HETEROTROPHIC CARBON DIOXIDE FIXATION BY *EUGLENA*

by

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ABSTRACT

Heterotrophic (dark, non-photosynthetic) carbon dioxide fixation was measured in the phytoflagellate *Euglena gracilis*. Variation in heterotrophic carbon dioxide fixation depends on the phase of batch growth and the mode of nutrition. A sharp increase in heterotrophic carbon dioxide fixation occurs during the mid- to late-logarithmic growth periods in *Euglena* growing photoautotrophically (with carbon dioxide as carbon source, light as energy source) and heterotrophically (in the dark with glucose as sole carbon and energy source). Cells growing heterotrophically with acetate or ethanol as sole carbon source do not increase their heterotrophic carbon dioxide fixation during the growth cycle. Addition of acetate to cultures of *Euglena* causes a reduction in dark carbon dioxide fixation. The results are consistent with the hypothesis that heterotrophic carbon dioxide fixation in *Euglena* functions in anaplerotic replenishment of the tricarboxylic acid cycle.

The regulation of these changes in heterotrophic carbon dioxide fixation was shown to be controlled by exogenous ammonium in a complex fashion. Ammonium stimulates heterotrophic carbon dioxide fixation after a period of ammonium deprivation. The kinetics of the regulatory effects of the ammonium ion on dark carbon dioxide fixation are presented.

A survey into the activities of carboxylating enzymes from both autotrophically and heterotrophically grown *Euglena* was conducted. The heterotrophic cultures were supplied with either glucose or acetate as substrate. PEP carboxykinase (E.C.4.1.1.38) and (E.C.4.1.1.49) could not be detected in any of the cultures tested. A trace amount of PEP

carboxykinase (E.C.4.1.1.32) is present in the acetate grown cells only and a trace amount of pyruvate carboxylase (E.C.6.4.1.1.) is present in the glucose grown cells only. Malate enzyme (E.C.1.1.1.40), PEP carboxylase (E.C.4.1.1.31) and acetylcoenzyme A carboxylase (E.C.6.4.1.2) are present in all cells tested. Ammonium stimulation causes a small increase in specific activity of all the enzymes except acetyl-CoA carboxylase. The largest increase occurs in PEP carboxylase, but the increase is not sufficient to account for observed increases in whole cell dark carbon dioxide fixation after ammonium stimulation.

Two isoenzymes of PEP carboxylase were purified from each other from both ammonium stimulated and non-stimulated cells. There are no significant differences between elution profiles of isoenzymes from ammonium stimulated and control cells. There are no significant differences between elution profiles of isoenzymes from autotrophic and heterotrophic cells. The kinetics of the regulation of the two isoenzymes by malate, citrate, succinate and 3-phosphoglycerate are presented.

The products of heterotrophic carbon dioxide fixation by ammonium stimulated and control cells were identified and measured by chromatography. Ammonium stimulates the biosynthesis of glutamine, glycine, serine and alanine.

DEFINITIONS AND EXPLANATIONS

Autrophically grown *Euglena* Batch cultures of *Euglena* grown in continuous light, with carbon dioxide as sole carbon source. Such a culture is also referred to variously as an autotrophic culture, autotrophically grown cells, autotrophic cells or autogrophic *Euglena*.

Heterotrophically grown *Euglena* A batch culture of *Euglena* grown in complete darkness, with a complex organic carbon source (usually glucose or acetate). Such a culture is also referred to variously as a heterotrophic culture, heterotrophically grown cells, heterotrophic cells or heterotrophic *Euglena*.

Heterotrophic carbon dioxide fixation is defined as the non-photosynthetic (dark) fixation of gaseous carbon dioxide to form a new carbon-carbon bond. It does not necessarily imply heterotrophic growth. It is also referred to as dark carbon dioxide fixation. To avoid unnecessary repitition of these terms, the abbreviations carbon dioxide fixation, dark fixation and fixation are sometimes used. These terms never imply photosynthetic carbon dioxide fixation, which is always referred to as photosynthesis.

ABBREVIATIONS

A-CoA	acetyl coenzyme A
ADP	adenosine diphosphate
ATP	adenosine triphosphate
cpm	counts per minute
GDP	guanosine diphosphate
IDP	inosine diphosphate
MDH	malate dehydrogenase
ME	malate enzyme
NADH	alpha-nicotinamide adenine dinucleotide, reduced
NADP	alpha-nicotinamide adenine dinucleotide, phosphate
OAA	oxaloacetate
PEP	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxykinase
PK	pyruvate kinase
POPOP	1,4-bis-(2-(5 phenoxyloxazoly))-benzene
PPO	2,5-diphenyloxazole
RDP	ribulose diphosphate
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid
Tris	tris(hydroxymethyl)aminomethane

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INTRODUCTION

Euglena has been and continues to be exploited in biological research laboratories for several reasons, most important of which are its ease of experimental manipulation and growth, and its biochemical adaptability. The level of adaptability is unusually high for a eukaryote organism. This dissertation describes an investigation into heterotrophic (as opposed to photosynthetic) carbon dioxide fixation in *Euglena*.

The existence of heterotrophic carbon dioxide fixation, or the dark assimilation of gaseous carbon dioxide into metabolism, has been recognised for about 40 years. During this period it has become evident that this fixation is the sum total of a plethora of biochemical carboxylations, catalysed by a range of different enzymes. Different cells and tissues possess a variety of enzymes catalysing different reactions. In all cells or tissues which have been critically investigated, some form of dark carbon dioxide fixation has been found to occur, indicating that it is probably ubiquitous and of basic importance. In some of these cases the function or functions of the carboxylations have been elucidated, and in other cases they are still not known.

In *Euglena* relatively little attention has been directed to the problems of heterotrophic carbon dioxide fixation. This is in spite of the fact that over 20 years ago it was shown that there may be unusual features in this organism's carbon dioxide fixation. The enzymology of the fixation is still not fully worked out and control processes are still obscure.

It was observed that heterotrophic carbon dioxide fixation in *Euglena* whole cells varies considerably during growth cycles (M.J. Peak, personal

communication). Such modifications reflect a regulating mechanism controlling heterotrophic carbon dioxide fixation. It was decided to use this observation as starting point to further elucidate functions and control mechanisms of *Euglena* heterotrophic carbon dioxide fixation.

THE GENUS *EUGLENA*

At the base of the plant and animal kingdoms are unicellular pigmented organisms, swimming freely by means of flagellae. These organisms, which are unique in that they photosynthesize like plants but are classified by zoologists among the animals, are the phytoflagellates. Recent protozoological classification distinguishes ten orders of phytoflagellates, one of which is the euglenoids, containing both pigmented and colourless forms. Euglenoid flagellates, in particular the green genus *Euglena* have been the subject of biological study for many years. Buetow (1968) edited a comprehensive survey of studies on *Euglena* taxonomy, morphology, behaviour, physiology and biochemistry.

Morphology and taxonomy *Euglena* is a widespread genus, inhabiting a variety of environments. Most species live in fresh water habitats such as ditches, ponds and streams, particularly those contaminated by animals or decaying vegetable matter. Members of the genus are free-living, single celled organisms, possessing two flagellae, one external and one internal. Different species vary considerably in body shape, from nearly spherical to extremely elongated or leaf-like. Characteristics common to all species include the presence of paramylon as the carbohydrate storage product, the lack of any known sexual processes, and the pellicle, which can be flexible or rigid, surrounding the body. The helical or screw symmetry which is characteristic of euglenoids is shown most clearly by the arrangement of the pellicle.

The taxonomy of *Euglena* is problematic. Most species, in the light, possess green chloroplasts and live photoautotrophically, in a plant-like fashion. However, *Euglena* is also capable of heterotrophic nutrition

and can grow equally efficiently on a variety of substrates in the absence of light, in which case the fully elaborated green chloroplasts are absent. Thus it has been classified variously as a mastigophoran protozoan, possessing green chromatophores, or as a green alga. Margulis (1970) suggested that *Euglena* may be polyphyletic in origin, and that the chloroplasts may originally have been prokaryotic blue-green algae, which became associated with a mastigophoran protozoan and entered into an endosymbiotic relationship with it. The animal part of the union derived benefit from the association since it obtained fixed carbon from the photosynthesis of the plant. Cavalier-Smith (1975) recently described a different theory whereby plastids evolved from pre-existing cytosolic structures.

Biochemistry and physiology of *Euglena* *Euglena* is widely used as an organism for biological research. Two characteristics of *Euglena* render it particularly suitable as a laboratory organism, firstly it can readily be cultured in the laboratory under a variety of culture conditions, and secondly, its biochemical and physiological flexibility can be exploited experimentally. Of all species of *Euglena*, the most commonly used in the laboratory is *Euglena gracilis*, in particular *Euglena gracilis* Z and *Euglena gracilis* var *bacillaris*.

Cook (1968) described experimental methods for the culture of *Euglena*, and Hutner *et al.* (1966) discussed various culture media. Important in physiological and biochemical research is the fact that *Euglena* can be cultured axenically, and on completely defined media. As euglenoid flagellates have simple chemical requirements, these media need not necessarily be complex. Various different methods can be employed to culture *Euglena*, depending on the need of the investigator. Cook (1968)

describes these methods in detail. The two most common ways in which *Euglena* is grown in the laboratory are either in simple batch culture, or synchronously. Cook and James (1960) described how autotrophic cultures of *Euglena* can be made to synchronize their division in the laboratory by exposure to appropriate, alternating periods of light and dark. The divisions become synchronized, occurring in the dark periods. Heterotrophic cultures may be made to divide synchronously by means of heat shocks (Pogo and Arce, 1964). When grown in batch culture, autotrophic *Euglena* receive continuous illumination with supplied carbon dioxide, whereas heterotrophic growth is always in complete darkness with supplied substrate.

Batch culture has been described as 'simple'. However, in fact more is involved in a growth cycle than simply an increase in numbers until the limits of the medium are reached. *Euglena* in batch growth behave in an extremely complex fashion, changing radically as they go through the different phases of growth. Cook (1968) pointed out that changes in physiology, morphology and biochemistry of *Euglena* during growth have been recognised by several investigators, for example decrease per cell of total protein, dry weight and RNA with culture age (Buetow and Levedahl, 1962), and decrease in oxygen consumption of cells grown on glucose (Cook and Heinrich, 1968). Because of such changes it is important that stages of growth be taken into account in any investigation into *Euglena* physiology and biochemistry. This is emphasized by Cook (1968), "Changes in the average *Euglena* that occur as the culture grows clearly make it a risky business to reproduce experiments that could be influenced by such changes.". However, changes in *Euglena* during growth cycles can also be a useful probe in biochemical investigation. This has been found to be the case during previous work.

For instance it was found that the specific activities of both the cytosolic isoenzyme of malate dehydrogenase, (E.C.1.1.1.37)* and of malate enzyme (E.C.1.1.1.40), increase severalfold during the growth cycle of *Euglena gracilis* Z grown heterotrophically on glucose. No increase in the specific activity of these enzymes was measured if the cells were grown autotrophically. These findings gave some insight into the question of the function of these enzymes in *Euglena* (Peak *et al.*, 1972b, 1973).

Euglena is able to withstand large changes in pH and can grow efficiently at pH values ranging from 3 to 9, although in general it grows faster in acid media. Cook and Heinrich (1965) studied the effect of initial pH of the medium on growth of *Euglena gracilis* Z, using the salt medium of Cramer and Myers (1952). They found no growth below pH 5 when acetate was sole carbon source. However, in the present study, *Euglena gracilis* Z were successfully cultured on acetate at pH 3.3 (see Materials and Methods).

Nutritional versatility is a feature of euglenoids. Most have the ability to modify their metabolism in such a way that they can grow equally efficiently photoautotrophically (i.e. fixing carbon dioxide with the aid of light) or heterotrophically (i.e. on complex organic carbon substrates, not necessarily in the light). Heterotrophic nutrition may be osmotrophic (intake of soluble food material) or phagotrophic (ingestion of particulate matter). In response to environmental change they can adapt rapidly to either the autotrophic or heterotrophic mode of life. Such changes can be controlled experimentally, and the organism induced to alter its mode of life so that modifications in metabolism can be

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* Nomenclature and numbering of enzymes is according to the Enzyme Commissions of 1965 (Barman, 1969) and 1972 (Florkin and Stotz, 1973).

studied. Peak *et al.* (1972b, 1973) studied changes in metabolism resulting from the abrupt transfer of *Euglena* from autotrophic culture to heterotrophic culture and *vice versa*. Such transfers were termed transpositions. When grown under autotrophic conditions, *Euglena* possess several plant-like characteristics, most notable of which is the presence of fully developed chloroplasts functioning in photosynthesis (approximately 30 in *E. gracilis*). Also observed in autotrophic cells is a plant-like spectrum of lipid content (Rosenberg, 1963). About 30% of the lipid found in autotrophic cells is alphalinolenic acid, which is a typical plant chloroplast fatty acid. This fatty acid is barely present in heterotrophic cells. If autotrophic cells are experimentally transferred to the dark and provided with a suitable complex carbon substrate, growth continues heterotrophically. Apparently the manufacture of new chloroplast material ceases and the chloroplasts regress, losing their chlorophyll and the organization of the photosynthetic lamellae (Ben Shaul *et al.*, 1965). After several generations of growth in the dark the cells become fully etiolated and have lost all their chlorophyll, the chloroplasts being present as proplastids, which are apparently simple sacs containing protochlorophyll(ide), but lacking internal structure (Epstein and Schiff, 1961; Schiff *et al.*, 1961b). During continued heterotrophic growth of the cells the proplastids continue to divide, maintaining the original number per cell.

Heterotrophic *Euglena* are in a temporarily etiolated or bleached condition and if they are placed in the light the proplastids rapidly elaborate once again into mature, functional chloroplasts. This process, which is known as "greening", is stimulated by light alone and can occur in the absence of carbon dioxide or other carbon source i.e. without cell division taking place. (However, both light and carbon dioxide are necessary for a

complete transposition to autotrophic growth). The details of the elaboration of the chloroplast structures and related matters during greening have been the subject of extensive study. Early electron micrographs illustrating the process were presented by Epstein and Schiff (1961) and by Ben-Shaul *et al.* (1964). Buetow (1968) devotes four chapters of his book to the subject of chlorophyll and the chloroplast in *Euglena*, and since then a considerable further body of work has been reported in the literature.

The transposition of *Euglena* from an autotrophic to a heterotrophic mode of life, and *vice versa*, is accompanied by other metabolic changes which have been relatively little researched in comparison with chloroplast changes. Peak *et al.* (1972a and b, 1973) studied metabolic changes associated with transpositions, in particular changes in the activities of certain enzymes. It was found for instance, that malate enzyme and cytosolic malate dehydrogenase increased considerably when autotrophic *Euglena* were transposed to heterotrophic conditions, whereas the reverse transposition was accompanied by sharp decreases in the enzymes' specific activities. The differences observed in enzyme activity between heterotrophic and autotrophic *Euglena* proved to be of importance in a consideration of the function of the enzymes (Peak *et al.*, 1972, 1973). In another comparison Rosenberg (1963) studied the differences in fatty acid composition between autotrophic and heterotrophic *Euglena*. He found a major difference in the proportion of alpha-linolenic acid as described above.

Whereas *Euglena* is only temporarily bleached by heterotrophic growth in the dark, and will "regreen" if given light, it is possible to induce permanent bleaching in the laboratory by various treatments such as

exposure to streptomycin, ultraviolet light or heat (Mego, 1968). These various treatments may cause bleaching in different ways. For instance, plastids are more sensitive to streptomycin or ultraviolet light compared with the cell nucleus, and can be selectively inhibited by these agents. In some cases the treatment may be reversible (Schiff *et al.*, 1961a) but continuation of the treatment through a number of generations can produce permanently bleached cells which are incapable of forming functional chloroplasts. This bleaching is sometimes thought to be due to genetic mutation, in which case the plastids are biochemically inhibited so that they can no longer become fully functional chloroplasts, but remain as non-functional inclusions (Gibor and Herron, 1968). However, ultraviolet treatment in some cases appears to block completely the replication of plastids so that after successive generations they are completely diluted out of the cells in the culture (Schiff *et al.*, 1961b). Permanent bleaching by heat may occur in a similar manner. At 34^{o}C the division rate of the plastids is inhibited, while cell division continues at the normal rate, so that after many generations of growth at this temperature no plastids remain in the cells. The bleached mutant used in the present study, *Euglena gracilis* Z SB3, was obtained by streptomycin treatment.

When cultured heterotrophically many Euglenae are able to grow on a wide variety of different substrates. *Euglena gracilis* Z in particular is very versatile in this regard, and has been found to grow successfully on glucose, fructose, malate, pyruvate, succinate, glutamate, acetate or ethanol as sole carbon source (Peak *et al.*, 1972b). Further, it has been shown that the cells can be induced to modify their metabolism not only by transposition from autotrophic to heterotrophic mode of growth, but also by varying the carbon source for heterotrophic growth. For instance, *Euglena gracilis* Z grown on glutamate have levels of mitochondrial malate

dehydrogenase twice as high as cells grown on glucose (Peak *et al.*, 1972b). Such a modification might act as a lever for an investigation into the regulation of this isoenzyme. Of interest with relation to the present study is the fact that *Euglena* is a member of a small group of eukaryotes known as the "acetate flagellates" which are able to utilize and grow on 2-carbon compounds as sole carbon source. For instance *Euglena* can grow on acetate or ethanol and thus must be able to synthesize 6-carbon sugars from these compounds. Heinrich and Cook (1967) have shown that growth on 2-carbon substrates affects the respiratory physiology of *Euglena gracilis*. A notable modification to growth on 2-carbon substrates such as acetate is the utilization of the glyoxylate shunt or bypass described below (Kornberg, 1966), which is catalysed by the enzymes isocitrate lyase (E.C.4.1.3.1.) and malate synthase (E.C.4.1.3.2.). These enzymes were first demonstrated in *Euglena* by Reeves *et al.* (1962). Cook and Carver (1966) showed that culturing *Euglena gracilis* on acetate induces the glyoxylate cycle enzymes in cells previously grown either autotrophically or heterotrophically on glucose. Also, Graves *et al.* (1975) demonstrated these glyoxylate enzymes in cells grown in the dark on ethanol.

HETEROTROPHIC CARBON DIOXIDE FIXATION

This subject was reviewed by Wood and Stjernholm (1962) and again by Wood and Utter (1965). The phenomenon of heterotrophic carbon dioxide fixation has only been known for the past 40 years, but it is now recognised as being of importance in a wide range of cells and tissues.

The formation and breakage of the covalent carbon-carbon bond, with one of the reactants being carbon dioxide, is basic to life. The formation of a large proportion of organic material is due to carboxylations which occur during photosynthesis in autotrophic organisms. Here light energy is used to form the covalent bond. Ultimately, all the carbon dioxide fixed in photosynthesis will be liberated again through the processes of respiration, either in an autotrophic plant cell or a heterotrophic animal cell.

The processes of photosynthesis and respiration, which involve large exchanges of gas, have been known for many years. The possibility of carboxylations other than those of photosynthesis occurring in metabolism was not considered by early investigators and in fact it was assumed that heterotrophic organisms were unable to utilize carbon dioxide at all. It is now known that both autotrophic organisms and heterotrophic organisms do fix carbon dioxide non-photosynthetically, but that these carboxylations were not detected because they are masked by the relatively larger exchanges of carbon dioxide occurring either in photosynthesis or respiration.

It was not until radioactive isotopes became available, in 1940, that it was possible to demonstrate heterotrophic uptake of carbon dioxide in many different cells and tissues using tracer techniques. Although the tissue

under investigation might actually be releasing more carbon dioxide than it is fixing, the use of labelled carbon dioxide picks out the carboxylation reactions. Since heterotrophic carbon dioxide fixation reactions are independent of light as energy source and can occur in the dark, the energy to form the covalent bond must come from within the cell.

In actual fact, heterotrophic carbon dioxide fixation was demonstrated before the advent of tracers, but only as the result of an unusual type of metabolism manifested by propionic acid bacteria. In 1936 Wood and Werkman, while conducting quantitative determinations of the products of glycerol fermentation, demonstrated a net uptake of carbon dioxide by these organisms, in contrast to other heterotrophic bacteria in which a net output of carbon dioxide had always been seen. At first the exceptional result with propionic acid bacteria was considered to be an artefact. However, it was subsequently discovered that more carbons had been produced by the fermentation, in the form of succinate and propionate, than were originally added in the form of glycerol, the excess being obtained from gaseous carbon dioxide.

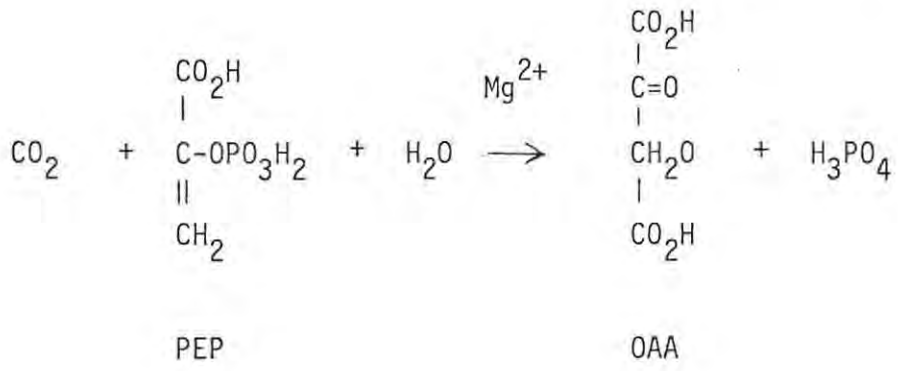
The metabolism of propionic acid bacteria is a special case, but with the use of radioactive tracers heterotrophic carbon dioxide fixation was demonstrated in many different organisms. Early workers measured carbon dioxide uptake by heterotrophic bacteria (Barker *et al.*, 1940); moulds (Foster *et al.*, 1942); and animal tissues (Evans and Slotin, 1940), and following these discoveries the heterotrophic assimilation of carbon dioxide by bacteria and mammalian tissues was extensively studied. Moreover, heterotrophic carbon dioxide fixation was found not to be limited to heterotrophic organisms, but was demonstrated in both higher and lower plants. By placing green algae in the dark, non-photosynthetic

uptake of labelled carbon dioxide could be measured. Heterotrophic carbon dioxide uptake was measured in this way in *Nostoc* and *Scenedesmus* (Holm-Hansen, 1956) and in *Chlorella* by Moses *et al.* (1959). In higher plants, for example Ting and Dugger (1965, 1967) have investigated carbon dioxide fixation by various root tissues. Having demonstrated the existence of heterotrophic carbon dioxide fixation it became evident that it is of fundamental importance in the physiology of, for example, many microorganisms, since lack of carbon dioxide in the environment caused an inhibition of growth (Hutner and Provasoli, 1951). Wood and Utter (1965) in their review stated that "... most, if not all, heterotrophic cells are dependent on carbon dioxide fixation reactions.".

Heterotrophic carbon dioxide fixation reactions Basically, heterotrophic carbon dioxide fixation as measured in a cell or tissue using tracer, is the net sum of all the carboxylations occurring in that tissue. However, as investigation into heterotrophic carboxylations continued, different reactions were distinguished and it became clear that there are many different routes of heterotrophic carbon dioxide fixation, catalysed by different enzymes. Moreover, these routes are not, as earlier workers had thought, the same as those of photosynthesis. The routes and enzymes may vary from organism to organism and from tissue to tissue. The following is a selected list of important heterotrophic carbon dioxide fixation reactions and the enzymes catalysing them.

(A) Phosphoenolpyruvate carboxylase (E.C.4.1.1.31)

This enzyme catalyses the formation of oxaloacetate from phosphoenolpyruvate. The reaction, which is essentially unindirectional, leads to the formation of orthophosphate.



PEP OAA

This enzyme was first discovered in spinach leaves by Bandurski and Greiner (1953) and was soon shown to be present in many higher plants (Tchen and Vennesland, 1955; Mazelis and Vennesland, 1957). It has been observed in *Thiobacillus thiooxidans* (Suzuki and Werkman, 1957), *Escherichia coli* (Ashworth and Kornberg, 1963) and *Salmonella typhimurium* (Theodore and Engelsberg, 1964). There is no evidence that this enzyme occurs in animals.

(B) Phosphoenolpyruvate carboxykinases

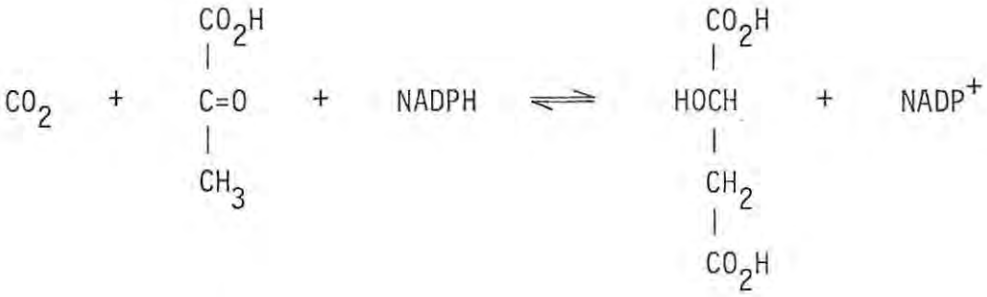
These enzymes also catalyse the carboxylation of phosphoenolpyruvate to oxaloacetate, but the reactions are reversible and phosphate acceptors are required. Three PEP carboxykinase enzymes have been distinguished on the basis of the phosphate substrate specificity. A GDP requiring enzyme (E.C.4.1.1.32) was discovered in chicken liver by Utter and Kurahashi (1953) and has been observed in liver and kidney of several animal species, where it is usually found in the mitochondria. The phosphate acceptor, GDP, can be replaced by IDP (Utter *et al.*, 1954; Kurahashi *et al.*, 1957). An ADP requiring PEP carboxykinase (E.C.4.1.1.49) appears to be limited to plants (Tchen and Vennesland, 1955; Mazelis and Vennesland, 1957), yeast (Cannata and Stoppani, 1959) and bacteria (Suzuki and Werkman, 1958; Diesterhaft *et al.*, 1973). Another PEP carboxykinase (E.C.4.1.1.38) has a requirement for inorganic phosphate as a phosphate

acceptor. Also referred to as PEP carboxytransphosphorylase, it was discovered by Siu and Wood (1963) and so far appears to be specific to propionic acid bacteria (Kaziro and Ochoa, 1964).

There is some confusion in the literature regarding the nomenclature and classification of PEP carboxylase and the PEP carboxykinase enzymes.

(C) Malate enzyme (E.C.1.1.1.40)

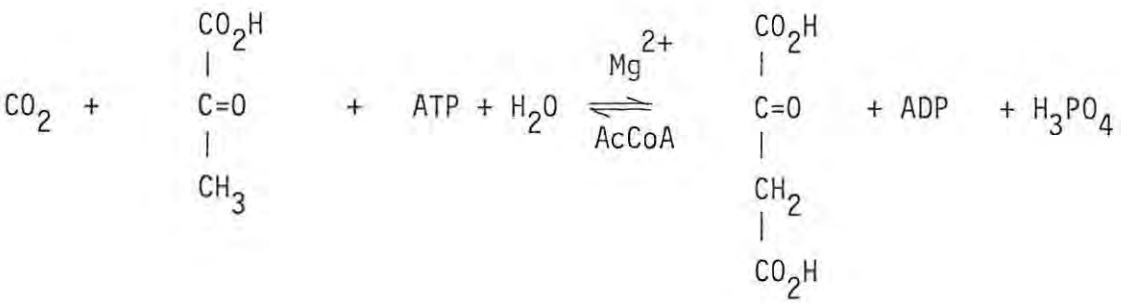
This enzyme catalyses the reversible carboxylation of pyruvate to malate. It is specific for NADP.



This enzyme has been shown to occur in all organisms and tissues so far examined, being found in animal tissues such as liver, where it was first discovered (Ochoa *et al.*, 1947), plants (Conn *et al.*, 1949), in bacteria such as *Lactobacillus arabinosus* (Korkes *et al.*, 1950) and many more.

(D) Pyruvate carboxylase (E.C.6.4.1.1.)

This enzyme catalyses the formation of oxaloacetate from pyruvate.

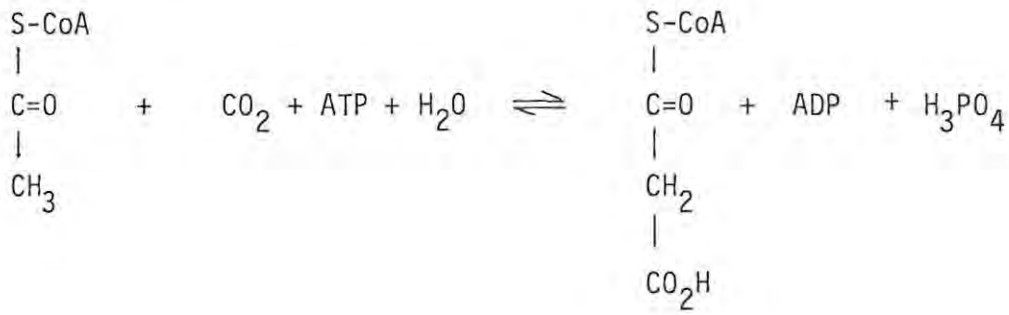


It was first described in chicken liver by Utter and Keech (1960). In this tissue it is confined to the mitochondria, however it has been found to be present in both mitochondria and cytosol in rat liver and adipose tissue (Resheff *et al.*, 1969). Pyruvate carboxylase was at first thought to be confined to animal tissues, however it has since been found in yeast (Losada *et al.*, 1964) and in *Bacillus subtilis* (Diesterhaft *et al.*, 1973)

Pyruvate carboxylase is a biotin containing enzyme and has a requirement for acetyl-CoA. However, an apparently analagous enzyme has been reported from *Pseudomonas citronellolis*, which does not require acetyl-CoA (Seubert and Remberger, 1961), and no requirement for acetyl-CoA was reported for the pyruvate carboxylase found in the cytosol of *Verticillium albo-atrum* (Hartman and Keen, 1973).

(E) Acetyl-CoA carboxylase (E.C.6.4.1.2)

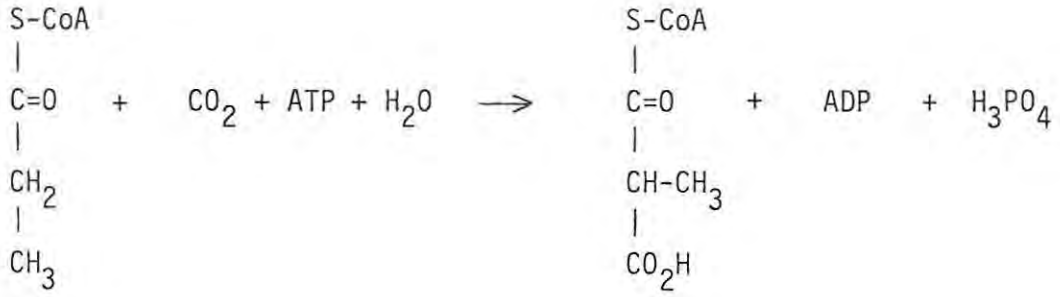
This biotin containing enzyme catalyses the carboxylation



of acetyl-CoA to form malonyl-CoA. Wakil (1958) first described the enzyme in liver.

(F) Propionyl-CoA carboxylase (E.C.6.4.1.3.)

This enzyme, also biotin containing, catalyses a similar reaction, the carboxylation of propionyl-CoA to methylmalonyl-CoA.



It was discovered in pig heart by Flavin *et al.* (1955).

Possible functions of heterotrophic carbon dioxide fixation reactions

It is now known that heterotrophic carboxylations occur at several sites in metabolism and the functions of carboxylations may vary from one cell to another. In some cells heterotrophic carbon dioxide fixation may have one single overriding function, in others there may be several functions occurring at the same time. Further, the functions may vary within a cell from time to time depending on the particular metabolic requirements. In some cases the metabolic functions of dark carboxylations are well worked out; in others they are still obscure. Listed below are possible functions of dark carboxylations in a range of situations.

(A) Fatty acid biosynthesis

The possible involvement of carbon dioxide fixation in fatty acid biosynthesis was not suspected by earlier workers, although some time ago it was considered possible that bicarbonate might be of importance in the formation of fatty acids from acetate (Brady and Gurin, 1950). Working with liver, Wakil (1962) clearly showed that the first step in fatty acid biosynthesis is a carbon dioxide fixation, catalysed by acetyl-CoA carboxylase.

$$CO_2 + \text{acetyl-CoA} + ATP + H_2O \rightleftharpoons \text{malonyl-CoA} + ADP + H_3PO_4$$

The malonyl-CoA formed in this reaction is a chain lengthening substrate in the formation of fatty acid chains, the condensation reactions being catalysed by the enzyme complex, fatty acid synthetase. Malate enzyme may also be involved in fatty acid biosynthesis in a more indirect fashion, as one of a series of enzymes which regulate the necessary supply of protons. This possibility is described under pyridine nucleotide ratio control, below.

(B) Gluconeogenesis

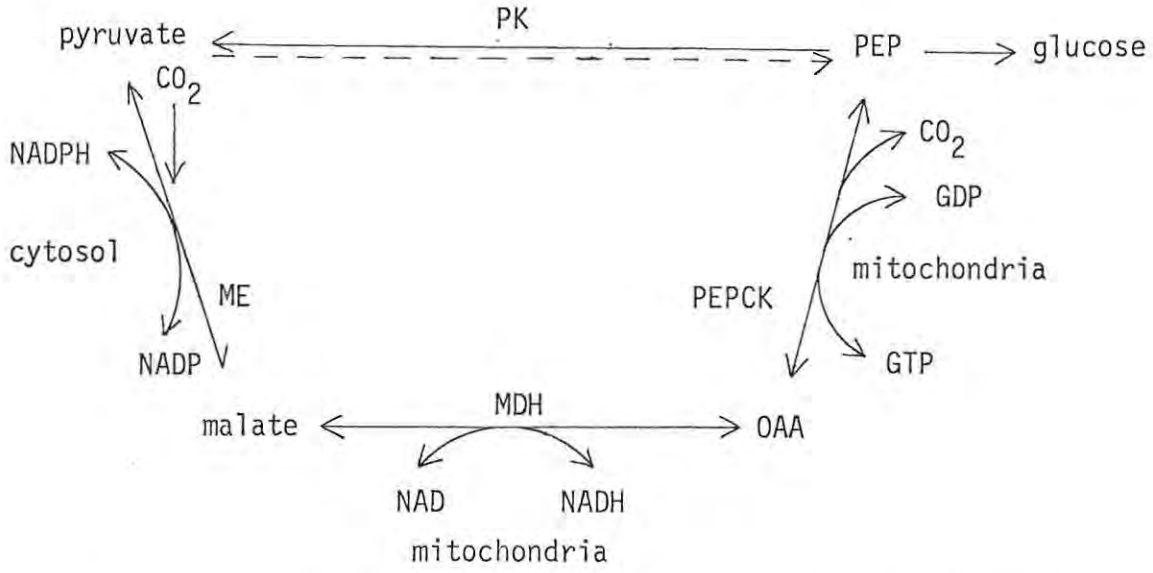
The resynthesis of glucose by reverse glycolysis is of particular importance in the liver, where glycogen is synthesized often from lactate via pyruvate. It also presumably occurs in organisms such as *Euglena* when it is growing solely on 2-carbon substrates.

Heterotrophic carbon dioxide fixation was implicated in gluconeogenesis by early workers who noted the incorporation of $^{13}CO_2$ into liver glycogen, but the metabolic site of the carboxylation was not known. The formation of PEP from pyruvate, a key reaction in gluconeogenesis, had been shown to take place in the mitochondria. Originally this was thought to be merely a reverse of the pyruvate kinase reaction of glycolysis

$$\text{PEP} + \text{ADP} \underset{\leftarrow}{\overset{Mg^{2+},\ K^{+}}{\rightleftharpoons}} \text{Pyruvate} + \text{ATP}$$

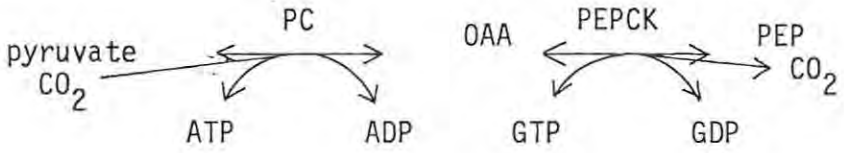
but the reaction in the direction of PEP is energetically unfavourable (Krebs, 1954), in fact Conn and Stumpf (1976) state that it is irreversible under physiological conditions. When both malate enzyme (Ochoa *et al.* 1947) and PEP carboxykinase (E.C.1.1.1.32) (Utter and Kurahashi, 1953) were discovered in liver it was considered possible that a bypass system

involving the carboxylation of pyruvate to malate by malate enzyme could provide an alternative route.



It was proposed initially that mitochondrial malate dehydrogenase could catalyse the intermediate reaction, converting malate to oxaloacetate. In this bypass malate enzyme would function as a carboxylating enzyme, whereas PEP carboxykinase would have a decarboxylating function . However, a possible objection to this scheme was the fact that the activity of malate enzyme in the liver is very low compared with that of PEP carboxykinase, and moreover malate enzyme is located in the cytosol and not the mitochondria (Utter, 1963). This would necessitate some form of shuttle.

With the discovery of another carboxylating enzyme in liver, pyruvate carboxylase, which converts pyruvate directly to oxaloacetate (Utter and Keech, 1960), an alternative bypass was postulated for the formation of PEP from pyruvate in liver gluconeogenesis (Utter, 1963).



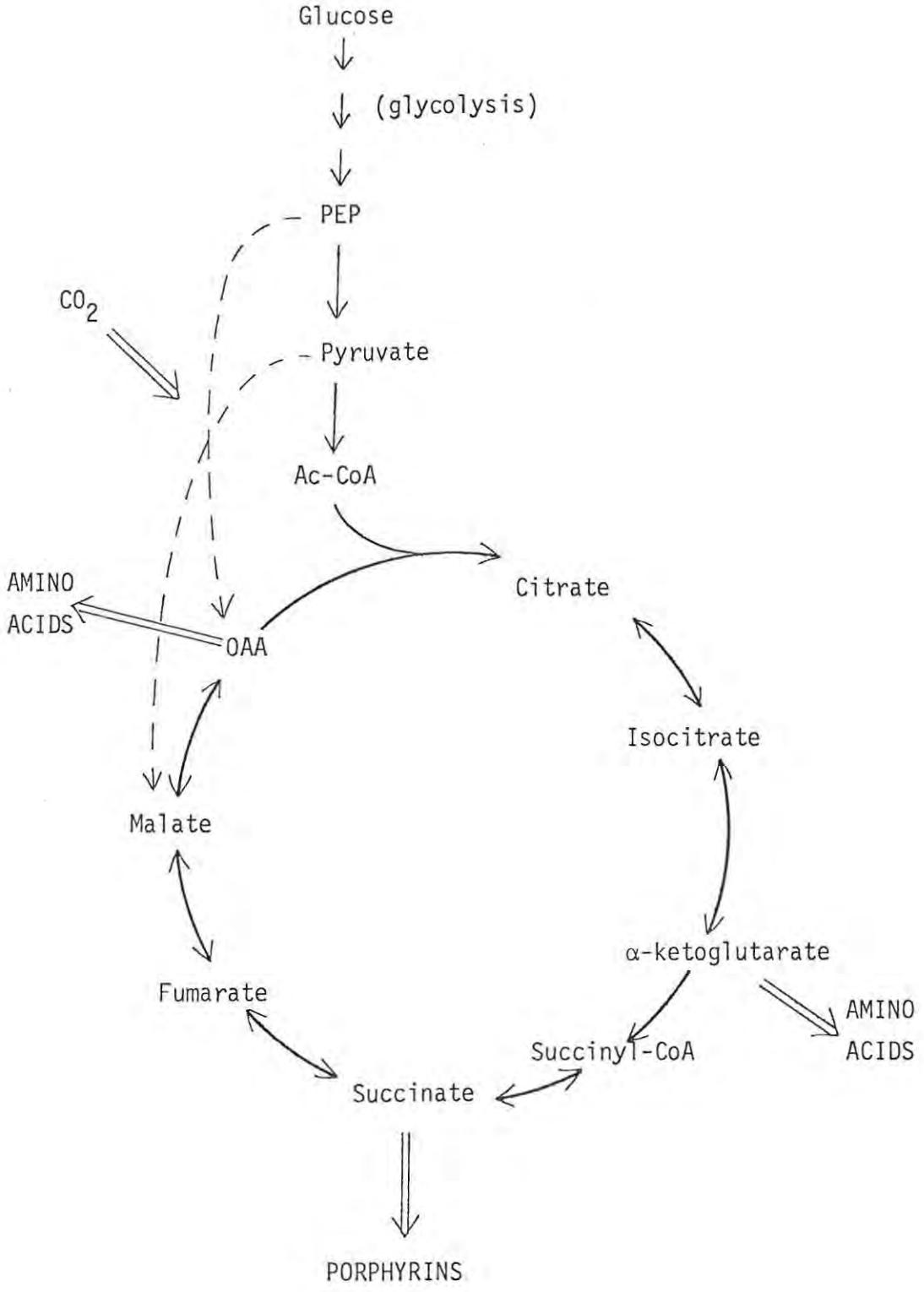
Pyruvate carboxylase occurs in large amounts in the mitochondria of both liver and kidney, and thus would be able to perform this function in these tissues. However, the enzyme has so far been described only in animals and a few microorganisms (Losada *et al.*, 1964; Diesterhaft *et al.*, 1973). In this bypass scheme, PEP carboxykinase has a decarboxylating function. There is in actual fact no total fixation of carbon fioxide as a result of these two reactions, but the carbon dioxide can be considered to have a catalytic function providing a bypass mechanism for an energetically unfavourable step (Wood and Utter, 1965).

(C) Pyridine nucleotide ratio control

It has been suggested that malate enzyme may function in fatty acid synthesis by acting in a transdehydrogenating system (Peak *et al.*, 1973). The conversion of malate to pyruvate, catalysed by malate enzyme, is thought to provide a source of NADPH, at the expense of NADH, as a source of protons for fatty acid synthesis in *Euglena*. Also possibly involved in this scheme are PEP carboxylase and cytosol malate dehydrogenase. This possibility is described in detail by Peak *et al.*, (1973). Danner and Ting (1967) also postulated that malate enzyme may function in the direction of decarboxylation as a source of NADPH in corn roots. The hypothesis will be considered more fully under Discussion.

(D) Anaplerosis

Kornberg (1966) introduced the concept and significance of anaplerotic reactions. The processes of catabolism and anabolism meet in a central cyclic pathway, the tricarboxylic acid cycle. The diagram shows a representation of this cycle and its amphibolic nature.



The products of catabolism enter the cycle, for example glucose is converted to pyruvate by glycolysis. Pyruvate is converted to acetyl-CoA which enters the cycle by condensing with oxaloacetate to form citrate. Each "turn" of the cycle produces a molecule of oxaloacetate to condense with another molecule of acetyl-CoA.

The tricarboxylic acid cycle is the main starting source of certain compounds essential for biosyntheses. An important example is α-ketoglutarate, which provides the α-ketoacid carbon skeleton for the biosynthesis, by amination, of amino acids such as glutamate, glutamine, ornithine, citrulline, arginine, proline and hydroxyproline, the majority of which are ultimately incorporated into protein. Similarly oxaloacetate can be aminated to form aspartate, and succinate is an important starting point in the formation of phorphyrins.

For each molecule drained from the cycle for anabolic purposes, one less molecule of oxaloacetate will be produced, resulting in a diminished cycle, since oxaloacetate is the starting molecule for another "turn" of the cycle. Thus, without replenishment of drained compounds the cycle would eventually stop. It is essential for continuation of the cycle that for each molecule drained, the tricarboxylic acid cycle be stoichiometrically replenished. Reactions are needed which will synthesize compounds to feed into the cycle, and the need for these reactions will presumably be greater at a time of rapid growth. Kornberg (1966) referred to such postulated reactions as anaplerotic reactions. He proposed that heterotrophic carbon dioxide fixation reactions function in this way as shown in the diagram.

The enzymes listed as A - D above, all catalyse reactions which produce 4-carbon acids such as oxaloacetate or malate, from the 3-carbon compounds, pyruvate or PEP, and therefore could function in anaplerotic replenishment. However, as Kornberg (1966) stated, "The existence of this plethora of carboxylating enzymes raises the question whether an anaplerotic function can be ascribed to any one enzyme, and, if it can, whether a role can also be found for the others." There is little direct evidence for the involvement of carboxylating enzymes in anaplerotic replenishment. However, this is often assumed to be the function of such an enzyme if evidence of an alternative function is lacking. This is the situation in *Euglena* as described below.

Direct evidence for the involvement of heterotrophic carboxylations in anaplerosis was obtained in the Enterobacteriaceae. Using mutants of *Salmonella typhimurium* (Theodore and Engelsberg, 1964) and *Escherichia coli* (Ashworth and Kornberg, 1963) it was clearly shown that PEP carboxylase catalyses an anaplerotic reaction. These organisms are normally able to grow on glucose, glycerol or pyruvate, but mutants lacking active PEP carboxylase could not grow on any of these substrates unless supplied also with tricarboxylic acid cycle intermediates. Thus the function of PEP carboxylase in the wild type must be to replenish the cycle. Since malate enzyme and PEP carboxykinase (E.C.4.1.1.49) were present in both wild type and mutant, these enzymes are clearly not implicated in the replenishment of the tricarboxylic acid cycle in these organisms.

As regards other groups, Kornberg (1966) stated that it is generally believed that in mammalian systems the enzyme pyruvate carboxylase has an anaplerotic function and is also involved in gluconeogenesis. Evidence supporting the anaplerotic function of yeast pyruvate carboxylase

has been reported (Losada *et al.*, 1964). Walker (1962) in his review of carboxylation reactions in plants, considered it likely that, in higher plants, PEP carboxylase fulfills an anaplerotic function, forming oxaloacetate for entry into the tricarboxylic acid cycle. The oxaloacetate could be stored as malate if not used immediately. However, Mukerji and Ting (1971) maintained that, although it is assumed that PEP carboxylase has this function in certain microbial systems, in higher plants such as corn roots, the enzyme does not appear to function in this way. They indicated that the malate aproduced in the adjacent reactions catalysed by the cytosol enzymes PEP carboxylase and soluble malate dehydrogenase is not in equilibrium with malate in the mitochondria, and it appears as though there are two separate malate pools. Furthermore, Ting and Dugger (1967) found that the carbon dioxide fixed heterotrophically by corn roots did not appear to find its way into biosyntheses.

Thus although the importance of anaplerotic reactions is certain, in many cases the actual enzyme(s) involved in these reactions is not finally established.

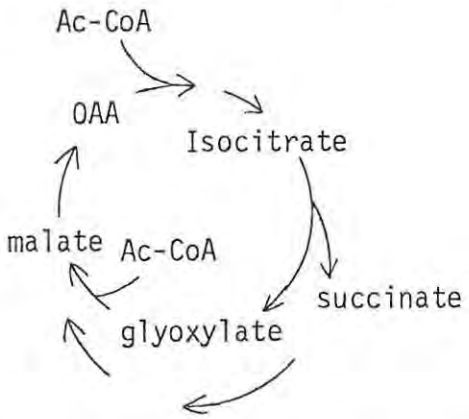
A special form of anaplerotic replenishment described by Kornberg (1966) is the glyoxylate cycle. Microorganisms which are able to utilize 2-carbon compounds such as acetate or ethanol as sole carbon source may use the glyoxylate shunt or cycle to build 4-carbon acids from 2-carbon compounds. The two reactions

$$\text{isocitrate} \longleftrightarrow \text{succinate} + \text{glyoxylate}$$

$$\text{acetyl-CoA} + \text{glyoxylate} + H_2O \longrightarrow \text{malate} + \text{CoA}$$

in combination lead to the formation of two units of 4-carbon dicarboxylic acid, succinate and malate, from one of acetate and one of isocitrate.

The extra 4-carbon acid thus formed can replenish the tricarboxylic acid cycle, obviating a requirement for other anaplerotic reactions.

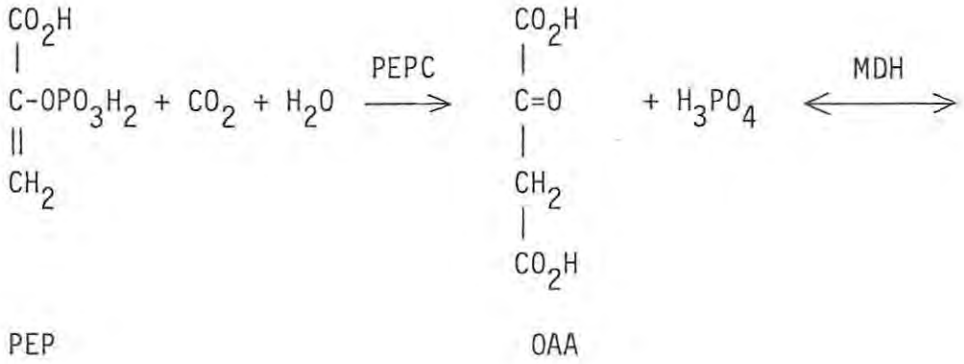


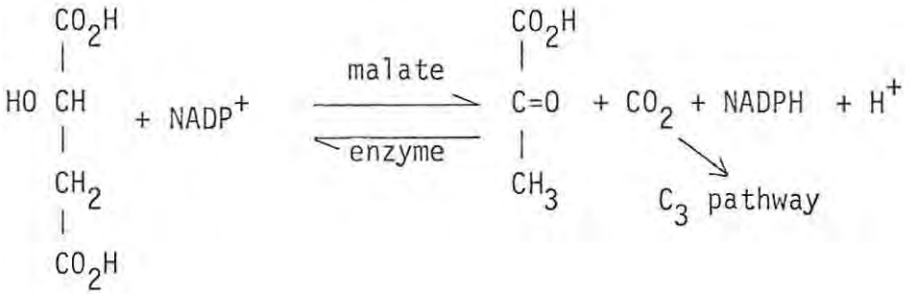
The two enzymes necessary to catalyse these reactions are present in acetate utilizing microorganisms. They are isocitrate lyase, which was discovered by Campbell *et al.* (1953) in *Pseudomonas aeruginosa*, and malate synthase, discovered in extracts of *Escherichia coli* by Wong and Ajl (1956). In the absence of 2-carbon units the enzymes are present only in small amounts. Exposure of the cells to 2-carbon compounds induces them (Kornberg, 1959; Rosenberger, 1962). In cases where the glyoxylate cycle is functioning the need for other anaplerotic reactions will be reduced, since the cycle is being continually replenished. Thus, in the case of the *Escherichia coli* and *Salmonella typhimurium* mutants described above, the mutants lacking PEP carboxylase were able to grow on acetate without addition of Krebs cycle intermediates.

(E) C_4 pathway of photosynthesis and Crassulacean acid metabolism

A specialised function of heterotrophic carbon dioxide fixation is found in certain tropical plants (the C_4 plants) in which the initial product of photosynthesis was found to be oxaloacetate (Hatch and Slack, 1966). These plants are thought to be able to conserve water by fixing carbon dioxide initially, by means of the PEP carboxylase reaction, into

oxaloacetate. PEP carboxylase has a higher affinity for carbon dioxide than does ribulose -1,5-diphosphate carboxylase (E.C.4.1.1.39) and is able to function efficiently under conditions of reduced carbon dioxide availability associated with restricted stomatal opening. The oxaloacetate is then thought to be converted to malate (malate dehydrogenase), and malate enzyme releases the carbon dioxide, which may then be taken into the classical Calvin cycle, using ribulose-1,5-diphosphate carboxylase as the catalyst.





L-malate pyruvate

A similar strategem is found in the Crassulaceae which fix atmospheric carbon dioxide into oxaloacetate (using PEP carboxylase) and store it as malate during the night. By subsequent decarboxylation the following day (malate enzyme) carbon dioxide is made available for Calvin cycle photosynthesis (Quieroz, 1974).

(F) The metabolism of propionic acid

This represents another specialized function of heterotrophic carboxylation. The reaction catalysed by propionyl-CoA carboxylase

$$\text{propionyl-CoA} + CO_2 + \text{ATP} + H_2O \longrightarrow \text{methylmalonyl-CoA} + \text{ADP} + H_3PO_4$$

which was elucidated by Flavin and Ochoa (1957) and others, has been shown to be the first step in a series of reactions whereby animals and some bacteria (Gibson and Knight, 1961) but not plants, metabolise propionate to pyruvate. Carbon dioxide fixation is an essential part of the process, whereas in the plant sequence the carbon dioxide fixation reaction is not involved.

HETEROTROPHIC CARBON DIOXIDE FIXATION IN *EUGLENA*

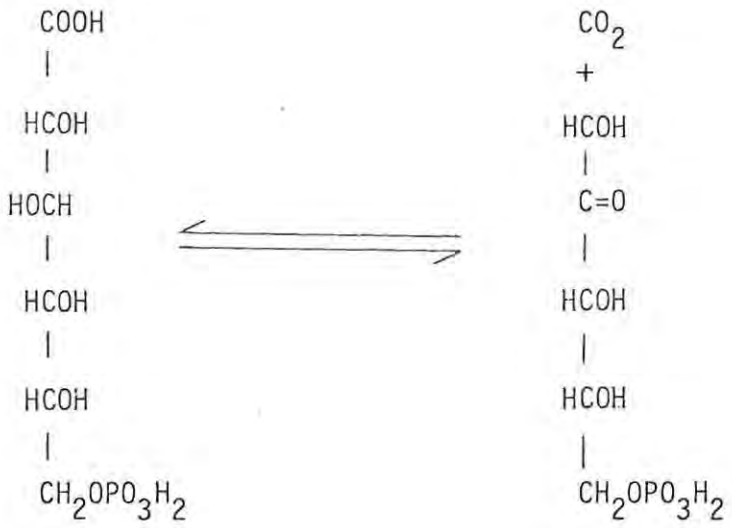
Physiological and biochemical processes in this organism are of particular interest due to its interesting position in phylogeny. There are few publications concerned with heterotrophic carbon dioxide fixation in *Euglena*, and it is apparent from these that the regulation and functions of the fixation in this organism remain obscure.

Levehahl (1968) reviewed early work on dark carbon dioxide fixation in *Euglena*. The first work to be reported in this field was a kinetic study by Lynch and Calvin (1953) who found that the products of dark fixation in *Euglena gracilis* differed from those of all other micro-organisms that they had tested previously (Lynch and Calvin, 1952). They found that, in addition to the usual products of heterotrophic carbon dioxide fixation, such as malate, citrate and aspartate, in *Euglena* many of the products of photosynthesis such as sugar monophosphates were also found. In fact these accounted for the major port of the radioactivity recovered (Table 1). It was considered possible that the sugar phosphates may have been formed as a result of preillumination photosynthesis. It had previously been shown that in *Chlorella* and *Scenedesmus* photosynthesis could continue for a short time after removal of light (Calvin, *et al.*, 1951). However, the formation of photosynthetic products by dark fixation in *Euglena* was apparently not due to such a preillumination effect, since holding the cells in the dark for up to 16 hours before adding radioactive carbon dioxide did not alter the spectrum of products of fixation. The dark fixation of carbon dioxide into phosphorylated sugars, plus the fact that fixation rates were biphasic, led Lynch and Calvin (1953) to suggest that *Euglena* may fix carbon dioxide heterotrophically by two routes, one being identical or similar to the path of photosynthetic

Table 1. Ethanol soluble products of heterotrophic carbon dioxide fixation by *Euglena gracilis*. Reproduced from Lynch and Calvin (1953).

Product	% 80% EtOH Soluble Activity
Fumarate	1.2
Succinate	21.3
Malate	4.8
Citrate	5.0
Glutamate	6.5
Aspartate	2.7
Alanine	2.7
Phosphopyruvate	1.1
Serine and glycine	12.4
Phosphoglycerate	4.6
Sugar monophosphates	19.8
Nucleotides	5.4

carbon dioxide fixation i.e. direct incorporation of carbon dioxide into phosphoglyceric acid. They speculated that *Euglena* may be unique in this respect, and that this may be due to the fact that its phylogenetic position may be at the point at which the animal kingdom separated from the plant kingdom. Moses *et al.* (1959) found similarities between the dark fixation pattern of the fungus *Zygorrynchus moelleri* and *Euglena*. They suggested that the reaction by which carbon dioxide enters the sugar monophosphates may be via phosphogluconic acid i.e. by a reversal of the decarboxylation of 6-phosphogluconic acid to pentose phosphate.



6-phosphogluconic acid ribulose-5-phosphate

Since then no work has been reported which elucidates further the possible paths of carbon dioxide incorporation into sugar phosphates.

Moses *et al.* (1959) also suggested that the fixation of carbon dioxide into the tricarboxylic acid cycle compounds might proceed via the carboxylation of pyruvate to oxaloacetate or malate. At that time however, no carboxylating enzymes had been detected in *Euglena*, so their conclusions were tentative.

The importance of heterotrophic carbon dioxide fixation in the metabolism of *Euglena* was further documented by Tremmel and Levedahl (1966) and Levedahl (1966, 1967), who measured the effect of exogenously supplied carbon dioxide on the growth and metabolism of *Euglena*. They used the streptomycin-bleached strain, *Euglena gracilis* var *bacillaris* SML-1, grown on either acetate or succinate as sole substrate. The significance of their findings is not always clear. Firstly, Tremmel and Levedahl (1966) showed that the bleached *Euglena* did in fact utulize carbon dioxide heterotrophically for growth, however the extent to which carbon dioxide affected growth depended on the substrate on which the cells were growing. Exogenous supplied carbon dioxide had small effects on growth when the cells were growing on acetate, however the effect was prominent when they were growing on succinate. In the latter case the carbon dioxide increased peak populations by as much as five-fold. Subsequently Levedahl (1966, 1967) reported measurements on the incorporation of carbon dioxide by the bleached *Euglena* as well as the fate of the carbon atoms from the respective substrates (succinate and acetate). He showed that carbon dioxide was fixed into essentially the same early products whether succinate or acetate was used as substrate. However, carbon dioxide altered the fate of the carbons of succinate when this was the substrate. In the presence of carbon dioxide, the carbon atoms of succinate were fixed into the compounds typical of those produced by heterotrophic carbon dioxide fixation such as phosphorylated sugars and malate, as found previously by Lynch and Calvin (1953). In the absence of carbon dioxide the carbon atoms of succinate were rapidly fixed into lipid. However, the presence or absence of carbon dioxide had no effect on the fate of the carbons of acetate. The meaning of these results is still not clear. Levedahl suggested that they implied that there may be a close relationship between the incorporation of carbon dioxide and the

metabolism of succinate, but not that of acetate. In his review he made the suggestion that the findings were consistent with the possibility that in *Euglena* carbon dioxide is fixed principally through a reaction involving phosphoenolpyruvate which is converted to succinate via the tricarboxylic acid cycle (Levedahl, 1968).

Since the review of Levedahl (1968) a few reports have been published on heterotrophic carbon dioxide fixation in *Euglena*, mostly concerned with the enzymology of the process. Heinrich and Cook (1967) compared the respiratory physiology of *Euglena gracilis* Z grown heterotrophically on either glucose or acetate. They observed that the rate of dark carbon dioxide fixation was approximately twice as fast when cells were growing on glucose as when they were growing on acetate. A partial separation of the products revealed that in both cases practically all of the radioactive carbon was found in an ethanol soluble fraction and a protein fraction. However, dark carbon dioxide fixation was not the primary concern of the investigation and was not pursued further. They measured the specific activity of malate enzyme and showed it to be higher in the cells growing on acetate, suggesting that the primary function of this enzyme in *Euglena* is not in heterotrophic carboxylation.

Codd and Merrett (1971) measured whole cell dark carbon dioxide fixation in cells grown in light/dark synchronous cultures. The rate of fixation increased in the light phase and decreased in the dark phase. PEP carboxylase (named phosphopyruvate carboxylase by them) activity also varied with the phase of the synchronous cycle, but the changes did not parallel changes in dark carbon dioxide fixation. The main products of the heterotrophic fixation of carbon dioxide were C_4 dicarboxylic acids especially malate. They concluded that the enzyme is involved in anaplerotic replenishment.

PEP carboxylase was first measured in *Euglena* by Ohmann and Plhak (1969). They conducted a major investigation into the properties of a purified preparation of PEP carboxylase. Several differences were noted between the properties of the *Euglena* enzyme and that which had previously been isolated from *Escherichia coli*, for instance the *Euglena* enzyme was not stimulated by acetyl-CoA nor inhibited by malate. Of all the compounds tested only oxaloacetate, citrate and isocitrate affected the activity of the purified enzyme. Ohmann (1969) claimed to have measured PEP carboxykinase (E.C.4.1.1.32) in *Euglena* in a previous unpublished study. He stated that the activity of this enzyme was found to be higher in cultures growing heterotrophically on acetate than in autotrophic or glucose heterotrophic cultures. In the case of PEP carboxylase the reverse situation obtained. Codd and Merrett (1971) did not detect PEP carboxykinase in the light/dark synchronized cultures of *Euglena*. Perl (1974) on the basis of the study by Codd and Merrett (1971) stated that PEP carboxylase had been shown by them to be the enzyme responsible for heterotrophic carbon dioxide fixation in *Euglena*. He extracted and partially purified two active fractions of PEP carboxylase from *Euglena*, one of which was found to be predominant in autotrophically grown cells, the other in heterotrophs. Preliminary kinetic properties of the partially purified isoenzymes and the effects of various inhibitors were presented. In contrast to Ohmann and Plhak (1969) he demonstrated inhibition of *Euglena* PEP carboxylase by α-ketoglutarate, malate, succinate, citrate and 3-phosphoglycerate. However, the concentrations of inhibitor used by Perl were considerably higher than those used by Ohmann and Phlak.

Ohmann and Plhak (1969), also Codd and Merrett (1971), assumed that PEP

carboxylase in *Euglena* has an anaplerotic function. Peak *et al.* (1973) argued that PEP carboxylase, together with malate enzyme and cytosol malate dehydrogenase might function in the regulation of the ratio of reduced pyridine nucleotide coenzymes. This might be of importance in regulating the supply of NADPH, specific for lipid biosynthesis, at the expense of NADH. This has since also been proposed by Wolpert and Ernst-Fonberg (1975).

It is clear that basic questions regarding the process, regulation and functions of heterotrophic carbon dioxide fixation in *Euglena* are still obscure. Nor is the enzymology of the fixation fully understood. Although it appears probable that PEP carboxylase is involved in heterotrophic carbon dioxide fixation, its functional significance is not yet proven. The observation by Peak (personal communication) that the amount of dark fixation varies considerably with the stage of the growth cycle in *Euglena* suggested that some hitherto undescribed regulatory mechanism exists. It was felt that this observation could be a starting point for investigating the control of heterotrophic carbon dioxide fixation in *Euglena*. From this point it was hoped to gain further insight into the function of *Euglena* dark carbon dioxide fixation.

MATERIALS AND METHODS

Euglena strains and culture methods Unless otherwise stated this investigation was carried out using *Euglena gracilis* Z. In describing the results this strain will be referred to as *Euglena*, except in the one instance where it is necessary to distinguish it from the streptomycin bleached strain *Euglena gracilis* Z SB3. *Euglena gracilis* Z was originally obtained in 1970 from Dr J.F. Preston, Microbiology Department, Yale University. Since that time it has been maintained and subcultured on slants of Difco *Euglena* agar, and more recently as stabs in 0.3% agar containing 0.2% peptone, 0.2 mg/ml Na acetate and 0.02 mg/ml vitamin B_{12}. The streptomycin bleached strain of *Euglena gracilis* Z SB3 was obtained from Dr W.R. Evans, Charles F. Kettering Laboratory, and maintained in the same manner.

For experimental purposes, both strains were cultured, either autotrophically or heterotrophically, at 25°C using a mineral medium modified from the low pH autotrophic medium of Hutner *et al.* (1966). The major components of this medium are, in mg/ml, KH_2PO_4 (0.15); $MgSO_4.7H_2O$ (0.3); $MgCo_3$ (0.3); $CaCO_3$ (0.02); EDTA (0.2); NH_4Cl (0.3). The carbonates were dissolved in concentrated HCl before addition. The trace elements were added as described by Hutner *et al.* (1966), and the pH adjusted to 3.3. This medium is referred to in the text as mineral medium.

Autotrophic cultures were grown in 500 ml sidearm flasks with a narrow glass inlet tube entering the bottom of the flask. Carbon dioxide (5% in air) was bubbled continuously through the culture at a rate of approximately 24 bubbles per minute. The culture was further agitated by means of a magnetic stirrer to prevent the cells from aggregating on

the glass. During the early stages of the work the cultures were illuminated by a bank of Sylvania cool white fluorescent tubes, placed at a distance of 2 feet from the culture flasks. In later experiments, due to a move to another laboratory, the cultures were grown in a constant environment room, illuminated continuously by means of overhead lights, Sylvania cool white fluorescent tubes, alternating with rows of incandescent lamps. The light energy reaching the cultures was 2200 foot candles. Heterotrophic cultures of both *Euglena gracilis* Z and SB3 were grown in 1 litre flat Pyrex culture flasks. The flasks were shaken by an orbital shaker in complete darkness. For heterotrophic growth the mineral medium was supplemented with an organic carbon source, either glucose (1%), ethanol (1%) or Na acetate (0.5%).

Cell numbers were estimated turbidometrically using a Klett-Summerson colorimeter, having established at the outset the relationship between turbidity and cell numbers using a cytometer. Cultures were always diluted to a concentration at which the relationship between turbidity and cell numbers was linear.

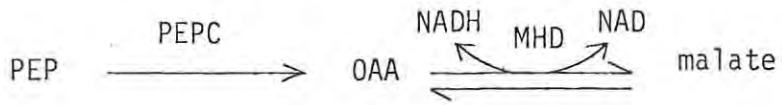
Measurement of heterotrophic carbon dioxide fixation Autotrophic or heterotrophic *Euglena* were grown to the appropriate cell density, harvested, washed by centrifugation and resuspended in the appropriate medium. Cells were always resuspended to the same concentration (7.5×10^5 cells/ml in the case of autotrophically grown *Euglena*, 1.0×10^6 cells/ml in the case of heterotrophic cells.) Aliquots of cell suspension (1 ml) were pipetted into foil wrapped brown flasks which were sealed air tight with serum stoppers. One µCi $Na_2^{14}CO_3$ (specific activity 54.9 mCi/mmole) was injected into each flask with a Hamilton

syringe. (In a preliminary experiment fixation was measured in a series of flasks which had been injected with various amounts of $Na_2^{14}CO_3$. The number of counts fixed by the cells was found to increase with increasing quantities of labelled carbonate up to 1 µCi. Further increases in labelled carbonate did not result in an increase in number of counts fixed. Thus by injecting 1 µCi it was ensured that the amount of radioactive carbon dioxide available was not limiting.) The low pH of the medium ensured that $^{14}CO_2$ would be released. The flasks were shaken in the dark for 20 mins at 25°C. (Preliminary experiments had shown that dark carbon dioxide fixation is linear over this period, so that initial rates of incorporation were being measured). The reaction was terminated by injection of 0.1 ml 100% (w/v) trichloracetic acid. Aliquots of the cell suspension (0.1 ml) were pipetted onto 1.5 x 1.5 cm squares of filter paper. After drying under an infrared lamp, the filter papers were transferred to liquid scintillation vials containing 8 ml scintillation solution (8 gm PPO, 0.2 gm POPOP, 2 l toluene, 1 l triton-X-100). The vials containing filter papers were counted using a Beckman liuqid scintillation counter. It was shown in preliminary experiments that quenching by the filter paper squares was negligible. No attempt was made to separate or fractionate the suspension before counting, thus the counts obtained were a measure of total teterotrophic carbon dioxide fixation.

Enzyme assay methods - preparation of crude extracts *Euglena* were grown to the desired cell density, harvested, and resuspended in cold 0.1 M Tris buffer, pH 7.5, to a concentration of 2 x 10^6 cells/ml. Cells were disrupted by 60 sec sonication, using a Wave Energy Systems Ultratip sonic generator and probe. The cell suspension was kept cold in an ice bath, and overheating was further prevented by sonicating in two 30 sec bursts.

The suspension of disrupted cells was clarified by centrifugation at 18,000 rpm for 20 min at $0^{o}C$, and the supernatant was used as a source of soluble enzyme.

PEP carboxylase (E.C.4.1.1.31) was assayed according to the method of Mukerji and Ting (1971). The reaction mixture contained in a final volume of 3.0 ml, 0.05 M Tris buffer, pH 8.5; 2.5 mM $NaHCO_3$; 6.67 mM $MgCl_2$; 0.134 mM NADH and 1 mM PEP. The disappearance of NADH was followed at 340 nm with a Beckman DU spectrophotometer and recorder. The oxidation of NADH is due to the coupled reactions:



the first catalysed by PEP carboxylase and the second by malate dehydrogenase. Since malate dehydrogenase is present in *Euglena* in considerable excess compared with PEP carboxylase the concentration of PEP carboxylase will be rate limiting, and there was no need to add extraneous malate dehydrogenase except in the case of the purified PEP carboxylase. PEP carboxylase was also assayed radiometrically using a method modified from that of Perl (1974). The reaction mixture contained, in a final volume of 2 ml, 0.05 M Tris buffer pH 8.5; 5 mM $MgCl_2$; 0.402 mM NADH; 1.5 mM PEP. These reagents together with 0.2 ml crude extract were added to the reaction flasks which were then stoppered with serum stoppers and 2 µCi $Na_2^{14}CO_3$ was injected into each flask. After 20 mins incubation at $25^{o}C$ the reaction was terminated by addition of 0.2 ml 100% (w/v) trichloracetic acid. Aliquots of the mixture were counted as described above.

PEP carboxykinase (E.C.4.1.1.32) and (E.C.4.1.1.38) were assayed spectrophotometrically according to the method of Codd and Merrett (1971).

The reaction mixture was the same as for PEP carboxylase except that it was supplemented with 1 umole GDP or 5 umoles Na_2HPO_4 respectively. The PEP carboxykinase activities were then calculated by difference.

PEP carboxykinase (E.C.4.1.1.49) was assayed by a method modified from that of Cazzulo *et al.* (1968). The reaction mixture contained, in a final volume of 3 ml, 0.05 M borax succinic acid buffer pH 5.4; 2.5 mM $NaHCO_3$; 617 mM $MnCl_2.4H_2O$; 0.134 mM NADH; 1 mM PEP and 1 umole ADP.

Pyruvate carboxylase (E.C.6.4.1.1.) was measured according to the method described by Codd and Merrett (1971). The reaction mixture contained, in a final volume of 3 ml, 0.05 M Tris buffer pH 8.5; 2.5 mM $NaHCO_3$; 6.67 mM $MgCl_2$; 0.134 mM NADH; 1.5 mM Ac-CoA; 1 mM Na pyruvate and 1 umole ATP.

Malate enzyme (E.C.1.1.1.40) was measured according to the method of Mukerji and Ting (1968). The reaction mixture contained in a final volume of 3 ml, 0.05 M TES buffer pH 7.0; 0.4 mM $MnCl_2$; 0.1 mM NADP and 0.5 mM L-malate. The initial rate of appearance of NADPH was followed at 340 nm. A correction was applied for endogenous reduction of NADP before addition of malate.

Acetyl-CoA carboxylase (E.C.6.4.1.2) was assayed radiometrically by a method modified from that of Wolpert and Ernst-Fonberg (1975). The assay mixture contained, in a final volume of 2 ml, 0.1 M imidazole buffer pH 7.5; 8 mM $MgCl_2$; 2 mM dithiothreitol; 0.66 mM acetyl-CoA; 4 mM ATP; 0.2 mg bovine serum albumen. After a 2 min warming period, 2 µCi $Na_2^{14}CO_3$ was injected into each flask, and the flasks incubated for 20 mins at 30°C. Termination of reaction and counting of aliquots were as described above.

Purification of PEP carboxylase isoenzymes from each other The method used was essentially the same as that of Perl (1974) except that a linear gradient was used instead of the stepwise gradient used by Perl. Cells (200 ml) were harvested, washed by centrifugation and resuspended in 5 ml 0.05 M Tris buffer pH 8.4, containing 0.1 mM mercaptoethanol. Cells were disrupted by 60 sec sonication as described above, and the suspension clarified by centrifugation at 18,000 rpm for 20 min at 0^{o}C.

Subsequent steps in the pruification were performed at low temperature (5^{o}-7^{o}C). The crude extract was brought to 20% saturation with solid ammonium sulphate (130 mg/ml) and stirred for 30 min with a magnetic stirrer. After centrifugation at 18,000 rpm for 20 min at 0^{o}C the precipitated protein fraction was discarded and the supernatant brought to 50% saturation with ammonium sulphate (220 mg/ml) and stirred for 30 min. After centrifuging as before the pellet was redissolved in 1 ml of 5 mM Tris buffer pH 8.2 and the solution applied to a Sephadex G-25 column (1 x 10 cm), and eluted with the same buffer. The high molecular weight fraction was collected and applied to a DEAE-cellulose column (1.6 x 6 cm) previously equilibrated with 5 mM Tris buffer pH 8.2. The proteins were eluted by a linear gradient of 5 - 1000 mM Tris buffer at pH 8.2. Fractions (2.5 ml) were collected and assayed for PEP carboxylase activity.

Other assay methods Total soluble protein in the cell extracts was measured by the method of Lowry *et al.* (1951). Ammonium concentration in the growth medium of *Euglena* was measured according to the method described by Vogel (1951), using a commercial preparation of Nessler's reagent.

Separation of products of heterotrophic carbon dioxide fixation *Euglena* were grown to appropriate cell densities (3.0×10^5 cells/ml for autotrophic cells, 5.0×10^5 cells/ml for heterotrophic cells). 100 ml cells were harvested, washed and resuspended in 10 ml of the appropriate medium. Each cell suspension was pipetted into a brown, foil-wrapped flask which was then sealed with a serum stopper. 50 µCi $Na_2^{14}CO_3$ (specific activity 54.9 mCi/mmole) was injected with a Hamilton syringe and the flasks incubated at 25°C. After 10 min the entire contents of each flask was poured into 40 ml boiling absolute EtOH. The disrupted cells were filtered and the filtrate evaporated to dryness and redissolved in 2 ml 80% EtOH.

50 µl aliquots were applied to 20 x 20 squares of Whatman No. 1 chromatography paper or Gelman polysilicic acid gel impregnated glass fibre sheets and subjected to 2-way ascending chromatography using the solvent system of Benson *et al.* (1950). Radioactive areas were located by use of X-ray film. Individual compounds were tentatively identified by comparison with standards, and then eluted and subjected to co-chromatography with standards for final identification. Radioactive 'spots' were cut out of the papers and counted in a Beckman liquid scintillation counter to determine the amount of label incorporated into each compound.

Chemicals Sodium (^{14}C) carbonate was obtained from The Radiochemical Centre, Amersham, England. Scintillation toluene, PPO, POPOP, and Triton-X-100 were obtained from Beckman Instruments Incorporated. Triton-X-100 was also obtained from Packard Instrument Company, Incorporated. All biochemicals were supplied by either Sigma Chemical Company or British Drug Houses, and inorganic salts were obtained from British Drug Houses or Merck.

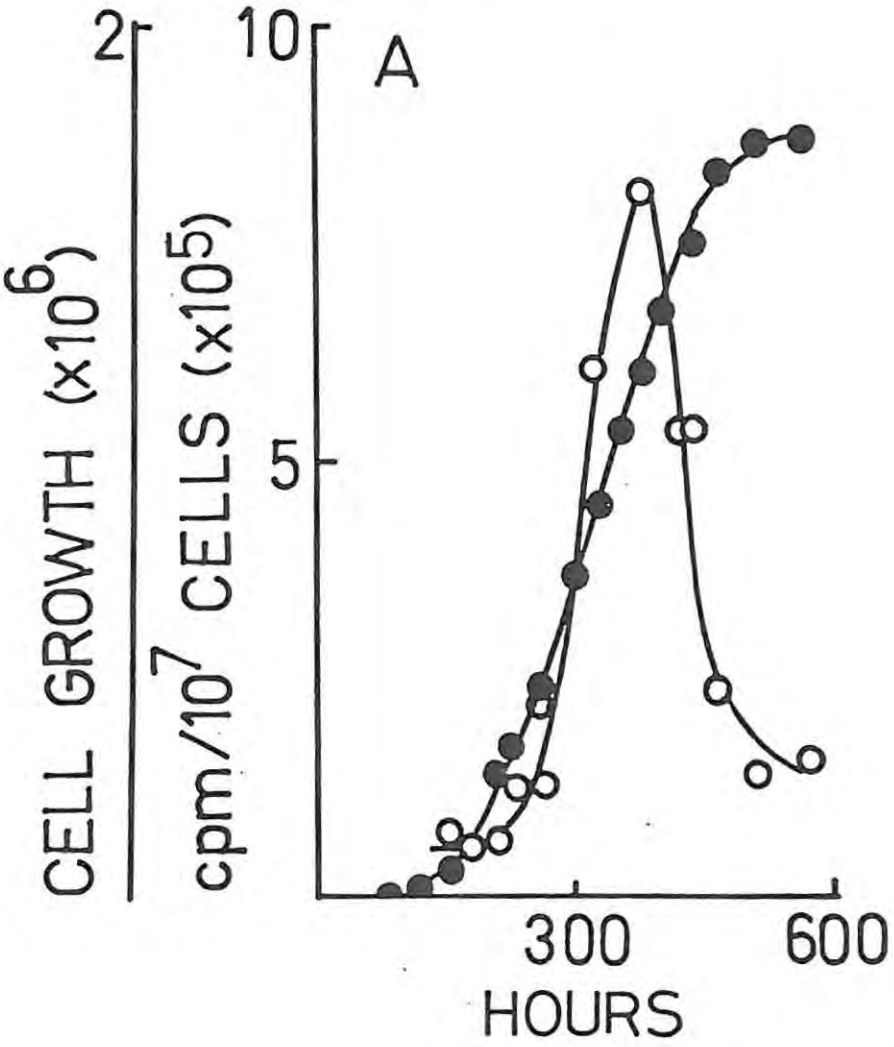


Figure 1. Variations in heterotrophic carbon dioxide fixation during the growth cycle of *Euglena gracilis* Z grown photoautotrophically. The growth of the culture, expressed as cell/ml, is shown by the closed circles. Open circles represent heterotrophic carbon dioxide fixation.

RESULTS

Variations in heterotrophic carbon dioxide fixation by *Euglena* during batch growth cycles A series of experiments was performed in order to verify, and to document more fully, the earlier finding that the amount of carbon dioxide fixed in the dark by *Euglena* increases towards the end of batch growth cycles. It was decided to measure heterotrophic carbon dioxide fixation at intervals throughout entire growth cycles so that any changes would be observed.

For these experiments *Euglena* were cultured under three different environmental conditions - (a) autotrophically, in the light, with carbon dioxide as sole carbon source; (b) heterotrophically, in the dark, with a 6-carbon substrate (glucose); (c) heterotrophically, in the dark, with a 2-carbon substrate (acetate or ethanol). At intervals during the growth cycle (usually every 24 hours, but more frequently during the period of logarithmic growth) aliquots of cells were harvested, resuspended to the correct cell density in fresh mineral medium, and carbon dioxide fixation measured in complete darkness as described in Materials and Methods.

(A) Heterotrophic carbon dioxide fixation by autotrophically grown *Euglena*

Fixation of carbon dioxide in the dark by cells harvested at intervals during an autotrophic growth cycle is shown in Figure 1. During the lag phase 10^6 cells fixed less than 10,000 cpm over the 20 minute period. During the logarithmic growth phase fixation increased ten fold. The highest fixation was recorded during the later part of the logarithmic growth period. Thereafter dark carbon dioxide fixation decreased, returning to almost the original level by the time the culture was in stationary phase. This result confirms the original observation by M.J. Peak.

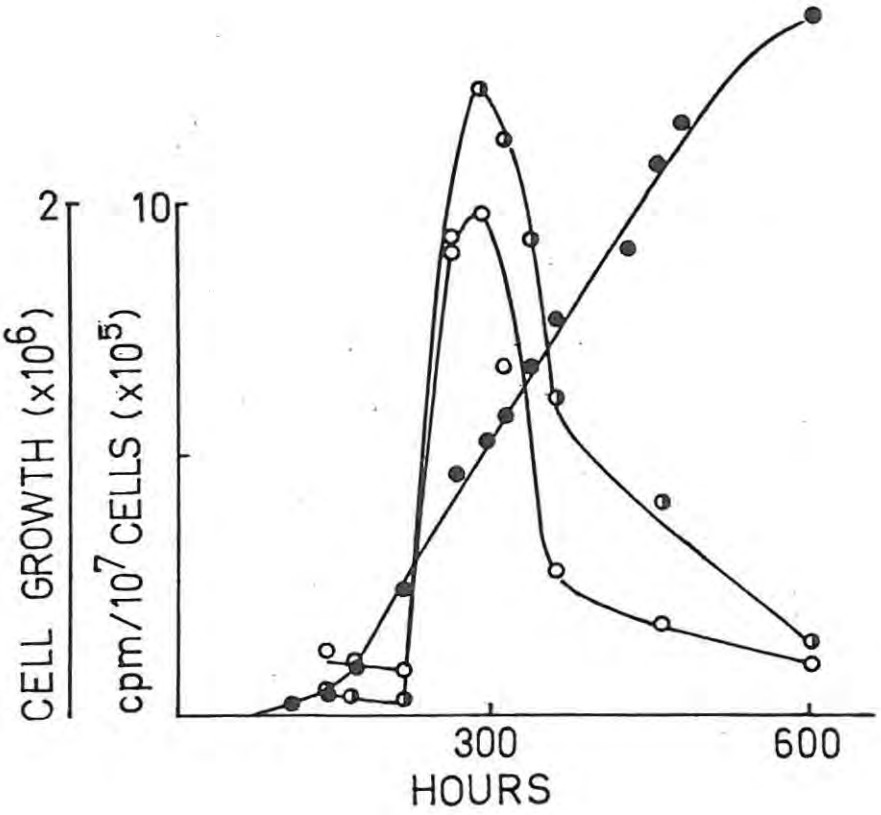


Figure 2. Effect of dark holding in mineral medium on heterotrophic carbon dioxide fixation during an autotrophic growth cycle of *Euglena*. Half-filled circles represent heterotrophic carbon dioxide fixation after 1 hour dark holding. Other symbols as in Figure 1.

In the experiment just described care was taken to exclude all light during the incubation of the cells with $Na_2^{14}CO_3$ lest dark carbon dioxide fixation should be masked by photosynthetic uptake. However, as already stated, preillumination photosynthesis was observed in *Chlorella* (Calvin et al., 1951). Although Lynch and Calvin (1953) found no evidence of such preillumination photosynthesis in *Euglena*, an experiment was performed to test whether the increase in dark carbon dioxide fixation measured during the logarithmic growth phase was the result of a photosynthetic component. Heterotrophic carbon dioxide fixation was again measured at intervals during the growth cycle of an autotrophic culture. Each aliquot of cells, after resuspension in fresh medium, was subdivided into two. After pipetting the samples into foil-wrapped brown flasks and sealing them one sample was placed in the dark and $Na_2^{14}CO_3$ was immediately injected. The other sample was held in the dark for 60 minutes before addition of the labelled carbonate. In both cases the reaction was terminated as usual, 20 minutes after addition of $Na_2^{14}CO_3$. If preillumination photosynthesis was taking place it would be expected that the sample which had been held in the dark for 60 minutes would fix less carbon dioxide since the reducing power would have been diminished by the time labelled carbonate was added. The results of the experiment are shown in Figure 2. Although dark holding the cells prior to measurement of carbon dioxide fixation reduced the fixation measured during lag phase, throughout the remainder of the cycle fixation was enhanced by dark holding in mineral medium. This switch-over phenomenon was observed consistently in several experiments. It can be inferred from the result shown in Figure 2 that the increase in heterotrophic carbon dioxide fixation measured during logarithmic growth phase is probably not due to preillumination photosynthesis. This was confirmed in later experiments using dark grown and permanently bleached cells. However, the results did not preclude the possibility that a component of the carbon

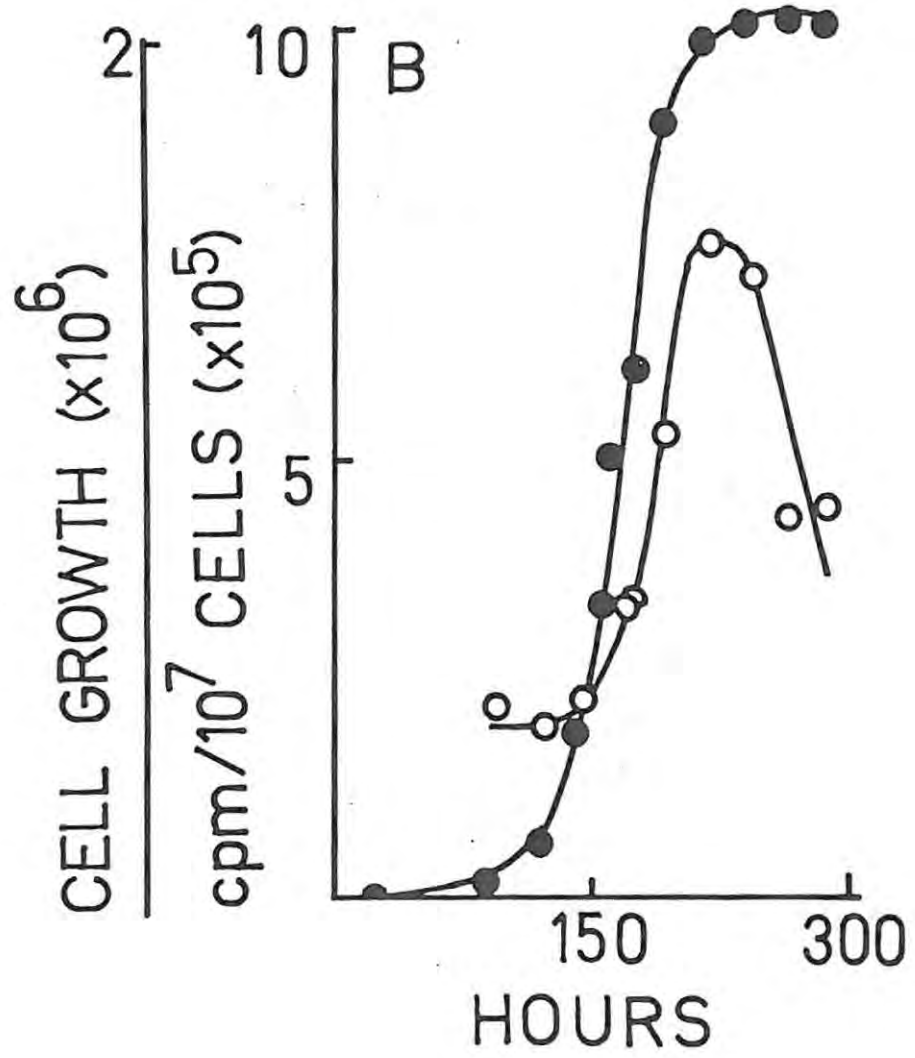


Figure 3. Variations in heterotrophic carbon dioxide fixation during the growth cycle of *Euglena gracilis* Z grown heterotrophically with 1% glucose as sole carbon source. Symbols as in Figure 1.

dioxide fixation measured during lag phase was a preillumination photosynthesis. Thus, in subsequent experiments, unless otherwise stated, heterotrophic carbon dioxide fixation by autotrophic cells was always measured after 60 minutes dark holding. The significance of the enhancement of fixation following dark holding in mineral medium which was measured in the later part of the growth cycle will be discussed below.

(B) Heterotrophic carbon dioxide fixation by *Euglena* grown heterotrophically on a 6-carbon source

Figure 3 shows dark carbon dioxide fixation measured throughout the growth cycle of a heterotrophic culture of *Euglena* which was grown in the dark and supplied with 1% glucose as sole carbon source. As in the case of autotrophically grown cells, dark carbon dioxide fixation increased transiently during the growth cycle. However, slight differences were observed between heterotrophic carbon dioxide fixation patterns in the two cultures, shown in Figures 1 and 3. The variation in fixation was of greater magnitude in autotrophically grown cells, which exhibited a tenfold increase in fixation during the growth cycle, whereas heterotrophically grown cells increased their dark carbon dioxide fixation only fourfold. Secondly, heterotrophic carbon dioxide fixation in autotrophically grown *Euglena* reached a peak when the cells were still in the phase of logarithmic growth, and had already declined by the time they entered stationary phase. In the case of heterotrophically grown cells dark fixation only reached its highest level at the end of the phase of logarithmic growth, and began to decline when the culture was already in stationary phase. The reasons for these kinetic differences are not clear, but it is possible that they are related to the fact that heterotrophically grown *Euglena* had a shorter period of logarithmic growth than autotrophically grown cells (Figures 1 and 3). Observations made during some subsequent experiments may support

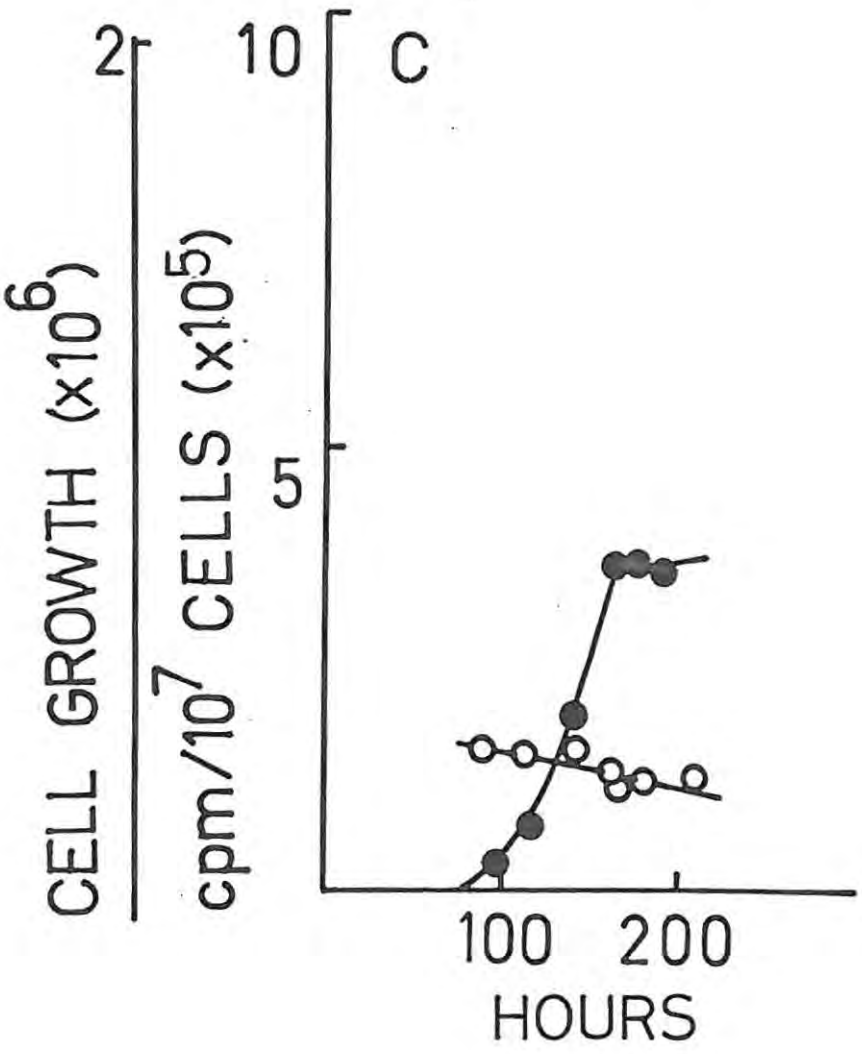


Figure 4. Variations in heterotrophic carbon dioxide fixation during the growth cycle of *Euglena gracilis* Z cultured heterotrophically, with 0.5% sodium acetate as sole carbon source. Symbols as in Figure 1.

this possibility. As described in Materials and Methods, in the later part of this study the autotrophically grown *Euglena* were cultured in a constant environment room with overhead fluorescent and incandescent illumination. This type of environment resulted in a shorter growth cycle compared with the earlier method whereby the cultures were illuminated from the side by a bank of fluorescent lights, and it was observed that under the later conditions heterotrophic carbon dioxide fixation only reached its highest level towards the end of the logarithmic growth phase (Figure 16).

Notwithstanding these kinetic differences, the basic similarity with regard to variation of heterotrophic carbon dioxide fixation between *Euglena* grown autotrophically and *Euglena* grown heterotrophically on glucose, confirm that as already indicated by Figure 2, the changes are not related to photosynthetic processes, since the cells grown in the dark on glucose lacked functional chloroplasts. This conclusion is further supported by experiments using a permanently bleached strain, described below (Table 3).

(C) Heterotrophic carbon dioxide fixation by *Euglena* cultured heterotrophically on a 2-carbon source

Euglena was grown in the dark with 0.5% Na acetate as sole carbon source, and dark carbon dioxide fixation was measured throughout the growth cycle as described above. Figure 4 shows that this culture exhibited no increase in fixation during the course of the growth cycle. There was an apparent slight decline in dark carbon dioxide fixation during the cycle. A similar experiment was performed using *Euglena* which had been cultured heterotrophically on 1% ethanol as sole carbon source. Heterotrophic carbon dioxide fixation by *Euglena* grown on ethanol was more variable than for other culture modes for reasons which are not understood. Thus the results of three replicate experiments are shown in Figure 5. As in the case of the cells cultured

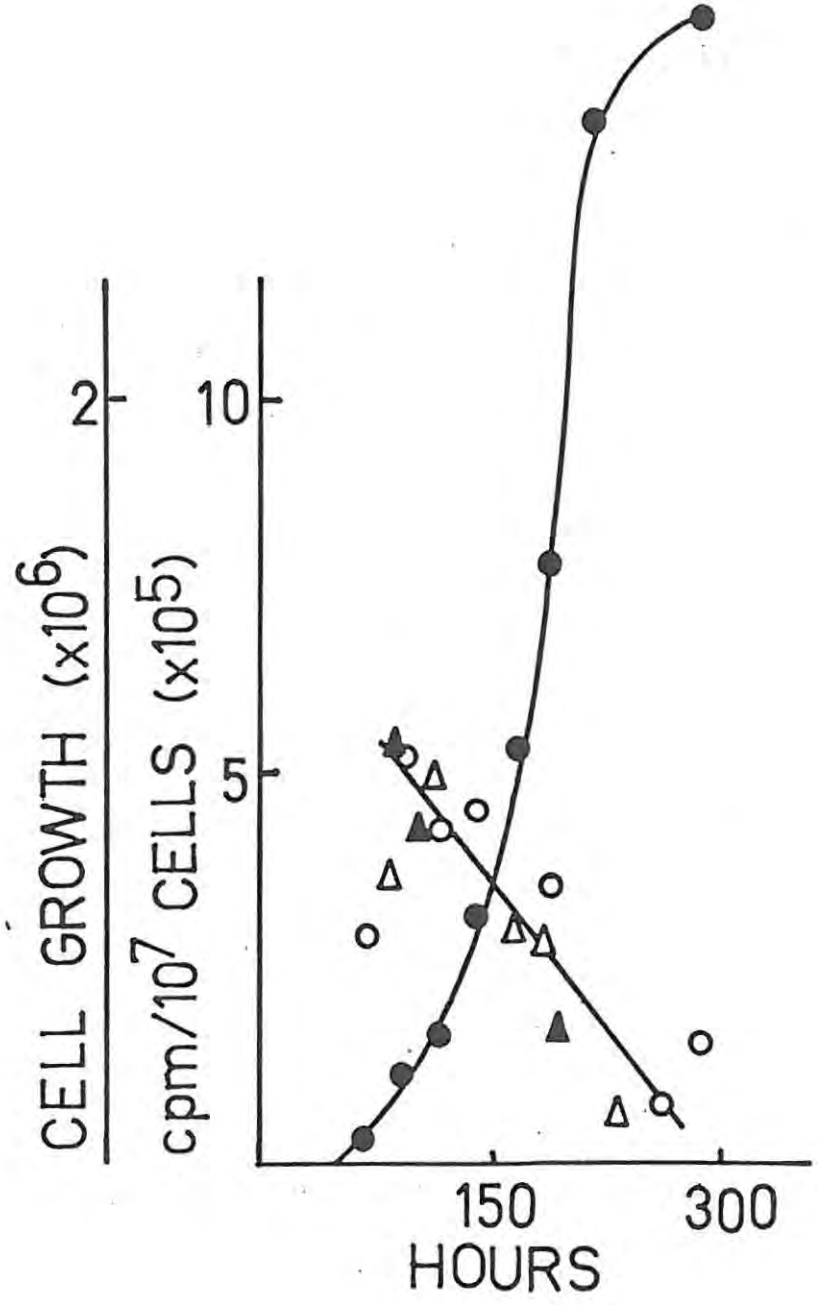


Figure 5. Variations in heterotrophic carbon dioxide fixation during the growth cycle of *Euglena gracilis* Z cultured heterotrophically with 1% ethanol as sole carbon source. Closed circles represent the growth of the culture. Open circles and open and closed triangles represent heterotrophic carbon dioxide fixation measured in three separate experiments.

on acetate, there was no increase in carbon dioxide fixation during the growth cycle, but a decline.

Repression of heterotrophic carbon dioxide fixation by acetate The results described above indicated that *Euglena* growing on acetate or ethanol may have a reduced dependence on heterotrophic carbon dioxide fixation during growth compared with autotrophic or glucose heterotrophic cells. Heinrich and Cook (1967) observed that *Euglena* cultured on acetate fixed less carbon dioxide than did cells cultured on glucose. The effect of acetate upon heterotrophic carbon dioxide fixation by a glucose grown culture was measured and the results are shown in Figure 6. Sodium acetate (0.5% final concentration) was added to a glucose culture at the stage of growth (shown by the arrow) when the dark carbon dioxide fixation was increasing. Following the addition of acetate heterotrophic carbon dioxide fixation decreased, to a level that was less than that of the lag phase cells and also less than that observed for cells growing on acetate alone, shown in Figure 4. The cells continued to grow after addition of acetate and the peak population was greater than that of the control culture which continued to grow on glucose alone, indicating the utilization of acetate as substrate. The control glucose cells continued the normally observed increase and decrease of dark carbon dioxide fixation. A similar experiment was performed using autotrophically grown *Euglena*. In this case, however, as shown in Figure 7, addition of acetate did not cause a rapid repression of heterotrophic carbon dioxide fixation, as had been the case with the glucose grown cells, but only a slight reduction compared with the control culture.

Regulation of heterotrophic carbon dioxide fixation in *Euglena* during batch growth cycles The experiments described above confirmed that

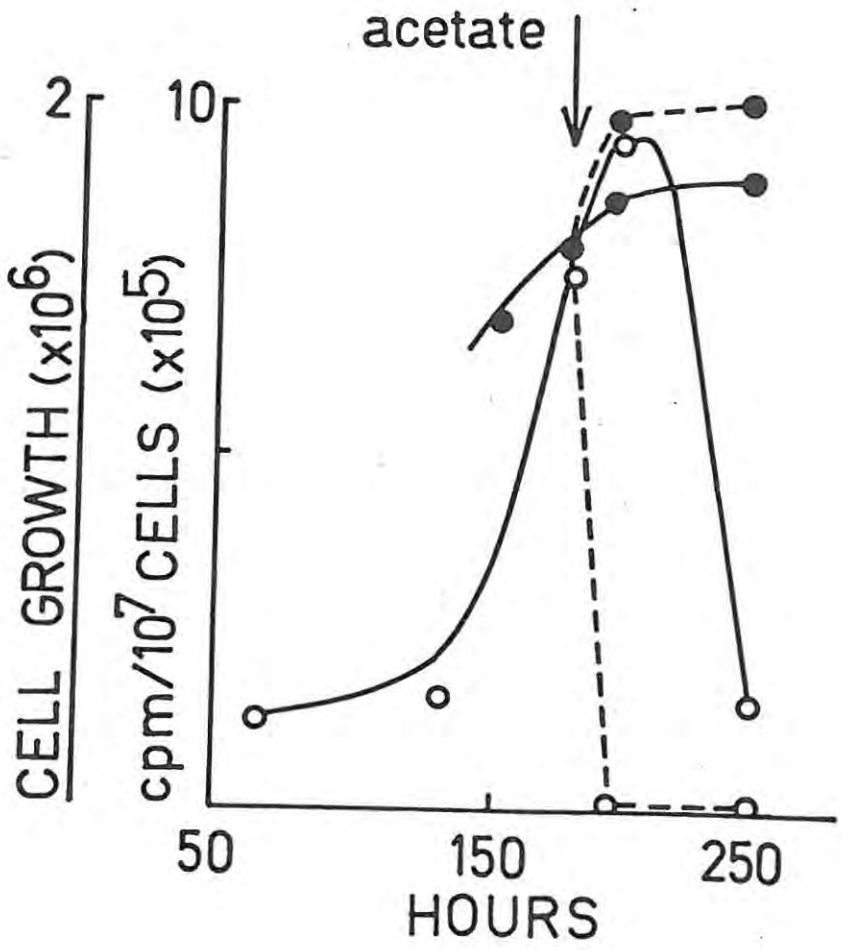


Figure 6. Effect of acetate (final concentration 0.5%) on heterotrophic carbon dioxide fixation by a glucose heterotrophic culture. The arrow indicates the time of addition of acetate. Closed circles represent cell growth; open circles represent carbon dioxide fixation. Unbroken lines represent the control culture; broken lines represent the culture to which acetate was added. Early growth points are omitted for clarity.

heterotrophic carbon dioxide fixation varies during batch growth cycles of *Euglena*, grown both autotrophically and heterotrophically on glucose. The cause of this variation was not yet known. Possibly the transient elevations of dark fixation observed during growth cycles are controlled by growth-induced changes in the extracellular medium. Cells from the later stages of the growth cycle may be stimulated to fix larger amounts of carbon dioxide than early phase cells by some factor or factors in their exogenous medium not present in medium at an early phase of the growth cycle. If this is the case it might be possible to stimulate early stage cells to fix larger amounts of carbon dioxide by exposing them to cell-free medium obtained from a culture in the later stage of growth.

Experiments were performed to test this possibility in both autotrophically and heterotrophically (glucose) grown *Euglena*. The experimental procedure for both types of culture was the same. *Euglena* were grown to the mid to late logarithmic growth stage, the stage of maximum dark carbon dioxide fixation. Cells were removed by centrifugation and the cell-free medium thus obtained was stored at -14°C. This medium is referred to as "late-growth phase medium". Another culture was grown to an early stage of the growth cycle when heterotrophic carbon dioxide fixation was low. Such an early phase culture is referred to as "early phase cells". The early phase cells were split into two aliquots. The control aliquot was grown as usual. Cells in the other, test aliquot were harvested by centrifugation and resuspended to the same cell density in the appropriate late-growth phase medium (i.e. early phase autotrophic cells were resuspended in autotrophic late-growth phase medium and early phase heterotrophic cells were resuspended in heterotrophic late-growth phase medium). Both test and control cultures were then exposed to identical culture conditions (light and 5% carbon dioxide in the case of the autotrophic cultures; complete darkness in the

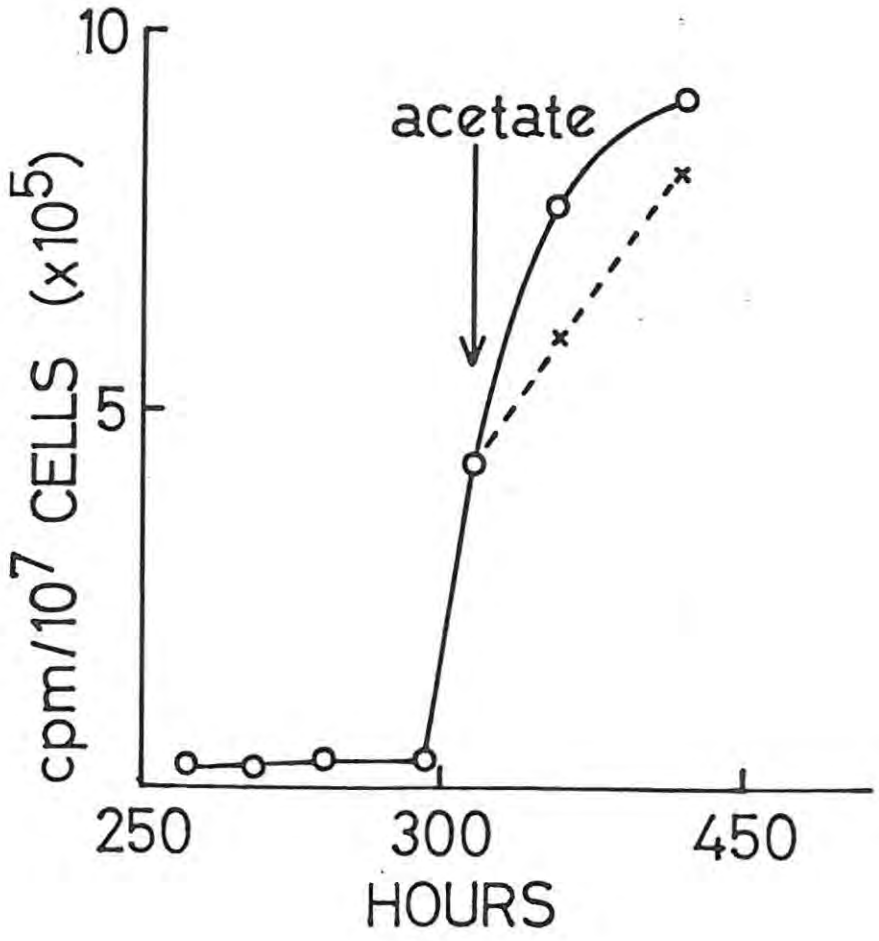


Figure 7. Effect of acetate (final concentration 0.5%) on heterotrophic carbon dioxide fixation by an autotrophic culture. The arrow indicates the time of addition of acetate. Open circles represent heterotrophic carbon dioxide fixation by control cells; crosses represent fixation by the culture after the addition of acetate. The growth curve is omitted for clarity.

case of the heterotrophic *Euglena*). At various intervals thereafter aliquots of both test and control cultures were harvested, cells collected by centrifugation and resuspended in fresh medium, and heterotrophic carbon dioxide fixation measured as usual.

Figure 8A and B show the results of these experiments with autotrophic and heterotrophic *Euglena* respectively. In both cases transfer of an early phase culture to late-growth phase medium for 24 hours resulted in an increase in heterotrophic carbon dioxide fixation compared with the control cells. In two subsequent measurements on autotrophic test cells after 59 hours and 72 hours in late-growth phase medium further increases in heterotrophic carbon dioxide fixation were observed. Control fixation remained constant during this entire period. Further measurements of dark carbon dioxide fixation by the test culture of heterotrophically grown *Euglena* after 49 hours and 82 hours exposure to late-growth phase medium showed a slight decline in fixation compared with the 24 hour measurement. However, the levels of fixation were still severalfold higher than was the control cell fixation at this stage.

For both autotrophic and heterotrophic cells the results shown in Figure 8 demonstrated that exposure to late-growth phase medium caused young cells to increase their dark carbon dioxide fixation. Thus it appeared that late-growth phase medium differs from fresh medium in some way; and that this difference affects heterotrophic carbon dioxide fixation. It was possible that, by exposing late-growth phase cells to fresh medium the reverse process could be demonstrated i.e. the fresh medium might cause late-growth phase cells to decrease fixation. The experiment to test this was performed using autotrophic cells only. The culture was split into two aliquots, this time at the stage during the logarithmic growth phase when heterotrophic

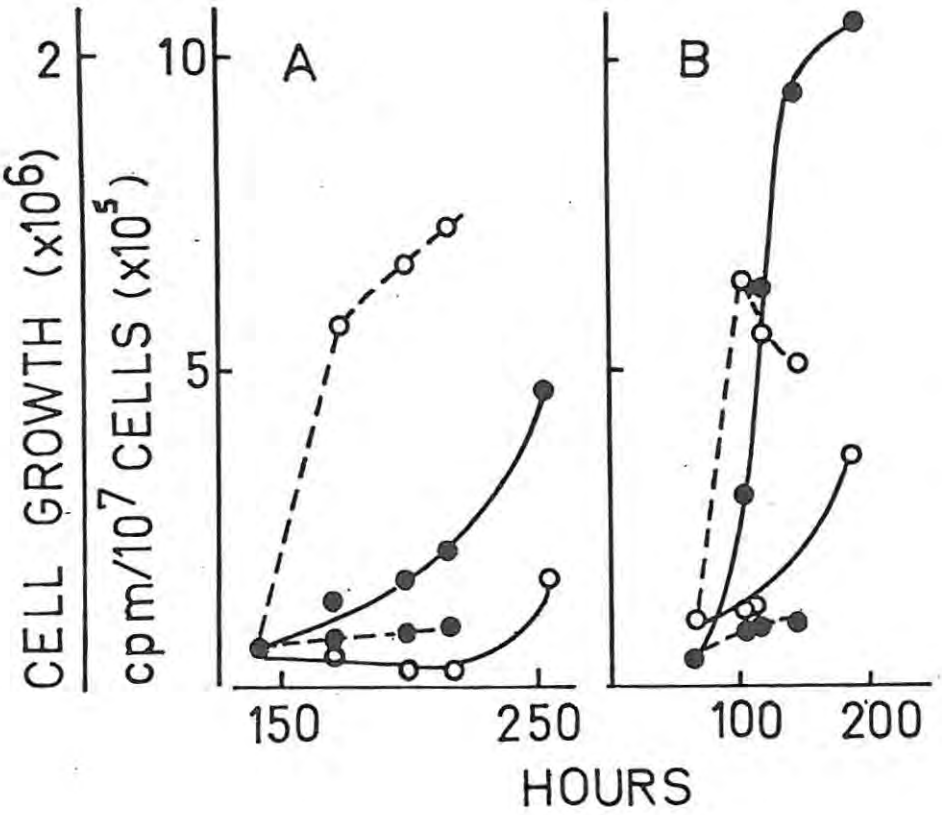


Figure 8. Effect of late-growth phase medium on heterotrophic carbon dioxide fixation by an early phase culture of *Euglena*. Figure 8A shows the results for autotrophically grown *Euglena;* Figure 8B shows the results for *Euglena* grown heterotrophically on glucose. Closed circles represent cell growth; open circles represent heterotrophic carbon dioxide fixation. Solid lines represent the control culture; broken lines represent the early phase cells which had been resuspended in late-growth phase medium.

carbon dioxide fixation had increased to half maximum as shown by the arrow in Figure 9. The control half of the culture continued normal growth. The test half was centrifuged and the cells resuspended to the same cell density in fresh medium. The culture was then allowed to continue growth under normal conditions of light and carbon dioxide. Heterotrophic carbon dioxide fixation of both the control and test cultures was measured at intervals in the usual way. Figure 9 shows the results of this experiment. After 24 hours in fresh medium heterotrophic carbon dioxide fixation by the test cells decreased to a lower level than that of lag phase cells. This decrease in fixation was clearly not the decrease normally observed towards the end of the logarithmic growth phase since 24 hours after the culture was divided, the control culture had further increased its fixation. Subsequent measurements on the control culture showed the normal decline in carbon dioxide fixation. The test culture continued to grow, reaching a higher peak population than the control culture, accompanied by a rise and subsequent decrease in heterotrophic carbon dioxide fixation as the fresh medium presumably changed in its turn as a result of the cell growth.

It appeared from these results that the ability of exogenous medium to stimulate heterotrophic carbon dioxide fixation by *Euglena* is a function of the 'age' of the medium, in other words, the cell density of the culture from which the medium was obtained. The precise stage of the growth cycle when the medium developed the ability to stimulate fixation, and the duration of the stimulating ability in relation to the age of the medium were then determined. At various stages during the growth cycle of an autotrophic culture two aliquots of the culture were harvested. One aliquot of cells was used for a normal control measurement of heterotrophic carbon dioxide fixation. (Fixation increased as usual during the logarithmic growth phase, reached a peak level at the end of logarithmic growth and declined during stationary phase, as shown in Figure 10.) The other

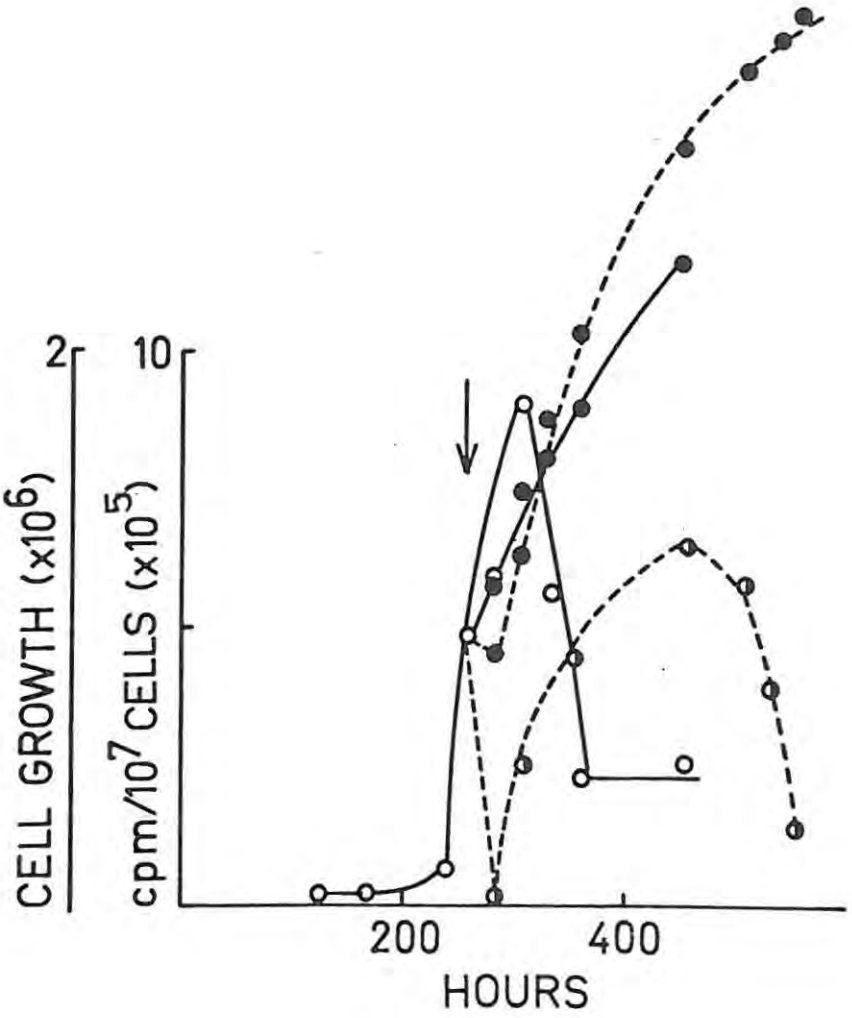


Figure 9. The effect of fresh medium on heterotrophic carbon dioxide fixation by late-growth phase cells from an autotrophically grown culture of *Euglena gracilis* Z. Open circles (solid line) show heterotrophic carbon dioxide fixation by the control culture throughout the growth cycle. Closed circles (solid line) show the growth of the control culture after the culture had been divided in half; early growth points are omitted for clarity. Half-filled circles and closed circles (joined by broken line) represent heterotrophic carbon dioxide fixation and growth respectively of the experimental culture.

aliquot was centrifuged and the cell-free medium (used medium) retained. Thus a series of used media taken from various stages throughout the entire growth cycle was obtained. Each of these used media was then tested for its ability to stimulate early phase cells from a separate autotrophic culture to fix carbon dioxide. Aliquots of cells from an early phase culture were resuspended in one of the used media for 24 hours and heterotrophic carbon dioxide fixation then measured in fresh medium in the normal way. The results of these measurements are shown in Figure 10 by crosses, each cross being represented on the graph at the corresponding stage of the control growth cycle from which the used medium was obtained. The figure demonstrates that the ability of the used media to stimulate dark carbon dixoide fixation by early phase cells parallelled the amount of fixation by the original culture from which the used medium was obtained. Medium from an early phase, low-fixing culture did not stimulate dark carbon dioxide fixation by early phase cells. The greatest stimulation was caused by used medium from a culture which was fixing carbon dioxide at the peak level. Subsequently there was a decline in fixation-stimulating ability concomitant with the decline in fixation of the control cells during stationary phase.

All the experiments described in this section clearly showed that the exogenous medium does have a regulatory effect on *Euglena* heterotrophic carbon dioxide fixation. It appeared that early phase cells fixed comparatively small amounts of carbon dioxide because they were in contact with fresh medium, and that with the onset of rapid cell growth the medium was changed in some manner which caused heterotrophic carbon dioxide fixation to increase. The next stage in this investigation was to determine what change, occurring in the medium during the course of the growth cycle, was controlling heterotrophic carbon dioxide fixation.

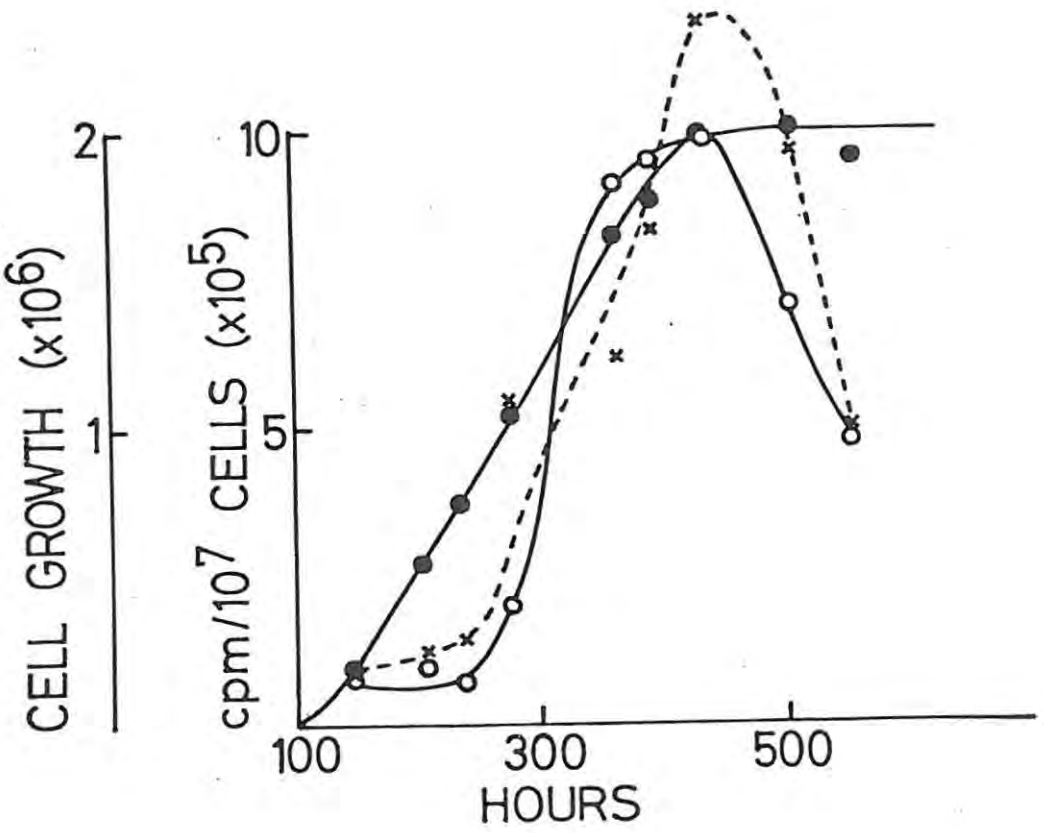


Figure 10. Ability of used medium from various stages of an autotrophic growth cycle of *Euglena gracilis* Z to stimulate heterotrophic carbon dioxide fixation by an early phase culture. Closed circles and open circles (solid line) represent growth and heterotrophic carbon dioxide fixation respectively of the control culture. The effects of the used media (separated from this culture) upon heterotrophic carbon dioxide fixation by early phase cells (crosses, broken line) are shown on the graph at the times at which the used media were sampled.

Investigations into the fixation stimulating ability of *Euglena* growth medium

A series of tests was conducted to characterize the fixation stimulating ability of late-growth phase medium. Samples of late-growth phase medium were obtained from an autotrophic culture as described above. One sample, the control, was not treated, one was boiled for 10 minutes and cooled, one was dialysed overnight against fresh medium and one was lyophylised and then reconstituted in distilled water. Each of these samples of late-growth phase medium was assayed for its ability to stimulate heterotrophic carbon dioxide fixation by early phase *Euglena*. Cells were harvested from an autotrophic culture at a cell density of 1.5×10^5 cells/ml, washed by centrifugation and resuspended to the same cell density in one of the test media. After 24 hours in the test medium (under normal conditions of light and carbon dioxide) heterotrophic carbon dioxide fixation was measured in fresh medium in the normal way. (The cell density after 24 hours in the test medium was never more than 2×10^5 cells/ml, well below the density at which heterotrophic carbon dioxide fixation normally starts to increase, as shown by Figure 1.) The results of the carbon dioxide fixation measurements are shown in Figure 11. For comparison, bar A shows the normal amount of fixation by early phase cultures, taken from previous experiments. The vertical line shows the range of variation of 8 measurements. Bar B shows the striking elevation of dark carbon dioxide fixation after 24 hours in late-growth phase medium. Boiling the late-growth phase medium had no significant effect on its ability to stimulate early phase cells to increase carbon dioxide fixation (bar C), whereas after dialysis against fresh medium the late-growth phase medium lost the stimulating ability (bar D). Lypophilization of the late-growth phase medium and reconstitution in distilled water did not significantly impair fixation-stimulating ability (bar E).

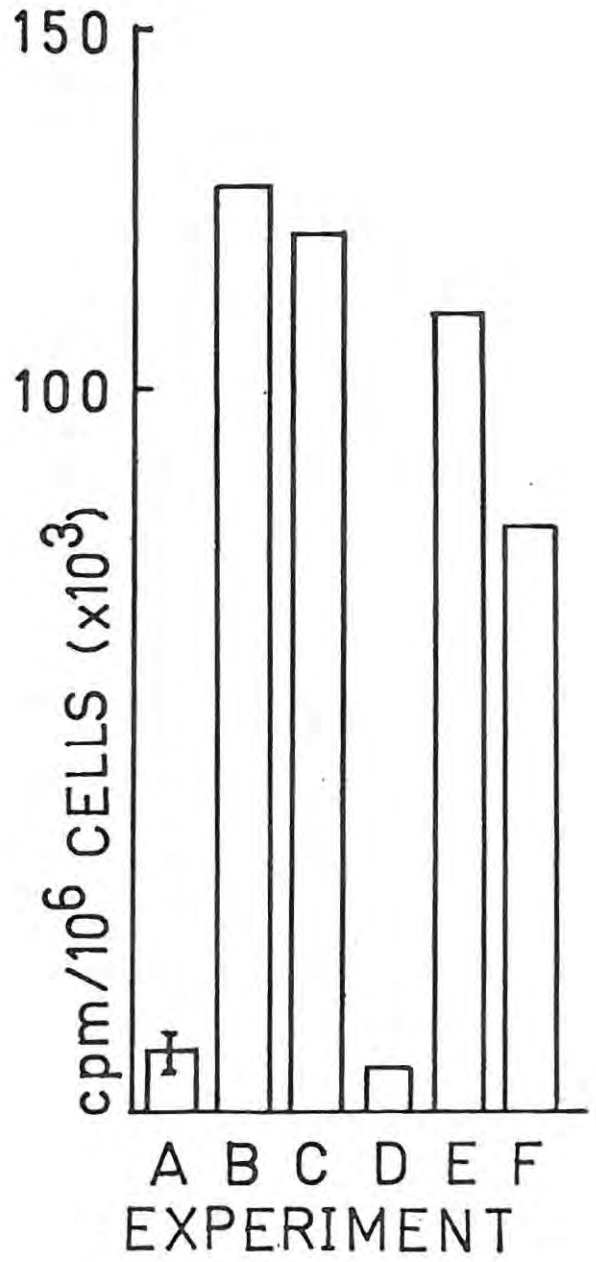


Figure 11. Heterotrophic carbon dioxide fixation by an early phase culture of autotrophically grown *Euglena*, after 24 hours treatment in various media. Fixation was measured in fresh medium.

A. Normal fixation by early phase cells. The vertical line shows the range of variation of 8 measurements.

B. Fixation after 24 hours in late-growth phase medium.

C. Fixation after 24 hours in late-growth phase medium, previously boiled for 10 minutes and cooled.

D. Fixation after 24 hours in late-growth phase medium, dialysed overnight against fresh medium.

E. Fixation after 24 hours in late-growth phase medium which had been lyophilised and reconstituted.

F. Fixation after 24 hours in fresh medium diluted 1:20 with distilled water.

These results indicated that the factor in *Euglena* growth medium which controls heterotrophic carbon dioxide fixation is a small, dialysable molecule (or molecules). The results are consistent with one of two possible alternatives. Either a small molecule (which must also be heat stable) could be added to the medium during growth, by cellular excretion, which molecule affects the amount of dark carbon dioxide fixation by the cells; or else a small molecule might be removed from the medium by the cells during growth, the lack of which affects heterotrophic carbon dioxide fixation. An indication as to which of these possible changes in the medium controls the level of fixation was obtained by suspending early phase cells for 24 hours in diluted fresh medium (1:20 dilution in distilled water) before measuring heterotrophic carbon dioxide fixation. Figure 11, bar F, shows that this treatment also resulted in a severalfold increase in fixation, albeit to a lower level than that caused by exposure to late-growth phase medium. This result indicated the latter alternative, thus it appeared that late-growth phase medium affects heterotrophic carbon dioxide fixation because it is deficient in a factor which has been removed by the cells during growth. Two types of experiment showed that this factor is ammonium ion.

Regulation of *Euglena* heterotrophic carbon dioxide fixation by ammonium - (1) ammonium starvation In order to simulate the effect of late-growth phase medium on heterotrophic carbon dioxide fixation by early phase cells, a series of test media were prepared each comprising the normal mineral medium used for culturing *Euglena*, but deficient in one or more of the components. The possibility that late-growth phase medium might affect fixation because its pH is different from fresh medium was also considered. Late-growth phase medium has a pH of 2.6, compared with 3.3 for fresh medium. The media were tested, in exactly the same manner as described in the preceding

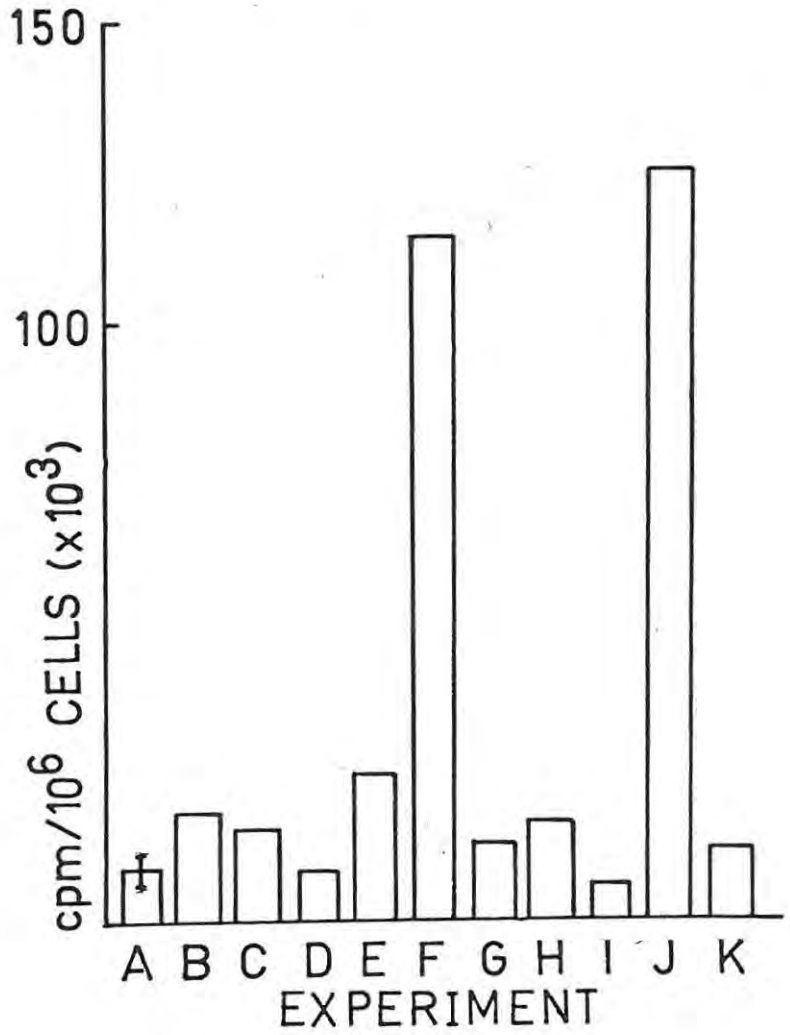


Figure 12. Heterotrophic carbon dioxide fixation by early phase cells from an autotrophically grown culture of *Euglena* after 24 hours in various media.

A. Normal fixation by early phase cells as shown in Figure 11. The remaining histograms represent fixation after 24 hours in:-

B. Complete mineral medium, pH adjusted to 2.6.

C. Mineral medium deficient in all trace metals.

D. Mineral medium deficient in vitamins B_1 and B_{12}.

E. Mineral medium deficient in Ca^{++}, Mg^{++} and EDTA.

F. Mineral medium deficient in NH_4Cl and KH_2PO_4.

G. Mineral medium deficient in orthophosphate, pH adjusted with KOH.

H. Mineral medium deficient in orthophosphate, pH adjusted with NaOH.

I. Mineral medium in which KH_2PO_4 was replaced by Na_2HPO_4.

J. Mineral medium deficient in NH_4Cl.

K. Late-growth phase medium supplemented with NH_4Cl.

section, for their ability to stimulate carbon dioxide fixation by early phase cells. The results of these tests are shown in Figure 12. Bar A shows the amount of carbon dioxide normally fixed by early phase cultures. Exposure of early phase cells to fresh medium, pH 2.6 (bar B) had a small effect on subsequent carbon dioxide fixation by the cells. This effect was not large enough to account for the stimulation of fixation by late-growth phase medium. Similarly, exposure of early phase cells to test media lacking all trace metals (bar C), vitamins (bar D) or the divalent cations Ca^{++} and Mg^{++} together with EDTA (bar E) did not have significant effects upon subsequent heterotrophic carbon dioxide fixation. Exposure of early phase cells for 24 hours to the test medium lacking both NH_4Cl and KH_2PO_4 (bar F) caused a striking increase in dark carbon dioxide fixation, similar to that observed after 24 hours in late-growth phase medium. Thus it appeared that either or both of these salts was important in control of carbon dioxide fixation in *Euglena*. Further experiments tested the effect of exposure to medium lacking phosphate ion (bar G), medium lacking KH_2PO_4 but containing Na^+ (bar H) and medium lacking K^+ only (bar I) on subsequent dark carbon dioxide fixation. None of these treatments affected the amount of fixation by the early phase cells when it was measured in the normal way. Exposure to a test medium deficient in NH_4Cl only, (bar J) caused more than 10 fold increase in subsequent dark fixation. These results indicated that late-growth phase medium affects heterotrophic carbon dioxide fixation by early phase cells because it is deficient in ammonium (medium J did in fact contain Cl^- from other sources, so this ion was presumed to be unimportant in regulation of dark carbon dioxide fixation. Subsequent tests confirmed this). In a final confirmatory experiment early phase cells were resuspended for 24 hours in late-growth phase medium which had been supplemented with 5.62 mM NH_4Cl (the concentration in fresh mineral medium) and heterotrophic carbon dioxide fixation was then measured in the normal way. As shown by bar K,

addition of NH_4Cl to late-growth phase medium completely destroyed its ability to stimulate carbon dioxide fixation by early phase cultures, when assayed in the normal way.

The series of tests described above were all conducted using autotrophically grown *Euglena*. The effect of suspending early phase heterotrophic cells in mineral medium (containing 1% glucose) deficient in NH_4Cl for 24 hours before measurement of heterotrophic carbon dioxide fixation was also measured. As was the case in autotrophic cells, fixation after this treatment was increased compared with that of a control early phase culture. The extent of the stimulation was less than in the case of autotrophic *Euglena*, a 5.4 fold increase compared with more than 10 fold increase in the case of autotrophic cells, shown in Figure 12J. This is consistent with the earlier observation that, during the growth cycle of *Euglena* grown heterotrophically on glucose, dark fixation increased only 4 fold, compared with a 10 fold increase during the autotrophic growth cycle (Figures 1 and 3).

Variation in ammonium concentration during batch growth cycles in *Euglena*

Thus far it had been established firstly that heterotrophic carbon dioxide fixation changes during growth cycles of *Euglena* grown autotrophically and heterotrophically on glucose, and it was reasoned that these changes might be caused by growth-induced changes in the exogenous medium. Secondly it had been shown that the condition of the growth medium did indeed affect the amount of carbon dioxide fixed by *Euglena* in that cell free medium from cells which were fixing large amounts of carbon dioxide (late-growth phase medium) was able to stimulate early phase cells, which normally fixed smaller amounts of carbon dioxide, to increase their dark fixation, and *vice versa*. Finally, it was shown that mineral medium deficient in ammonium was also able to stimulate early phase cells, so that they subsequently fixed increased amounts of carbon dioxide.

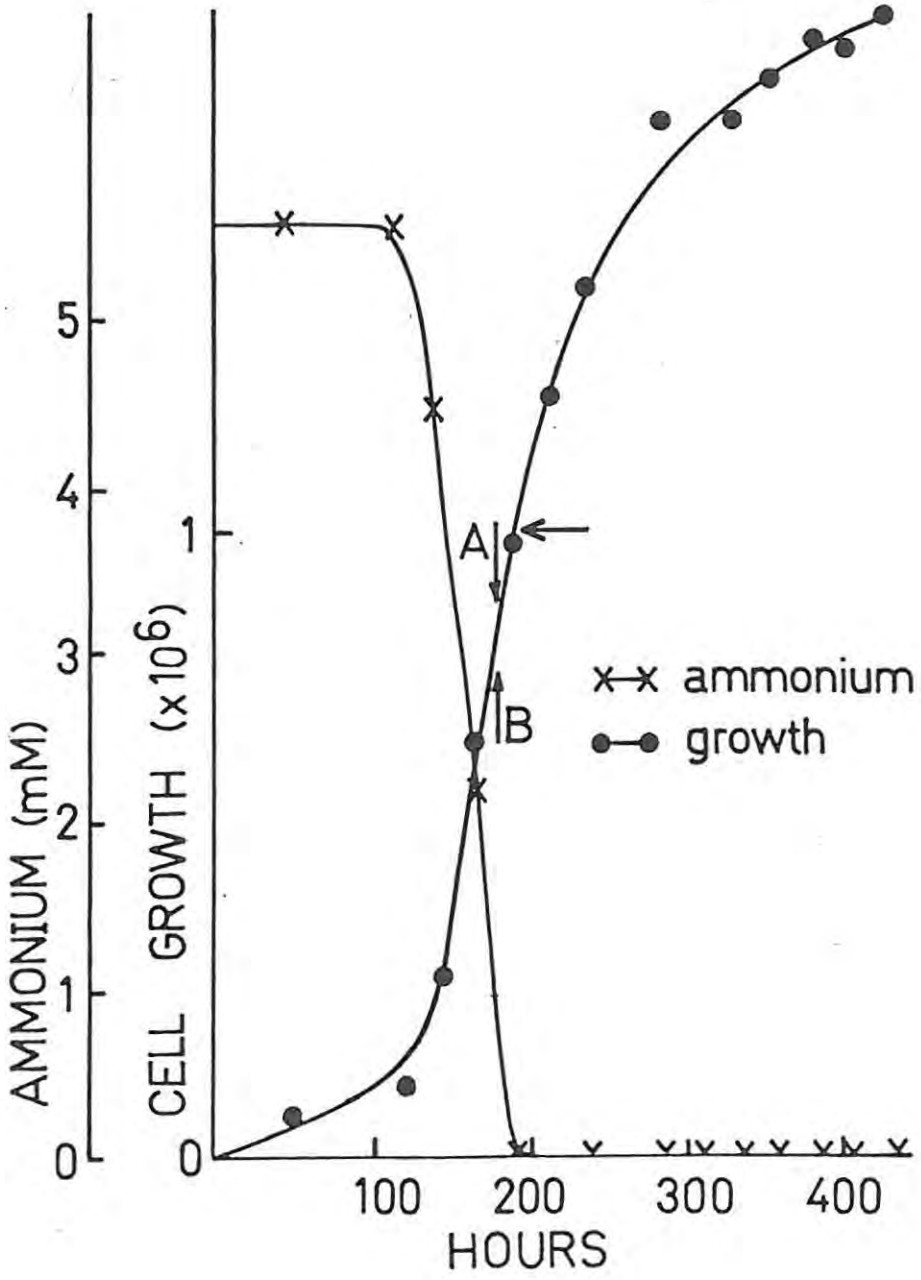


Figure 13. Concentration of exogenous ammonium throughout the growth cycle of autotrophically grown *Euglena*. Closed circles represent cell growth; crosses represent concentration of ammonium expressed as mmoles/l ammonium chloride. The horizontal arrow indicates the stage of the growth cycle when ammonium was depleted. The arrows A and B are explained under Figure 19 in the text.

These findings indicated that the reason for the increase in heterotrophic carbon dioxide fixation exhibited by *Euglena* during the logarithmic growth phase is because the exogenous medium becomes deficient in ammonium, due to the uptake of the ion by the cells. It remained to be established whether ammonium is in fact removed from the medium during the growth cycle in such a way as to correlate with the observed increases in heterotrophic carbon dioxide fixation. Thus the concentration of exogenous ammonium in the medium was measured throughout the growth cycle of *Euglena* growing both autotrophically and heterotrophically on glucose. Cultures were established under the normal growth conditions. At intervals during the growth cycle aliquots of the culture were harvested, the cells removed by centrifugation,and the residual exogenous ammonium concentration measured in the cell-free supernatants. Figure 13 shows the results of these measurements for an autotrophic culture. The concentration of ammonium remained constant during the lag period. Onset of the logarithmic growth phase was accompanied by a drop in ammonium concentration such that, by the mid logarithmic growth phase, no measureable ammonium remained in the medium. Comparing this result with Figure 1, it is clear that the disappearance of ammonium from the medium correlated closely with the time of the increase in heterotrophic carbon dioxide fixation during the growth cycle. The stage of the growth cycle when ammonium was completely depleted (according to the assay method used) is shown in Figure 13 by the horizontal arrow. (The significance of arrows A and B will be described in connection with Figure 19).

Figure 14 shows the results of the same measurements with a heterotrophic culture grown on glucose. As in the case of autotrophic cells, exogenous ammonium concentration remained constant during the lag period, followed by a decrease at the commencement of the logarithmic growth phase. Also, as with autotrophic cells, the time of maximum carbon dioxide fixation

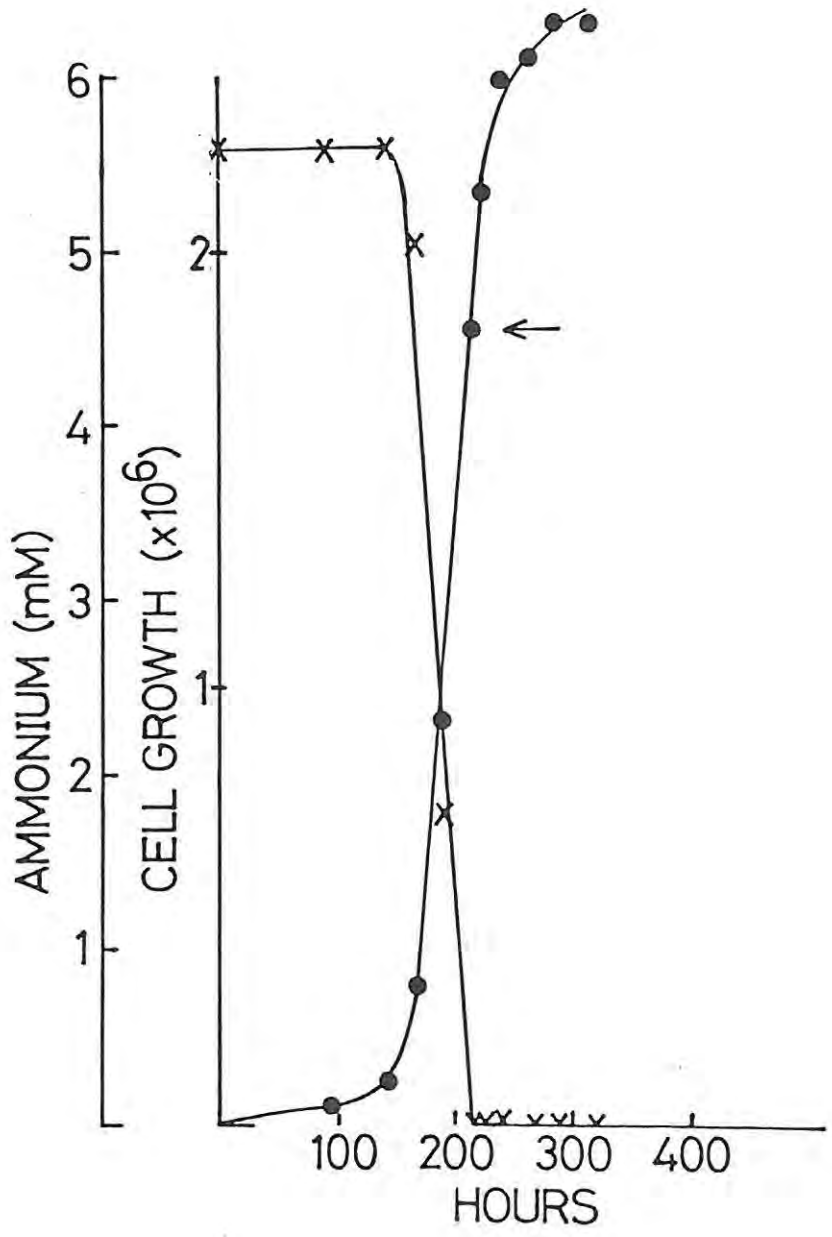


Figure 14. Concentration of exogenous ammonium throughout the growth cycle of heterotrophically grown *Euglena*. Symbols as in Figure 13.

(shown in Figure 3) correlated closely with the time of complete depletion of ammonium during the growth cycle. These events occurred later in the growth cycle in heterotrophic cells compared with autotrophic cells.

Regulation of *Euglena* heterotrophic carbon dioxide fixation by ammonium - (2) ammonium replenishment The fact that the important change in the medium during growth affecting heterotrophic carbon dioxide fixation was shown to be the removal of a substance rather than the addition of some stimulatory molecule, raised a further question. In all the experiments described so far, heterotrophic carbon dioxide fixation was measured by harvesting the cells and resuspending them in complete fresh medium before addition of labelled carbonate, as described above. This was also the method used by the majority of workers who previously investigated heterotrophic fixation in *Euglena* and also in *Chlorella* (Levedahl, 1966, 1967; Codd and Merrett, 1971). Thus in the experiments reported above the cells had been subjected to two distinct treatments before measurement of heterotrophic carbon dioxide fixation.

Firstly they had been starved of exogenous ammonium, either normally during the course of the growth cycle as shown in Figures 13 and 14, or experimentally by resuspension for 24 hours in late-growth phase medium or ammonium-free prepared medium. Secondly, before the injection of $Na_2^{14}CO_3$, the cells had been resuspended in fresh medium, and thus their supply of exogenous ammonium had been replenished. The possibility therefore existed that the cells had been stimulated to fix more carbon dioxide not only by the removal of ammonium from the environment but by its subsequent replenishment (or possibly that of another compound).

To investigate this possibility a series of tests was carried out to compare the carbon dioxide fixation of ammonium starved *Euglena* with and without

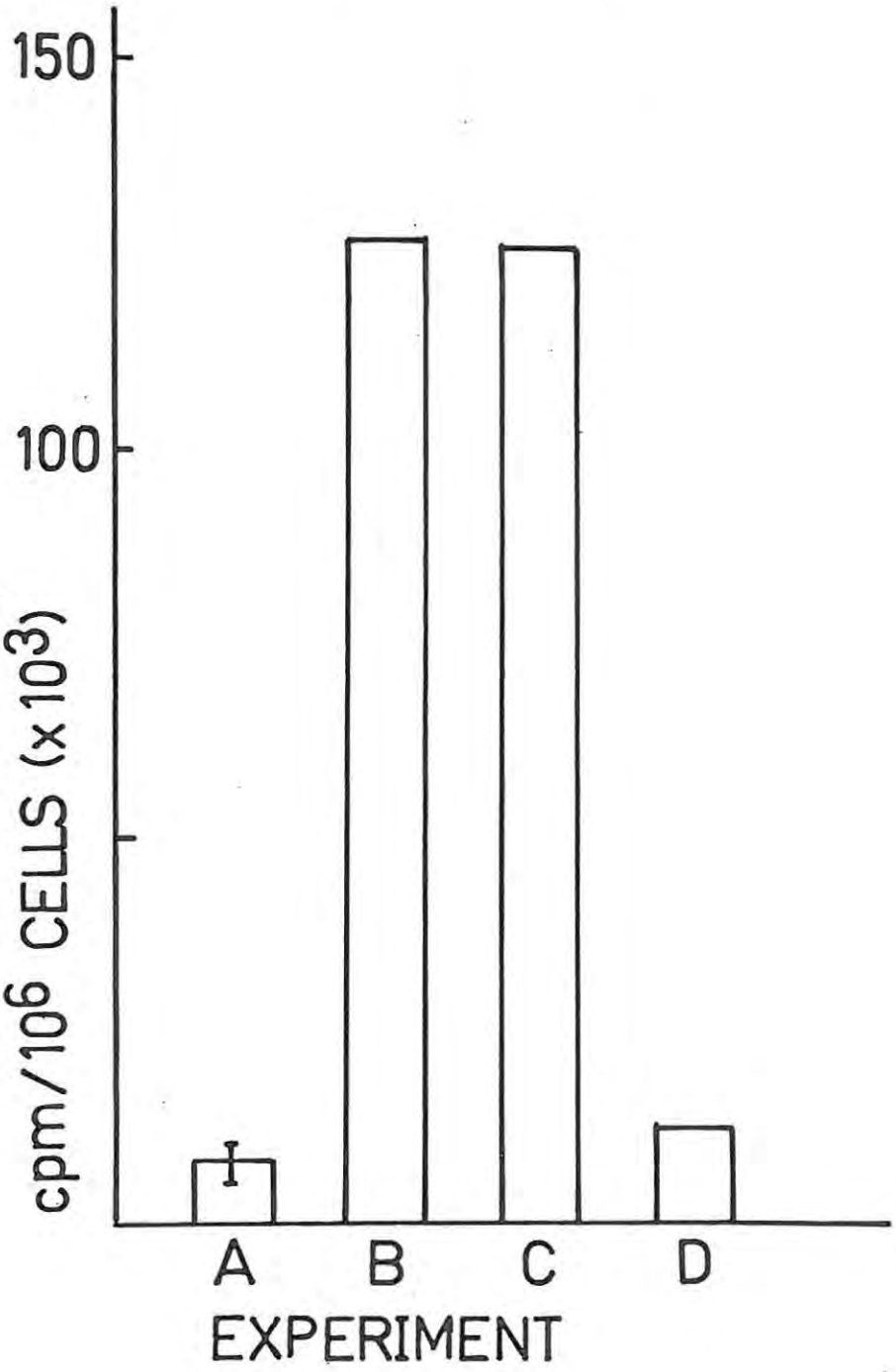


Figure 15. Heterotrophic carbon dioxide fixation by early phase autotrophic cells measured under various conditions.

A. Normal fixation by early phase cells as shown in Figure 11.

B. Fixation by early phase cells after 24 hours ammonium deprivation, fixation measured in fresh medium.

C. As for B, but fixation measured in phosphate buffer, pH 4.7, containing 10 mM NH_4Cl.

D. As for B, but fixation measured in phosphate buffer.

subsequent ammonium replenishment. An early phase autotrophic culture was deprived of ammonium by resuspending the cells in ammonium-free medium for 24 hours as described previously. After the 24 hour starvation period the culture was divided into three aliquots. The cells were transferred by centrifugation to either fresh medium, phosphate buffer (1 mM KH_2PO_4, pH 4.7) or the phosphate buffer containing 10 mM NH_4Cl, for measurement of dark fixation. The results of these experiments are shown in Figure 15. Bar A shows low levels of heterotrophic carbon dioxide fixation by early phase control cultures as described for Figures 11 and 12. Bar B shows fixation by an early phase culture which had been starved of ammonium for 24 hours and then resuspended as usual in fresh medium before injection of $Na_2^{14}CO_3$. The normal increase in heterotrophic carbon dioxide fixation by the ammonium starved culture occurred. Bar C shows that the same dramatic increase in fixation was observed if the ammonium deprived cells were resuspended in phosphate buffer containing 10 mM NH_4Cl before injection of labelled carbonate. In both these cases the cells were resupplied with ammonium following the starvation period. However, when heterotrophic carbon dioxide fixation by ammonium starved cells was measured in phosphate buffer alone (bar D) no significant increase over the control level was observed. This experiment clearly showed that the increases in heterotrophic carbon dioxide fixation were not caused by ammonium deprivation alone, but that subsequent replenishment of the cells with ammonium after the deprivation caused the large increases in dark fixation.

The tests described above showed that early phase *Euglena* which were starved of ammonium experimentally only showed increased heterotrophic carbon dioxide fixation if they were resupplied with ammonium. Similarly, *Euglena* which are deprived of ammonium during logarithmic growth should also increase dark fixation only if they are first resupplied with more ammonium. This was

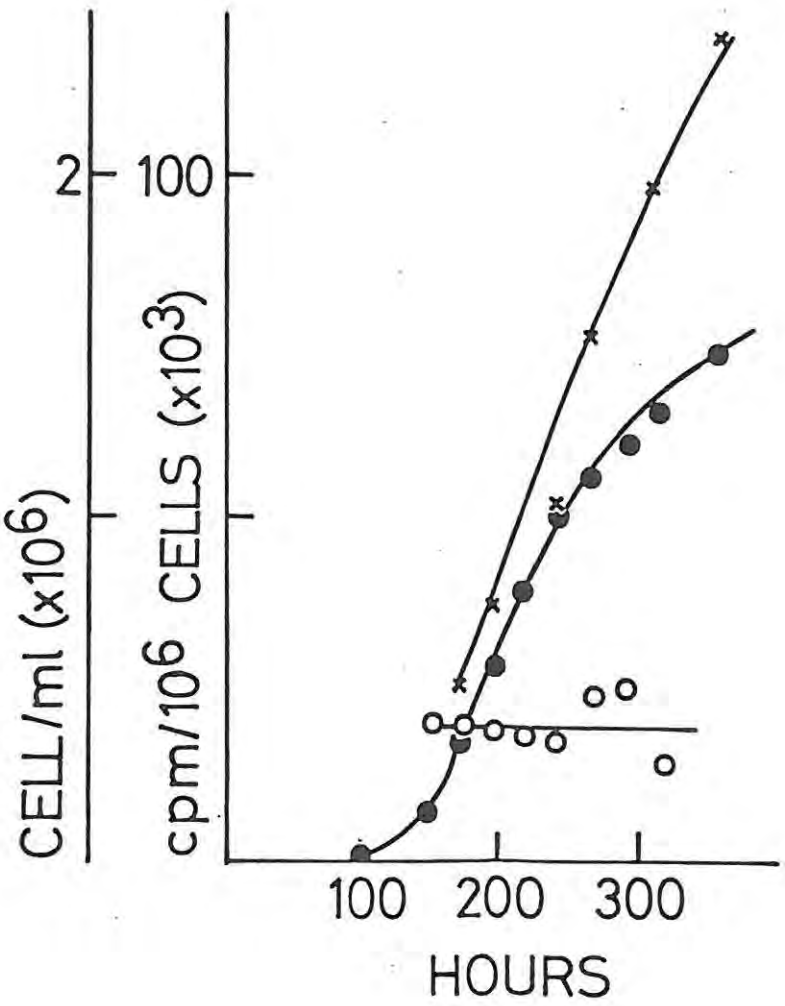


Figure 16. Heterotrophic carbon dioxide fixation during the growth cycle of *Euglena gracilis* Z grown autotrophically. Open circles represent fixation measured in phosphate buffer, pH 4.7; crosses represent fixation measured in phosphate buffer containing 10 mM NH_4Cl. Closed circles represent cell growth.

tested for in both autotrophic and heterotrophic (glucose) cultures of *Euglena*. The two cultures were established in the usual way. At intervals throughout the growth cycles aliquots of cells were harvested from the cultures. The aliquots were divided into two, washed and resuspended in either phosphate buffer alone or in phosphate buffer containing 10 mM NH_4Cl, for measurement of heterotrophic carbon dioxide fixation. The results with an autotrophic culture are shown by Figure 16. There was an increase in fixation during the logarithmic growth phase only when the measurements were made in the presence of NH_4Cl. For cells resuspended in phosphate buffer no increase was observed. In this case dark carbon dioxide fixation remained constant throughout the logarithmic growth period. Figure 17 shows the results of a similar experiment with a culture of *Euglena* grown heterotrophically on glucose. As in the case of the autotrophic culture, no significant change in fixation was observed during the growth cycle if the measurement was carried out in phosphate buffer. When the cells were resuspended in phosphate buffer containing 10 mM NH_4Cl before addition of labelled carbonate, the normally observed transient increase in fixation was obtained.

Since the ammonium deprived cells only increased dark carbon dioxide fixation if they were resupplied with ammonium, and since non-starved cells supplied with ammonium did not increase fixation, it was possible that ammonium starved cells might take up supplied ammonium more readily than non-starved cells. In order to investigate this possibility an early phase autotrophic culture was divided into two. One half (the control) was washed and resuspended in phosphate buffer containing 10 mM NH_4Cl. Labelled carbonate was immediately injected and heterotrophic carbon dioxide fixation measured as usual. Immediately before termination of the reaction with trichloracetic acid an aliquot of the suspension was sampled and the cells removed from the aliquot by centrifugation. The

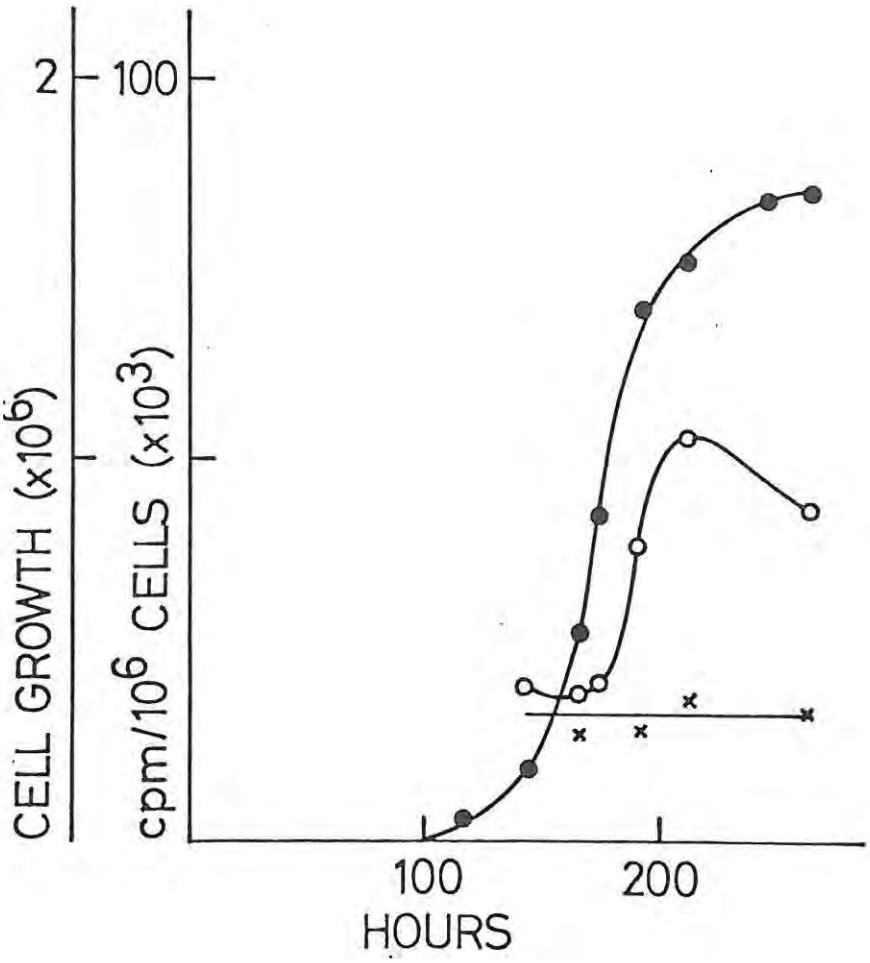


Figure 17. Heterotrophic carbon dioxide fixation during the growth cycle of *Euglena gracilis* Z grown heterotrophically (glucose). Crosses represent fixation measured in phosphate buffer, pH 4.7; open circles represent fixation measured in phosphate buffer containing 10 mM NH_4Cl. Closed circles represent cell growth.

Table 2. Comparison of heterotrophic carbon dioxide fixation and ammonium uptake between ammonium starved and non-starved *Euglena*.

	CO_2 Fixation in 20 Minutes (cpm/10^6 cells)	NH_4^+ Uptake in 20 Minutes (μmoles/10^6 cells)
Control cells	16,037	0
Ammonium starved cells	80,894	1.25

Cells were supplied with 10 mM NH_4Cl before measurement of heterotrophic carbon dioxide fixation.

ammonium content of the supernatant was assayed in order to determine uptake of ammonium from the medium during the fixation period. The second half of the culture was starved of ammonium for 24 hours and then treated in the same manner as the control. The results are presented in Table 2. The ammonium starved cells showed the usual increase in heterotrophic carbon dioxide fixation when supplied with more ammonium, compared with the control cells. Further, the ammonium starved cells removed ammonium from the medium during the 20 minute fixation period (1.25 umoles of ammonium was taken up by 10^6 cells), whereas there was no measureable uptake of ammonium during this period by the non-starved cells. Therefore, ammonium stimulated heterotrophic carbon dioxide fixation is accompanied by uptake of ammonium by the cells.

Measurements of the stimulatory effect of nitrate on heterotrophic carbon dioxide fixation after ammonium starvation for 24 hours were carried out, using autotrophically growing early phase cells. Nitrate had a smaller stimulatory effect (approximately 2.5 fold increase) compared with the effect of ammonium (10 fold increase).

Heterotrophic carbon dioxide fixation by a permanently bleached *Euglena*

The effect of ammonium deprivation and restoration on heterotrophic carbon dioxide fixation by a permanently bleached strain of *Euglena, E.gracilis* Z SB3, was measured and the results compared with those obtained from the normal heterotrophically (glucose) grown *Euglena gracilis* Z under the same conditions. Dark carbon dioxide fixation by early phase cells was measured after various ammonium treatments. Firstly, in order to demonstrate the effect of ammonium starvation alone upon subsequent carbon dioxide fixation, an aliquot of early phase cells (1.5×10^5 cells/ml) was harvested and divided into two in the usual way. Dark carbon dioxide fixation by

Table 3. Comparison of heterotrophic carbon dioxide fixation by early phase *Euglena gracilis* Z and *Euglena gracilis* Z SB3 (streptomycin bleached), measured under various conditions.

	Euglena gracilis Z	*Euglena gracilis* Z SB3
A	13,400	6,876
B	11,169	3,946
C	26,470	24,263
D	54,410	52,924

A. Without NH_4^+ starvation, fixation measured in 1 mM KH_2PO_4.

B. After NH_4^+ starvation, fixation measured in 1 mM KH_2PO_4.

C. Without NH_4^+ starvation, fixation measured in 10 mM NH_4Cl in KH_2PO_4.

D. After NH_4^+ starvation, fixation measured in 10 mM NH_4Cl in KH_2PO_4.

the control half was measured immediately in phosphate buffer. The other half was starved of ammonium for 24 hours and fixation then measured in phosphate buffer i.e. the cells were starved of ammonium but not replenished. The results, shown in Table 3A and B were essentially the same for both permanently bleached and temporarily etiolated strains. In neither case did ammonium starvation lead to increased heterotrophic carbon dioxide fixation in the absence of ammonium replenishment. Secondly, in order to demonstrate the effect of ammonium starvation followed by replenishment, an aliquot of early phase cells was divided as before. Dark fixation by the control half was measured immediately in phosphate buffer containing 10 mM NH_4Cl. The other half was starved of ammonium for 24 hours and fixation then measured in phosphate buffer containing 10 mM NH_4Cl. Comparison of these results (Table 3C and D) shows that for both strains ammonium starvation followed by replenishment resulted in a more than 2 fold increase in heterotrophic carbon dioxide fixation compared with the non-starved cells. The extent of stimulation was smaller for the heterotrophic and bleached cells than for autotrophic cells shown by Figure 15. This phenomenon was noted previously. Table 3 also shows that 10 mM NH_4Cl stimulated heterotrophic carbon dioxide fixation even without prior ammonium deprivation (comparing A and C). The observation is discussed below (Figure 21).

Kinetic characterization of the effects of exogenous ammonium on heterotrophic carbon dioxide fication in *Euglena* A series of kinetic studies was performed in order to characterize quantitatively the effect of exogenous ammonium upon *Euglena* heterotrophic carbon dioxide fixation. For the purposes of these studies the 'ammonium effect' was considered as two distinct processes. Thus depriving the cells of ammonium either experimentally or during the course of a growth cycle had the effect of

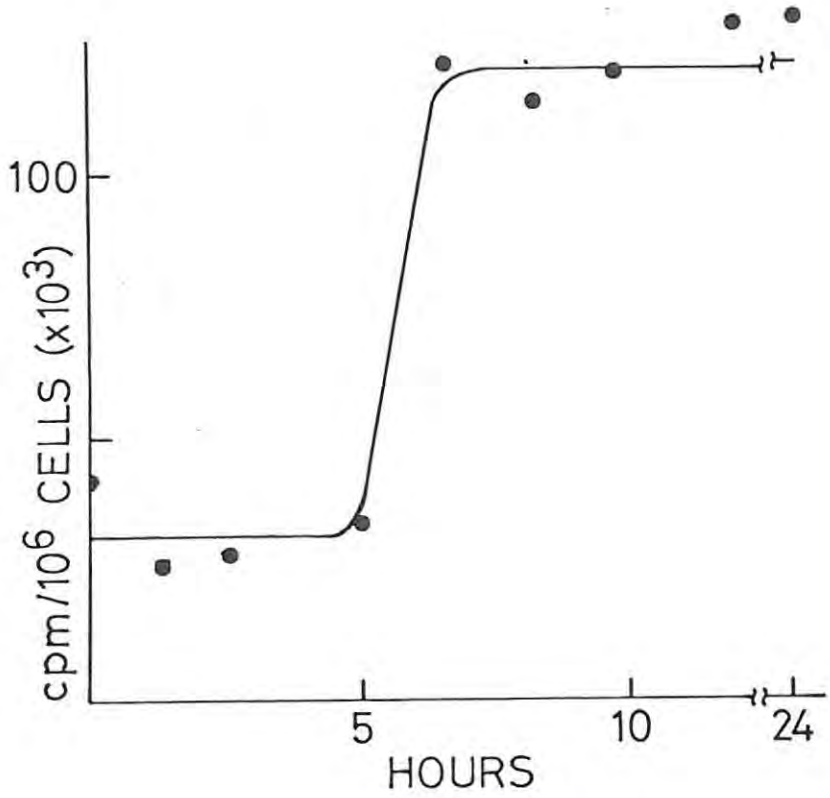


Figure 18. Heterotrophic carbon dioxide fixation by early phase autotrophic cells, measured in phosphate buffer containing 10 mM NH_4Cl, following various periods of ammonium deprivation. Abscissa represents length of ammonium deprivation period.

sensitizing the cells, such that the subsequent replenishment of the medium with more ammonium caused a *stimulation* of heterotrophic carbon dioxide fixation. The following kinetics were measured:

1) The time of ammonium starvation required to sensitize early phase cells
2) The concentration of ammonium required to sensitize early phase cells
3) The concentration of ammonium required to stimulate heterotrophic carbon dioxide fixation by ammonium deprived (sensitized) cells
4) The concentration of ammonium required to stimulate heterotrophic carbon dioxide fixation by non-sensitized cells
5) The time of exposure to ammonium required to stimulate heterotrophic carbon dioxide fixation by sensitized cells.

In all cases early phase autotrophic cells obtained from cultures grown to a cell density of 1.5×10^5 cells/ml were used. (Such cultures, after 24 hours normal growth, did not show increased heterotrophic carbon dioxide fixation.)

1) In all the experiments described above in which cells were deprived of ammonium before measurement of heterotrophic carbon dioxide fixation, the time of ammonium deprivation was 24 hours. It was important to measure the rate of development of sensitivity of the cells to stimulation of heterotrophic carbon dioxide fixation by resupplied ammonium. An early phase culture was harvested and resuspended in ammonium-free medium. At intervals thereafter heterotrophic carbon dioxide fixation was measured in phosphate buffer containing 10 mM NH_4Cl. The results of this experiment are shown in Figure 18. Ammonium starvation up to 5 hours had no sensitizing effect on the cells and carbon dioxide fixation in the presence of added ammonium remained constant. Between 5 and 7 hours after the cells were placed in ammonium free medium, addition of ammonium stimulated

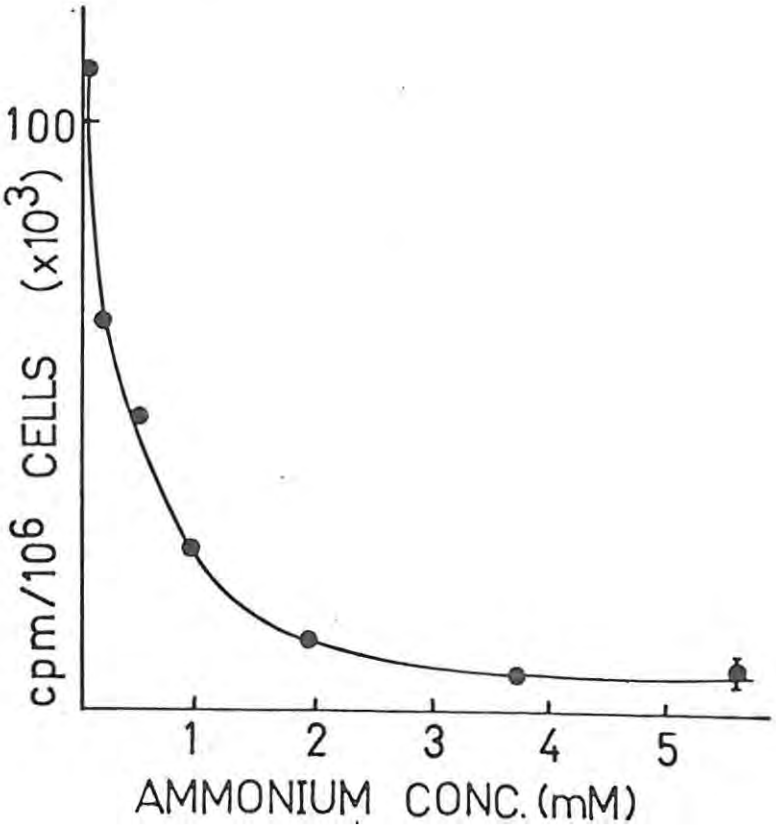


Figure 19. Heterotrophic carbon dioxide fixation by early phase autotrophic cells, measured in fresh medium, following 24 hours in media containing various concentrations of NH_4Cl. Abscissa represents concentration of NH_4Cl in which cells were suspended for the deprivation period.

the cells to increase fixation to the high levels observed previously. Longer periods of starvation caused only negligible further increases.

2) A series of media was prepared, each containing a different concentration of NH_4Cl, ranging from zero to 5.62 mM, which is the concentration in fresh medium. Cells from an early phase culture were harvested and resuspended to the same cell density in the various media. After 24 hours, heterotrophic carbon dioxide fixation was measured in fresh medium i.e. in the presence of 5.62 mM NH_4Cl. The results are shown in Figure 19. Medium containing more than 2 mM NH_4Cl did not sensitize early phase *Euglena* and heterotrophic carbon dioxide fixation measured after 24 hours in such medium was low. Exposure to medium containing 2 mM NH_4Cl caused a slight increase in subsequent carbon dioxide fixation. Below this concentration the sensitizing effect of the medium increased sharply and the highest level of fixation was recorded using cells which had been completely starved of ammonium.

These results provided a more quantitative correlation between degree of ammonium depletion and degree of subsequent stimulation of heterotrophic carbon dioxide fixation by resupplied ammonium. In Figure 13 the rate of depletion of ammonium from the exogenous medium during an autotrophic growth cycle was demonstrated. Removal of ammonium from the medium appeared to correlate in time with the increase in heterotrophic carbon dioxide fixation occuring during the growth cycle. From Figure 19 the concentration of ammonium that caused half maximal stimulation of fixation when the cells were subsequently reexposed to 5.6 mM ammonium ("$V_{max/2}$") was 0.5 mM. In the experiment shown in Figure 13, ammonium in the medium had dropped to this "$V_{max/2}$" value when the culture had grown to a density of 9×10^5 cells/ml (indicated on the figure by the arrow A). From the mean of the results of four experiments in which heterotrophic carbon

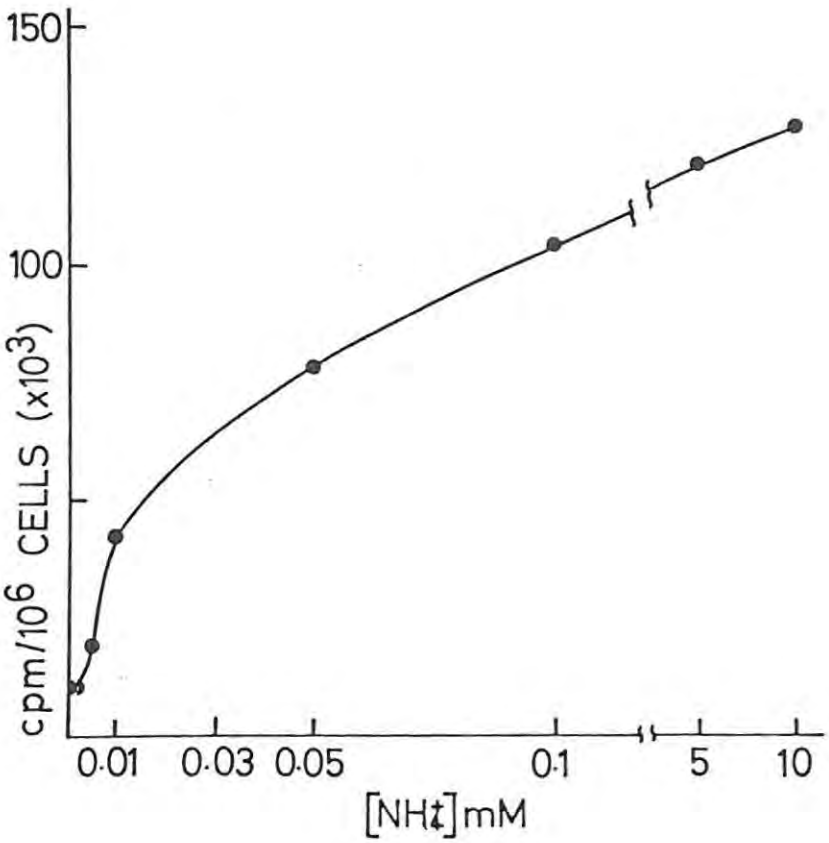


Figure 20. Heterotrophic carbon dioxide fixation by early phase autotrophic cells, measured in phosphate buffer containing various concentrations of NH_4Cl, following 24 hours ammonium deprivation. Abscissa represents concentration of NH_4Cl used for measurement of fixation.

dioxide fixation was measured throughout autotrophic growth cycles, it was estimated that fixation reached half its maximum level when the culture had a cell density of 8.2×10^5 cells/ml (indicated on the figure by the arrow B). Given a certain experimental variability in growth cycle profiles of heterotrophic carbon dioxide fixation changes, these half maximal values are in close proximity.

3) An early phase culture was deprived of ammonium for 24 hours in the normal way. A series of phosphate buffers, pH 4.7, was prepared for measurement of heterotrophic carbon dioxide fixation, each containing a different concentration of NH_4Cl. After the period of ammonium starvation cells were harvested and the stimulation of fixation by the various concentrations of ammonium was measured. The results are shown in Figure 20. A slightly sigmoid kinetic was obtained such that 0.001 mM NH_4Cl had the same effect as zero NH_4Cl. Above 0.001 mM NH_4Cl the fixation increased rapidly with ammonium concentration. The V_{max} was not obtained at 10 mM NH_4Cl, the highest concentration used in this analysis.

Attempts to analyse the data shown in Figures 19 and 20 by the method of Lineweaver and Burke (1934) and by other methods were unsuccessful. The resulting graphs were far from linear. This shows that these are not classical hyperbolic rate curves. It was presumed that the relationship between ammonium and heterotrophic carbon dioxide fixation was too complex to permit this type of analysis. However, the results clearly showed two aspects of this relationship. Firstly, decreasing the amount of exogenous ammonium in the environment of *Euglena* caused an increased sensitization of the cells to stimulation of heterotrophic carbon dioxide fixation by added ammonium. Complete ammonium deprivation caused the highest degree of sensitization. Secondly, cells which were completely

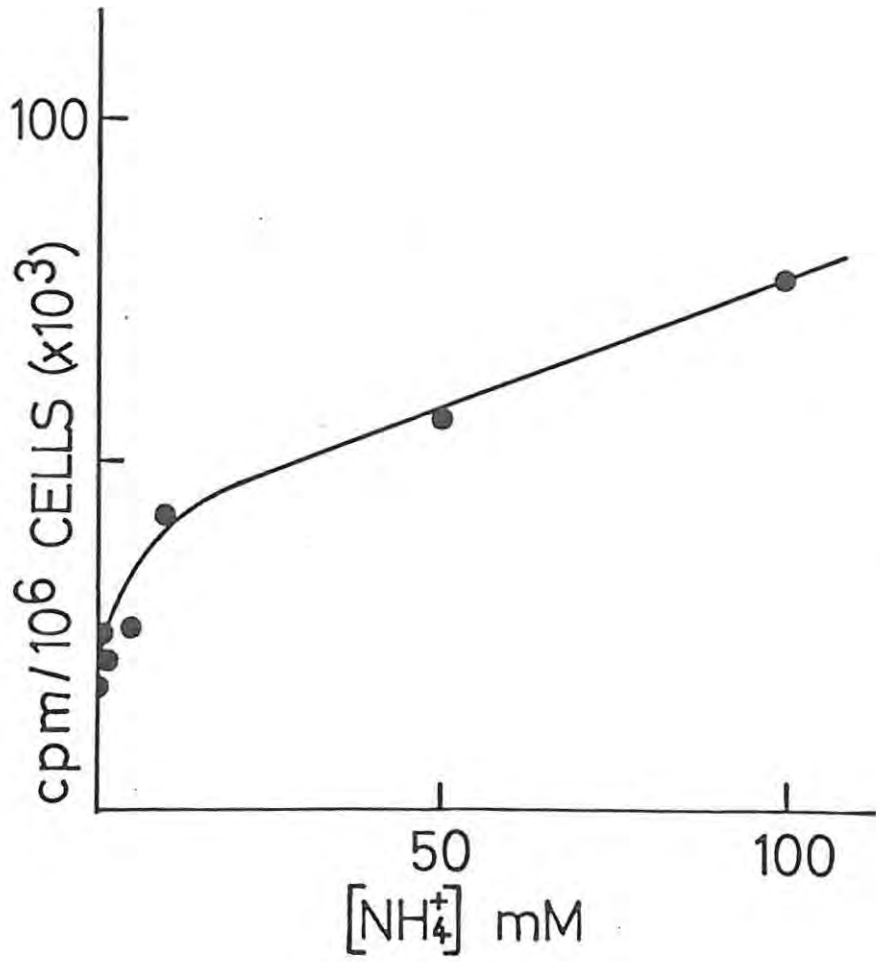


Figure 21. Heterotrophic carbon dioxide fixation by early phase, non-sensitized autotrophic cells, measured in phosphate buffer containing various concentrations of NH_4Cl. Abscissa represents concentration of NH_4Cl used for measurement of fixation.

starved of ammonium were stimulated to fix more carbon dioxide by very low concentrations of added ammonium such as 5 μM NH_4^+. Increasing the concentration of added ammonium caused increased stimulation of heterotrophic carbon dioxide fixation.

4) As an extension of the second aspect of the relationship between ammonium and heterotrophic carbon dioxide fixation described above, it was possible that by increasing the concentration of ammonium above that which was normally used in the experiments described above, even cells not deprived of ammonium could be "forced" to increase heterotrophic carbon dioxide fixation. To test this a series of phosphate buffers was prepared containing a range of concentrations of NH_4Cl up to 100 mM, and heterotrophic carbon dioxide fixation by an early phase, non-sensitized culture was measured in each of these. The results are shown in Figure 21. When supplied with ammonium at concentrations up to 5 mM, dark fixation by cells which had not been sensitized by ammonium deprivation did not increase, but remained constant within the limits of experimental variation as previously observed. When the concentration of NH_4Cl exceeded 10 mM the amount of carbon dioxide fixed increased as a linear function of the increased ammonium concentration, up to 100 mM NH_4Cl. However, comparison of Figures 20 and 21 shows that, although heterotrophic carbon dioxide fixation could be stimulated by sufficiently high concentrations of added ammonium without a sensitization period of ammonium deprivation, the stimulating effect of 100 mM NH_4Cl in this case was not as great as the stimulating effect of 5.62 mM NH_4Cl on cells previously sensitized by deprivation of ammonium.

The results shown in Figure 21 confirm the previous observation (Table 3) that heterotrophic carbon dioxide fixation by two strains of *Euglena*, a

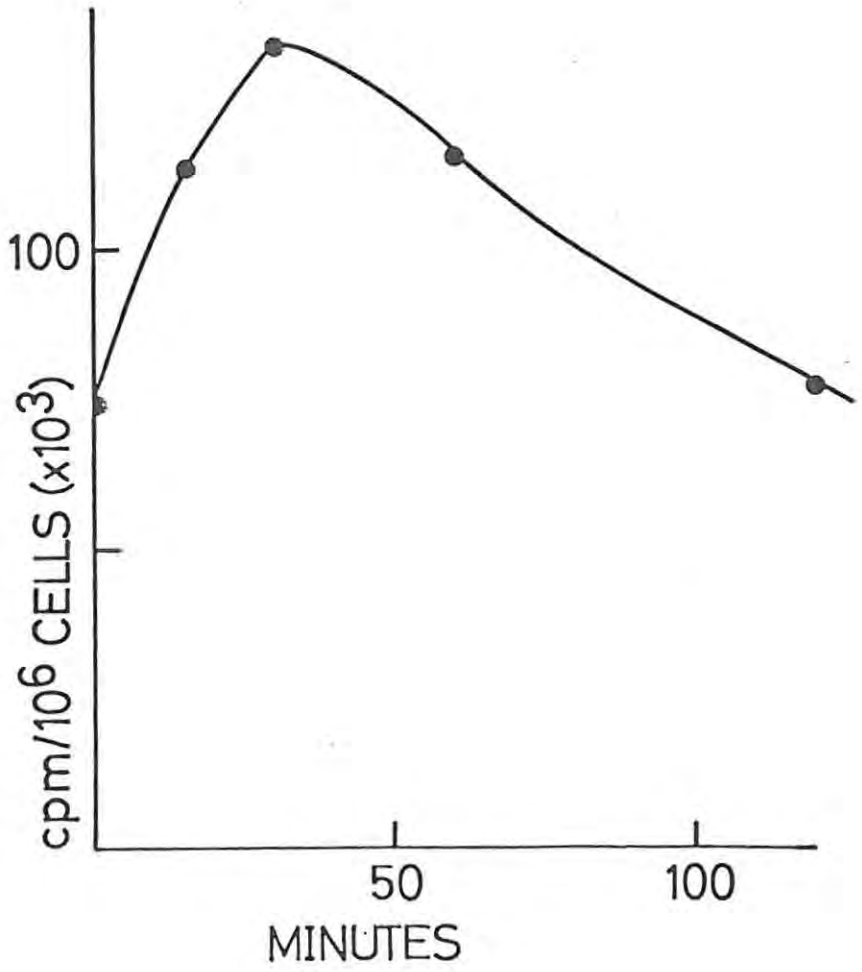


Figure 22. Heterotrophic carbon dioxide fixation by early phase autotrophic cells, measured after various periods of holding in phosphate buffer containing 10 mM NH_4Cl, following 24 hours ammonium deprivation. Abscissa represents length of holding period.

bleached strain and a temporarily etiolated strain, was greater if the fixation was measured in the presence of 10 mM NH_4Cl than if measured in phosphate buffer alone, even without prior ammonium deprivation.

5) It was important to examine any changes in heterotrophic carbon dioxide fixation that might occur with time after the cells were resupplied with stimulating ammonium. In this experiment an early phase culture was deprived of ammonium for 24 hours. Cells were then harvested, resuspended in phosphate buffer containing 10 mM NH_4Cl and aliquots were held in the dark. At time zero (i.e. immediately after resupplying the cells with ammonium) and at intervals thereafter, labelled carbonate was added and carbon dioxide fixation measured in the usual way. The results are shown in Figure 22. After zero holding time (this measurement actually represents the amount of carbon dioxide fixed in the 20 minute period immediately following resupply of ammonium) carbon dioxide fixation was increased compared with the normal level of fixation by non-sensitized cells in the presence of added ammonium. Heterotrophic carbon dioxide fixation increased further with dark holding up to 30 minutes and thereafter declined. However, even after 120 minutes holding the amount of carbon dioxide fixed by the cells was still elevated, compared with the amount fixed by non-sensitized cells.

Carboxylating enzymes in *Euglena* Heterotrophic carbon dioxide fixation reactions in *Euglena* must be catalysed by one or more carboxylating enzymes. As noted in the Introduction, the pathways of heterotrophic carbon dioxide fixation are still not fully elucidated and the enzymology of the carboxylations is still not clearly understood. The new knowledge described above, that heterotrophic carbon dioxide fixation in *Euglena* is regulated by ammonium, provided a possible tool for elucidating further some of these enigmatic areas.

Table 4. Carboxylating enzymes in *Euglena gracilis* Z.

Enzyme (E.C. number)	Ammonium stimulation	Specific activity as NAD(P)H/min/mg soluble protein (μmoles x 10^2)		
		Autotrophic	Glucose	Acetate
PEP carboxykinase	-	0	0	0
(E.C.4.1.1.38)	+	0	0	0
PEP carboxykinase	-	0	0	0
(E.C.4.1.1.49)	+	0	0	0
PEP carboxykinase	-	0	0	0.17
(E.C.4.1.1.32)	+	0	0	-
Pyruvate carboxylase	-	0	0.14	-
(E.C.6.4.1.1.)	+	0	0.20	-
PEP carboxylase	-	1.83	0.67	0.64
(E.C.4.1.1.31)	+	2.85	0.90	-
Malate enzyme	-	0.68	5.05	12.88
(E.C.1.1.1.40)	+	0.84	6.26	-

		Specific activity as cpm/mg soluble protein	
		Autotrophic	Glucose
PEP carboxylase (E.C.4.1.1.31)	-	174,650	54,456
Acetyl-Co carboxylase	-	32, 766	32,533
(E.C.6.4.1.2)	+	20, 186	23,850

A survey into the activities of various carboxylating enzymes in cell-free extracts of *Euglena* was carried out. Enzyme activities in crude extracts prepared from cells which had stimulated heterotrophic carbon dioxide fixation (i.e. cells which had been deprived of ammonium for 24 hours and then provided with 10 mM ammonium for 30 minutes before preparation of the extract) were compared with activities in crude extracts from control, non-stimulated cells. Crude extracts were prepared from cells growing autotrophically and heterotrophically on either glucose or acetate. The results of all these assays are shown in Table 4.

Under the experimental conditions employed no PEP carboxykinase (E.C. 4.1.1.38 and E.C.4.1.1.49) activity was detected in any of the cultures tested. However, a trace of PEP carboxykinase (E.C.4.1.1.32, GDP requiring) activity was present in the extract from the acetate grown heterotrophic culture. A trace amount of pyruvate carboxylase activity was measured in the glucose heterotrophic culture only. The most active carboxylating enzymes found in *Euglena* were PEP carboxylase, malate enzyme and acetyl-CoA carboxylase. Malate enzyme was found to be active in all types of culture tested, however, the extract of autotrophic cells had about 8 fold less activity than that from glucose heterotrophic cells, and the highest specific activity was measured in the extract from acetate grown cells which were found to have more than 2 fold more activity than the glucose culture. PEP carboxylase activity was also measured in all three extracts, however, in this case the specific activity in the autotrophic extract was greater than in the extracts from either of the heterotrophic cultures (glucose or acetate grown). Acetyl-CoA carboxylase activity was detected in both autotrophic and heterotrophic (glucose) cultures of *Euglena*, with similar specific activities in both extracts. When the specific activities of acetyl-CoA carboxylase were compared with those of PEP carboxylase it was

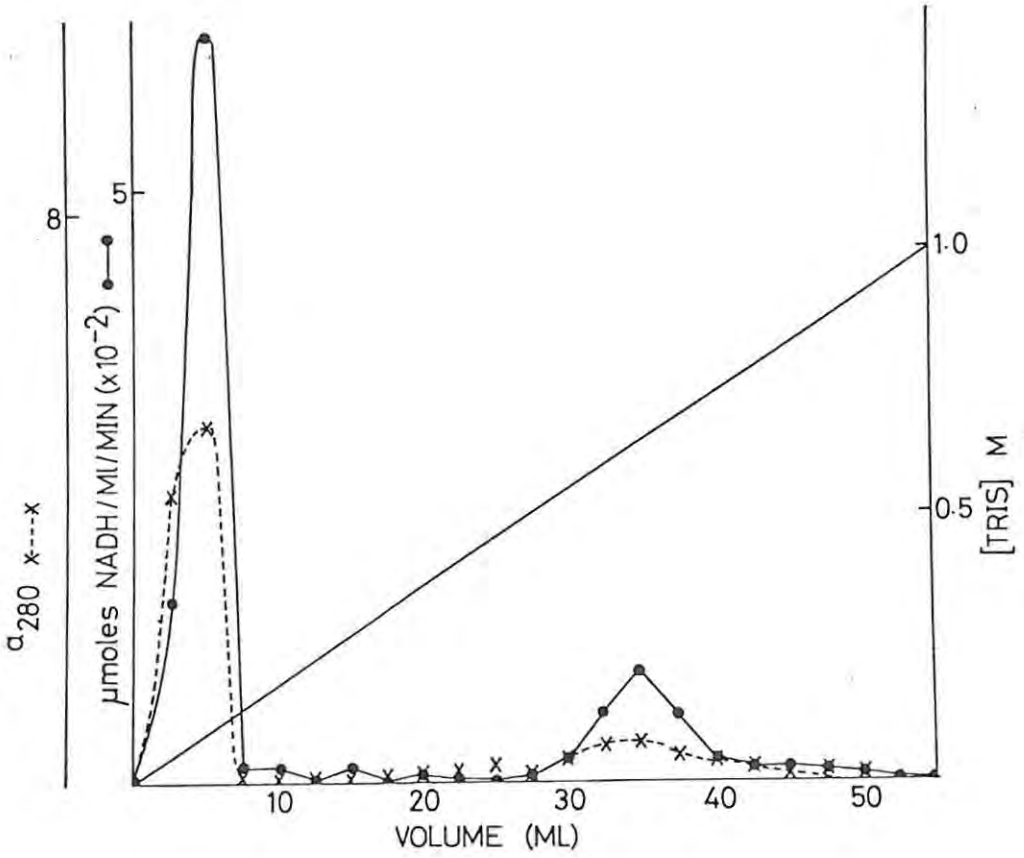


Figure 23. Chromatography on DEAE cellulose of PEP carboxylase from heterotrophically grown *Euglena*. Closed circles represent enzyme activity; crosses represent relative protein concentration as measured by the optical density at 280 nm. The straight line represents Tris concentration.

found that the PEP carboxylase specific activity was approximately 5 fold greater than that of acetyl-CoA carboxylase in autotrophic cells. This comparison was made possible because both enzymes were assayed radiometrically. In the case of glucose heterotrophic cells the specific activity of PEP carboxylase measured radiometrically was approximately 2 fold greater than that of acetyl-CoA carboxylase.

Table 4 also shows the effect of the ammonium treatment (starvation for 24 hours, followed by replenishment by 10 mM NH_4Cl before preparation of the extract) on the specific activities of carboxylating enzymes in crude extracts of *Euglena*. After the ammonium treatment the specific activities of all the enzymes tested showed increased activities, with the exception of acetyl CoA carboxylase, whose specific activity decreased after the ammonium treatment. PEP carboxylase specific activity showed the largest increase after the ammonium treatment, and the increase in specific activity of this enzyme was greatest ($\pm$ 1.6 fold) in the case of the autotrophic culture.

Partial purification of PEP carboxylase from *Euglena* and separation of isoenzymes For reasons described in the Discussion below it was decided to continue the investigation by further study of PEP carboxylase. Figure 23 shows a typical elution profile of the enzyme obtained from DEAE cellulose chromatography of the 20-25% ammonium sulphate protein fraction from a heterotrophic culture of *Euglena*, after Sephadex G 25 chromatography. The first active fraction to elute from the column, isoenzyme 1, barely adsorbed onto the cellulose. Isoenzyme 1 accounted for 73% of the recovered activity. The second active fraction, isoenzyme 2, which accounted for the remainder of the recovered activity eluted at a concentration of 0.65 M Tris. Also shown by Figure 23 is the relative protein concentration as measured by the optical density of the samples

Table 5. Purification of PEP carboxylase from *Euglena*.

Source	Activity /ml/min ($x10^2$)	Protein mg/ml	Specific activity	Total activity	Yield %	Prop. total activity %
Heterotrophic culture						
Crude extract	6.2	4.66	1.33	31.0		
Peak 1, DEAE cellulose	5.33	0.93	6.73	14.4	63.4	73
Peak 2, DEAE cellulose	0.6	0.5	1.2	5.24		27
Autotrophic culture						
Crude extract	5.0	4.0	1.24	24.8		
Peak 1	4.9	1.12	4.38	12.4	66.0	75.6
Peak 2	1.1	0.88	1.25	4.0		24.4

Activity units are µmoles NADH oxidised. Both cultures were harvested at the mid-logarithmic growth phase. The specific activities of the separated isoenzyme represent the most active fraction. Total activities were calculated by pooling all active fractions.

at 280 nm. The profile of protein concentration showed two peaks which corresponded to the isoenzyme 1 and 2 peaks.

In the case of an autotrophic culture, two isoenzymes also eluted from the DEAE cellulose at the same Tris concentrations as for the heterotrophic culture. The proportion of activity in each peak was also similar to that observed for the heterotrophic culture. Details of a typical purification of the two isoenzymes from each other from the two cultures are shown in Table 5, and the similarities between the two types of culture are apparent. In both autotrophic and heterotrophic cultures isoenzyme 1 accounted for approximately 75% of the recovered activity, and the percentage total activity recovered from the original crude extract was also almost identical in the two cultures. In the case of the heterotrophic culture the specific activity of isoenzyme 1 was 5 times greater than that of the crude extract, representing a 5 fold purification of the enzyme. A 3.5 fold purification was obtained in the case of isoenzyme 1 from the autotrophic culture. In neither case was any purification of isoenzyme 2 measured, due to the low activity and instability of this isoenzyme (described below). The elution profile of PEP carboxylase isoenzymes from a control early phase culture was compared with that of a culture whose heterotrophic carbon dioxide fixation was stimulated by ammonium treatment as described above. In both stimulated and non-stimulated cells two isoenzymes eluted from the DEAE cellulose and ammonium treatment had no effect on the Tris concentration at which the isoenzymes eluted, or the proportion of activity in each isoenzyme.

Both isoenzymes were unstable in Tris, especially isoenzyme 2 which retained less than 10% of its activity after 24 hours at -14^{o}C. The stability of isoenzyme 1 was inconsistent from experiment to experiment,

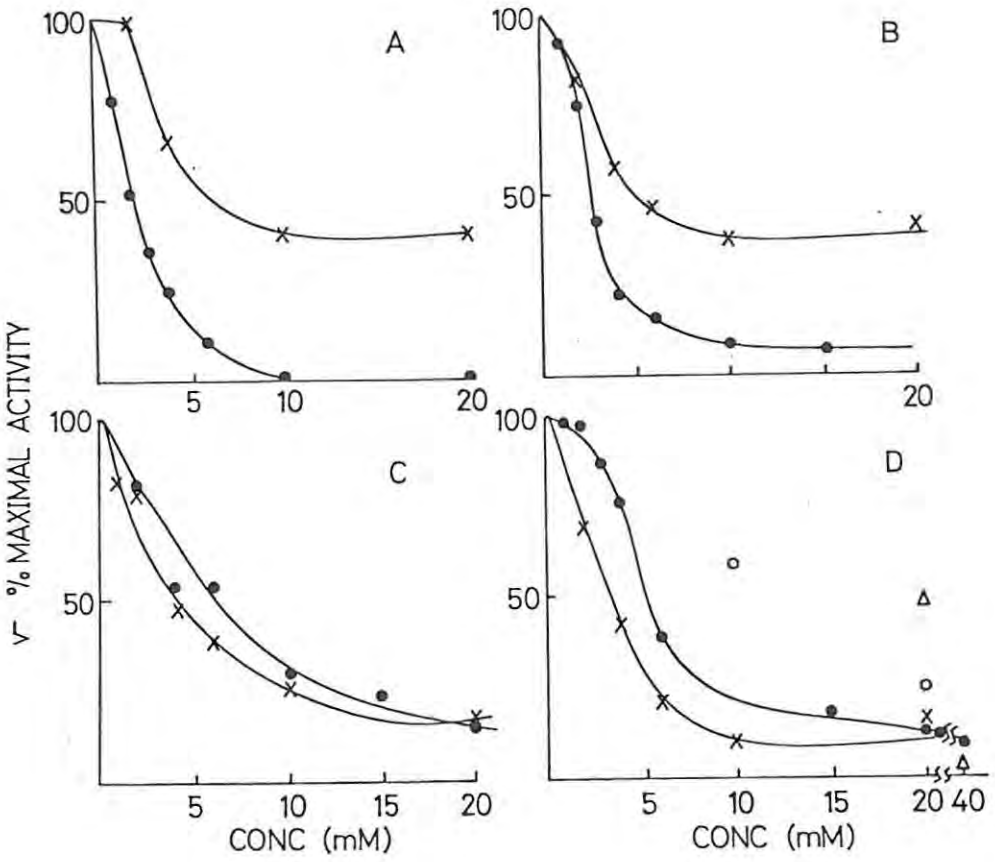


Figure 24. Effect of various inhibitory substances on isoenzymes of PEP carboxylase from *Euglena*.

A. Effect of malate on isoenzymes from heterotrophic cells.

B. Effect of malate on isoenzymes from autotrophic cells.

C. Effect of citrate on isoenzymes from heterotrophic cells.

D. Effect of citrate, succinate and 3-phosphoglycerate on isoenzymes from autotrophic cells.

Closed circles represent inhibition of isoemzyme 1; crosses represent inhibition of isoenzyme 2. Open circles represent inhibition of isoenzyme 1 by succinate; triangles represent inhibition of isoenzyme 1 by 3-phosphoglycerate.

and it normally lost 25-50% of its activity after 24 hours at -14°C. Ethanol (2%) stabilized crude extracts kept at -14°C such that no loss in activity was observed after 14 days of storage. However, the ethanol was less effective at stabilizing the separated isoenzymes. In one experiment isoenzyme 1 lost 33% of its activity and isoenzyme 2 lost 53% in 24 hours when stored at -14°C in the present of 2% ethanol. Other stabilizing agents such as glycerol, mercaptoethanol and dithiothreitol were tested but were equally ineffective at stabilizing the separated isoenzyme.

Kinetics of PEP carboxylase isoenzymes from *Euglena* The effects of various substances upon the partially purified PEP carboxylase isoenzymes from *Euglena* were studied. In crude extracts an apparent noncompetitive inhibition by glutamate was obtained, using the colorimetric assay. This was not observed for the two isoenzymes nor when the radiometric assay was used. The apparent inhibition of PEP carboxylase by glutamate was subsequently shown to be an artefact of the colorimetric assay, due to other enzymes present in the crude competing for the substrate (OAA). The effect of ammonium on the activity of PEP carboxylase was tested under a variety of conditions, but no effect was observed at concentrations of up to 50 mM NH_4Cl.

The effect of various inhibitors on the activity of the two isoenzymes from both autotrophic and heterotrophic *Euglena* is shown in Figure 24. Malate, citrate, succinate and 3-phosphoglycerate were found to inhibit PEP carboxylase activity. The results obtained for autotrophic and heterotrophic cells were essentially similar. Isoenzyme 1 activity was inhibited about 15-20% by 1 mM malate, and at a

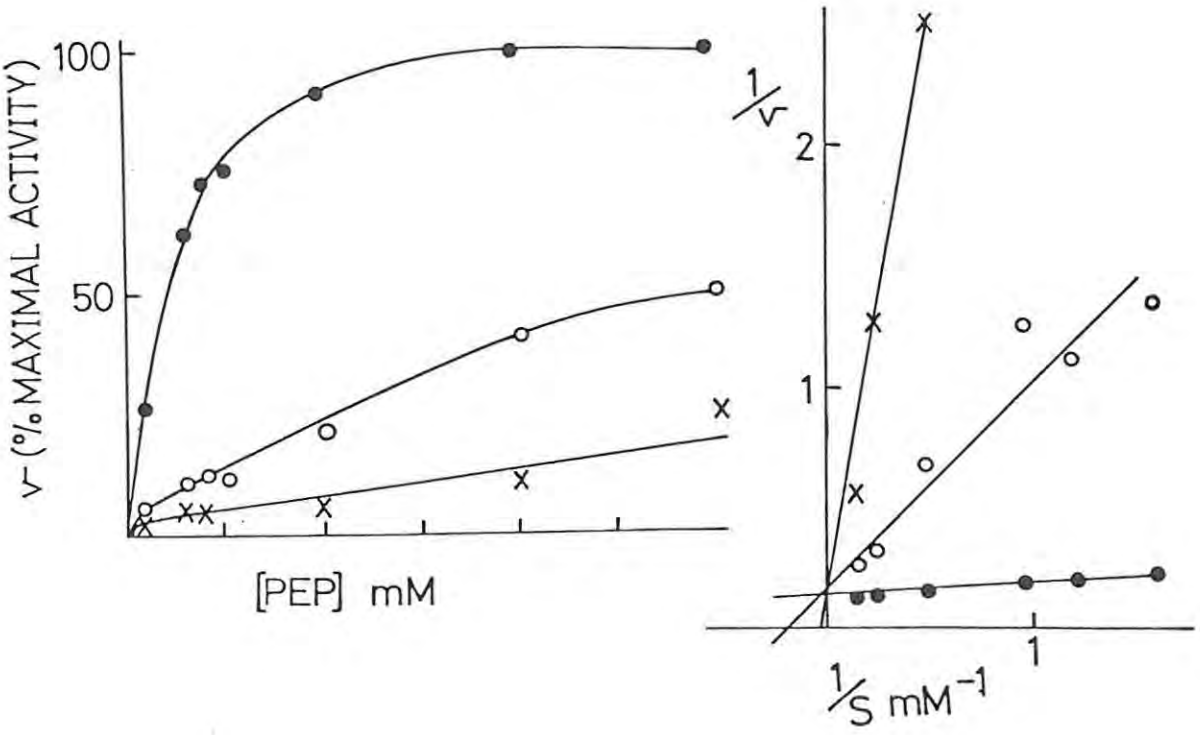


Figure 25. Rate curves and double reciprocal plots showing the kinetics of inhibition of PEP carboxylase (isoenzyme 1) from *Euglena* by malate with respect to PEP. Closed circles represent initial reaction rates in the absences of malate; open circles represent reaction rates in the presence of 4 mM malate; crosses represent reaction rates in the presence of 10 mM malate.

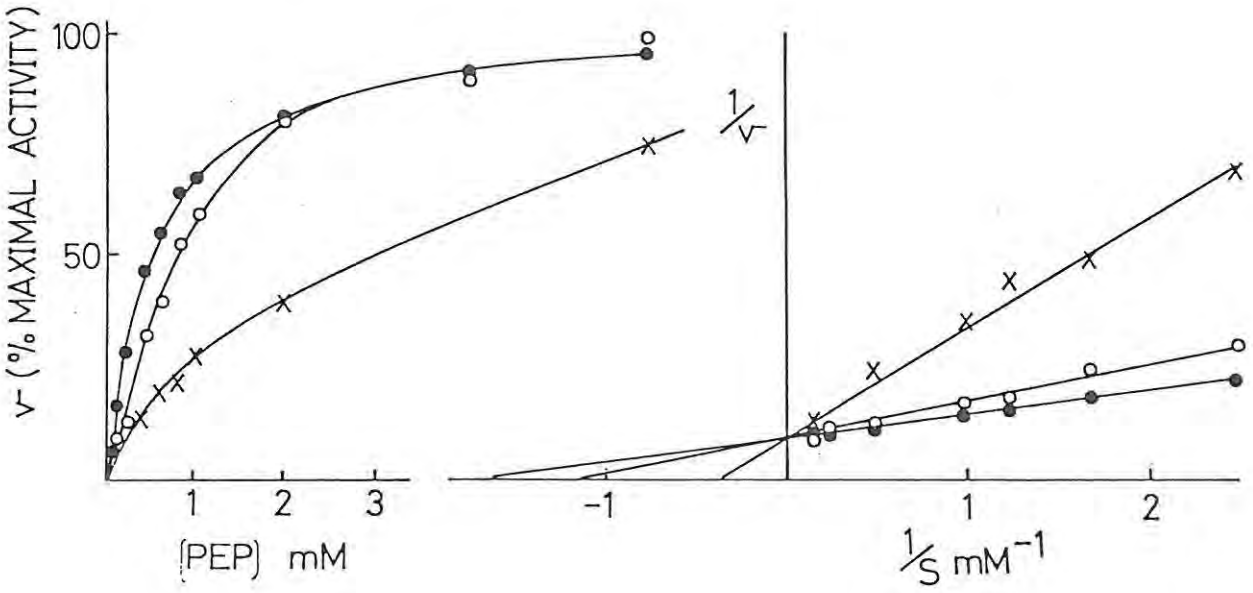


Figure 26. Rate curves and double reciprocal plots showing the kinetics of inhibition of PEP carboxylase (isoenzyme 1) from *Euglena* by citrate with respect to PEP. Closed circles represent initial reaction rates in the absence of citrate; open circles represent reaction rates in the presence of 3 mM citrate; crosses represent reaction rates in the presence of 10 mM citrate.

concentration of 10 mM was virtually totally inhibited. Isoenzyme 2 was also inhibited by malate, such that, at a concentration of 10 mM malate, enzyme activity was 60% inhibited. *Euglena* PEP carboxylase isoenzymes were also shown to be inhibited by citrate. As shown in Figure 24 the pattern of inhibition by citrate also was essentially similar for the enzymes from autotrophic and heterotrophic cells. Inhibition increased sharply up to a concentration of 20 mM citrate (10-15% maximum activity remaining). Further increases in citrate concentration did not increase inhibition significantly. Also shown in Figure 24 is evidence that succinate and 3-phosphoglycerate inhibit isoenzyme 1.

Analysis of the inhibition of isoenzyme 1 by malate and citrate by the conventional method of Lineweaver and Burke (1934) is shown in Figures 25 and 26. The analysis revealed that malate and citrate inhibited by classical competitive inhibition, since K_m values increased with inhibitor, whereas V_{max} was constant at different concentrations of the inhibitors. Analysis of the data by the Hanes technique (s plotted against s/v) (Wong, 1975) gave essentially the same information.

Effect of malate on heterotrophic carbon dioxide fixation by whole cells

Since PEP carboxylase from *Euglena* was shown to be inhibited by malate, it was possible that malate might inhibit whole cell heterotrophic carbon dioxide fixation. In order to investigate this possibility heterotrophic carbon dioxide fixation was measured in both autotrophic and heterotrophic cells, with and without stimulation of fixation by ammonium treatment. Before addition of labelled carbonate cells were exposed for 60 minutes to various concentrations of malate. The effects of malate on whole cell fixation in both ammonium stimulated and non-stimulated cells is shown in Figure 27. Malate affected the cells' heterotrophic carbon

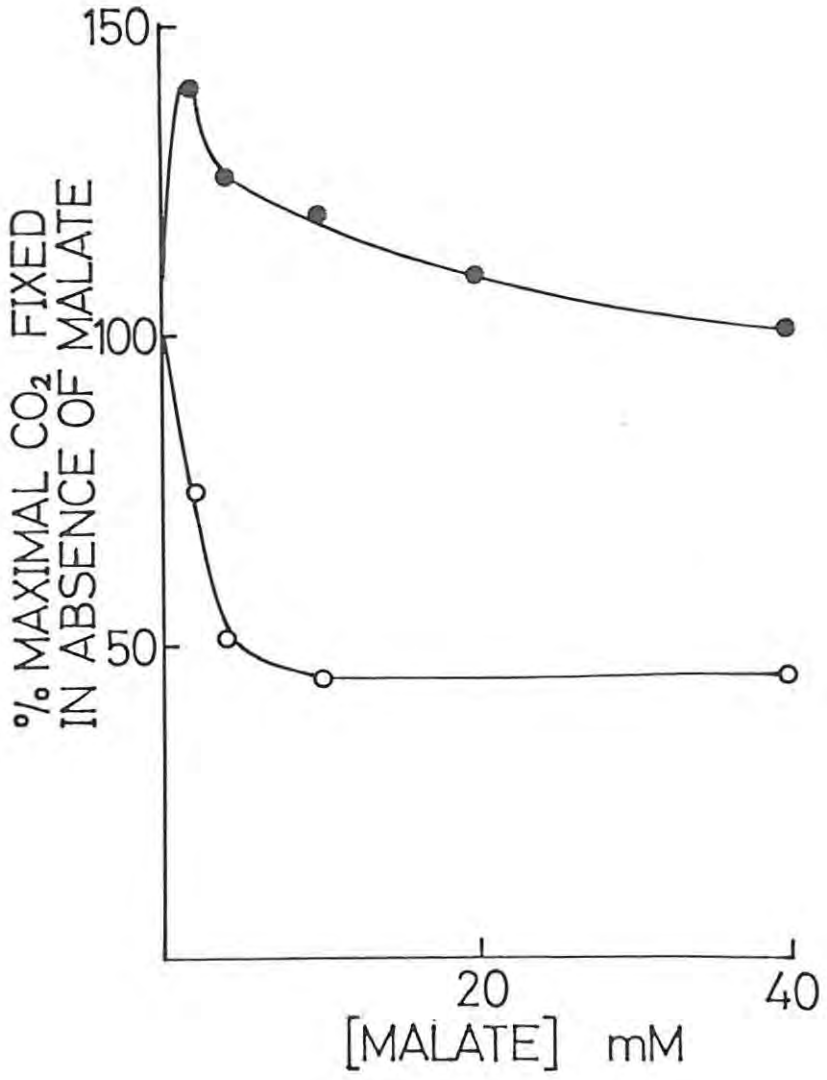


Figure 27. Heterotrophic carbon dioxide fixation by early phase heterotrophic *Euglena* after 1 hour holding in the presence of various concentrations of malate. Closed circles represent heterotrophic carbon dioxide fixation by control cells; open circles represent heterotrophic carbon dioxide fixation by cells which were stimulated by ammonium treatment.

dioxide fixation differently in the two types of cells. In the case of the control (unstimulated) cells there was stimulation of fixation by a low concentration of malate, and a decrease of the stimulation at the higher concentrations, such that at 40 mM malate, the fixation was the same as at zero malate concentration. In the case of the ammonium stimulated cells, malate inhibited heterotrophic carbon dioxide fixation such that 40 mM malate caused 45% inhibition.

The products of heterotrophic carbon dioxide fixation The radioactive products formed after 10 minutes dark carbon dioxide fixation were separated by two dimensional chromatography as described in Materials and Methods. A tracing of an autoradiograph of a typical chromatogram is shown in Figure 28. The radioactive compounds separated as discrete spots. In the case of glutamate considerable trailing occurred, as was observed by Moses *et al.* (1959). Labelled carbon dioxide was incorporated into amino acids, phosphorylated compounds, tricarboxylic acid cycle intermediates and nucleotides. The sugar phosphates, which include 3-phosphoglycerate in the separation illustrated, barely migrated from the origin. The other phosphorylated compound, PEP, migrated with solvent 2 but not with solvent 1. Two low activity compounds were not positively identified by co-chromatography, but on the basis of their migration characteristics are possibly nucleotides.

The radioactive spots were cut from the chromatograms, eluted by 1 ml volumes of 80% ethanol, and aliquots counted. The remainder of the eluates was used for identification of the compounds by co-chromatography. Table 6 shows the amount of radioactivity incorporated into the various compounds both for control cells and for cells stimulated by the normal ammonium treatment. Figure 29 shows the same results as Table 6, presented in the form of a histogram. The total amount of radioactivity incorporated by

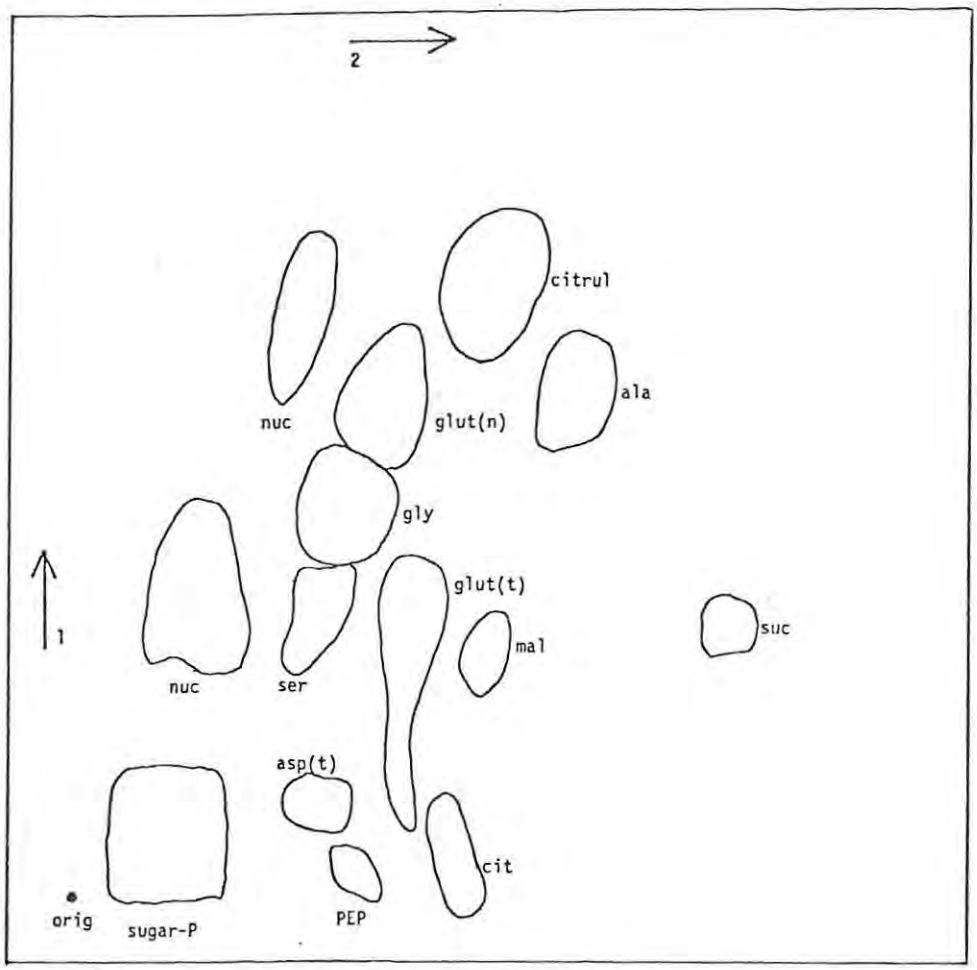


Figure 28. Alcohol soluble products of heterotrophic carbon dioxide fixation (10 minutes) by an autotrophic culture of *Euglena*, stimulated by the normal ammonium treatment. The products were separated chromatographically as described in the text and the chromatograms exposed to X-ray film for 4-5 months. The radioactive areas were traced from the autoradiogram. The tracing was reduced by 1/3. Ala - alanine; asp(t) - aspartate; cit - citrate; citrul - citrulline; glut(n) - glutamine; glut(t) - glutamate; gly - glycine; mal - malate; nuc - nucleotides; orig - origin; PEP - phosphoenolpyruvate; ser - serine; suc - succinate; sugar-P - sugar phosphates.

Table 6. Alcohol soluble products of heterotrophic carbon dioxide fixation, with and without ammonium stimulation.

Compound	Total (Counts/10^6 cells) Control	Ammonium Treatment
Glycine	467	17,533
Glutamine	1,720	17,493
Glutamate	12,810	
Alanine	1,067	8,347
Serine	0	7,587
Aspartate	2,133	2,667
Citrulline	0	470
Citrate	133	1,533
Malate	0	360
Succinate	0	120
Sugar phosphates	280	2,427
Phospho*enol*pyruvate	1,840	1,333
Unidentified nucleotides	0	4,787
Total counts/10^6 cells	20,450	80,830

Products of heterotrophic carbon dioxide fixation were separated and identified as described in the text. Chromatograms were exposed to X-ray film for 4-6 months for localization of radioactive areas. These were eluted from the chromatograms as described and aliquots counted by liquid scintillation for calculation of the total counts incorporated into each compound.

the stimulated cells was fourfold greater than the total amount fixed by the control cells. Quantitative differences between this result and the previously described effect of ammonium stimulation on heterotrophic carbon dioxide fixation by autotrophic cells (a tenfold stimulation, for example Figure 15) may be the result of different techniques used, especially as the cell densities used in the two experiments were different.

Table 6 shows that in both control and stimulated cells between 85 and 90% of the labelled carbon dioxide was incorporated into amino acids. In the control culture the major product was glutamate, which accounted for 64% of the total radioactivity incorporated. The second highest activity was found in aspartate, which accounted for 10% of the total counts. Some radioactivity was found in phosphoenolpyruvate, 3-phosphoglycerate and sugar monophosphates. The amount of labelled carbon dioxide incorporated into tricarboxylic acid cycle intermediates was comparatively small. The only acid detected was citrate, accounting for 0.6% of the total counts.

Generally ammonium treatment caused an increase in the amount of labelled carbon dioxide incorporated into the various compounds. The largest increases were in the counts found in glutamine, glycine, alanine, serine and the unidentified nucleotides. Smaller increases were observed after ammonium treatment for all the other compounds with the exception of PEP. Some compounds, not detected in the control cell separation, were labelled after ammonium treatment, for example malate, succinate and serine.

In both control and stimulated cells glutamate was a major product of dark carbon dioxide fixation. Ammonium treatment stimulated incorporation of carbon dioxide into glutamate by only 25%, compared with for instance 97% stimulation in the case of glycine.

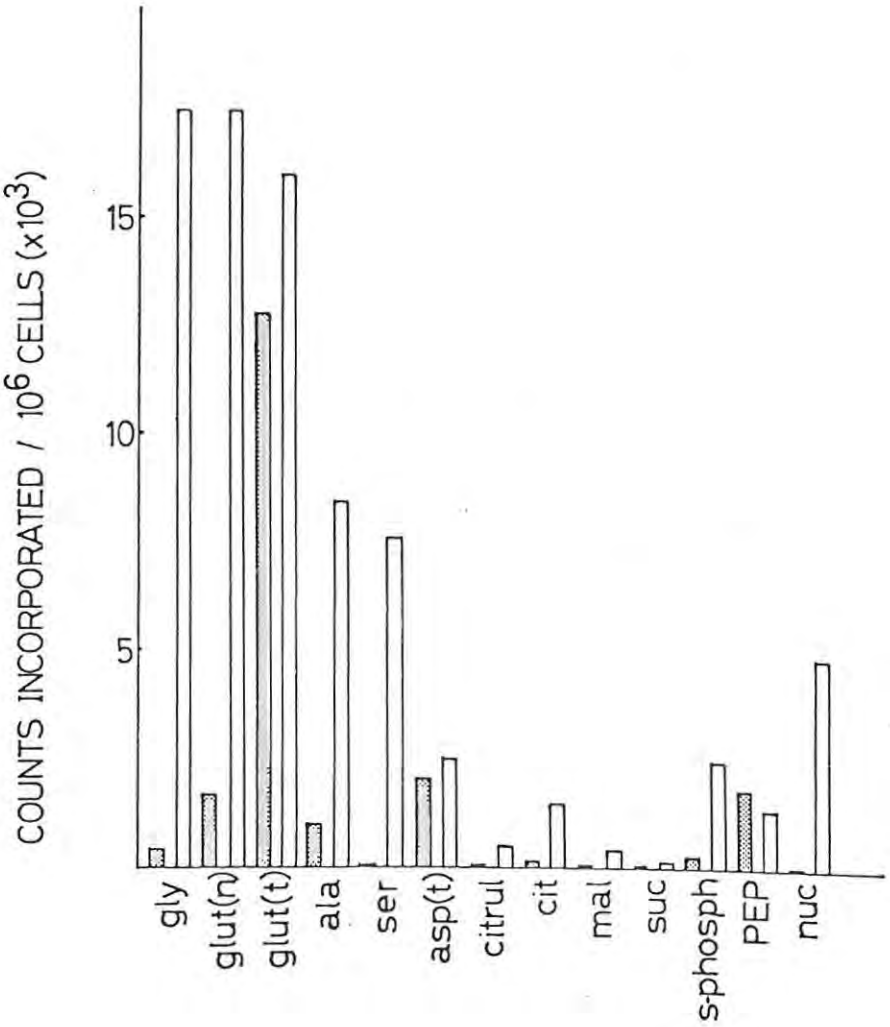


Figure 29. Comparison of the distribution of ^{14}C from heterotrophic carbon dioxide fixation in *Euglena* by a control culture and a culture stimulated by ammonium treatment. The cultures were both grown autotrophically. Shaded bars represent incorporation of $^{14}CO_2$ by the control culture; open bars represent incorporation by the ammonium treated culture. Abbreviations as for Figure 28.

DISCUSSION

In this investigation into heterotrophic carbon dioxide fixation in *Euglena*, discoveries were made which gave new insight into aspects of the function of this non-photosynthetic fixation and its regulation at the enzyme level. Changes in heterotrophic carbon dioxide fixation during growth cycles were confirmed and documented, and it was subsequently established that the amount of carbon dioxide fixed by *Euglena* is controlled in a complex fashion by ammonium. This is the first time that such a regulatory mechanism has been described in these cells. As a result of this finding it became possible to control *Euglena* heterotrophic carbon dioxide fixation experimentally. For instance, if early phase cells were first starved of ammonium and then supplied with more ammonium, heterotrophic carbon dioxide fixation was stimulated. This enabled comparisons between, for instance, properties of carboxylating enzymes and products of heterotrophic carbon dioxide fixation when cells were either stimulated by ammonium treatment or non-stimulated (control cells). This relationship between nitrogen metabolism and heterotrophic carboxylation, discovered in *Euglena* in this study, may be a general phenomenon in free-living organisms. Syrett (1956) observed that nitrogen starved *Chlorella* showed an increase in dark carbon dioxide fixation if supplied with ammonium. The effect was also found by Morris *et al.* (1971) with various marine algae.

Involvement of photosynthetic carbon dioxide fixation in heterotrophic carbon dioxide fixation The observation that heterotrophic carbon dioxide fixation was increased in the later stages of the growth cycle of *Euglena* formed the starting point for this study. The observation was confirmed first in an autotrophic culture as shown in Figure 1. Several experiments showed that the increase in fixation is not related to photosynthetic

processes. It was shown that dark holding cells in mineral medium for 60 minutes prior to measurement of carbon dioxide fixation did not remove the increase in fixation observed during the logarithmic growth phase but caused a larger increase than was measured in the absence of dark holding (Figure 2). If the increase in carbon dioxide fixation was due to a preillumination photosynthetic component it is likely that 60 minutes dark holding would have removed or decreased this component. Further confirmation was provided by the fact that similar changes in heterotrophic carbon dioxide fixation were measured during the growth cycle of cells growing heterotrohpically on glucose (Figure 3). The glucose grown cells were etiolated and lacked functional chloroplasts, possessing only proplastids which lack the ability to photosynthesize. The changes in heterotrophic carbon dioxide fixation during growth cycles were shown to be regulated by ammonium and it was shown that similar changes in carbon dioxide fixation, also regulated by ammonium, occur in a permanently bleached strain, *E.gracilis* Z SB3 (Table 3). This strain, as a result of streptomycin treatment, is incapable of ever forming functional chloroplasts. Permanent bleaching was discussed in the introductory section (p. 8 and 9).

Isotope dilution The possibility that the observed changes in heterotrophic carbon dioxide fixation during growth cycles were artefacts due to isotope dilution was discounted for several reasons. Such artefacts could have occurred if the environment of the early phase cells contained more carbon dioxide than that of the later phase cells, altering the ratio of added labelled carbon dioxide to unlabelled carbon dioxide. This was unlikely in the first place because in the autotrophic cultures carbon dioxide was kept at 5% throughout the growth cycle. Secondly, in an experiment heterotrophic carbon dioxide fixation was compared in both early phase cells (non-stimulated) and in cells from the late logarithmic growth phase

(stimulated) in the presence of increasing quantities of added, non-labelled carbon dioxide. Similar rates of decrease of amount of labelled carbon dioxide incorporated were obtained in the two cases. This indicated that the original ratio of labelled to unlabelled carbon dioxide is similar in both types of cells. Finally it was established that the change in heterotrophic carbon dioxide fixation is an ammonium-induced phenomenon, correlating with ammonium changes in the medium during growth, and controllable at any stage of the growth cycle by ammonium manipulation.

It is possible that the decline in fixation with time shown in Figure 22, discussed below, may be due to isotope dilution. However, this particular possibility was not deemed of sufficient importance to pursue.

Two components of heterotrophic carbon dioxide fixation It was shown (Figures 16 and 17) that heterotrophic carbon dioxide fixation in *Euglena* comprises two components. There is a minor component which is always observed at a constant background level. This component was demonstrated by measuring heterotrophic carbon dioxide fixation throughout growth cycles in the presence of phosphate buffer alone (i.e. with the cells not stimulated by ammonium) and it was shown to remain constant throughout the growth cycle. The second, major moeity of fixation was shown to be controllable in a complex fashion by ammonium and could also be controlled experimentally. Details of the control by ammonium are discussed below. Lynch and Calvin (1953) suggested that *Euglena* may fix carbon dioxide heterotrophically by two routes. It is possible that these two supposed routes correspond to the two components of dark fixation demonstrated in the present study, but certain findings, discussed below, make this appear unlikely.

Involvement of heterotrophic carbon dioxide fixation in *Euglena* in anaplerosis

Several possible functions of heterotrophic carbon dioxide fixation reactions were described in the introductory section (p. 17). Apart from specialized functions, for example the metabolism of propionate, four possible functions of heterotrophic carboxylations were described. These are fatty acid biosynthesis, pyridine nucleotide ratio control, gluconeogenesis and anaplerotic replenishment of the tricarboxylic acid cycle. Several discoveries in this study strongly support anaplerotic replenishment as a function of the major, ammonium dependent component of heterotrophic carbon dioxide fixation in *Euglena*. These important discoveries are firstly, the increase in heterotrophic carbon dioxide fixation during the logarithmic growth phase, secondly, the repression of dark carbon dioxide fixation by acetate, thirdly, the link between heterotrophic carbon dioxide fixation and ammonium and finally, the effect of ammonium on the products of heterotrophic carbon dioxide fixation.

(A) Changes in heterotrophic carbon dioxide fixation during growth cycles

In the first instance it was found that autotrophic cells and cells growing heterotrophically on glucose show increases in heterotrophic carbon dioxide fixation during the logarithmic growth period as shown in Figures 1 and 3. It was later established that the reason for the increase in fixation is the depletion of exogenous ammonium in the medium during growth (shown in Figures 13 and 14) followed by resupplying ammonium for measurement of heterotrophic carbon dioxide fixation. The implications of the regulation by ammonium are discussed below (C). The observed changes in dark fixation during growth cycles were discussed by Peak and Peak (1976). It was considered probable that there is an increased demand for anaplerotic reactions when the cells are growing and biosyntheses, particularly of protein and nucleic acids, are taking place at an increased rate, with concomitant

drainage of precursors such as α-ketoglutarate from the tricarboxylic acid cycle. There seems to be no reason why gluconeogenesis would increase at certain stages of growth cycles since the cells are supplied with substrate (carbon dioxide in the case of autotrophic cells, glucose in the case of heterotrophic cells). Also, although Peak *et al.* (1973) discussed an increased requirement for fatty acid biosynthesis towards the end of the growth cycle in heterotrophically grown cells with a concomitant demand for increased NADPH, there appeared to be no such increased demand in autotrophic cells. However both autotrophic and heterotrophic (glucose) cells show increased heterotrophic carbon dioxide fixation during the logarithmic growth phase. Furthermore, the increase in the case of the autotrophic culture is greater than that for glucose-grown cells. These observations make it appear unlikely that a major function of heterotrophic carbon dioxide fixation in *Euglena* is fatty acid biosynthesis or pyridine nucleotide ratio control, which may be concerned with fatty acid biosynthesis (Peak *et al.*, 1973).

(B) The effect of acetate on heterotrophic carbon dioxide fixation in *Euglena*

Further evidence supporting an anaplerotic role for the major, regulable component of heterotrophic carbon dioxide fixation was obtained from the effects of acetate on the fixation. When heterotrophic carbon dioxide fixation was measured throughout growth cycles of *Euglena* growing on acetate, no increases during the logarithmic growth phase were observed. Similar results were obtained for cultures growing on ethanol (Figures 4 and 5). Moreover, it was found that acetate, added to a glucose grown culture, repressed heterotrophic carbon dioxide fixation to a level where it was barely detectable under the experimental conditions employed (Figure 6). It is known that organisms which can grow on 2-carbon substrates utilize

the glyoxylate cycle, by means of which one molecule of malate and one of succinate are formed from one molecule of isocitrate and one of acetyl-CoA (Kornberg, 1966). This process was described in the introductory section (p. 24). Functioning of the glyoxylate cycle obviates the need for other anaplerotic reactions. *Euglena* is able to grow heterotrophically on acetate and under these conditions the glyoxylate cycle is functional. Cook and Carver (1966) and Heinrich and Cook (1967) demonstrated that culturing *Euglena* on acetate leads to the induction of the glyoxylate cycle enzymes, isocitrate lyase and malate synthase. The same enzymes are induced when the cells are growing on ethanol. Cook and Carver (1966) found the level of malate synthase in these cells to be almost as high as for acetate grown cells, and Graves *et al.* (1975) reported the presence of "glyoxysomes" containing the glyoxylate cycle enzymes in *Euglena* grown on ethanol. Thus if heterotrophic carbon dioxide fixation reactions function in *Euglena* mainly as anaplerotic replenishment reactions, increased requirement for these reactions during the logarithmic growth phase of cultures growing on acetate or ethanol would not be expected, since there would be a ready supply of the cycle intermediates, malate and succinate due to the activity of the glyoxylate cycle. The proposed interactions between the tricarboxylic acid cycle, heterotrophic carbon dioxide fixation reactions and the glyoxylate cycle are summarized in Figure 30. Intermediates of the tricarboxylic acid cycle are drained for anabolic sequences, and carbon dioxide fixation reactions leading to oxaloacetate or malate could replenish the cycle, for example the carboxylation of PEP to oxaloacetate. When *Euglena* are grown on acetate or ethanol the alternate means of replenishing the cycle with 4-carbon intermediates malate and succinate would function, i.e. the glyoxylate cycle.

The finding that heterotrophic carboxylations are repressed by acetate not only supports anaplerotic replenishment as a function of these carboxylations,

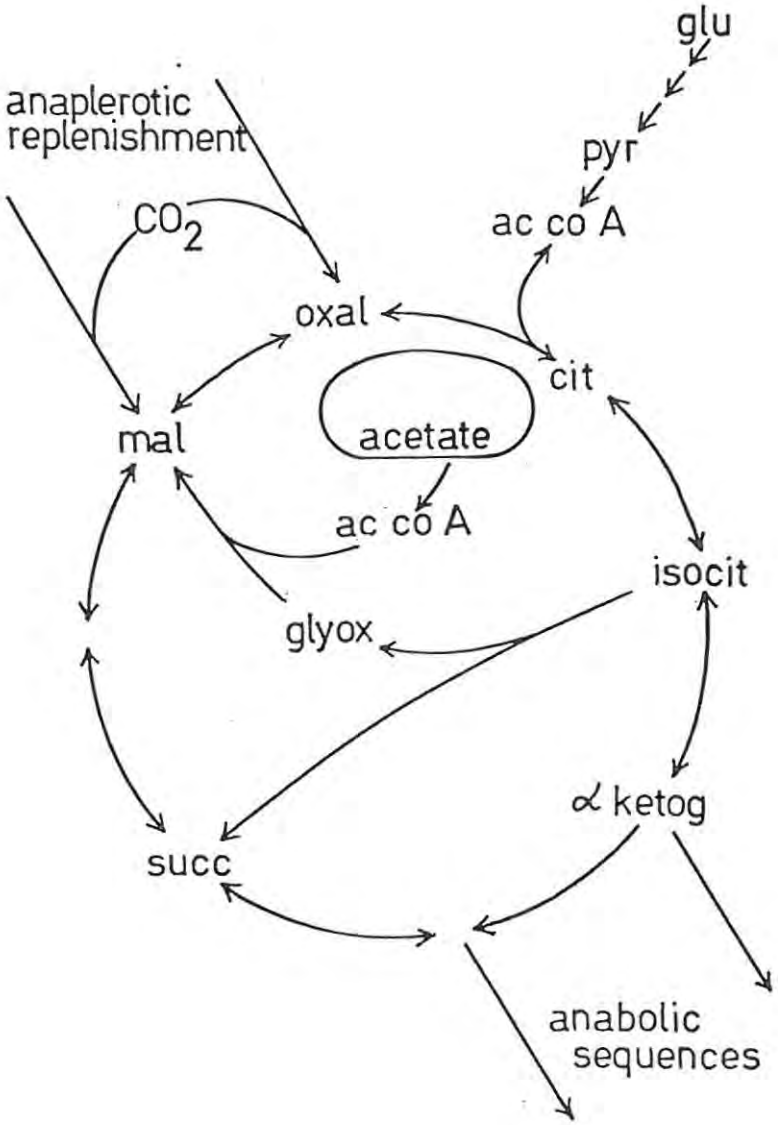


Figure 30. Schematic representation of drainage of the tricarboxylic acid cycle and of its anaplerotic replenishment. The alternate replacement of the intermediates by acetate-induced glyoxylate cycle is also shown.

but also further decreases the possibility that the major function of heterotrophic carbon dioxide fixation is in gluconeogenesis, since cells growing on acetate would presumably exhibit more gluconeogenesis than glucose grown cells. Acetate grown cells require to synthesize 6-carbon sugars from 2-carbon substrates by the gluconeogenetic pathway. As shown in Figure 6, carbon dioxide fixation was repressed by acetate within 24 hours. Little information is available on the time course of glyoxylate cycle induction in heterotrophically grown *Euglena*. Cook and Carver (1966) found that addition of acetate to autotrophic *Euglena* had completed the induction of malate synthase in 12 hours, while Collins and Merrett (1975) who added acetate to an autotrophic culture which was then transferred to the dark, showed that the specific activity of malate synthase continued to increase for up to 48 hours after transfer to heterotrophic growth on acetate.

An aspect of the "acetate effect" may be concerned with an earlier observation made in this study (shown in Figures 1 and 3) that autotrophic cells increase heterotrophic carbon dioxide fixation 10 fold during the growth cycle, whereas glucose grown cells show only a 4-fold increase. (The same difference between the two types of culture was observed when heterotrophic carbon dioxide fixation was stimulated by "ammonium treatment"). It was shown in Figure 6 that addition of acetate to a glucose culture represses heterotrophic carbon dioxide fixation to a barely detectable level, even in the continued presence of glucose, whereas Figure 7 showed that addition of acetate to an autotrophic culture causes a comparatively small decrease in heterotrophic carbon dioxide fixation. These results are consistent with earlier work where the activities of glyoxylate cycle enzymes were measured under various conditions. For example, Heinrich and Cook (1967) found that glucose did not inhibit the synthesis of malate synthase but Cook (1965) found that light inhibited respiratory adaptation of *Euglena* to acetate,

and Cook and Carver (1966) found that light inhibited the induction of glyoxylate cycle enzymes by acetate. Further, it was shown that *Euglena* grown heterotrophically on glucose alone contain measurable amounts of malate synthase and isocitrate lyase activities, but that these enzymes are not detectable in *Euglena* grown autotrophically (Heinrich and Cook, 1967; Peak, 1972). Thus it is possible that glucose grown *Euglena* (but not *Euglena* grown autotrophically) are able to utilize the glyoxylate cycle partially and are thus not so dependent on heterotrophic carboxylations for anaplerosis as are autotrophically grown cells. This consideration also supports anaplerosis as a major function of heterotrophic carbon dioxide fixation.

(C) The regulation of heterotrophic carbon dioxide fixation by ammonium
Further evidence supporting the involvement of *Euglena* heterotrophic carbon dioxide fixation in anaplerotic replenishment was provided by the finding that one component of the fixation is regulated by ammonium, described by Peak and Peak (1977). It was found that if ammonium was supplied to cells previously starved of that ion, the cells' heterotrophic carbon dioxide fixation was increased. It is evident that the function of this component of dark fixation is intimately linked to ammonium. This does not support an involvement of this component in gluconeogenesis, since this is not closely involved with nitrogen metabolism. For the same reason it is unlikely that fatty acid biosynthesis is a major function of the ammonium regulable component of dark carbon dioxide fixation. However, if heterotrophic carboxylations function in anaplerosis of the tricarboxylic acid cycle, a link with ammonium supply would be feasible, since ammonium is necessary for instance for amino acid biosyntheses by aminations, and the biosyntheses of some amino acids requires tricarboxylic acid cycle intermediates such as α-ketoglutarate as carbon skeletons. It is possible that as exogenous ammonium becomes depleted biosynthesis of amino acids

diminishes. Replenishment of the medium with more ammonium may allow for an upsurge of amino acid biosyntheses, some of which cause concomitant drainage of tricarboxylic acid cycle intermediates, thus necessitating anaplerotic replenishment. This possibility is supported by the fact that ammonium starved cells, when supplied with more ammonium, take up ammonium from the medium concomitant with an increase of their heterotrophic carbon dioxide fixation, whereas non-starved cells do not take up ammonium when it is supplied, nor do they increase heterotrophic carbon dioxide fixation (Table 2).

No direct measurements of an upsurge in amino acid biosynthesis after provision of ammonium have been reported for *Euglena*. Reisner *et al.* (1960) found that supplying ammonium to *Chlorella* previously starved of nitrogen caused an increase in formation of amino acids, in some cases measureable within 15 minutes after supplying ammonium. The largest increases were observed in the production of glutamine, glutamate, alanine, asparagine and aspartate. Stimulation of amino acid biosynthesis by ammonium is further discussed below.

(D) Stimulation of glutamine production by ammonium treatment

If ammonium stimulates heterotrophic carbon dioxide fixation in order to replenish the tricarboxylic acid cycle during periods of increased amino acid biosynthesis, it is likely that the extra carbon dioxide fixed will be found in amino acids derived from tricarboxylic acid cycle intermediates. When fixation products of control cells were compared by means of chromatography with products of cells whose heterotrophic carbon dioxide fixation had been stimulated by ammonium treatment, a high proportion of the extra counts were found in glutamine (Table 6, Figure 29). A smaller increase in incorporation of labelled carbon dioxide into glutamate was also observed. This indicated that the supplied ammonium stimulated formation of these amino acids and that carbon dioxide was entering them via the tricarboxylic

acid cycle, since α-ketoglutarate provides the carbon skeleton for the synthesis of glutamate and glutamine.

Other pathways of heterotrophic carbon dioxide fixation Figures 28 and 29 and Table 6 also showed that ammonium stimulation causes increased incorporation of labelled carbon dioxide into other amino acids not derived from the tricarboxylic acid cycle intermediates, for example glycine, serine and alanine which are all derived from the glycolytic pathway. The largest increase in incorporation of labelled carbon dioxide after ammonium treatment is into glycine. Glycine and serine are synthesized from 3-phosphoglycerate (which was also observed to be one product of dark carbon dioxide fixation) and alanine from pyruvate. (The amino groups for the formation of these amino acids, as well as glutamine, are derived from glutamate. This may be the reason why glutamate does not show as large an increase after ammonium treatment as the other amino acids. Possibly glutamate does not accumulate because it is being utilized to provide amino groups for the synthesis of glutamine, glycine etc.) Incorporation of carbon dioxide into glycine and serine during dark carbon dioxide fixation was also observed by Lynch and Calvin (1953) as was the incorporation into sugar phosphates and 3-phosphoglycerate. The fact that ammonium stimulates the dark fixation of carbon dioxide into 3-phosphoglycerate and into glycine and serine which are both derived from this compound may confirm the suggestion by Lynch and Calvin that *Euglena* fixes carbon dioxide heterotrophically by more than one route. However, the exact path by which carbon dioxide first enters 3-phosphoglycerate is still not certain.

A possible route, described in the introductory section (p. 30), was suggested by Moses *et al.* (1959). They proposed that carbon dioxide might enter 3-phosphoglycerate by way of 3-phosphogluconic acid, by a reversal of the decarboxylation of this compound to pentose phosphate. Although incorporation

of the labelled carbon dioxide into 3-phosphogluconate has not so far been shown in *Euglena*, Moses *et al.* (1959) observed that this compound is an early product of dark carbon dioxide fixation in *Zygorrynchus moelleri*. Although the reaction in the direction of decarboxylation is favoured, the reaction has been shown to be reversible in yeast (Horecker and Smyrniotis, 1952) so this could be a feasible route for the entry of carbon dioxide into 3-phosphoglycerate. The enzyme catalysing the reaction, phosphogluconate dehydrogenase (E.C.1.1.1.44) has been shown to be present in *Euglena* (Smillie, 1968). Another possibility is that originally suggested by Lynch and Calvin (1953) that carbon dioxide enters 3-phosphoglycerate directly as in photosynthesis but without using light energy. Although the exact pathway for the incorporation of carbon dioxide is still not known, the results shown in Figure 29 and Table 6 indicate that heterotrophic carbon dioxide fixation, as well as functioning in anaplerotic replenishment, is also involved in the formation of amino acids such as glycine and alanine, and that this component of the fixation is also regulated by ammonium. No further investigation into this component of heterotrophic carbon dioxide fixation in *Euglena* was carried out in this study.

Another possible pathway for heterotrophic incorporation of carbon dioxide in *Euglena* was suggested by the fact that a small percentage of labelled carbon dioxide was fixed into citrulline in the ammonium stimulated cells. Hiller (1964) found that ammonium stimulated dark carbon dioxide fixation in *Chlorella* and that in the presence of ammonium more carbon dioxide was fixed into citrulline and arginine, whereas in control cells the main products of dark carbon dioxide fixation were associated with the tricarboxylic acid cycle. He suggested that the results were consistent with the possibility that, in *Chlorella*, the presence of ammonium stimulates not only the carboxylation of phosphoenolpyruvate to oxaloacetate, but also the carboxylation of ornithine to citrulline and hence arginine, using some of the reactions of

the urea cycle. In mammals the first step in urea synthesis is the formation of carbamyl phosphate involving carbon dioxide, ammonia and ATP. It is probable that these reactions also occur in *Euglena*, since this pathway is a route for the formation of arginine. However, as far as is known the enzymes catalysing these reactions have not been detected in *Euglena*.

The significance of the smaller, non-ammonium regulable component of dark fixation in *Euglena* is still not clear. As already described (Figure 16 and 17) cells which have not been stimulated by ammonium treatment fix carbon dioxide in the dark at a constant level (about 1/10 the level of fixation of stimulated cells in the case of autotrophic *Euglena*). Since it has been shown that incorporation of carbon dioxide into glycine and serine, via 3-phosphoglycerate, is stimulated by ammonium it does not appear as if this pathway of heterotrophic carbon dioxide fixation corresponds to the smaller, basal component of fixation, as suggested above. However it is possible that this smaller component may at least in part, have the same function as the major moeity. It may represent the basal amount of anaplerotic replenishment required by the cell in the absence of ammonium stimulation. The fact that glutamate and aspartate were found to be major products of heterotrophic carbon dioxide fixation in non-stimulated cells supports this possibility. However, although gluconeogenesis and fatty acid biosynthesis are not considered to be the major functions of heterotrophic carbon dioxide fixation in *Euglena* they must occur in this organism. (Although gluconeogenesis, as described in the introductory section, involves both a carboxylation and a decarboxylation step, early workers measured incorporation of labelled carbon dioxide into liver glycogen (Wood and Utter, 1965).) The non-ammonium regulable component of fixation may encompass these functions. More work is necessary before this can be elucidated.

Implications of the regulation of heterotrophic carbon dioxide fixation by ammonium

(A) Previous work on heterotrophic carbon dioxide fixation in *Euglena*

The effect of exogenous ammonion on *Euglena* heterotrophic carbon dioxide fixation was apparently not known to previous workers and may have affected some of their results. By working with cells from the mid-logarithmic growth phase, at which stage exogenous ammonium is considerably depleted (Figure 14) and measuring fixation in fresh medium, the cells would be in the stimulated condition. Thus the finding by Heinrich and Cook (1967) that glucose grown *Euglena* fixed twice as much carbon dioxide as acetate grown cells may have been due to the fact that fixation by the glucose grown cells was stimulated but not that by the acetate grown cells. In the present study it was found that the amount of heterotrophic carbon dioxide fixation by non-stimulated glucose grown cells is practically the same as for acetate grown cells (Figures 3 and 4).

Another instance of a possible ammonium effect is in the results found by Codd and Merrett (1971) who measured dark carbon dioxide fixation by *Euglena* at various phases of a synchronous cycle. The fixation was measured in fresh whole medium, which contained ammonium. They found that fixation increased during the light phase of the cycle, reached a peak in early dark phase and then declined. A possible explanation for their results may be that ammonium in the medium was increasingly depleted by the cells up until their synchronous division during the dark phase.

(B) Kinetic analysis of regulation of heterotrophic carbon dioxide fixation by ammonium

The kinetic studies, the results of which are presented in Figures 18 - 22, revealed complex control mechanisms whereby the cell apparently avoids

unnecessary carbon dioxide fixation. By this means the cell presumably conserves energy since cellular energy is required for heterotrophic carbon dioxide fixation in order to form the covalent bond. For instance Figure 19 showed that complete ammonium starvation is necessary before heterotrophic carbon dioxide fixation is increased to maximum level after replenishment with ammonium. Thus it may be that, provided the cells have some ammonium available for biosyntheses, stimulation of heterotrophic carbon dioxide fixation by added ammonium is inhibited. However, as shown in Figure 21, when cells are provided with very high concentrations of ammonium this inhibition appears to be overridden. Once cells have been completely starved of ammonium however, only trace amounts of ammonium are required to stimulate heterotrophic carbon dioxide fixation, indicating the capacity for renewed aminoacid biosynthesis after ammonium starvation.

As regards the time course of ammonium starvation and subsequent replenishment, it was shown in Figure 18 that a 7 hour period after removal of all exogenous ammonium from the medium is necessary to sensitize cells to stimulation by added ammonium. This result is consistent with that shown in Figure 19 and also suggests that the presence of even small amounts of ammonium inhibits the stimulation of heterotrophic carbon dioxide fixation by added ammonium. Possibly after 7 hours the level of amino acid biosynthesis has diminished to such an extent that stimulation of dark fixation is no longer inhibited.

Once cells are fully sensitized by ammonium starvation, stimulation of heterotrophic carbon dioxide fixation by added ammonium is a rapid event. Figure 22 shows an increase in fixation in the first 20 minutes after resupplying ammonium. However, larger increases in fixation occur if cells are held for periods of up to 30 minutes in the presence of

ammonium before measuring fixation. Possibly there is an increase in amino acid biosynthesis with time after ammonium is supplied to the starved cells. It is probable that the increased heterotrophic carbon dioxide fixation measured after 60 minutes dark holding in mineral medium, shown in Figure 2, represents the same effect. Also shown in Figure 22 is the fact that heterotrophic carbon dioxide fixation declines compared with the maximum level if the cells are held in the presence of ammonium for longer than 30 minutes before measurement of fixation. A possible reason for this is that amino acid biosynthesis declines because the fresh supply of ammonium has been utilized after 30 minutes, and this may cause a concomitant decline in stimulation of fixation. It was shown in Table 2 that ammonium starved cells take up 1.25 umoles of NH_4Cl from the medium per 10^6 cells during a 20 minute period of carbon dioxide fixation, whereas no uptake by non-starved cells was measured. Another possibility is that the decline in measured fixation is an isotope dilution effect, possibly due to an increase in proportion of unlabelled carbon dioxide caused by respiration. The effect of ammonium replenishment on respiration of ammonium-starved *Euglena* has not been investigated. However, Syrett (1953) found increases in respiration of nitrogen starved *Chlorella* when ammonium was resupplied.

(C) Significance of the nitrogen compound supplied

When ammonium starved cells are resupplied with nitrate instead of ammonium (at the same molarity) an increase in dark carbon dioxide fixation is observed, as reported in the results (p. 77) but of a smaller magnitude compared with ammonium stimulation. This may be because *Euglena* may not utilize nitrate so readily as ammonium. Little work has been reported on the ability of *Euglena* to utilize various sources of nitrogen. Cramer and Myers (1952) reported that *Euglena gracilis* var *bacillaris* was not

able to grow on nitrate as sole carbon source. In *Chlorella* Syrett (1956) showed that nitrate has a smaller stimulating effect on heterotrophic carbon dioxide fixation by nitrogen starved cells than ammonium. Later he reviewed the subject of nitrogen assimilation by algae (Syrett, 1962). He stated that most algae use ammonium in preference to nitrate, and that if both are supplied the ammonium will be used first. This preferential utilization of ammonium is caused by the repression of nitrate reductases by ammonium. It is possible that a similar situation may exist in *Euglena* (however, since algae are plants and *Euglena* can live as an animal this may not necessarily be the case). This possibility has not been explored.

A mechanism for the regulation of heterotrophic carbon dioxide fixation by ammonium This section is concerned only with the component of heterotrophic carbon dioxide fixation involved in anaplerosis. With the knowledge that heterotrophic carbon dioxide fixation in *Euglena* can be regulated experimentally by ammonium, it was possible that 'ammonium treatment' might have a direct effect on the carboxylating enzyme(s) responsible for dark carbon dioxide fixation. In order to ascertain whether ammonium treatment resulted in induction of carboxylating enzymes activities of various carboxylating enzymes were measured in extracts from both ammonium stimulated and non-stimulated cells, using the normal regime for ammonium stimulation. As shown in Table 4 there is an increase in specific activity of PEP carboxylase after ammonium stimulation but it is not large enough to account for the entire increase in heterotrophic carbon dioxide fixation following ammonium treatment. For example, the specific activity of PEP carboxylase from autotrophic cells increased 1.6 fold following ammonium stimulation of the cells, whereas it has been shown repeatedly, for example in Figure 15, that ammonium stimulation of autotrophic cells causes a 10 fold increase in

heterotrophic carbon dioxide fixation. However, part of the increase in heterotrophic carbon dioxide fixation caused by ammonium treatment represents an increase in formation of glycine, serine and alanine, which is unlikely to be catalysed by PEP carboxylase, so it would not be expected that PEP carboxylase would increase to such an extent as does whole cell heterotrophic carbon dioxide fixation following ammonium treatment. Although the effect of ammonium treatment on PEP carboxylase is small, the information compiled in Table 4 points to the fact that PEP carboxylase is a major enzyme responsible for an important component of heterotrophic carbon dioxide fixation in *Euglena*. The findings made in the present study on whole cell carbon dioxide fixation in *Euglena* suggest, as described above, that a major function of dark carbon dioxide fixation in this organism is anaplerotic replenishment of the tricarboxylic acid cycle. PEP carboxylase could function in this way, as was assumed by Ohmann and Plhak (1969), also by Codd and Merrett (1971).

It is shown in Table 4 that PEP carboxylase is present in extracts from *Euglena* grown autotrophically and heterotrophically on glucose or acetate. Moreover the specific activity of PEP carboxylase from autotrophic cells was found to be 3 times greater than that from the glucose heterotrophic cells. It has been observed frequently in this study that ammonium stimulated autotrophic cells fix more carbon dioxide heterotrophically (an average value is 130 x 10^3 cpm/10^6 cells) than do heterotrophic cells (average value 60 x 10^3 cpm/10^6 cells). Also autotrophic cells show a greater increase in heterotrophic carbon dioxide fixation after ammonium treatment, 10 fold increase compared with 4-5 fold increase in heterotrophic cells. One reason for the apparently greater requirement for heterotrophic carbon dioxide fixation reactions in autotrophic cells is possibly the complete absence of glyoxylate cycle activity discussed above. The acetate

grown cells contain slightly less PEP carboxylase activity than the glucose grown cells, which also correlates with the reduced requirement for heterotrophic carbon dioxide fixation reactions in cells growing on 2-carbon substrates. Thus the specific activities of PEP carboxylase in *Euglena* cultured under various conditions correlate with the observed whole cell heterotrophic carbon dioxide fixation.

As regards malate enzyme, although this enzyme was also found to be present in all types of culture, the specific activities do not correlate with the observed whole cell heterotrophic carbon dioxide fixation. Malate enzyme was found to be $\pm$ 8 times more active in glucose heterotrophic cells than in autotrophic cells, confirming earlier results of Peak *et al.*, (1973). They previously established that malate enzyme activity in *Euglena* is reduced by light (Peak *et al.* 1972a). The activity of malate enzyme in acetate grown cells was found to be twice that for glucose grown cells (the fact that malate enzyme is more active in acetate, compared with glucose grown cells was also observed by Heinrich and Cook, 1967). Clearly there is no correlation between malate enzyme activity and requirement for heterotrophic carbon dioxide fixation reactions. The function and regulation of malate enzyme has been the subject of previous study (Peak *et al.*, 1973). It was shown that malate enzyme was more active in glucose grown heterotrophic cells than in autotrophic cells. Also, in autotrophic cultures there was no change in malate enzyme specific activity throughout the growth cycle, whereas it increased fourfold during heterotrophic growth cycles. It was found that these changes correlated with an increased requirement for fatty acid biosynthesis towards the end of the growth cycle in heterotrophic cells, and it was suggested that malate enzyme in *Euglena* functions in the direction of decarboxylation, serving to provide a source of NADPH for fatty acid

biosynthesis. It is possible that the high activity of malate enzyme found in acetate grown cells also supports this hypothesis, since acetate is a precursor of fatty acids. These considerations together with the results in Table 4 render it improbable that malate enzyme is important in heterotrophic carbon dioxide fixation in *Euglena*.

Of the other enzymes measured, only acetyl-CoA carboxylase was found to be active in autotrophic cells. The absolute activity of acetyl-CoA carboxylase is less than that of PEP carboxylase. Acetyl-CoA carboxylase is known to be involved in fatty acid biosynthesis. As discussed above, it is unlikely that the major, ammonium regulable moeity of heterotrophic carbon dioxide fixation in *Euglena* has this function. This is also indicated by the fact that ammonium treatment decreased the specific activity of acetyl-CoA carboxylase in both autotrophic and heterotrophic cells. Acetyl-CoA carboxylase is probably involved in a proportion of the measured dark carbon dioxide fixation, possibly the smaller non-regulable moeity, as discussed above, however this was not investigated further. The expense of assaying this enzyme was beyond the economic scope of this project.

Pyruvate carboxylase activity was observed in glucose heterotrophic cultures. As far as is known this is the first positive report of pyruvate carboxylase from this organism. Codd and Merrett (1971) did not detect pyruvate carboxylase activity in their extract, however they worked only with light/dark synchronized cultures. Since pyruvate carboxylase is present in only trace amounts in the glucose culture, according to the method used, and is not detectable in autotrophic cells, it was not considered to be of major importance in *Euglena* heterotrophic carbon dioxide fixation and was not investigated further. Similarly,

PEP carboxykinase (E.C.4.1.1.32) is barely evident in the acetate culture only and therefore can not be the enzyme responsible for heterotrophic carbon dioxide fixation as measured in this study. Ohmann (1969) reported having measured PEP carboxykinase in *Euglena* in previous unpublished work, and stated that the activity was found to be higher in acetate grown cultures than in glucose heterotrophic or autotrophic cultures. It is probable that PEP carboxykinase functions in *Euglena* in gluconeogenesis which is of particular importance in the acetate grown cells which have to build up 6-carbon sugars from 2-carbon substrates. As described in the introductory section (p.18) PEP carboxykinase is considered to function in gluconeogenesis in the decarboxylation step, catalysing the formation of PEP from oxaloacetate. As discussed above, gluconeogenesis is not considered to be the function of the major, ammonium regulable moeity of heterotrophic carbon dioxide fixation in *Euglena*.

For these reasons it appeared that PEP carboxylase is the enzyme responsible for at least the part of the major moeity of heterotrophic carbon dioxide fixation in *Euglena*, functioning in anaplerotic replenishment and leading to the biosynthesis of glutamine and glutamate. The properties of this particular enzyme were thus investigated further. Although the increase in specific activity of PEP carboxylase after ammonium stimulation of the cells is not sufficient to account for the increase in heterotrophic carbon dioxide fixation caused by ammonium stimulation, it was possible that ammonium stimulation might cause for example a modification in the distribution of PEP carboxylase isoenzymes. Perl (1974) separated two isoenzymes of PEP carboxylase from *Euglena*, but he was unaware of the stimulating effect of ammonium on heterotrophic carbon dioxide fixation. In this study, for the first time, PEP carboxylase isoenzymes were purified from cultures which had been previously stimulated by the ammonium treatment

in order to compare them with those from control cultures. The results as regards the effect of ammonium stimulation were negative since the ammonium treatment did not cause any changes in the distribution of PEP carboxylase isoenzymes. However Perl's results were largely confirmed and elaborated. One difference noted in the present study was in the proportion of the total activity recovered in the two isoenzymes. The present study indicated that in both autotrophic and heterotrophic cells the two isoenzymes are present in the same proportions i.e. 75% of the total recovered activity was in isoenzyme 1. Perl (1974) found that the distribution of recovered activity differed between autotrophic and heterotrophic cultures. In autotrophic cultures 60% of the activity was in isoenzyme 1 (his fraction A) whereas only 20% of the activity recovered from a heterotrophic culture was in isoenzyme 1. It may be significant, however, that in his separation of isoenzymes from the heterotrophic culture, only 19% of the original activity was recovered altogether.

The investigation into the effects of ammonium on the characteristics of PEP carboxylase isoenzymes revealed no significant effects as shown by DEAE cellulose chromatography. There is no significant difference between the control and ammonium stimulated cells as regards the number of isoenzymes eluting, the Tris concentration at which they elute or the proportion of recovered activity found in each isoenzyme. As shown in Table 4, ammonium treatment causes an increase in specific activity of PEP carboxylase, and this increase may occur in both isoenzymes. It was not possible to detect any such increase in the elution profiles, possibly because the difference in specific activity is too small and because of the instability of the isoenzymes. The fact that no new isoenzyme was found after ammonium treatment indicates that the increase in whole cell heterotrophic carbon dioxide fixation after ammonium treatment is not due

to the *de novo* biosynthesis or induction of another PEP carboxylase isoenzyme. However, the possibility that the ammonium treatment causes induction of the existing isoenzymes cannot be ruled out.

Another possibility is that the increase in whole cell heterotrophic carbon dioxide fixation may be due to stimulation of PEP carboxylase. If this is so, it does not seem likely that ammonium itself is the immediate regulatory ligand, since addition of NH_4Cl in concentrations up to 40 mM has no effect on PEP carboxylase activity *in vitro*. The stimulation of dark fixation may be due to changing metabolic pools resulting in an increase or decrease of stimulating or inhibiting metabolites respectively. A possible mechanism whereby this may occur is described below. A further possibility is that heterotrophic carbon dioxide fixation may be increased after ammonium treatment by reduction in the turnover rate of PEP carboxylase, since the prevailing concentration of a eukaryote enzyme depends both on the rate of its formation and of its degradation (Stadtman, 1970).

The inhibition of PEP carboxylase by various substrates was first measured by Ohmann and Plhak (1969) who worked with only one isoenzyme, and subsequently by Perl (1974) as described in the introductory section (p.33). The present study has essentially confirmed and elaborated the results of Perl, with certain inconsistencies. For example, inhibition of PEP carboxylase by malate, demonstrated by Perl, was confirmed (Figure 24). However, Perl only observed inhibition of isoenzyme II by malate, whereas isoenzyme I was stimulated. It was shown by Figure 27 that malate apparently stimulates whole cell carbon dioxide fixation by non-ammonium stimulated cells. The kinetics of the stimulation are similar to those of Perl (1974) i.e. an increase in fixation was caused by 1 mM malate, followed by a subsequent decline in fixation at higher malate concentrations.

This result is not understood, but since it was observed in the case of non-stimulated cells only it may possibly reflect a stimulatory effect of malate on the smaller, non-ammonium regulable component of heterotrophic carbon dioxide fixation.

The inhibition of PEP carboxylase by tricarboxylic acid cycle intermediates may provide support for a possible mechanism whereby ammonium stimulates heterotrophic carbon dioxide fixation in *Euglena* for anaplerotic replenishment. The relative importance of the control exerted by these intermediates in the metabolism of *Euglena* has yet to be established, however, one possibility is that high pool levels of malate, citrate and succinate could inhibit PEP carboxylase by negative feedback inhibition and so cause reduction of dark fixation. The introduction of ammonium to a previously ammonium starved culture might result in an increase in amino acid biosynthesis with concomitant draining of the tricarboxylic acid cycle. The diminishing levels of the intermediates might stop their inhibition of PEP carboxylase, thus allowing for the carboxylation of PEP to replenish the cycle. This control would be of a multivalent nature in that all the intermediates, malate, citrate, succinate, would have to be low before their inhibition of PEP carboxylase ceased. It is not known at this stage whether the multivalent inhibition is cumulative or concerted feedback inhibition. Control of the fixation of carbon dioxide into glycine, alanine and serine by ammonium is less well understood, since the enzyme involved in this particular carboxylation has not yet been studied.

The fact that similar inhibition kinetics were obtained for both the isoenzymes of PEP carboxylase (Figure 24) indicates that they may both be involved in the same function . The explanation for having two isoenzymes of PEP carboxylase both involved in anaplerotic replenishment remains

obscure. The possibility exists that one isoenzyme of PEP carboxylase is involved in the larger, ammonium regulable component of heterotrophic carbon dioxide fixation while the other is involved in the smaller, basal moeity. While this does not appear to be likely if the two components have different functions, it is also possible that the smaller component of fixation represents a constant basal level of anaplerosis as described above. One difficulty in further elucidating the reason for the presence of two isoenzymes lies in their instability. A means of stabilizing these isoenzymes must be found before more information can be obtained.

AFTERWORD

This investigation, following on from the initial observation that *Euglena* manifests marked modifications in whole cell dark carbon dioxide fixation during batch growth cycles, has resulted in increased understanding of the functions and regulation of these particular metabolic processes. The wide range of growth conditions under which this organism flourishes and the ease with which it can be manipulated in the laboratory have contributed to this increased insight. The use of abrupt changes in environmental conditions has again proved to be invaluable in elucidation of biochemical problems.

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