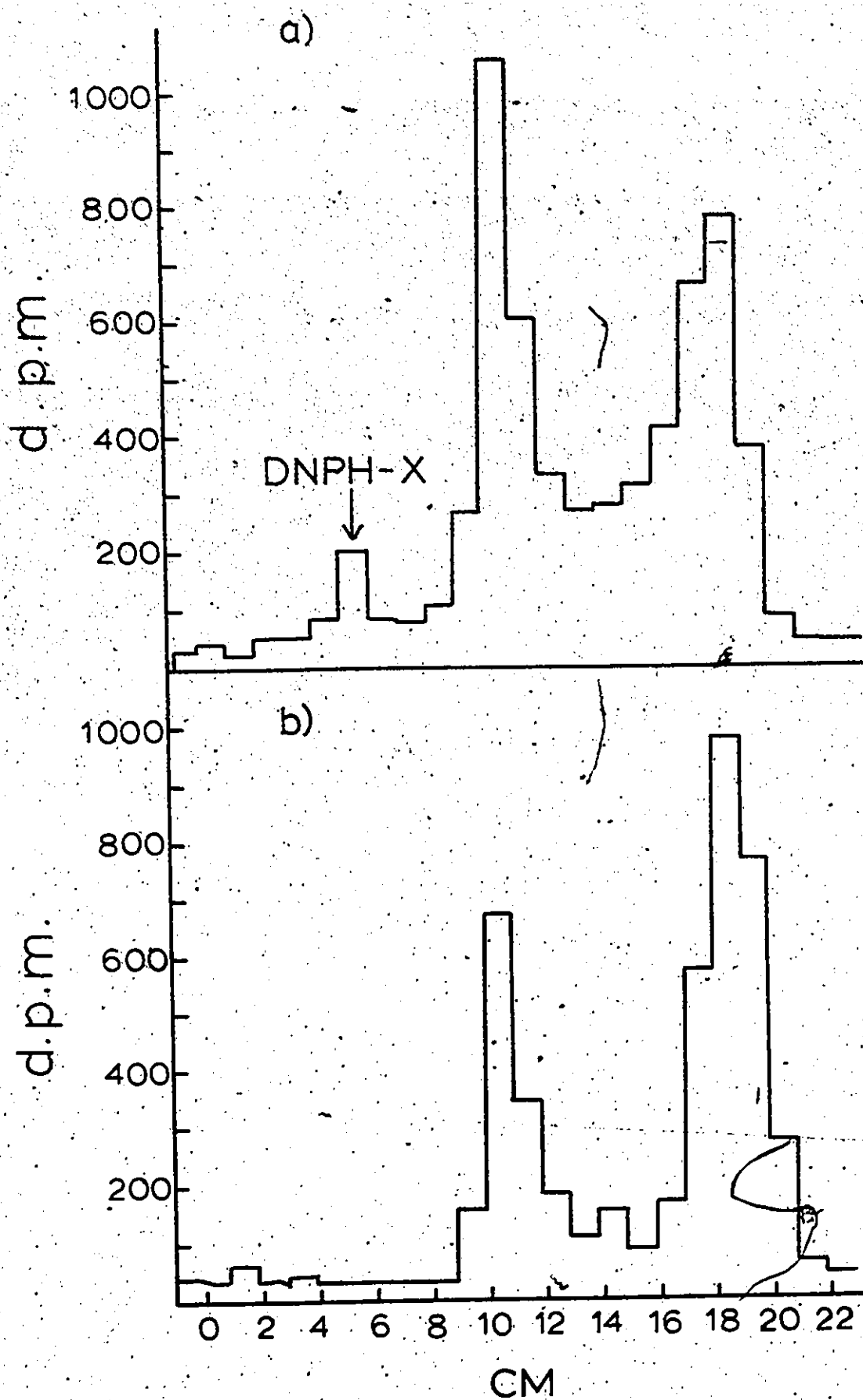


Figure 3.12

Growth medium and gas phase in assay	$\mu\text{moles } ^{14}\text{CO}_2/\text{hr}$	
	No TCA-AAP	TCA-AAP
Synthetic, Air	0.030	1.059
Synthetic, Nitrogen	0.018	1.065
Proteose-peptone, Air	0.051	1.523

Table 3.15: α -Ketoglutarate:glyoxylate carboligase activity from cells grown in synthetic or proteose-peptone medium.

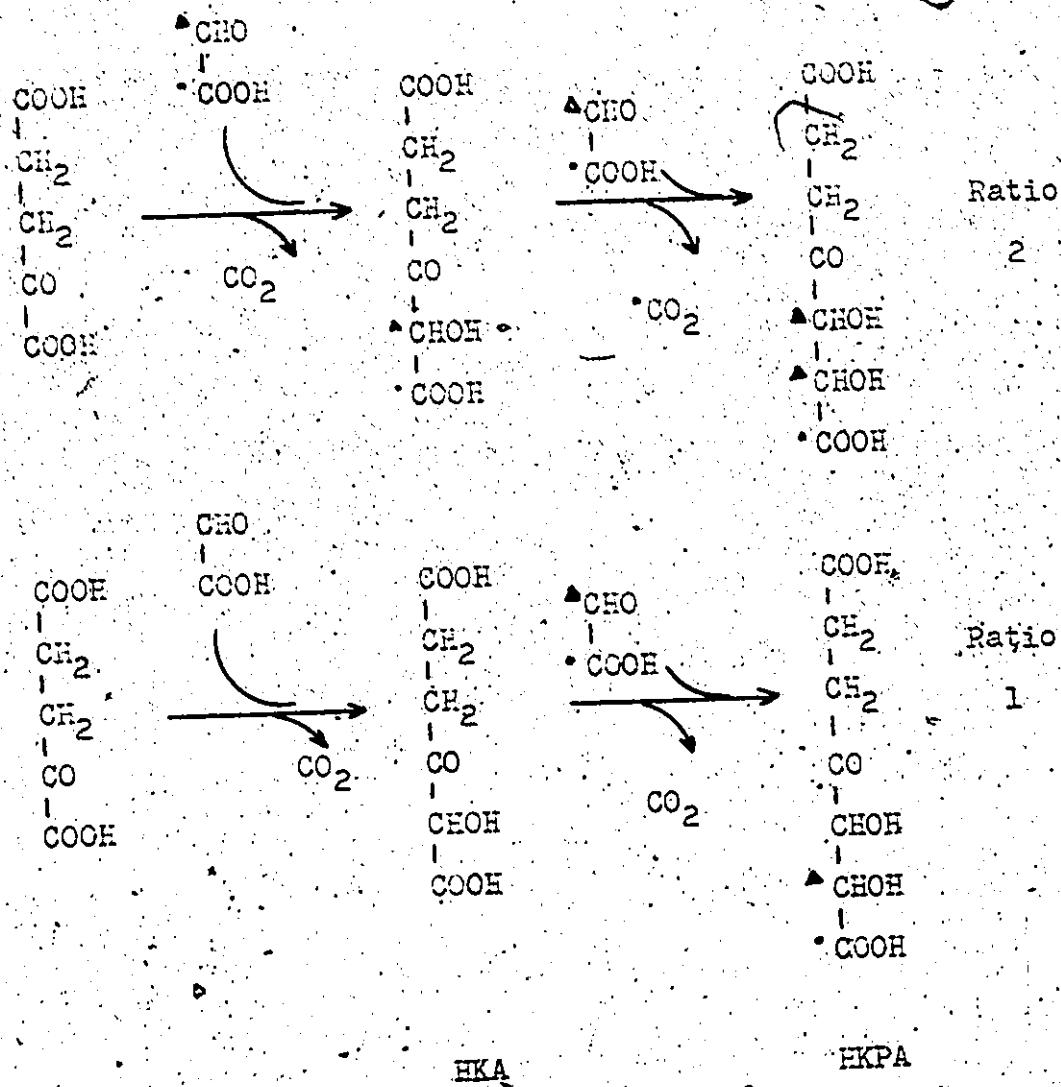
Mitochondrial fraction from the synthetic medium plus acetate (2.2 mg) or from proteose-peptone (2.4 mg) obtained by method B were incubated in the standard conditions but containing $4 \mu\text{moles}$ of $(1-^{14}\text{C})$ glyoxylate (189,068 d.p.m.) as the substrate labelled. $^{14}\text{CO}_2$ was estimated after 30 min of incubation and after the addition of TCA-AAP.

Substrate labelled and gas phase	O.D./ml 550nm	d.p.m.	d.p.m. per 1.312 O.D/ml	Ratio (2- ¹⁴ C)glyoxylat (1- ¹⁴ C)glyoxylat
(1- ¹⁴ C) glyoxylate, Air	1.312	2,337 (3,040)	2,337	
(2- ¹⁴ C) glyoxylate, Air	1.206	2,887 (3,712)	3,140	1.34
(1- ¹⁴ C) glyoxylate, N ₂	1.287	2,400	2,445	
(2- ¹⁴ C) glyoxylate, N ₂	1.187	2,950	3,260	1.33

Table 3.16: Labelling and yield of DNPH-X when (1-¹⁴C) or (2-¹⁴C) glyoxylate was the labelled substrate. Assay conditions and chromatography are described in the legend of Figure 3.11. DNPH-X was extracted after the second chromatography and the light absorption (O.D.) and radioactivity were estimated as described in Experimental Procedures. Figures in parenthesis correspond to the area under the radioactive peak of DNPH-X from chromatograms from another experiment. The d.p.m. have been corrected to the same specific radioactivity of glyoxylate in the assay mixtures which were 215,440 d.p.m. for (2-¹⁴C) glyoxylate and 189,068 d.p.m. for (1-¹⁴C) glyoxylate.

were measured it was found that the compound was labelled when either (1- ^{14}C) or (2- ^{14}C) glyoxylate was used as the labelled substrate. The ratio of the counts associated with it under these conditions was 1.35 (Table 3.16) under either air or nitrogen during the assay conditions. A similar ratio was obtained when the calculation was done directly from the distribution of radioactivity on the chromatograms (figures in parenthesis in Table 3.16).

This ratio, being near 1, indicates that both carbon atoms of the glyoxylate molecule were incorporated in the X compound, in accord with the results of Koch *et al.*, (1967). The unknown compound cannot be HKA for the reasons discussed previously. The fact that α -ketoglutarate and TPP were required for its formation, even when the carbolligase activity was somewhat lower from cells grown in the synthetic medium, suggests that the formation of this compound may be associated with the carbolligase reaction. The secondary product detected by Schlossberg *et al.*, (1970), HKPA, may be a possible candidate. This compound will give a ratio (2- ^{14}C /1- ^{14}C) equal to 2, however, if HKPA was also formed from the unlabelled HKA, also present in the reaction mixture, then the ratio would be smaller than 2 as shown below:



It is not obvious why this compound does not appear when mitochondrial fraction from cells grown in proteose-peptone medium were used as the enzyme source. It appeared only when mitochondrial fraction from synthetic medium was used.

Section 3.7: KINETICS OF α -KETOGLUTARATE:GLYOXYLATE
CARBOXYLASE.

3.7.1. Progress curve. The activity was linear up to 60 min under the standard assay conditions (Fig. 3.13).

3.7.2. Enzyme concentration curve. The activity was linear up to 5 mg of mitochondrial protein under the standard assay conditions (Fig. 3.14).

3.7.3. pH curve. The activity was maximal over a broad pH range from 6.5 to 7.0 and was 63% active at pH 7.6 and only 42% active at pH 6.0, in the presence of potassium phosphate buffer at 50 mM final concentration (Fig. 3.15). No differences were observed at pH 7.0 when the phosphate buffer varied from 20 to 60 mM.

3.7.4. Effect of glyoxylate concentration. A normal hyperbolic curve was obtained when glyoxylate concentration up to 2.4 mM was plotted versus velocity. The K_m calculated from the double reciprocal plot of substrate concentration versus initial velocity was 5 mM in the presence of 1 mM α -ketoglutarate (Fig. 3.16).

3.7.5. Effect of α -ketoglutarate concentration. A normal hyperbolic curve also was obtained when α -ketoglutarate

Figure 3.13: Progress curve. Mitochondrial fraction (1.2 mg) obtained by method A (section 2.2.4) was incubated in the standard conditions (section 2.2.5.A) with α -(1- ^{14}C) ketoglutarate (113,228 d.p.m.). After the indicated times PCA was added and the $^{14}\text{CO}_2$ estimated.

Figure 3.14: Enzyme concentration curve. Different amount of protein from mitochondrial fractions obtained by method A were incubated in the standard conditions (section 2.2.5.A) with (U- ^{14}C) glyoxylate (300,000 d.p.m.). After 1 hour of reaction PCA was added and the $^{14}\text{CO}_2$ estimated.

Figure 3.15: pH curve. The enzyme was assayed as described in Figure 3.14 and the pH of the potassium phosphate buffer was varied as indicated. The protein present during the assay was 3 mg.

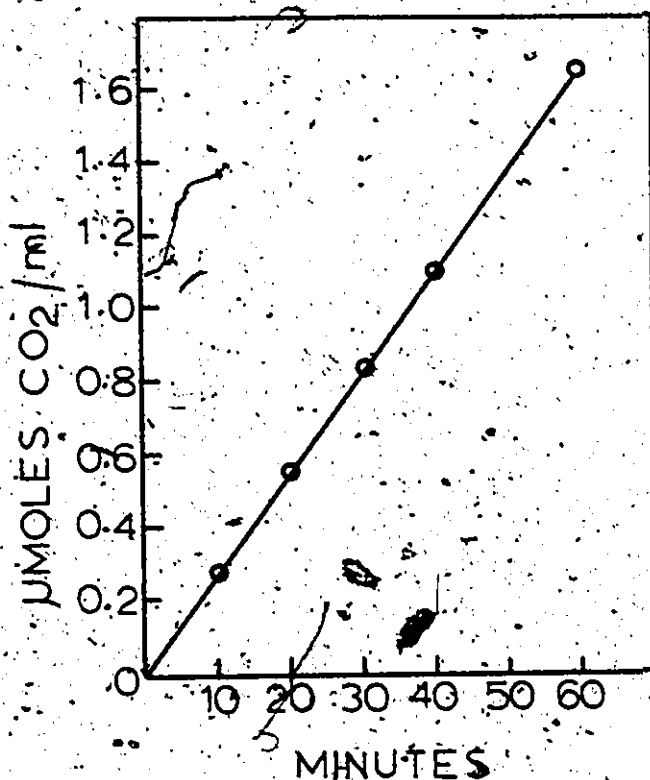


Figure 3.13 :Progress curve.

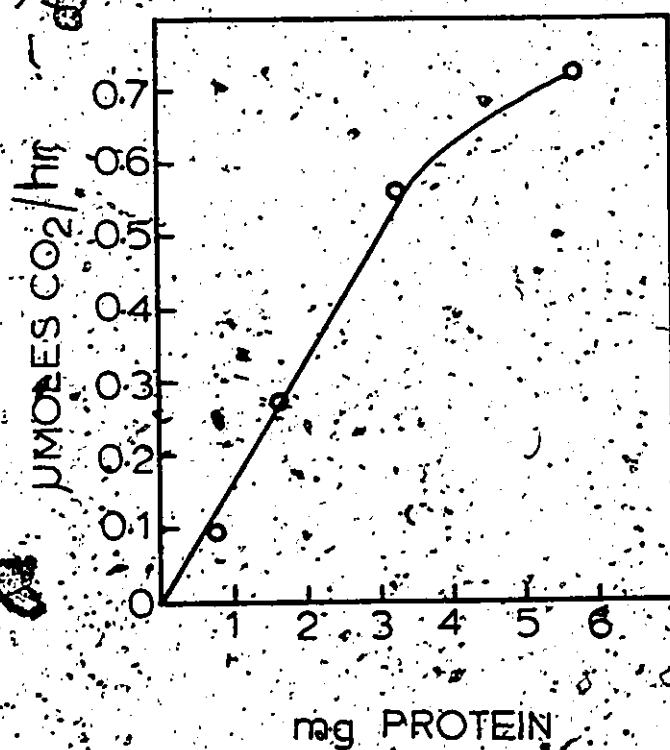


Figure 3.14: Enzyme concentration.

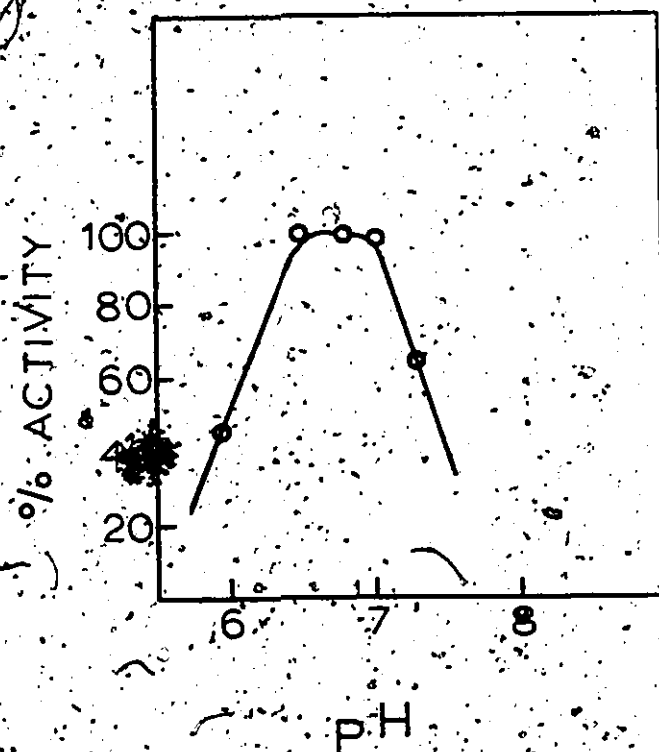
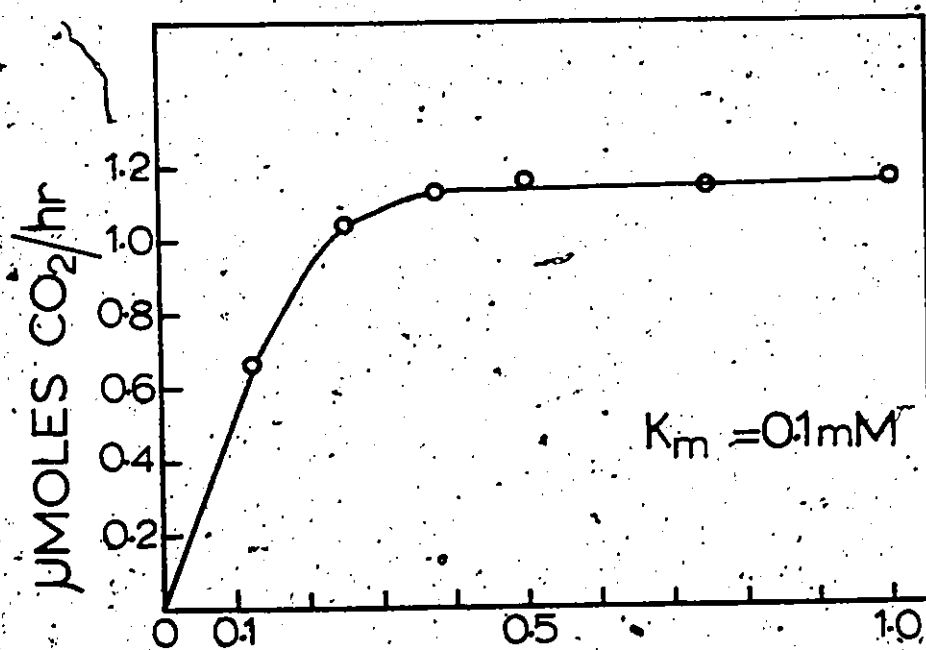
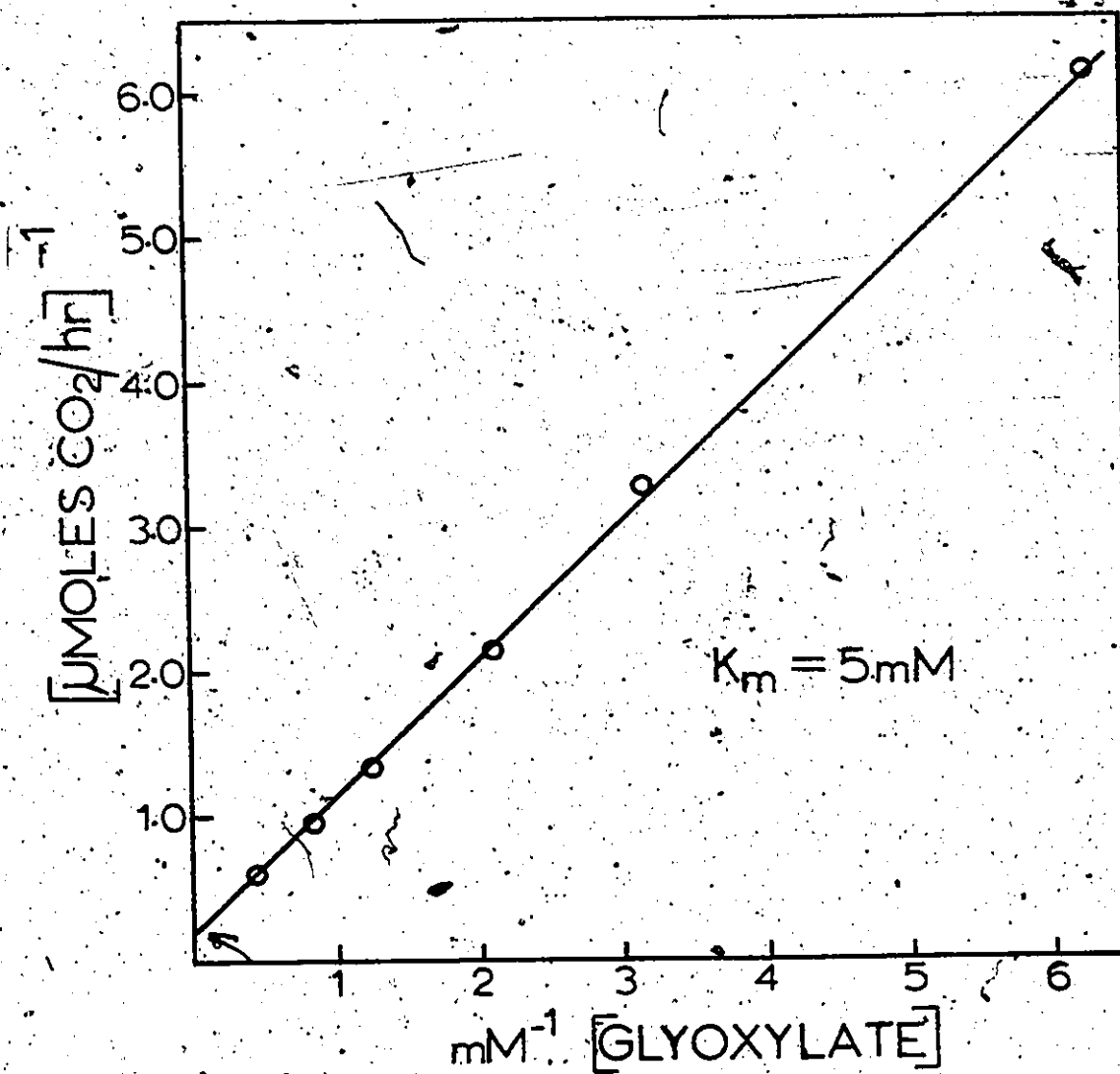


Figure 3.15: pH curve.

Figure 3.16: Effect of glyoxylate concentration on the initial velocity of α -ketoglutarate: glyoxylate carboligase reaction. Mitochondrial fractions (2.9 mg) were incubated as described in Figure 3.14 except that glyoxylate was varied while keeping the specific radioactivity constant. α -Ketoglutarate was present at a final concentration of 1 mM. The data were plotted according to Lineweaver and Burk (1934).

Figure 3.17: Effect of ketoglutarate concentration on the initial velocity of α -ketoglutarate: glyoxylate carboligase reaction. Mitochondrial fractions (1.5 mg) were incubated as described in Figure 3.16 except that α -ketoglutarate was varied. Glyoxylate was present at a final concentration of 1 mM.



Enzyme source	Purification state	pH optimum Buffer	pH	K_m α -Keto-glutarate/	K_m glyoxylate
Rat liver mitochondria (1)	85-fold	60mM Na-K phosphate	6.2		
Rat liver mitochondria extracts (2)	5-10-fold	28mM Na-K phosphate	6.8-7.0		
Pig-liver mitochondria (3)	5-fold	20mM K phosphate	7.0	3.6mM	16mM
<u>E. coli</u> (4)	Highly	33mM K phosphate	Broad	0.5mM	4.2mM
<u>R. sheroide</u> (5)	cell-free extracts	100mM K phosphate	at 6.5		
<u>M. phlei</u> (6)	340-fold	33mM K phosphate	7.2	2.0mM	3.2mM
Plant mitochondria (7)	100,000xg		6.3	0.2mM	

Table 3.17: pH optimum and K_m values of α -ketoglutarate:glyoxylate carboligase reported in the literature.

- (1) Koch and Stockstad (1966); (2) Nakada and Sund (1958); (3) Steward and Quayle (1967); (4) Kubasik et al., (1972); (5) Okuyama et al., (1965); (6) Yamasaki and Moriyama (1971); (7) Davies and Kenworthy (1970).

concentration was varied. The K_m calculated from the plots of substrate concentration versus initial velocity was 0.10 mM in the presence of 1 mM glyoxylate (Fig. 3.17).

As shown in Table 3.17 the optimal pH values reported in the literature for purified and partially purified α -ketoglutarate:glyoxylate carbolicase activity are in the range of 6.2 to 7.2. The K_m values reported in a few cases always showed that the enzyme has a higher affinity for α -ketoglutarate than for glyoxylate. Even though our preparation was not purified, these constants are quite in agreement with those reported in the literature.

Section 3.8 ATTEMPTED PURIFICATION OF α -KETOGLUTARATE: GLYOXYLATE CARBOLIGASE.

In all the experiments reported in this Thesis the whole mitochondrial fraction was used as the source for the enzyme. The purpose of this section is to present experiments conducted to obtain mitochondrial extracts and to purify the enzyme. In one experiment the activity of α -ketoglutarate decarboxylase was also assayed for the

purpose of establishing correlation between the two activities. As it has been pointed out in the Introduction (section 1.1.2.B) studies indicate that the carboxylase reaction is an activity of α -ketoglutarate decarboxylase and this matter will be discussed in more detail later in section 3.9.

Experimental procedures Mitochondrial fractions from cells grown in proteose-peptone medium were obtained by method A or B (section 2.2.4) and were stored at -15° for three days. The preparations were then thawed out and treated as outlined below.

(i) Freezing and thawing: quick freezing at -40° was achieved by immersing the tubes containing the mitochondrial fraction in acetone for 20 min, and then thawing under running tap water. This procedure was repeated three times. 0.1% Triton X-100 was present during this treatment in some experiments.

(ii) Sonication: the mitochondrial fraction was sonicated at 5 kcycycle for 30 sec or 120 sec in tubes immersed in a mixture of water and ice.

(iii) Preparation of acetone powder: Acetone powder

from mitochondria was prepared basically as described by Gunsalus, (1955), as follows: Mitochondrial fraction obtained by method B was suspended in 20 mM K-phosphate buffer, pH 7.0, supplemented with 1 mM BME. This suspension was added slowly with magnetic stirring to 20 volumes of acetone previously cooled to -20° .

During this procedure the beaker containing the acetone was immersed in a mixture of ice and salt (NaCl). After about 1 min the solvent was removed by filtration in a Buchner funnel using Whatman N^o1 filter paper and the precipitate was washed with 20 ml of acetone at -20° , and dried on the filter. The filter paper was then transferred to a vacuum dessicator containing phosphorus pentoxide where it was stored under vacuum at -15° until further treatment. Extracts from acetone powder were obtained by suspending the acetone powder in 50 mM K-phosphate, pH 7.0, supplemented with 1 mM BME. After homogenization in a glass homogenizer the suspension was stirred for 60 min at 0° , and centrifuged at 14,000 xg for 60 min (Koch and Stokstad, 1966).

Isoelectric precipitation from the acetone powder extract was conducted by dropwise addition of 1% acetic acid at 0° (Seaman 1953). The precipitate was suspended

in a small volume of 20 mM K-phosphate buffer, pH 7.0, supplemented with 1 mM BME. The supernatant fluid was neutralized with 1 N NaOH.

Ammonium sulphate fractionation: Solid ammonium sulphate was added slowly with magnetic stirring to extracts from acetone powder until 60% saturation was obtained. After 20 min the solution was centrifuged at 14,000xg for 30 min. The supernatant fluid was discarded and the pellet was suspended in 50 mM K-phosphate buffer, pH 7.0, with 1 mM BME and dialyzed overnight against 530 volumes of the same buffer (Koch and Stokstad, 1966; Saito et al., 1971).

Assay of α -ketoglutarate:glyoxylate carboligase and α -ketoglutarate decarboxylase were performed essentially as described in Methods (section 2.2.5).

Results and discussion. The effects of freezing and thawing and of sonication on α -ketoglutarate:glyoxylate carboligase and α -ketoglutarate decarboxylase are shown in Table 3.18. These activities assayed in thawed mitochondrial fraction, which had been kept for one week at -15° were not changed significantly by further freezing and thawing or sonication for 30 sec or 2 min.

Treatment	Units/ml			
	α -Ketoglutarate: glyoxylate carboligase		α -Ketoglutarate decarboxylase	
Mitochondria ^a	1.280	100	0.980	100
Supernatant fluid	0.280	22	0.220	22
Mitochondria ^b	1.260	98	1.030	105
Mitochondria ^c				
30 sec	1.240	97	0.940	96
120 sec	1.220	95	0.860	88

Table 3.18: Effect of freezing and thawing and of sonication on α -ketoglutarate:glyoxylate carboligase and on α -ketoglutarate decarboxylase activities.

Mitochondrial fraction obtained by method A, which was suspended in sucrose-phosphate-BME solution (section 2.2.4) and kept frozen for one week at -15° , was thawed and subjected three times to freezing and thawing or to sonication at 5 kcycle for 30 sec or 120 sec. An aliquot of the thawed mitochondrial fraction was centrifugated at $27,000 \times g$ for 30 min to obtain the mitochondrial supernatant fluid. The enzymes were assayed in the standard conditions containing 1.2 mg of protein for 30 min with α -(1- 14 C)ketoglutarate (132,212 d.p.m.).

^a Thawed once; ^b Frozen and thawed three times;

^c Sonicated.

If it is considered that both enzymatic activities are contained inside the mitochondria, then any treatment that can disrupt the mitochondrial membrane would result in an "activation" of the enzymes and therefore the results presented above may indicate that one freezing and thawing (thawing after storage) was enough to disrupt the mitochondrial membrane. When the thawed mitochondrial fraction was centrifuged at 25,000xg for 15 min (Table 3.13), only 22% of both activities were found in the mitochondrial soluble supernatant fluid. The recoveries of carboligase in supernatant fluids from mitochondria that have been subjected to repeated freezing and thawing and to detergent action were studied and are shown in Table 3.19. In this experiment, thawed mitochondrial fraction isolated by method B and suspended in 20 mM K-phosphate buffer, pH 7.0, with 1 mM BME, which had been kept for three days at -15° was divided into three equal portions. The portions were then frozen and thawed three times and are referred to as A, B, and C. Portions A and B were treated similarly except that portion B was allowed to stand for an additional 30 min with occasional mixing at 0°. This additional time at 0° did not result in significant changes in enzyme activity nor did it improve the recovery of the enzyme in the super-

Treatment	Vol. ml	Protein mg/ml	Units ml	Total Units	Units mg prot.	Yield %	Units/ml (after 22 hours)	Ratio of units
(A)								
F-T mitochondrial suspension	1.6	28.0	28.60	45.7	1.02	100	27.50	0.96
Supernatant fluid 1	1.5	8.3	8.88	13.3	1.07	29	8.22	0.93
Pellet	2.1	16.4	13.56	28.4	0.82	62		
Supernatant fluid 2	2.0	1.5	2.74	5.4	1.79	12		
Supernatant fluid 1 plus sucrose			8.40				8.10	0.97
(B)								
F-T mitochondrial suspension	1.6	27.6	28.34	45.4	0.97	100	23.50	0.83
Supernatant fluid 1	1.5	8.5	7.81	11.7	0.92	26	6.23	0.79
Pellet	2.3	15.0	13.27	30.5	0.88	67		
Supernatant fluid 2	2.2	1.5	3.12	6.8	2.15	15		
Supernatant fluid 1 plus sucrose			8.14				6.47	0.79
(C)								
F-T mitochondria plus Triton	1.6	26.6	27.10	43.4	1.02	100	23.54	0.87
Supernatant fluid 1	1.5	8.9	8.05	12.1	0.90	28	6.58	0.82
Pellet	2.3	15.3	13.39	30.8	0.87	71		
Supernatant fluid 2	2.2	1.8	3.16	6.9	1.90	16		
Supernatant fluid 1 plus sucrose			8.20				6.87	0.84

Table 3.19

continued...

Table 3.19: α -Ketoglutarate:glyoxylate carboligase release from mitochondria after freezing and thawing in the presence or absence of Triton X-100, and effects of sucrose and temperature on enzyme activity. Mitochondrial fraction treatment are described in the text. The enzyme was assayed in the standard conditions but with 10 μ moles of (1- 14 C) glyoxylate (222,727 d.p.m.) and 4 μ moles of α -ketoglutarate for 30 min. The reaction was stopped with TCA-AAP.

^a The fractions were assayed after storage for 22 hours at -15° in experiment A or at 0° in experiments B and C.

^b Represents the units determined after standing for 22 hours divided by the units determined immediately after obtaining the fractions.

F-T, freezing and thawing three times.

natant fluid obtained after centrifugation of the mitochondrial fraction (supernatant fluid 1) or from the similarly obtained pellet supernatant fluid (supernatant fluid 2) (Table 3.19, A and B). Therefore, the discussion will be limited only to portion B which will serve as a control for portion C in which 0.1% Triton X-100 was present during freezing and thawing.

Table 3.19 shows that upon centrifugation the supernatant fluid 1 contained about 26% of the units initially present in the mitochondrial preparation. When the pellet was extracted with 20 mM K-phosphate buffer, pH 7.0, with 1 mM BME and centrifuged, supernatant fluid 2 was obtained containing 15% of the enzyme units present in the original mitochondrial fraction. Only in supernatant fluid 2 was a moderate increase obtained in specific activity. Triton X-100 was completely ineffective in increasing the recovery in supernatant fluid 1 as shown in Table 3.19, C. Only 28% of the original units were found in supernatant fluid 1 and 16% in supernatant fluid 2. Again the same slight increase in specific activity was obtained in supernatant fluid 2.

These results suggest that in Tetrahymena the carbolligase and decarboxylase activities are bound to

some degree to membranes because freezing and thawing was not sufficient to release these activities and, in terms of the carbolligase activity, the nonionic detergent was also ineffective in this respect.

It has been pointed out that the decarboxylative condensation of α -ketoglutarate with glyoxylate may be an activity of the α -ketoglutarate decarboxylase component of the α -ketoglutarate dehydrogenase complex (section 1.1.2.B). The results of Table 3.18 tend to confirm this. However, it has been suggested that the α -ketoglutarate- and pyruvate-dehydrogenase complexes are bound loosely, if at all, to the mitochondrial membrane (Linn et al., 1972) because mild conditions i.e. freezing and thawing were effective in extracting these activities from kidney and heart mitochondria and results reported by other workers suggest that these complexes are located within the mitochondria matrix (Smoly et al., 1970; Brdiczka et al., 1968; Scinaitman and Greenawalt, 1968). More evidence that α -ketoglutarate:glyoxylate carbolligase and therefore possibly α -ketoglutarate decarboxylase may not be located in the mitochondrial matrix was obtained when the same extraction procedure for α -ketoglutarate-

and pyruvate dehydrogenase complex from kidney mitochondria (Linn et al., 1972) was utilized. In this procedure, thawed mitochondrial fraction was acidified with 0.1 N HCl to pH 6.5 and made 50 mM in NaCl, and the suspension was centrifuged at 22,000xg for 30 min. It was found as shown in Table 3.20, A, that only 30% of the carbolligase units were recovered in the mitochondrial extract and 32% in the pellet. However, 38% of the original activity was lost and this is reflected in the lower specific activities of the extract and the pellet.

Contradictory results on the location of the carbolligase have been reported. Steward and Quayle (1967) reported that the enzyme was concentrated in the soluble fraction within the pig liver mitochondria rather than the particulate fraction. In their experiment the mitochondria were passed through a Hughes press at -15° and the resulting extract contained 77% of the units present in the mitochondria with an increase in the specific activity of 1.82. On the other hand, five minutes sonication of the rat liver mitochondria at 10 kcycycle and a fractionation (105,000xg for 90 min) of the ~~pellet~~ ^{supernatant} revealed nearly equal distribution of the activity in the supernatant fluid and the pre-

cipitate (Kawasaki et al., 1966). Moriyama and Fui (1966) have reported that the enzyme was firmly bound to a particulate fraction in the freshly prepared cell extracts from Mycobacterium takeo and failed to be released by sonic oscillation or digitonin treatment, however, in a 50% ammonium sulphate fraction, on storage for a long period (several months) in the frozen state, the enzyme was solubilized to a considerable extent.

Extraction of the enzyme from mitochondrial acetone powder was also attempted as shown in Table 3.20; B and C. The extracts from acetone powder contained 47% and 43% of the units initially present in the mitochondrial fraction (B and C respectively). In the preparation of the acetone powder itself (C, total AP suspension) about 43% of the units were lost. No decrease in the specific activity of the extract with respect to the initial mitochondrial fraction was observed so that this procedure seems to be superior than the freezing and thawing treatment. Acetone-dried rat liver mitochondria were used as the source of the enzyme by Koch and Stokstad (1966) and by Saito et al., (1971).

The enzyme present in the mitochondrial fraction

	Vol		Protein		Total Prot.	Units		Total	Units	Yield
	ml	mg/ml	mg	mg	mg	ml	mg prot.	Units	mg prot.	%
Exp. 1: (A)										
Mitochondrial fraction	1.5	32	48			35.90		53.7	1.12	100
NaCl extract	1.3	14	18			12.58		16.4	0.91	30
Pellet	1.2	21	26			14.26		17.1	0.66	32
(B)										
Mitochondrial fraction	9.0	32	288			35.90		323.0	1.12	100
A.P. extract	10.4	10	101			14.60		152.0	1.50	47
Pellet	4.0	46	185			8.30		33.2	0.18	11
(C)										
Mitochondrial fraction	23	21	483			17.22		397.0	0.82	100
Total A.P. suspension	12.9	36	459			17.68		228.0	0.50	57
A.P. extract 1	12	16	193			14.23		171.0	0.88	43
Extract 2	5	7	34			1.68		8.4	0.25	2
Pellet	4	51	204			2.22		8.8	0.04	2
Exp. 2: (D)										
A.P. extract	10	10	97			14.60		146.0	1.50	100
pH 5.4 supernatant	12	7	85			0.53		6.2	0.07	4
Pellet	0.4	4	1			0.07		0.0	0.02	0
(E)										
A.P. extract	11.5	16	185			14.23		163.5	0.88	100
0-60% (NH ₄) ₂ SO ₄ dialyzed pellet	2.1	41	86			5.18		10.9	0.13	7

Table 3.20: Enzyme activity in mitochondria and mitochondria acetone powder. The enzyme was assayed as described in Table 3.19. The indicated treatment are described in Experimental procedures. A.P. acetone powder.

and in the supernatant fluids was stable at least for 22 hours when the fractions were stored at -15° (Table 3.19A). When these fractions were kept at 0° for 22 hours a slight inactivation, about 17% was observed (Table 3.19 B and C). Sucrose present at a final 0.25 M concentration did not enhance the stability of the soluble enzyme at this stage of purification. A stimulatory effect by sucrose was observed by Stewart and Quayle (1967) but at a higher purification level.

Precipitation of acetone powder extracts at pH 5.4 resulted in a complete inactivation of the enzyme as shown in Table 3.20, D. Only 0.02% of the units present in the extracts were found in the pellet and in the neutralized supernatant fluid 4% of the units were recovered.

When extracts from acetone powder were made 60% saturated in ammonium sulphate by the addition of the solid salt (39 gr/100ml) only 7% of the units present in the extracts were found in the dialyzed pellet, which had a very low specific activity (Table 3.20, E). The possibility that dialysis of the pellet obtained at 60% saturation in ammonium sulphate was not completely effective in removing the salt must be considered as an explanation of the poor recovery obtained. As shown in Table 3.21 the presence of

Ammonium sulphate, per cent		Units/ml	Inhibition, %
In assay	In pretreated enzyme		
--	--	6.01	0
0.5	--	5.56	7.5
5.0	--	1.25	79
0.5	1.0	5.10	15
5.0	10.0	0.80	87

Table 3.21: Effects of ammonium sulphate on enzyme activity. Appropriate aliquots of a saturated ammonium sulphate solution, pH 7.0, were either added to the enzyme alone or to the complete assay mixtures. After standing for 10 min at 0 the carbolligase activity was determined under the conditions described in Table 3.19. The enzyme source used was the supernatant fluid obtained by centrifugation at 25,000xg for 15 min of the frozen and thawed mitochondrial fraction (8.6 mg/ml from which 0.2 ml were assayed).

0.5% or 5% ammonium sulphate in assay mixtures resulted in 7.5% and 79% inactivation of the enzyme. It was also found that allowing the enzyme to stand in the presence of the salt for 10 min at 0° at final concentrations of 1% and 10% an additional inactivation was observed which resulted in a 15% and 85% inactivation, respectively, as compared to the untreated enzyme.

In conclusion, our attempts to release the enzyme from the mitochondria in a good yield for the purpose of purification and for correlation studies between the carbolicase and decarboxylase activities were unsuccessful. Correlation between the two activities was pursued with a different approach and this will be presented in the next section.

Section 3.9: CORRELATION BETWEEN α -KETOGLUTARATE:GLYOXYLATE CARBOLIGASE AND α -KETOGLUTARATE DECARBOXYLASE IN TETRAHYMENA.

Schlossberg et al., (1970) purified α -ketoglutarate: glyoxylate carbolicase about 100-fold from beef heart and, during the purification procedures α -ketoglutarate decarboxy-

lase and α -ketoglutarate:acetaldehyde carbolicase activities were parallel with that of α -ketoglutarate:glyoxylate carbolicase. Similar results were found with the purified α -ketoglutarate:glyoxylate carbolicase from extracts of Rhodospseudomonas spheroides, and acetone-dried liver mitochondria (Saito et al., (1971) and from extracts of Mycobacterium phlei (Yamasaki and Moriyama, 1971). The α -ketoglutarate dehydrogenase complex prepared from pig heart (Saito et al., 1971) and from E. coli (Kubasik et al., 1972) also catalyzed the carbolicase reaction between α -ketoglutarate and glyoxylate or some other aldehyde. The α -ketoglutarate decarboxylase subcomplex derived from the α -ketoglutarate dehydrogenase complex, by resolution with urea, also showed the carbolicase activity (Saito et al., 1971)

It is evident, as has been suggested by the above workers that the enzyme catalyzing the synergistic decarboxylation of α -ketoglutarate and glyoxylate is identical with α -ketoglutarate decarboxylase. In an attempt to study this aspect in Tetrahymena purification of α -ketoglutarate:glyoxylate carbolicase had, previously and unsuccessfully, been attempted (section 3.8). Therefore a different

approach was taken based on the assumption that any condition that could affect one activity would also affect the other if the two activities resided in a single protein.

3.9.1. Effects of nucleotide on enzyme activities.

The adenine nucleotide regulation of α -ketoglutarate dehydrogenase complex has been recently reported. In cauliflower florets the enzyme, which was 50-fold purified from the sonically disrupted mitochondria and which clearly prefers adenylate activators to other nucleotides, was strongly activated by AMP with a decrease in the apparent K_m for α -ketoglutarate (Wedding and Black, 1971). The stimulation was found to be exerted on the E_1 - E_2 sub-complex (section 1.3). Davies and Kenworthy (1970) appeared to indicate that the activating effect of AMP took place prior to the decarboxylation of α -ketoglutarate in the reaction. These workers measuring $^{14}CO_2$ production from d -(1- ^{14}C)-ketoglutarate by an extract of pea mitochondrial preparation, found a 4-fold increase in decarboxylation in the presence of 0.5 mM AMP.

In blowfly flight muscle, ATP inhibition of the partially purified enzyme has been reported to be overcome

by AMP and ADP (Hansford, 1972). Neither of these nucleotides activated the enzyme in the absence of ATP. The site of action of the adenine nucleotide within the enzyme complex was not identified, although the competitive inhibition by ATP with respect to α -ketoglutarate would suggest component E_1 . However, the spectrophotometric ferricyanide-linked assay did not fully confirm this suggestion, as the ATP inhibition was found to be greatly decreased.

In Acinetobacter Iwoffii, the overall activity of the complex was inhibited by NADH. AMP and ADP relieved this inhibition and also stimulated enzyme activity. This was observed with a 200,000xg supernatant fluid from A. Iwoffii (Weitzman, 1972) and it was confirmed with the purified enzyme (Parker and Weitzman, 1973). Assays specific for the first enzyme component (E_1) (following oxidative decarboxylation with dichlorophenol-indophenol as an electron acceptor) showed this to be the site of action of the adenylate. A generalization of the nucleotide effects on α -ketoglutarate dehydrogenase complex cannot yet be done, however, the above reports indicate the strong possibility that the enzyme, as other constitutive enzymes, may respond to the energy state of the cell. These effects have been generalized by Atkinson (1968) into a concept

designed as control through the energy charge of the cells. In this concept the energy charge is low when nucleoside monophosphates predominate and high when the triphosphates are in greatest abundance. Enzymes which are part of a system regenerating ATP are usually found to be activated by AMP and inhibited by ATP, and this is frequently observed when the enzyme being controlled does not use any of the nucleotides in its reaction. Since the E_1 component seems to be the site of action of AMP, the effect of this and other nucleotides on the carbolicase and decarboxylase activities was studied.

Experimental procedures. The mitochondrial fraction from cells grown in proteose-peptone medium were obtained by method A (section 2.2.4). α -Ketoglutarate:glyoxylate carbolicase was assayed in the standard conditions containing (U- ^{14}C) glyoxylate (283,000 d.p.m.) as the labelled substrate. After 1 hour of incubation the reaction was stopped by adding TCA-AAP (section 2.2.5.A). α -Ketoglutarate decarboxylase was assayed in the standard conditions (section 2.2.5.B). Nucleotides were present in the incubation mixtures at 1 mM final concentration.

Result and discussion. The adenine nucleotides

AMP, ADP and ATP and the guanine nucleotides GMP and GTP at 1 mM final concentration did not affect the activity of the carbolicase nor the activity of the decarboxylase (Table 3.22).

When lower concentrations of AMP (0.1 and 0.5 mM) were present in the incubation mixtures, no stimulatory effects were observed on α -ketoglutarate decarboxylase.

No effect of AMP, ADP or ATP was found on a partially purified carbolicase from R. spheroides (Okuyama et al., 1965) and on rat liver mitochondria suspension (Crawhall and Watts, 1962). Our results with Tetrahymena mitochondrial suspensions are similar and in this case a parallel assay of the α -ketoglutarate decarboxylase indicated that this enzyme was not affected by these nucleotide either. However, the E_1 component of the α -ketodehydrogenase complex seems to be the site of action of AMP, and the E_1 component when assayed for the carbolicase reaction was stimulated by AMP only in one case (Davies and Kenworthy, 1970) and ADP was the activating nucleotide for a partially purified carbolicase from extract of M. takeo (Moriyama and Yui, 1966). The fact that α -ketoglutarate decarboxylase in Tetrahymena was not activated by AMP or ADP may be due to the kind of

Additions	Enzyme activity (per cent of control)	
	α -ketoglutarate:	α -ketoglutarate
	glyoxylate carboligase	decarboxylase
--	100	100
AMP	95.3	95.0
ADP	92.5	94.5
ATP	90.5	89.0
GMP	99.0	100.0
GTP	93.5	105.0

Table 3.22: Effects of some nucleotides on enzyme activities. Both activities were assayed in the standard conditions as described in Experimental Procedures. The indicated nucleotides were present at 1 mM final concentration. The assays were done in duplicate. Protein present was 2.9 mg.

preparation utilized, as was the case with the enzyme from M. takeo (Noriyama and Yui, 1966) which was not activated by ADP when a particulate preparation was used, however, after solubilization ADP was effective in activating the enzyme assayed as the carbolicase. These workers suggested that the activation may result in a conformational change in the enzyme on addition of ADP, which was quite specific in enhancing the activity.

3.9.2 Effect of varying growth conditions on enzyme activities: static and agitated cells.

Experimental procedures. The "static" cells were grown without shaking in 500 ml flasks containing 250 ml proteose-peptone medium. The "agitated" cells were grown in 500 ml flasks containing 150 ml proteose-peptone medium with rotatory shaking at 120 r.p.m. After two and three days of growth the cells were counted and collected and the homogenate obtained by method A (section 2.2.4) was used as the enzyme source. The enzymes α -ketoglutarate: glyoxylate carbolicase and α -ketoglutarate decarboxylase were assayed under the standard conditions (section 2.2.5).

Results and discussion. Table 3.23 shows that the changes in the activity of α -ketoglutarate decarboxylase

Hours of growth	Carbolygase		Decarboxylase	
	Agitated	Static	Agitated	Static
48	4.2 (0.035)	22.8 (0.088)	2.5 (0.021)	15.6 (0.060)
72	5.6 (0.045)	11.8 (0.055)	4.1 (0.032)	8.8 (0.041)

Table 3.23: Activity units per 10^6 cells of α -ketoglutarate:glyoxylate carbolygase and α -ketoglutarate decarboxylase in the crude homogenate. Figures in parentheses indicate activity per milligram protein. The homogenate was the 500xg supernatant fluid. Agitated cells were grown with shaking in a rotary shaker set at 120 r.p.m. The carbolygase was assayed with α -(1- 14 C)ketoglutarate as the labelled substrate and the reaction was stopped with PCA.

due to varying growth conditions (static or agitated cells) or to age of cultures, are accompanied by quite similar changes in the carboligase activity. The "static" cells contained 5.4-fold and 6.2-fold more units of carboligase and decarboxylase, respectively, as compared to the "agitated" cells, after growth for 48 hours. The corresponding figure for cells grown for 72 hours is about 2. A marked decrease in the units in the "static" cells from 48 hours to 72 hours of growth was found with both activities.

The evidence is circumstantial but it points to the possibility that in Tetrahymena also the two activities may be linked. It should be noted that the "static" cells were much larger than the "agitated" cells and this probably accounts for the smaller differences for the different growth conditions when enzyme activities were expressed as specific activities rather than as activities per 10^6 cells.

3.9.3 Effect of varying growth conditions on enzyme activities: nutritional effects and thiamine deficiency.

Experimental procedures. In these experiments mitochondrial and supernatant fractions obtained by method B, from cells grown for 48 hours in synthetic medium (S.M.), synthetic medium supplemented with either acetate (S.M.A.) or glucose (S.M.G.) were utilized. Also cells deficient in thiamine were used. These organisms were grown in synthetic medium supplemented with acetate containing 0.2 μ g of thiamine per 100 ml medium, in this condition the growth was reduced to an 80% to that obtained when normal amounts of thiamine were present during growth (100 μ g/100ml) (section 2.2.14). The mitochondrial and supernatant fractions from the thiamine deficient organisms were harvested after 72 hours of growth and the activities compared to normal cells grown for the same period of time.

α -Ketoglutarate:glyoxylate carboligase was assayed in the standard conditions with 2 μ moles (1- 14 C) glyoxylate (178,483 d.p.m.) as the labelled substrate. Incubations were carried out for 30 min and the reaction was stopped with TCA-AAP. α -Ketoglutarate decarboxylase was assayed as described previously (section 2.2.5.B).

Total collection, extraction and counting procedures of DNPH- α -ketoglutarate were conducted as described

previously (section 2.2.10). This compound was obtained from assay mixtures containing 4 μ moles (2- 14 C) glyoxylate (245,744 d.p.m.) and 4 μ moles α -ketoglutarate. Discussion on this area will be presented in section 3.10.5.

Results and discussion. Table 3.24 shows the effects of the presence of acetate and glucose on α -ketoglutarate: glyoxylate carboligase and α -ketoglutarate decarboxylase activities. The two activities in the mitochondrial fraction from cells grown in synthetic medium were not significantly different from those found in the mitochondrial fraction from cells grown in medium supplemented with acetate or glucose. The activities in the supernatant fraction were about 17% and 12% respectively, of the activities found in the mitochondrial fraction from the cells grown in the synthetic medium. Acetate or glucose added to the growth medium did not affect the activities of the supernatant fraction.

Table 3.25, shows the effect of thiamine deficiency on both activities. The mitochondrial fraction from the deficient cells have only 51% of carboligase activity and 52% of decarboxylase activity of this fraction of normal cells in exp. 1, and 32% and 23% respectively, in experiment

Growth	Enzyme source	Carboligase Units/mgProt.	DNPH- α -keto- glutarate d.p.m./mgProt.	Decarboxylase Units/mgProt.
S.M.	Mitochondria	0.288(4)	1,565(3)	0.328(2)
	Supernatant	0.047(4)	228(2)	0.041(2)
S.M.A.	Mitochondria	0.324(2)	1,565	0.360(2)
	Supernatant	0.047(2)	250	0.040(2)
S.M.G.	Mitochondria	0.274(2)	1,446(2)	0.309
	Supernatant	0.048(2)	263(2)	0.039

Table 3.24: Effect of acetate and glucose on α -ketoglutarate: glyoxylate carboligase, on α -ketoglutarate decarboxylase and on α - 14 C ketoglutarate labelling. Cells were grown in synthetic medium (S.M.) and synthetic medium supplemented with either acetate (S.M.A.) or glucose (S.M.G.). α -Ketoglutarate: glyoxylate carboligase was assayed in the standard conditions in the presence of 1.5 mg of mitochondrial protein or about 1.5 mg of supernatant protein and (1- 14 C)glyoxylate (178,483 d.p.m.) as the labelled substrate. Incubations were carried out for 30 min and the reaction was stopped with TCA-AAP. α -Ketoglutarate decarboxylase was assayed in the standard conditions in the presence of 1.5 mg protein. DNPH- α - 14 C ketoglutarate was obtained from the assay mixtures for the carboligase reaction containing about 2.4 mg of mitochondrial protein or about 3.2 mg of supernatant protein and (2- 14 C)glyoxylate (245,744 d.p.m.). The values are means, whenever more than one experiment was performed. Numbers in parenthesis indicate the numbers of experiments.

Type of organism	Subcellular fraction	Carboligase Units/mgProt.	DNPH- α -keto-glutarate d.p.m./mgProt.	Decarboxylase Units/mgProt.
Experiment 1				
Normal	Mitochondria	0.343	1,780	0.344
	Supernatant	0.044		0.033
Defficient	Mitochondria	0.174	808	0.178
	Supernatant	0.044		0.044
Experiment 2				
Normal	Mitochondria	0.121	500	0.162
	Supernatant	0.016		0.012
Defficient	Mitochondria	0.039	135	0.037
	Supernatant	0.014		0.019

Table 3.25: Effect of thiamine deficiency on α -ketoglutarate: glyoxylate carboligase, on α -ketoglutarate decarboxylase and on α -ketoglutarate labelling. Organisms were grown in synthetic medium supplemented with acetate. Deficient organisms were grown on a medium containing 0.2 μ g of thiamine per 100ml and normal organism on the same medium containing 100 μ g of thiamine per 100 ml. The protein present in the enzyme assays was 0.87 mg (exp. 1) and 1 mg (exp. 2) of mitochondrial fraction from normal cells; 1.5 mg (exp. 1) and 1.9 mg (exp. 2) of supernatant fraction from normal cells; 1.48 mg (exp. 1) and 1 mg (exp. 2) of mitochondrial fractions from deficient cells; 1.5 mg (exp. 1) and 2.2 mg (exp. 2) of supernatant fraction from deficient cells. α -Ketoglutarate was obtained as described in Experimental Procedures, with 1.7 mg (exp. 1) and 4 mg (exp. 2) of protein from mitochondrial fraction of normal cells; or 2.9 mg (exp. 1) and 4 mg (exp. 2) from the mitochondrial fraction of deficient cells

2. Both activities in the supernatant fraction from deficient cells were comparable to those of normal cells.

These results also suggest that the α -ketoglutarate:glyoxylate reaction may be an activity of the α -ketoglutarate decarboxylase. It is important for cellular economy that a constitutive enzyme may be capable of a dual role in providing energy to the cell, by oxidizing α -ketoglutarate in the TCA cycle, and by maintaining a physiological concentration of metabolites which can be harmful to the cell, as in the case of glyoxylate.

α -Ketoglutarate decarboxylase appears to be regulated by AMP (section 3.9.1) and in one case it was reported (Davies and Kenworthy, 1970) that α -ketoglutarate:glyoxylate carboligase activity was also affected to a similar degree. It thus seems possible that the carboligase activity will be manifested when glyoxylate becomes accessible to the α -ketoglutarate dehydrogenase complex.

In thiamine deficiency the pyruvate dehydrogenase complex activity is decreased and both pyruvate (Peters, 1953) and glyoxylate (Liang, 1962) accumulate. This was attributed to excess tissue breakdown which allowed glyoxylate

to be formed from glycine (Liang, 1962). The finding that α -ketoglutarate:glyoxylate carbolicase were decreased by about 50% in the thiamine-deficient Tetrahymena cells suggests that the observed accumulation of glyoxylate was due to an enzyme defect in the utilization of glyoxylate involving the α -ketoglutarate dehydrogenase complex and possibly the pyruvate dehydrogenase complex as well. Both complexes are thiamine-dependent and capable of reacting with glyoxylate. However in mammalian tissues the reaction of glyoxylate with pyruvate is much slower than its reaction with α -ketoglutarate (Kubasik et al., 1972).

The low activity observed in the mitochondrial fraction from normal cells in experiment 2 (Table 3.25) may be due to prolonged storage (4 days at -15°) before being assayed. Normally, the maximum time elapsed between fractionation and assaying was about 12 hours at -15° .

Section 3.10: STUDIES ON THE ORIGIN OF α -KETOGLUTARATE REGENERATED IN THE α -KETOGLUTARATE:GLYOXYLATE CARBOLIGASE ASSAY MIXTURES.

It has been repeatedly observed that the DNPH derivative

corresponding to α -ketoglutarate was labelled even when unlabelled α -ketoglutarate was present in the assay mixtures. The origin of the label and the possible pathways responsible for its final transfer to the keto-acid are considered in this section.

3.10.1 Origin of α -ketoglutarate formed during the carbolligase reaction.

Experimental procedures. Cells were grown in proteose-peptone medium for about 60 hours. The cell extract was prepared by freezing and thawing (section 2.2.3) and the homogenate and mitochondrial fractions were obtained by fractionation method A (section 2.2.4). Incubations were carried out as described in section 2.2.5A. Substrate concentrations are indicated in the legends of the figures and tables. The reaction was stopped with PCA. DNPH derivatives were obtained either by crystal collection or by total extraction as described in section 2.2.10. Paper chromatography of DNPH derivatives was done using solvent systems 1 and 2 and the radioactivity distribution on the papers was obtained after cutting the paper in 1 cm long-segments and counting in the liquid scintillation counter (section 2.2.10).

In one experiment parallel preparations of mitochondrial fractions by method B were obtained from cells grown both in synthetic medium supplemented with acetate and in proteose-peptone medium. The assay conditions and further treatment of DNPH derivatives formed in the reaction mixtures are described in section 3.6. The photometric reading for the DNPH- α -ketoglutarate was at 420 nm (Friedemann and Haugen, 1943).

Results and discussion. Cell extract containing 21 mg protein was incubated under the assay conditions for the carboligase in the presence of 4 μ moles ($\text{U-}^{14}\text{C}$) glyoxylate and 10 μ moles α -ketoglutarate. Two radioactive peaks were obtained upon chromatography of DNPH derivatives (Fig. 3.18). The first peak was found in the position of the α -ketoglutarate derivative and the second peak in the position of the HLA derivative. When the enzyme was omitted from the reaction mixtures two radioactive peaks corresponding to the derivatives of glyoxylate isomers were obtained. In experiments where α -ketoglutarate was omitted from the reaction mixtures no radioactivity was detected in the position corresponding to the DNPH- α -ketoglutarate. These results were observed several times with the cell extract as enzyme source.

Figure 3.18: Radioactivity distribution of DNPH derivatives on paper chromatography. Cell extract (21 mg) was incubated in the standard conditions but with 4μ moles of ($U-^{14}C$)glyoxylate and 10μ moles α -keto-glutarate. After 1 hour incubation, DNPH derivatives were formed in the deproteinized assay mixtures and the crystals were collected as described in the Experimental Procedures. Paper chromatography was conducted in solvent system 1. Then the chromatograms were cut in 0.5 cm-long segments and counted. The cells were grown in proteose-peptone. a) From complete assay mixtures, b) From control assay (no enzyme).

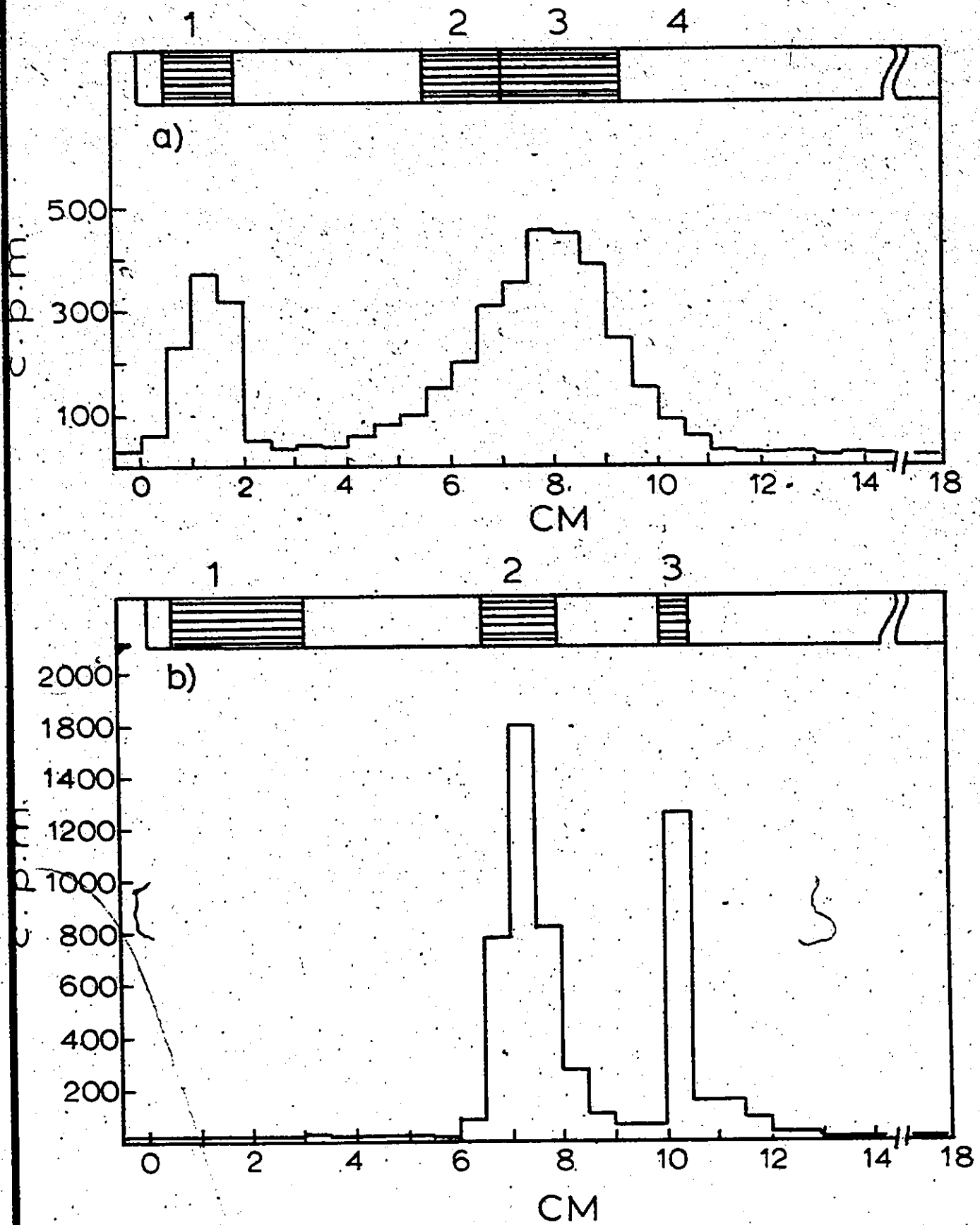


Figure 3.18

Enzyme source	$^{14}\text{CO}_2$ d.p.m.	DNPH- α -keto- glutarate, d.p.m.
Homogenate	91,882	6,598
Mitochondrial fraction	127,926	3,176

Table 3.26: Evolution of $^{14}\text{CO}_2$ and DNPH- α - ^{14}C ketoglutarate formation.

Homogenate (10.5 mg) and mitochondrial fraction (7 mg) obtained by method A from cells grown in proteose-peptone which had been kept at -15° for 2 months, were incubated in the standard conditions but with $4\mu\text{moles}$ of (U- ^{14}C) glyoxylate (568,000 d.p.m.) and $4\mu\text{moles}$ of α -ketoglutarate. After 1 hour the reaction was stopped with PCA. DNPH derivatives were formed in the deproteinized assay mixtures and a complete extraction was conducted. Paper chromatography was in solvent system 2. The amount of α - ^{14}C ketoglutarate derivative was calculated by adding up the radioactivity of the paper segments corresponding to the area of α -ketoglutarate on the chromatograms.

The mitochondrial fraction retained the ability to form ^{14}C - α -ketoglutarate, as indicated in Table 3.26. When the carbolligase was assayed with 4 μmoles ($\text{U-}^{14}\text{C}$) glyoxylate and 4 μmoles α -ketoglutarate, both the homogenate and the mitochondrial fractions formed α - ^{14}C -ketoglutarate. Evolution of $^{14}\text{CO}_2$ was also observed. It is important to note that with both solvent systems the radioactive peak migrated to the same position as DNPH- α -ketoglutarate.

These data suggest that one or both carbon atoms of glyoxylate were incorporated into the α -ketoglutarate molecule, and this process was promoted by the particulate fraction of the cells.

In order to find the origin of the ^{14}C carbon in the α -ketoglutarate molecule, the compound was studied by incubating specifically labelled glyoxylate substrate with mitochondrial preparations from cells grown in synthetic medium supplemented with acetate and in proteose-peptone medium. At the same time the effect of the presence of nitrogen and air in the gas phase during the reaction and the effect of TPP on the α - ^{14}C -ketoglutarate formed were investigated. As shown in Table 3.27 DNPH- α -ketoglutarate

Growth medium	Glyoxylate labelled	Gas phase	O.D. (420nm) per ml x 4×10^{-2}	DNPH-d-ketoglutarate d.p.m. per ml x 4×10^{-2}	2- 14 C per 1- 14 C
Synthetic medium plus acetate	1- 14 C	Air	0.382	67	70
	2- 14 C	Air	0.365	975	1,055
	2- 14 C	(TPP omitted)	0.620	425	270
	1- 14 C	N ₂	0.395	67	67
	2- 14 C	N ₂	0.395	675	675
Proteose-peptone medium	1- 14 C	Air	0.308	112	143
	2- 14 C	Air	0.208	657	1247

Table 3.27: Labelling of DNPH-d-ketoglutarate when (1- 14 C) or (2- 14 C) glyoxylate was the labelled substrate.

Assay conditions and DNPH derivatives chromatography are described in Table 3.16. d-Ketoglutarate derivative was extracted from the second chromatogram and the light absorption (O.D.) and radioactivity estimated (section 3.6.). The d.p.m. correspond to the total crystals collected. The d.p.m. have been corrected to the same specific radioactivity of glyoxylate in the assay mixtures.

was labelled mainly when (2-¹⁴C) glyoxylate was the labelled substrate. When (1-¹⁴C) glyoxylate was used as labelled substrate little radioactivity was found in α -ketoglutarate. The ratio of radioactivity of the α -ketoglutarate derivative obtained from (2-¹⁴C) glyoxylate over that obtained from (1-¹⁴C) glyoxylate varied from about 9 to 15 under the various conditions indicated in Table 3.27. A reduction of 57% was observed when TPP was omitted from the reaction mixture. Air in the gas phase stimulated by 45% the labelling in α -ketoglutarate.

These results indicate that under the conditions used for assay of the α -ketoglutarate:glyoxylate carboligase the C₂ carbon of glyoxylate was incorporated into the α -ketoglutarate molecule, and this incorporation was dependent on TPP, a cofactor which already has been demonstrated to be required for maximal activity of the carboligase reaction.

There are numerous reactions involving glyoxylate as substrate as has been presented in the Introduction and several possibilities that through some of those reactions labelled α -ketoglutarate may be regenerated from the C₂ carbon of glyoxylate. It was important to find which

carbon was labelled and this problem was investigated next.

3.10.2. Distribution of the label in the α -ketoglutarate molecule.

Experimental procedures. Mitochondrial fractions from cells grown in synthetic medium supplemented with either acetate or glucose were incubated under the standard conditions but with 4 μ moles (2- 14 C) glyoxylate (427,600 d.p.m.) and 4 μ moles α -ketoglutarate, for 1 hour under air or nitrogen as the gas phase. The reaction was stopped with PCA and DNPH derivatives formed in the deproteinized assay mixtures were collected by centrifugation of the crystals (experiment 1) or by total extraction (experiment 2). After chromatography of the DNPH derivatives for 24 hours in solvent system 1 the derivative of α -ketoglutarate was extracted and rechromatographed in the same system. Oxidation with acid-permanganate of DNPH- α -ketoglutarate was conducted as described in Methods (section 2.2.6). Oxidations of authentic DNPH- α -(1- 14 C) ketoglutarate and DNPH- α -(5- 14 C) ketoglutarate were also included in these experiments. The succinate formed in

the oxidation reaction was extracted, chromatographed and counted as described in section 2.2.6.

Results and discussion. When mitochondrial fractions from cells grown in synthetic medium supplemented with glucose were incubated under the assay conditions for the carbolligase reaction, with (2-¹⁴C) glyoxylate, under nitrogen or air as the gas phase, the DNPH- α -ketoglutarate became radioactive and its degradation to succinate and CO₂ by acid-permanganate oxidation established the location of ¹⁴C mostly in the 1-carboxyl carbon. As shown in Table 3.28, experiment 1, 87% of the radioactivity present in DNPH-¹⁴C- α -ketoglutarate from enzymatically formed α -ketoglutarate was recovered as CO₂ whether nitrogen or air was the gas phase during the enzymatic reaction. When the derivative from authentic α -(1-¹⁴C)-ketoglutarate was oxidized 96% of the label was recovered as CO₂. Only 1% was recovered as CO₂ when the derivative from authentic α -(5-¹⁴C)-ketoglutarate was oxidized. When the mitochondrial fraction from cells grown in the synthetic medium supplemented with glucose or acetate was used as the enzyme source about 80% of the label was recovered as CO₂. In no case could radioactivity be detected in chromatograms in the place of

Source of d-ketoglutarate	DNPH- α - 14 C ketoglutarate used for oxidation, d.p.m.	14 CO $_2$ -formed after KMnO $_4$ oxidation d.p.m.	% recovery
Experiment 1			
Mitochondria, N $_2$	499	434	87
Mitochondria, air	1,432	1,241	87
Authentic d-(1 14 C) keto-glutarate	2,625	2,522	96
Authentic d-(5 14 C) keto-glutarate	390	4	1
Experiment 2			
Mitochondria from:			
Cells grown in S.M.G.	3,290	2,768	84
Cells grown in S.M.A.	2940	2,304	80
Authentic d-(1 14 C) keto-glutarate	6,040	6,053	100
Authentic d-(5 14 C) keto-glutarate	2,220	136	6

Table 3.28: Permanganate oxidation of DNPH- α - 14 C ketoglutarate from enzymatically formed and authentic ketoacid.

Mitochondrial fraction (6.1 mg) from cells grown in synthetic medium supplemented with glucose (exp. 1) or mitochondrial fractions from cells grown in synthetic medium supplemented with glucose (S.M.G., 5.7 mg) or acetate (S.M.A., 7.5 mg) were incubated as described in Experimental Procedures. The DNPH- α -ketoglutarate extracted from the chromatograms was oxidized with KMnO $_4$ (section 2.2.6) and the 14 CO $_2$ estimated. Authentic d-ketoglutarate derivatives were also oxidized as described in section 2.2.6.

succinate which was formed during the permanganate oxidation.

As a result of these experiments it can be concluded that under the assay conditions of the carbolicase reaction carbon 2 of glyoxylate became the 1-carboxyl group of α -ketoglutarate.

As was pointed out previously there are numerous reactions involving glyoxylate as substrate that result in formation of TCA cycle intermediates, therefore, the possibility that under our experimental conditions α -ketoglutarate was formed by some of these reaction had to be considered.

In Tetrahymena the metabolism of glyoxylate (section 1.4.2) has been studied principally in relation to the glyoxylate cycle (Fig. 1.1). Accordingly, glyoxylate will condense with acetyl CoA by the action of malate synthase to form malate which is subsequently converted to isocitrate. Isocitrate may re-enter the glyoxylate cycle through the action of isocitrate lyase which yields succinate and glyoxylate. The succinate so formed will be labelled in the carboxyl group with the C_1 of glyoxylate and the inner carbons with the C_2 of glyoxylate as shown in Figure

3.19. Succinate via the TCA cycle enzymes will eventually yield α -ketoglutarate. This last compound then will be labelled with the two carbons from glyoxylate, and the ratio of C_2 carbon incorporated over the C_1 will be 2. This does not agree with our experiments which show (Table 3.27) that 9 to 15 times more incorporation into α -ketoglutarate occurs from (2- ^{14}C) than from (1- ^{14}C) glyoxylate.

Blum (1972) has presented evidence suggesting that glyoxylate added to growing Tetrahymena gives rise to CO_2 via the reactions of the active TCA cycle. This suggestion implies that the added glyoxylate does not re-enter the reaction of isocitrate lyase but rather is metabolized by the reactions of the TCA cycle. In this case the distribution of the labelled carbon atoms from glyoxylate after three or more turns of the TCA cycle (Figure 3.20) shows that α -ketoglutarate will be labelled only from the C_2 carbon of glyoxylate and one-third of the radioactivity in the α -ketoglutarate molecule will be located in the 1-carboxyl group. This is in qualitative agreement with the experiments of Table 3.27 which show that most of the label in α -ketoglutarate came from the

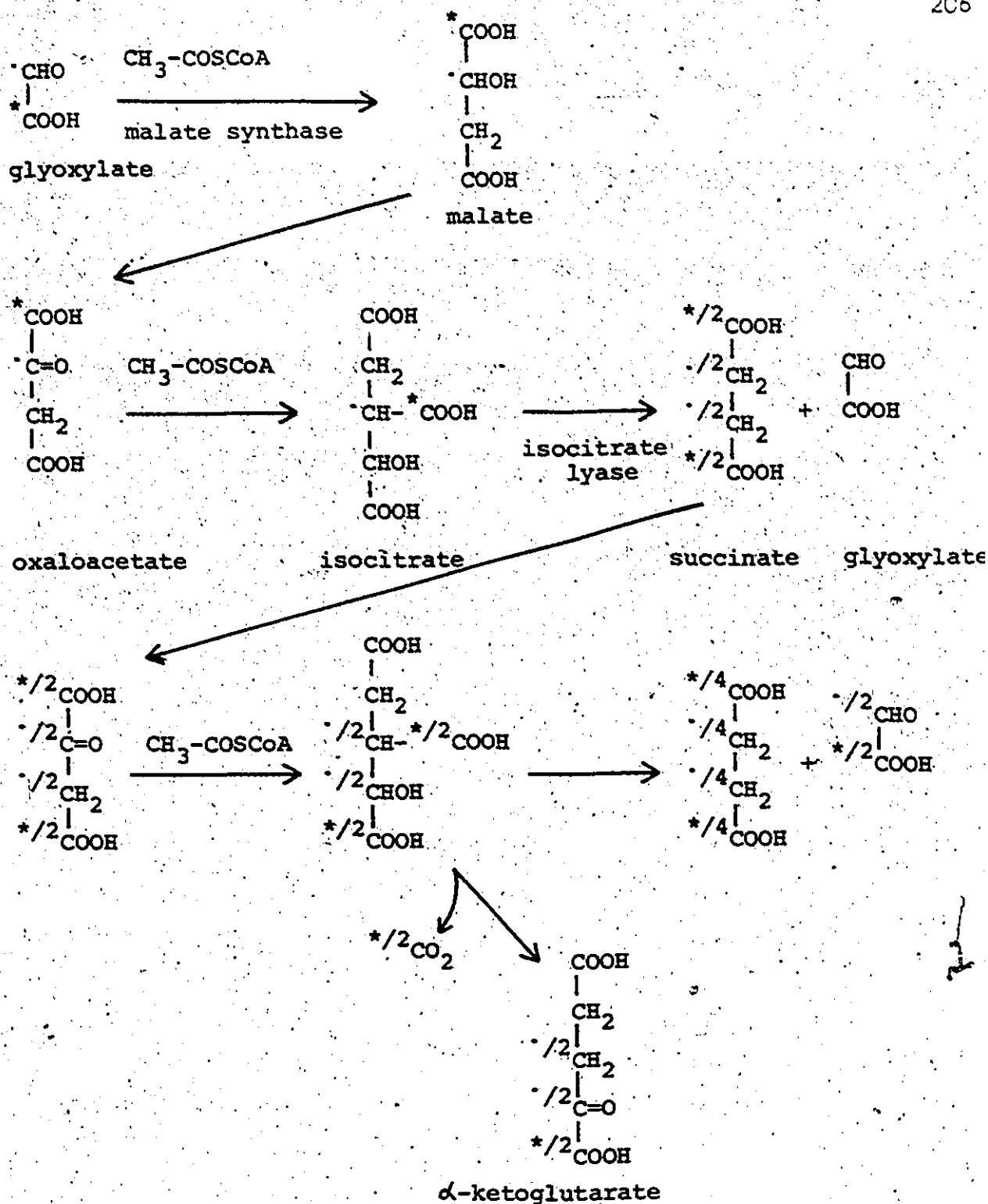


Figure 3.19: The distribution of carbon atoms from glyoxylate in TCA intermediates after participation of isocitrate lyase.

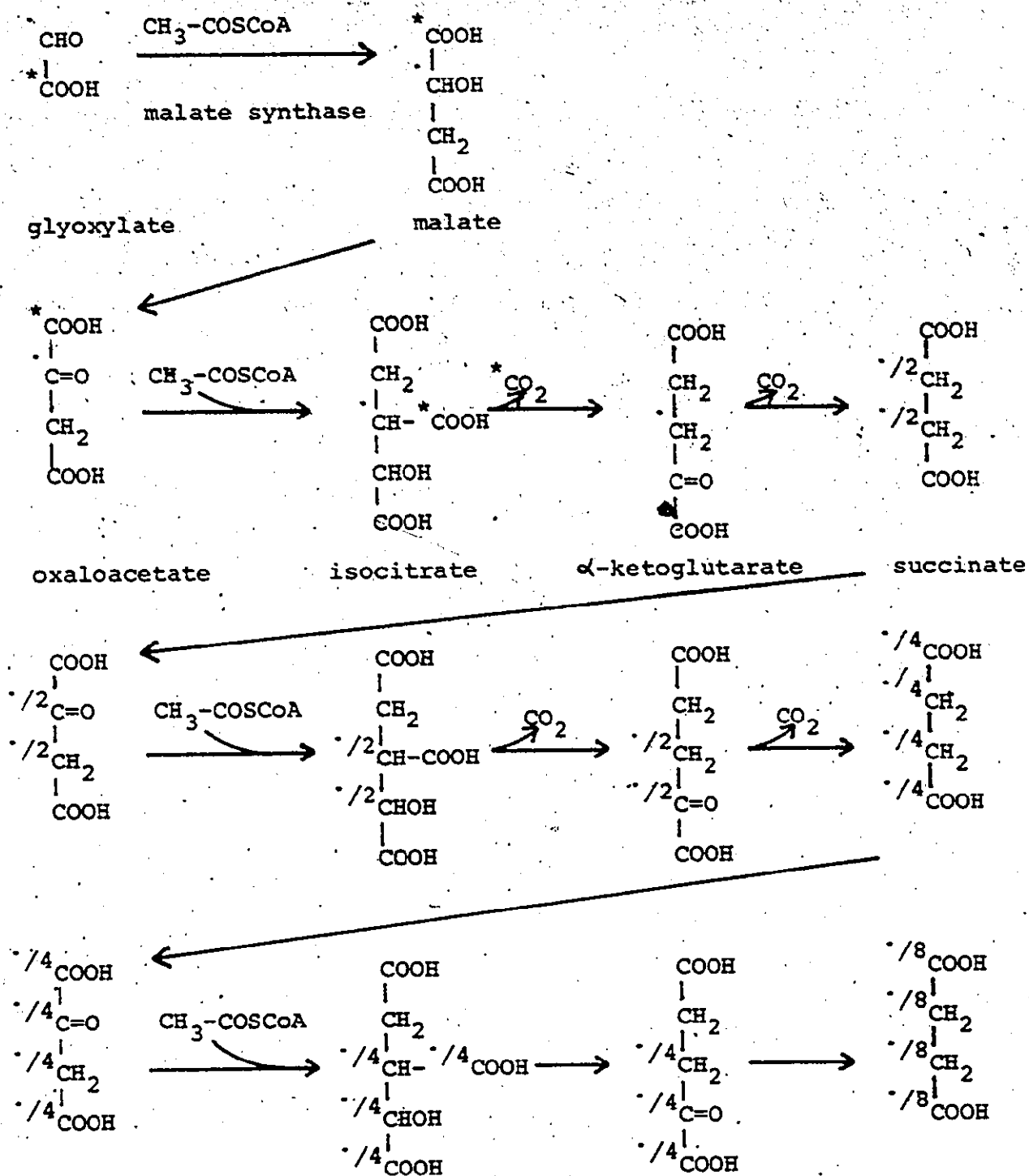


Figure 3.20: The distribution of the labelled carbon atoms from glyoxylate during three turns of the TCA cycle.

C₂ of glyoxylate. It does not agree; however, quantitatively with the experiments shown in Table 3.28 in which about 80-87% of the label was found in the C₁ of α -ketoglutarate when (2-¹⁴C) glyoxylate was the labelled substrate.

The possibility that the series of reactions known as the dicarboxylic acid cycle (DCA, Figure 1.3) of Kornberg and Sadler (1961), may be involved in the formation of (1-¹⁴C) ketoglutarate also must be considered. In Figure 3.21 the distribution of the labelled carbon from glyoxylate during two turns of the DCA cycle results in malate labelled as indicated. This malate after one turn in the TCA cycle will give α -ketoglutarate labelled only with the C₂ of glyoxylate, but 50% of the total label in the α -ketoglutarate molecule will be located in the 1-carboxyl group. Succinate labelled at the inner methylene carbons will give rise, by the reactions of the TCA cycle (Figure 3.20), to α -ketoglutarate carrying the label from the C₂ of glyoxylate, and of the total label of the molecule only one-third will be located in the 1-carboxyl group. Again this kind of distribution of the label does not agree with our results (Table 3.28).

In all three of the above possibilities a functional

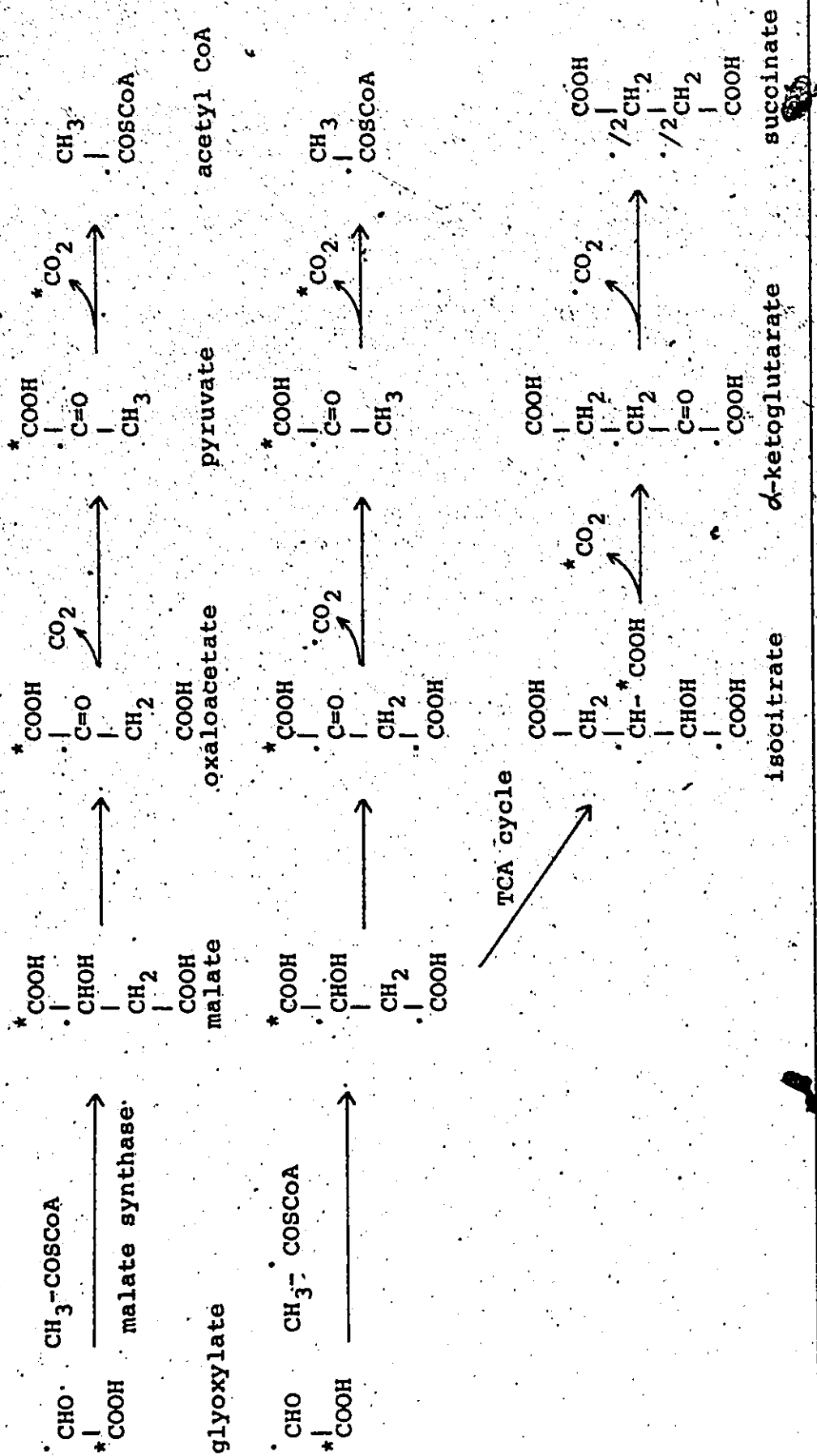


Figure 3.21: The distribution of the carbon atoms from glyoxylate in TCA intermediates after two turns of the DCA cycle.

malate synthase was required to catalyze the initial condensation of glyoxylate with acetyl CoA. This enzyme is repressed by glucose and stimulated by acetate (Hogg and Kornberg, 1973) in Tetrahymena. If indeed, malate synthase is involved as the first enzyme in the schemes shown in figures 3.19-3.21 one would expect suppression of isotopic incorporation into α -ketoglutarate in media supplemented with glucose and stimulation in media supplemented with acetate. The results of Table 3.28, however, show that growth in the presence of glucose or acetate resulted in mitochondria able to form labelled α -ketoglutarate to about the same extent. More detailed discussion on this topic is reserved for a later section (section 3.10.5).

Formation of TCA cycle intermediates from glyoxylate by reactions which do not involve the participation of malate synthase has also been reported and will be considered below. These last reactions may be important in animal tissues and in higher plants where malate synthase has not been conclusively demonstrated (section 1.1.1.A), as a possibility for the oxidation of glyoxylate.

Payes and Laties (1963a) have proposed a cyclic

mechanism for glyoxylate oxidation which is dependent on the catalytic participation of pyruvate as shown in Figure 3.22. The cycle is initiated by a condensation reaction between glyoxylate and pyruvate to form β -hydroxy- α -ketoglutarate (section 1.1.C). This last compound may be oxidatively decarboxylated to malate by an α -ketoacid dehydrogenase and a peroxidase. The former enzyme was found in rat liver mitochondrial preparations and in yeast and was not identical with α -ketoglutarate dehydrogenase. These reactions did require TPP and magnesium. The latter was not replaced by manganese.

If catalytic amounts of pyruvate were present in our mitochondrial preparations or were formed by the DCA cycle (Fig. 3.21) then α -ketoglutarate could be formed from glyoxylate (Figure 3.22) via malate. Malate formed after two turns of the cycle will be labelled in the manner shown which is identical with that obtained in the DCA cycle, (Fig. 3.21). For the reasons discussed in connection with the DCA cycle the thus labelled malate would give rise, via the reactions of the TCA cycle, to α -ketoglutarate labelled with the C_2 of glyoxylate and of the total label in the ketoacid only one-third will reside in the 1-carboxyl group.

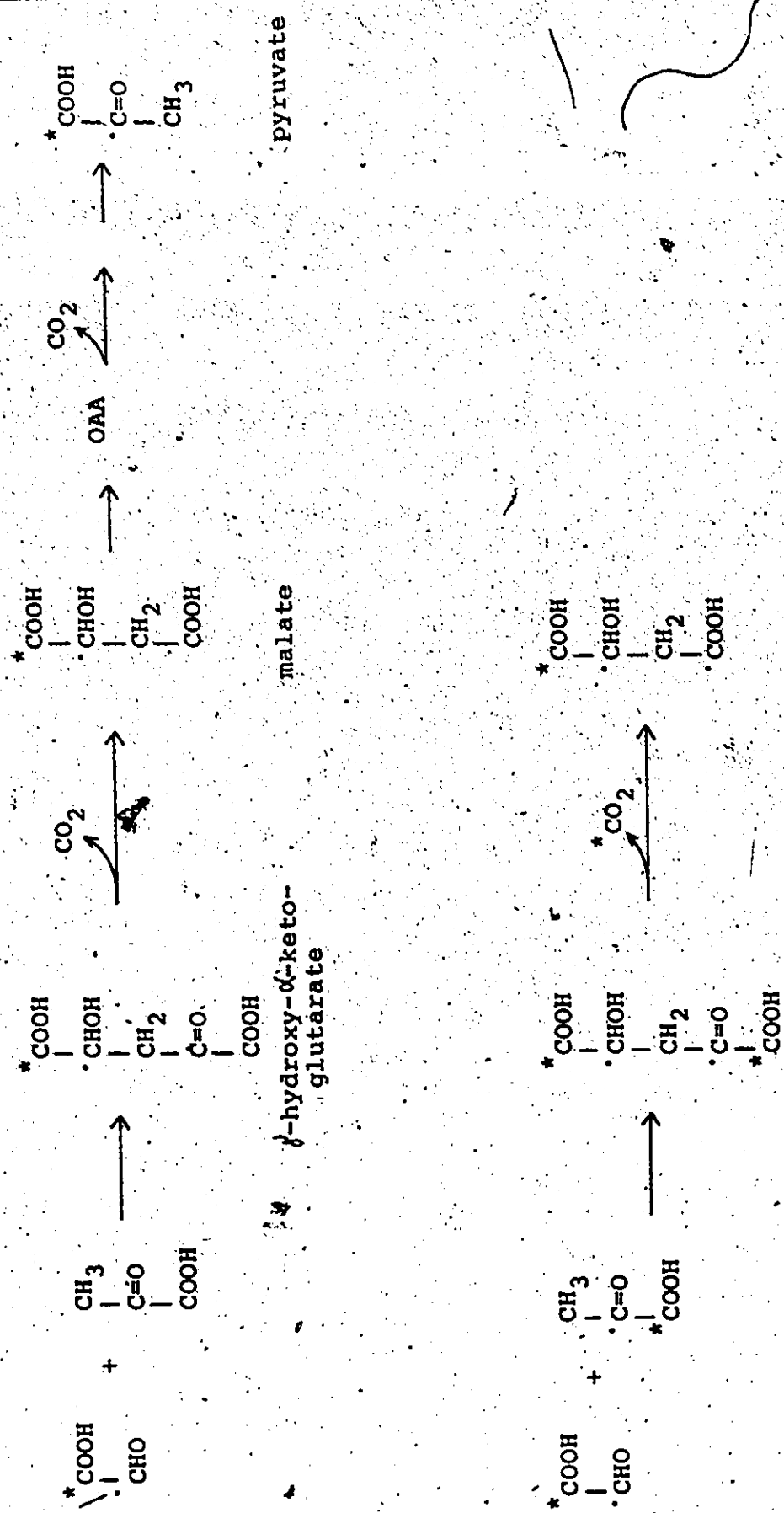
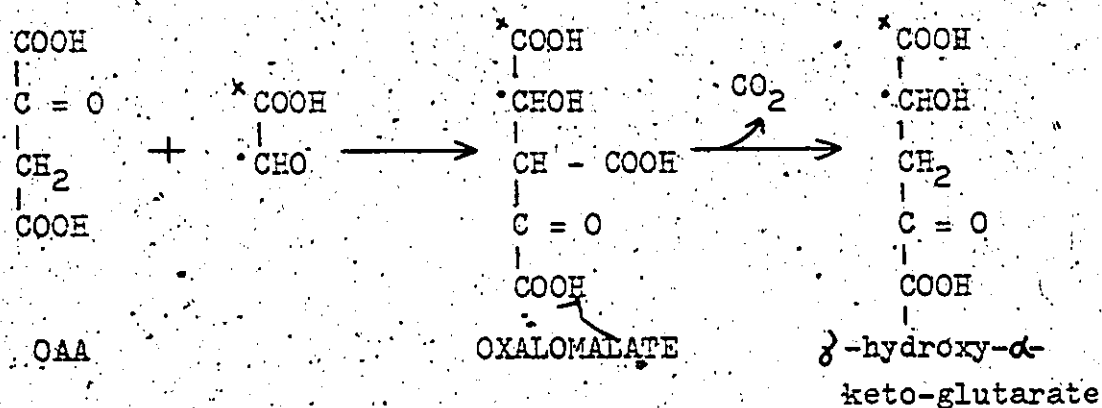


Figure 3.22: The distribution of the carbon atoms from glyoxylate in malate after two turns of the cycle in the pyruvate-dependent oxidation of glyoxylate.

γ -Hydroxy- α -ketoglutarate can also be formed from the nonenzymatic or enzymatic condensation of glyoxylate and oxaloacetate (section 1.1.1.D). The initial product of this condensation reaction is oxalomalate which decarboxylates to give γ -hydroxy- α -ketoglutarate. The evolved CO_2 corresponds to the β -carboxyl group of the oxalomalate molecule. Therefore the same distribution of radioactivity in α -ketoglutarate will be obtained as that discussed previously in connection with the pyruvate-dependent oxidation of glyoxylate (Figure 3.22).



Thus, none of the known pathways discussed in this section appears to account for the labelling of α -ketoglutarate from $(2\text{-}^{14}\text{C})$ glyoxylate in our system.

Recently, Wang et al., (1970) studied in vivo and in

vitro the metabolism of hydroxylevulinic acid (HLA), formed by decarboxylation of α -hydroxy- β -ketoadipic acid (HKA), the primary product of the α -ketoglutarate:glyoxylate carboligase reaction. Evidence was presented for the formation of ^{14}C -aspartate, ^{14}C -glutamate and ^{14}C - δ -aminolevulinic acid (ALA) when ^{14}C -HLA was incubated with sonicated rat liver mitochondria. Formation of these products suggested that HLA may be converted to α -ketoglutarate and ALA via δ , δ -dioxovalerate as indicated in Figure 5.23. The C_1 of HLA became C_5 of α -ketoglutarate, and aspartate can be formed from α -ketoglutarate via TCA cycle intermediates. Oxidation of ^{14}C -HLA to $^{14}\text{CO}_2$ in vivo was also shown in the rat.

α -(1- ^{14}C)ketoglutarate as well as α - ^{14}C -hydroxy-glutarate, were in fact detected by Okuyama et al. (1965) when cell extracts of Rhodoseudomonas spheroides were incubated under similar conditions for the carboligase reaction in the presence of (2- ^{14}C) glycine. The latter was presumably first converted to (2- ^{14}C) glyoxylate by transamination.

The natural occurrence of δ , δ -dioxovalerate (DOV), the intermediate proposed by Wang et al. (1970) in the

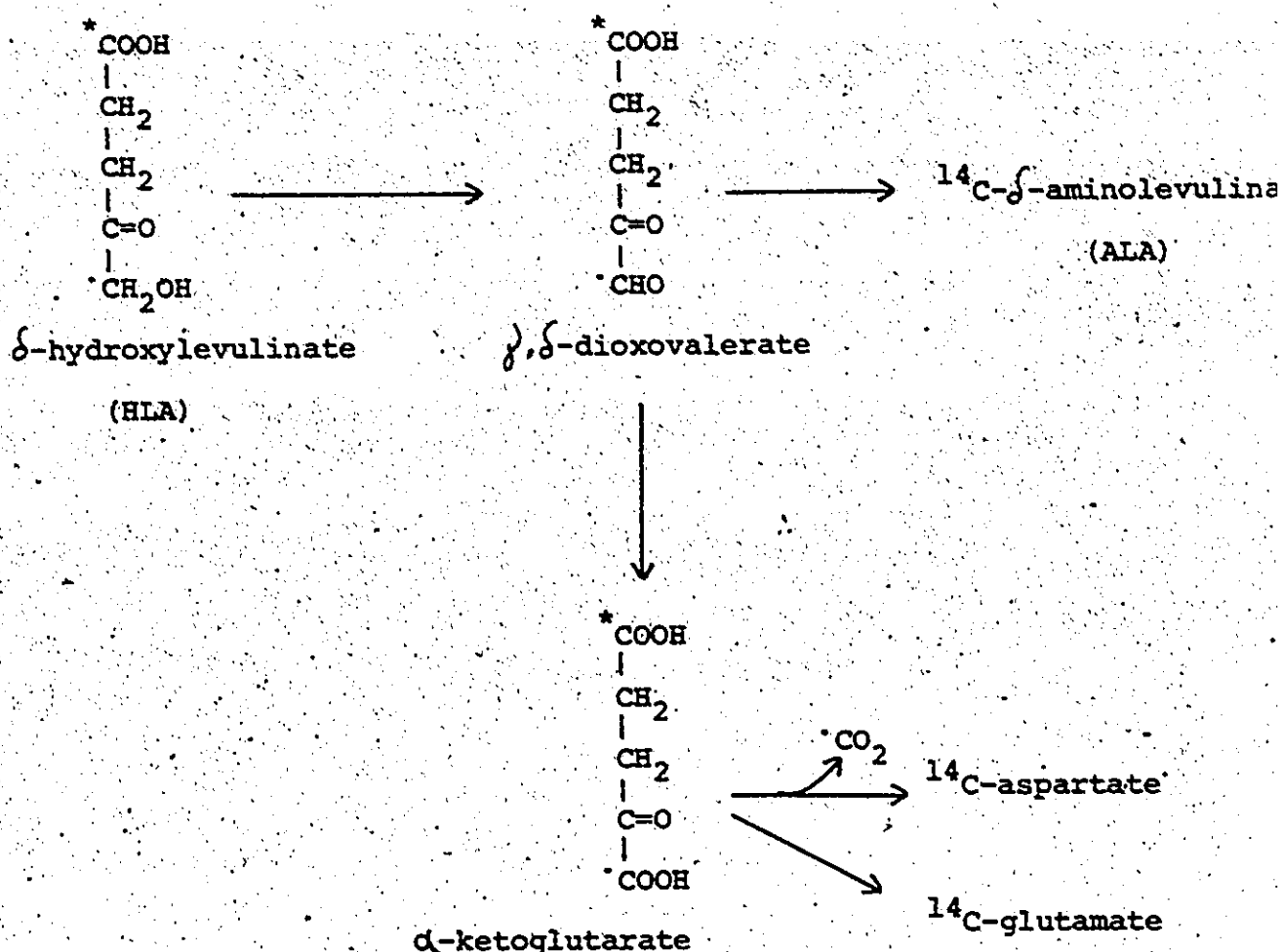


Figure 3.23: Metabolism of δ-hydroxylevulinic acid in the rat, as suggested by Wang *et al.*, (1970).

HLA was formed enzymatically under the assay conditions for α-ketoglutarate:glyoxylate carboligase; The compound was isolated from the incubation mixtures through column chromatography (Dowex 1x8, formate form). The (1- ^{14}C) label in HLA comes from (5- ^{14}C) ketoglutarate, and the (5- ^{14}C) in HLA comes from the (2- ^{14}C) glyoxylate. Aspartate was labelled only from (1- ^{14}C) HLA, and C_1 of HLA is not the C_1 of glutamate.

metabolism of HLA, and its interconversion with δ -aminolevulinic acid (ALA) in rats was demonstrated by Kissel and Heilmeyer (1969). A specific transaminase which is thought to be involved in this interconversion was shown to occur in a variety of organisms including mammals, photosynthetic and non-photosynthetic bacteria and algae (for review see Jerzykowski *et al.*, 1973).

DOV had been postulated previously as an intermediate in the formation of succinate-glycine cycle (Shemin and Russell, 1953; 1954). Shemin and Russell (1953) first pointed out the possibility that ALA might give rise to a "one carbon unit" and succinate after conversion to its aldehyde analogue, DOV, by way of a cyclic mechanism (Fig. 3.24). In search of this possibility Nemeth *et al.*, (1957) gave rats and pigeons (5- ^{14}C)ALA. Radioactivity was found in the ureido group of guanine and of uric acid and in formate. That is, this carbon atom appeared to function as a "one-carbon unit" while the remainder of the ALA molecule was recovered as succinate. Similar results have also been obtained using tissue slices with (5- ^{14}C)-DOV (Braunstein *et al.*, (1964).

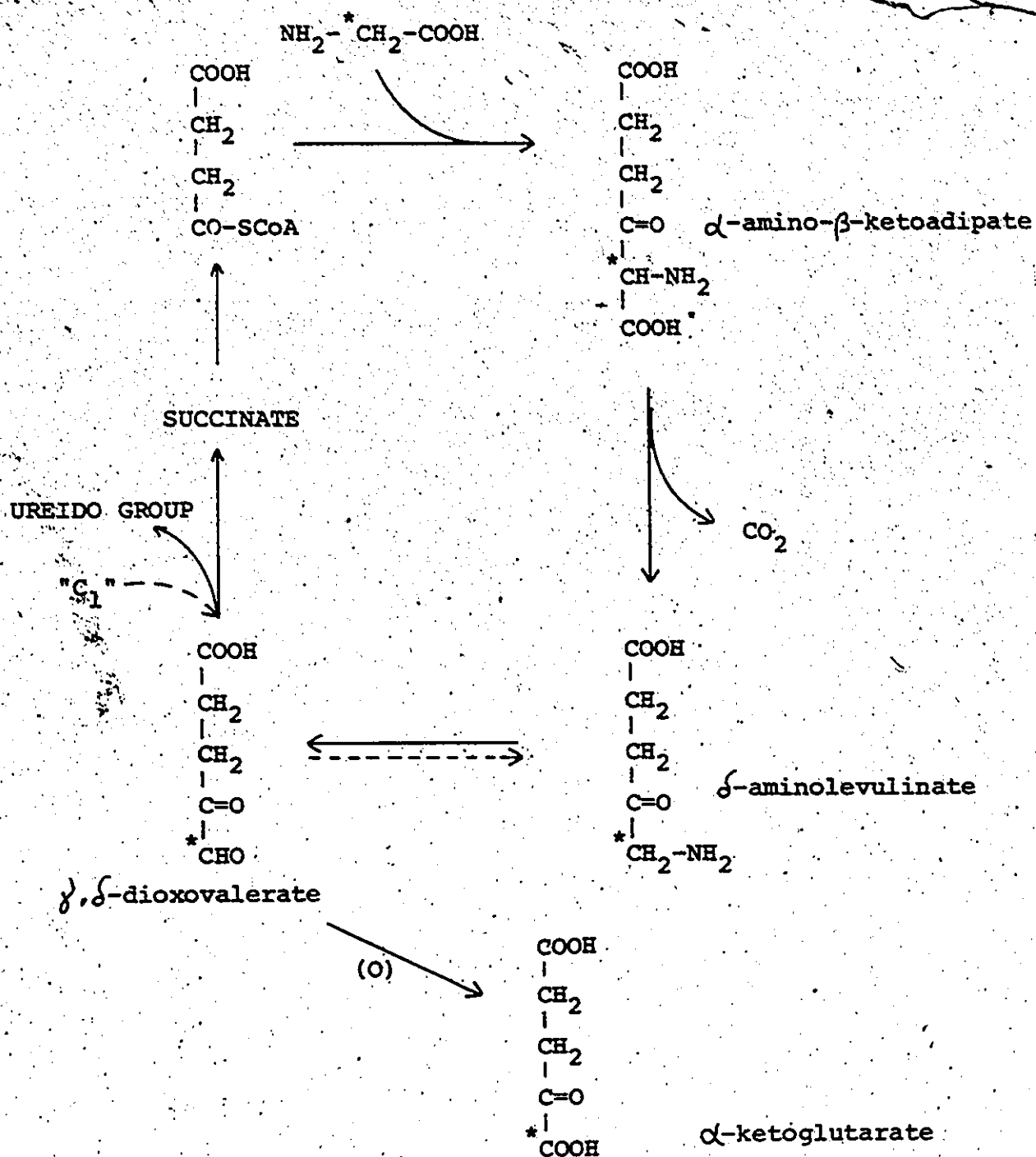


Figure 3.24: Succinate-glycine cycle. (Shemin and Russell, 1953).

Recently, Shigesada (1972) suggested the occurrence of a succinate-glycine cycle as represented in Figure 3.25, in Rhodospirillum rubrum. This work seems to give the first experimental evidence that this pathway operates as a whole in one organism, though some of the reaction steps involved have been found in various organisms. Shigesada did not study the possible occurrence of the alternative cycle originally proposed by Shemin and Russell (1953), which was postulated to catalyze conversion of the δ -carbon atom of ALA to a "one-carbon unit". The major metabolic products produced through the new pathway from (5- ^{14}C)ALA were δ -amino- γ -hydroxyvalerate, α -hydroxyglutarate and glutamate. The existence of the succinate-glycine cycle was further substantiated by demonstration of two enzyme activities, a glyoxalase system and D- α -hydroxyglutarate dehydrogenase, in cell-free extracts. Of the reaction steps in the proposed pathway, only the enzymatic mechanism of DOV formation from ALA was not elucidated; in accordance with the reported irreversibility of the transaminase reaction in extracts of R. spheroid (Neuberg and Tuner, 1963) which favors ALA formation.

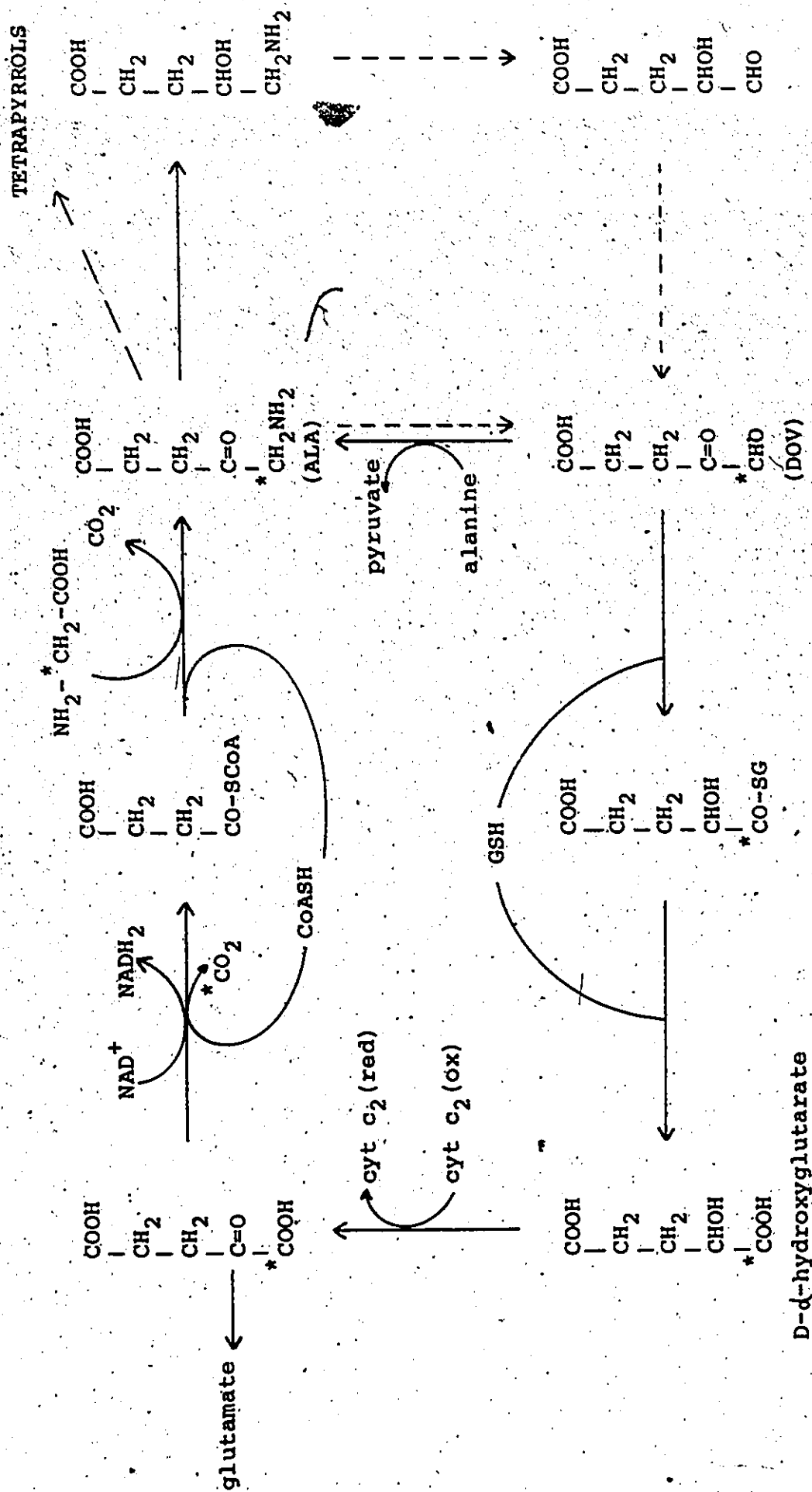


Figure 3.25: Succinate-glycine cycle as proposed by Shigesada (1972).

It was considered possible but not very likely that the succinate-glycine cycle might be responsible for the labelling of α -ketoglutarate in our experimental system (Fig. 3.18). This possibility would presuppose the conversion of added α -ketoglutarate and added labelled glyoxylate to succinyl-CoA and glycine, respectively, by mitochondrial enzymes with the participation of endogenous substrate and cofactors. An attempt to demonstrate the operation of the cycle in our system is described in the following sub-section.

3.10.3 On the participation of the succinate-glycine cycle on the formation of α -ketoglutarate.

Experimental procedures. Mitochondrial fractions were obtained from cells grown in synthetic medium supplemented with glucose, by method B (section 2.2.4).

Incubations were carried out under the standard assay conditions (section 2.2.5.A) but in the presence of 4 μ moles (2- 14 C) glyoxylate (222,800 c.p.m.) and 4 μ moles α -ketoglutarate or 4 μ moles (2- 14 C) glycine (112,800 c.p.m.) and 4 μ moles α -ketoglutarate. The mitochondrial fractions contained 5 mg.protein. The

reactions were stopped with either PCA (0.1 ml, 70%) or TCA (0.3 ml 50%). Total collection, extraction and counting of DNPH derivatives from the PCA-deproteinized reaction mixtures were conducted as described (section 2.2.10.B) and chromatography of the DNPH derivatives was done in solvent system 1.

Analysis of amino acids formed during the α -ketoglutarate: glyoxylate carboligase reaction was performed by column chromatography of the TCA-deproteinized reaction mixtures as described in section 2.2.13.

Results and discussion. When the TCA-deproteinized reaction mixtures were passed, without added ^{14}C -amino acid, through a column for amino acid chromatography, two radioactive peaks were obtained. The radioactivity, expressed in c.p.m., contained in these peaks is shown in Table 3.29. The most radioactive peak 1 appeared in a position similar to that obtained when authentic ^{14}C -glycine was chromatographed. Peak 2 was obtained at a position similar to that of authentic ^{14}C -aspartate. Verification of the identity of the amino acids was accomplished by co-chromatography of the deproteinized reaction mixtures with authentic labelled glycine and

Labelled substrate	DNPH- α -ketoglutarate	Peak 1, c.p.m.	Peak 2, c.p.m.
	d.p.m.	$+^{14}\text{C}$ glycine	$+^{14}\text{C}$ aspartate
(2- ^{14}C) glyoxylate	3,900	910 (2,173)	480 (960)
(2- ^{14}C) glycine	0		

Table 3.29: α -Ketoglutarate and amino acid formation.

(2- ^{14}C) glyoxylate or (2- ^{14}C) glycine were incubated as described in

Experimental Procedures. After 1 hour of reaction, PCA or TCA was added.

From the PCA-deproteinized assay mixtures total collection of

DNPH derivatives was done. The TCA-deproteinized assay mixtures were

analyzed for amino acids. 0.5 μ moles of ^{14}C -glycine (950 c.p.m.)

and 0.5 μ moles of ^{14}C -aspartate (1,800 c.p.m.) were added to

the deproteinized assay mixtures for the purpose of co-chromatography.

Figures in parentheses represent a second experiment.

aspartate. The radioactive elution pattern from this last chromatography again showed two peaks, and the increase in the counts of each peak corresponded to the additional counts from added authentic ^{14}C -glycine and ^{14}C -aspartate as is shown in Table 3.29.

These results indicate that during the assay of α -ketoglutarate:glyoxylate carboligase, transamination of glyoxylate with endogenous glutamate also occurred which resulted in glycine formation. The other amino acid obtained from the reaction mixtures behaved as aspartate on chromatography but it is not understood how (2- ^{14}C) glyoxylate might give rise to this amino acid under our conditions, since α -(1- ^{14}C) ketoglutarate could not give rise to labelled aspartate. On the other hand, DNPE- ^{14}C - α -ketoglutarate was obtained from assay mixtures with (2- ^{14}C) glyoxylate but not from mixtures with (2- ^{14}C) glycine (Table 3.29).

It can be concluded that although glycine could be formed by side reaction in the assay mixtures the succinate-glycine cycle was not involved in the formation of α -ketoglutarate under our experimental conditions.

3.10.4. A possible cycle to account for our observations.

The results and discussion in the previous two subsections have excluded the participation of malate synthase and ancillary reactions (section 3.10.2) and of the succinate-glycine cycle (section 3.10.3) in the incorporation of label from glyoxylate into α -ketoglutarate described in section 3.10.1 and 3.10.2.

A cycle similar to that suggested by Shigesada (1972) (Fig. 3.25), which does not depend on glycine and succinyl-CoA was considered as being potentially responsible for the incorporation of label from glyoxylate into α -ketoglutarate and is shown in Figure 3.26.

The glyoxalase enzyme system consists of two separable enzymatic steps in the conversion of methylglyoxal to lactic acid. Glyoxalase I, (S-lactoyl-glutathione methylglyoxal lyase, EC 4.4.1.5) and glyoxalase II, (A-2-hydroxyacyl-glutathione hydrolase, EC 3.1.2.6).

The glyoxalase system has been found to be widely distributed in animal tissues, plants and microorganisms (for review see Racker, 1951). The possible natural substrates for glyoxalase I and glyoxalase II include methylglyoxal (Racker, 1951), phosphohydroxypyruvic aldehyde (Weever and Lardy, 1961) and hydroxypyruvic aldehyde (Reeves and Ajl,

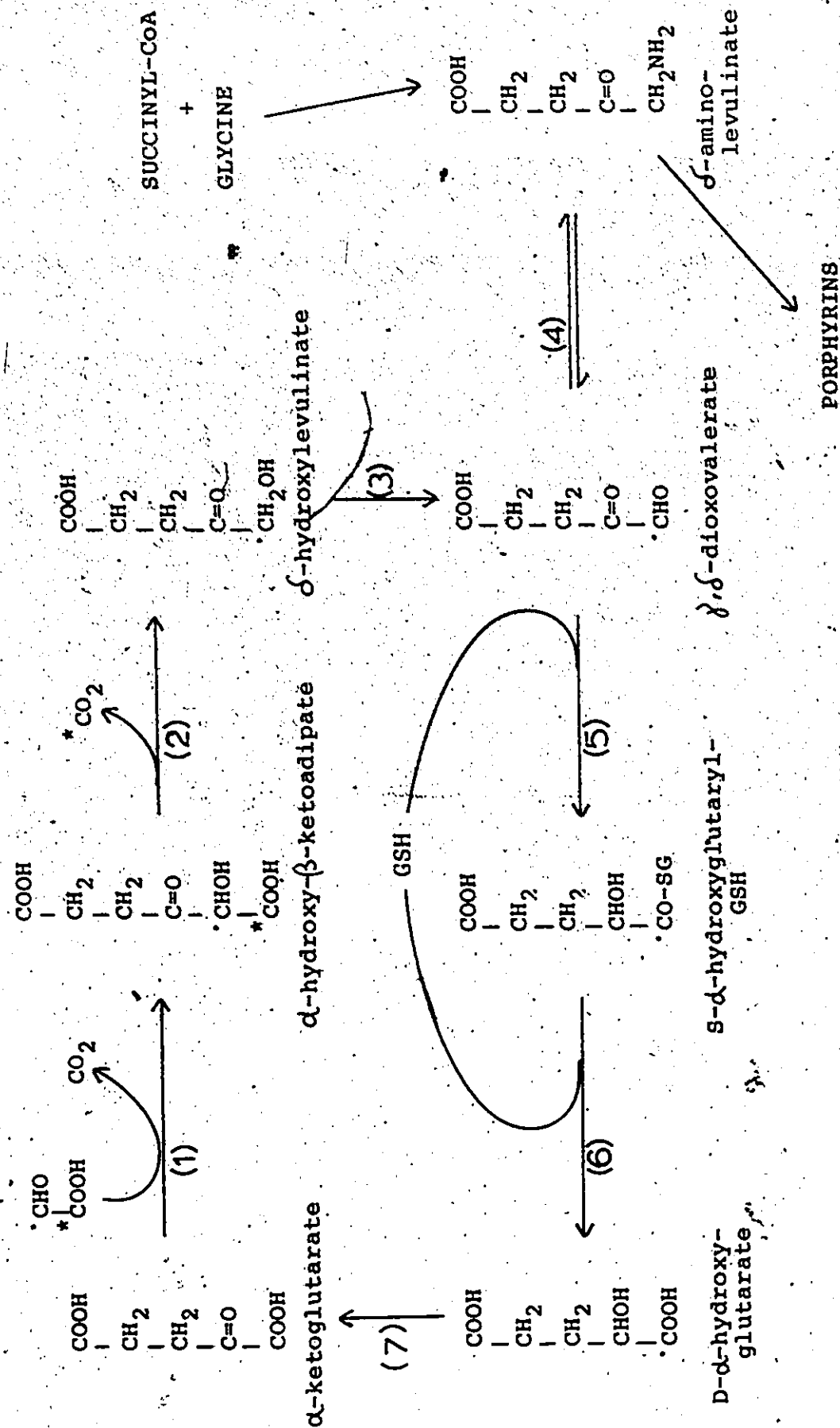


Figure 3.26: Proposed reaction for glyoxylate metabolism in mitochondria.

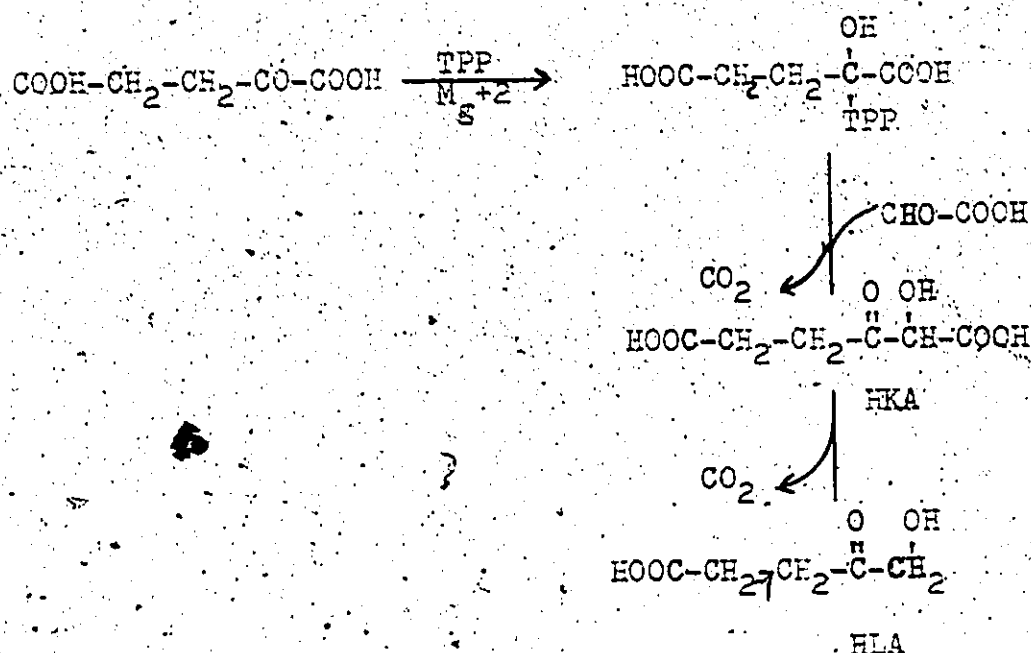
(1) α -ketoglutarate:glyoxylate carboxylase; (2) Spontaneous or enzymatic decarboxylation; (3) Oxidation of HLA, enzyme unknown; (5) and (6) Glyoxylase system; (7) α -Hydroxy acid dehydrogenase; (4) Alanine:dioxovalerate aminotransferase.

(1965). Recently, the enzymatic formation of the GSH ester of DOV by the action of purified glyoxalase II to form D- α -hydroxyglutarate has been reported (Jerzykowski et al., 1973). The reaction is irreversible. It was suggested that one of the metabolic functions of the glyoxalase system may be to provide a mechanism for the entry of DOV into the TCA cycle.

Partially purified D- α -hydroxy acid dehydrogenase from R. rubrum (Shigesada, 1972) appeared to be highly specific for α -hydroxyglutarate, and the D-isomer was a better substrate than the L-isomer. The enzyme reduced cytochrome C₂ obtained from R. rubrum as well as 2,6-dichlorophenol-indophenol but did not utilize NAD⁺ or NADP⁺ as a redox acceptor. A D- α -hydroxyacid dehydrogenase with wide specificity also has been reported in various mammals (Tubbs and Greville, 1961; Cammack, 1969) and in yeast (Boeri et al., 1960). The enzyme catalyzed the oxidation of lactate and straight-chain homologues, glycerate, malate and tartrate, and was specific for the D-isomers (Tubbs and Greville, 1961; Boeri et al., 1960). There seems to be a strong possibility that D- α -hydroxyglutarate is a natural substrate. The enzyme is a flavoprotein and no activity was detected with NAD⁺, NADP⁺ or oxygen as acceptors, though a weak

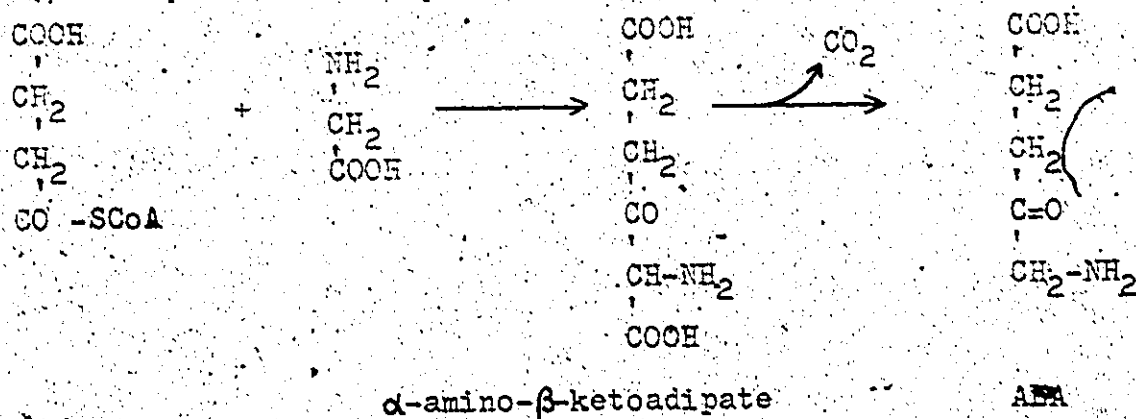
oxidase activity has been detected (Cammack, 1969). The enzyme was found located in mitochondria from various mammalian tissues (for review see Tubbs and Greville, 1961).

α -Ketoglutarate:glyoxylate carboligase catalyzes the formation of HKA as follows:



The complex of α -hydroxy- β -carboxylpropyl. TPP with the enzyme reacts with glyoxylate to yield HKA and CO_2 . The spontaneous decarboxylation of HKA then yields HLA. The above reaction mechanism is similar to that leading to the formation of δ -aminolevulinic acid catalyzed

by ALA synthase:



It is believed that α -amino- β -ketoadipate, which is a structural analogue of HKA, decarboxylates virtually instantaneously to yield ALA in neutral solutions (Laver et al., 1959). Recently, in studies on the mechanism and stereochemistry of δ -aminolevulinate synthase reaction, Zaman et al. (1973) postulated the formation of a Schiff base of α -amino- β -ketoadipate and pyridoxal phosphate. The latter intermediate may then yield ALA after decarboxylation either of the free or the bound α -amino- β -ketoadipate. It is possible that the limited evolution of CO_2 prior to the addition of acid to the reaction mixtures (which will be underestimated as a result of the partial dissolution of the CO_2 in the mixtures) represents the spontaneous decarboxylation

of HKA to HLA which is finally converted to α -ketoglutarate through the reactions of Fig. 3.26.

It is possible that a β -decarboxylase may exist to catalyze the decarboxylation of HKA, as is the case for acetoacetate decarboxylase; or that HKA may be decarboxylated by β -hydroxy- α -ketoglutarate aldolase (section 1.1.1.C) which recently has been found to catalyze the β -decarboxylation of oxaloacetate at 50% the rate of β -hydroxy- α -ketoglutarate cleavage (Kobes and Dekker, 1971a). Also β -decarboxylation of oxaloacetate was reported by 2-keto-3-deoxy-6-phosphogluconate aldolase (Ingram and Wood, 1966), but in this case the rate was 0.5% the rate of 2-keto-3-deoxy-phosphogluconate cleavage. The β -hydroxy- α -ketoglutarate aldolase activity localized in the mitochondrial fraction from rat liver, presented a pH optimum at 8.8, and about 40% of the activity was found at pH 7 (Maitra and Dekker, 1964). High concentrations (above 6 mM) of glyoxylate have been observed to inhibit the condensation reaction with pyruvate (Rosso and Adams, 1967). It has been suggested that glyoxylate is bound nonspecifically by virtue of its being an analogue of pyruvate and its binding by the enzyme may not be physiologically significant (Kobes and Dekker, 1971b) due to the usually low concentration of glyoxylate in mammalian tissues.

Since the equilibrium of the transaminase reaction (Fig. 3.25) is towards ALA production (Neuberger and Turner, 1965; Shigesada, 1972), it was suggested by Tait (1968) that ALA transaminase may be involved in ALA synthesis in some systems, particularly those where no ALA synthase activity has been detected. If this is true then DOV could arise from HLA and then be converted into ALA (Fig. 3.26, reaction 4) and further into porphyrins or, alternately, into TCA intermediates. This cyclic mechanism would ensure the complete oxidation of glyoxylate to CO_2 and would control the amount available for oxidation to oxalate (section 1.1.4).

Further studies on the possible cycle shown in Figure 3.26, require the detection of at least the glyoxalase system and D- α -hydroxyglutarate dehydrogenase. Their presence in Tetrahymena is very likely since they are widely distributed among organisms. HKA is not available commercially. Purification from enzymatic reaction mixtures is theoretically possible but no attempts were made to accomplish this. Instead a correlation study between α -ketoglutarate:glyoxylate carboligase activity and α -ketoglutarate formation was undertaken in order to establish whether factors affecting the carboligase activity also affected the labelling of α -ketoglutarate.

3.10.5 Correlation between α -ketoglutarate labelling and α -ketoglutarate:glyoxylate carboligase.

Experimental procedures. Cells were grown in synthetic medium and synthetic media supplemented with either acetate or glucose, as described in section 3.9.3. Thiamine deficient cells were obtained from cultures containing 0.2 μ g thiamine $\cdot$ HCl per 100 ml medium. Enzyme activity and DME- α -ketoglutarate extraction and counting were conducted as described in section 3.9.3.

Results and discussion. Table 3.24 shows the absence of effect in the presence of acetate and glucose during growth of the organisms on the labelling of α -ketoglutarate and on the activities of α -ketoglutarate:glyoxylate carboligase and α -ketoglutarate decarboxylase. The latter activity was discussed earlier (section 3.9.3). The activity of α -ketoglutarate:glyoxylate carboligase in the mitochondrial fraction from cells grown in synthetic medium was about the same as that found in the mitochondrial fractions from cells grown in media supplemented with acetate or glucose. The activity in the supernatant fraction was about 17% the activity found in the mitochondrial fraction from the cells grown in the synthetic medium. Acetate or glucose added to the growth medium did not affect the supernatant activity.

The α -ketoglutarate labelling in the mitochondrial fraction, measured in DNPH- ^{14}C -derivative, was not affected by glucose or acetate. The supernatant fractions from cells grown under these three conditions were also able to form labelled α -ketoglutarate, but the labelling was only about 15% of that obtained from the mitochondrial fractions and was also unaffected by glucose and acetate.

These results suggest that α -ketoglutarate:glyoxylate carboligase is a constitutive enzyme and that the labelling of α -ketoglutarate is brought about through steps which also seem not to be affected by the presence of acetate or glucose during growth. This is further evidence that malate synthase is not involved in the metabolism of glyoxylate, as was discussed earlier (section 3.10.2).

Table 3.25 (section 3.9.2) shows the effect of thiamine deficiency on the carboligase and on the formation of α -ketoglutarate. The mitochondrial fraction from deficient cells had only 51% carboligase activity of the normal cells in experiment 1, and 32% in experiment 2. The activity in the supernatant fraction from deficient cells was comparable to that found in normal cells. Less α -ketoglutarate was labelled when the mitochondrial fraction from deficient cells was used (Table 3.25). 45% of the control

value was found in experiment 1 and 27% in experiment 2.

It is interesting to note that thiamine deficiency reduced to practically equal degrees the activity of the carbolicase and the capacity for α -ketoglutarate labelling.

The results support our suggestion that α -ketoglutarate:glyoxylate carbolicase may be involved in the labelling of α -ketoglutarate when mitochondrial fractions from Tetrahymena are incubated with the substrates and cofactors for the carbolicase reaction.

CHAPTER 4 : GENERAL DISCUSSION.

The principal aim of the present research, namely the study of the α -ketoglutarate-dependent metabolism of glyoxylate in Tetrahymena was pursued by the initial demonstration of the enzymatic condensation between α -ketoglutarate and glyoxylate, catalyzed by the α -ketoglutarate:glyoxylate carboligase of mitochondrial preparations. The immediate product of the reaction, HKA, was not itself isolated, however, its decarboxylation product HLA was identified chromatographically.

Attempts to purify the enzyme failed but some kinetic studies with mitochondrial preparations revealed properties in general agreement with those reported for the enzyme from other sources.

The enzyme appears to be constitutive in that its activity did not change by the inclusion or omission during growth of nutrients such as acetate and glucose which affect a variety of other enzymes in Tetrahymena.

The carboligase of beef-heart mitochondria has been shown by others (Schlossberg et al., 1970) to reside in the α -ketoglutarate dehydrogenase complex. Although the

failure to purify the enzyme prevented us from demonstrating directly that this is also true of the Tetrahymena enzyme, indirect evidence supports the identity of carbolligase and the dehydrogenase complex. Thus it was found that thiamine deficiency and the degree of aeration (static or agitated cells) affected the carbolligase and the α -ketoglutarate decarboxylase in a coordinate manner. The physiological significance of this finding cannot be deduced at this time.

An unknown compound capable of forming an osazone with 2,4-dinitrophenylhydrazine was detected in the assay mixtures. It is likely to be α,β -dihydroxy- γ -ketopimelic acid which would indicate that a second molecule of glyoxylate condenses with HKA (section 1.1.2.B, Fig. 1.4).

An interesting aspect of our work was the finding that α -(1- ^{14}C) ketoglutarate was produced in the assay mixtures in the presence of unlabelled α -ketoglutarate and (2- ^{14}C) glyoxylate. This suggested, after exclusion of several other possibilities (section 3.10.2), the operation of a cycle responsible for the regeneration of α -ketoglutarate.

A hypothetical but not improbable cycle is presented to account for the regeneration of α -ketoglutarate (Fig. 3.26) based on our data and the work of others (Shigesada,

1972). The members of the cycle are known to occur physiologically. The required enzymes except those needed for the conversion of α -hydroxy- β -ketoacid to δ -hydroxylevulinate and the conversion of the latter to dioxovalerate (Fig. 3.26) have also been demonstrated in various organisms. No attempt was made for the detection of the intermediates or the enzymes, and thus the proposed cycle would have to be substantiated with much more specific work. However, it was important to provide evidence that the carboligase was involved in the cycle. Such evidence came from experiments designed to study the relationship between carboligase activity and capacity to form labelled α -ketoglutarate from (2- 14 C) glyoxylate in mitochondrial fractions. Thus it was found that thiamine-deficient cells provided mitochondria with a substantially reduced carboligase activity and a reduced capacity to produce labelled α -ketoglutarate. (section 3.10.5).

The physiological importance of the proposed pathway is unclear. Due to the presence of the glyoxylate pathway in the peroxisomes glyoxylate formed in these organelles would have to be transported to the mitochondria to enter the reactions of the cycle.

In rat liver mitochondria where formation of dioxovalerate was suggested from HLA (Wang et al., 1970)

the cycle might account, in part, for the metabolism of glyoxylate as supported by the increased amounts of glyoxylate formed in rats on thiamine-deficient diets (Liang, 1962). It is however possible that other thiamine-requiring enzymes acting on glyoxylate, such as the pyruvate dehydrogenase system (section 1.1.2.C), might be involved.

The importance of the cyclic operation is that catalytic amounts of α -ketoglutarate would be necessary for the oxidation of glyoxylate. Dioxovalerate might also serve as a precursor of porphyrins after conversion to ALA by transamination (Fig. 3.26). This last possibility, has been considered by Tait (1963). Since there are porphyrin-synthesizing tissues with no detectable δ -aminolevulinate synthase and considering the favourable equilibrium of transamination toward ALA formation (Neuberg and Tuner, 1963) this route for porphyrin biosynthesis becomes a distinct possibility.

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