PHOTORESPIRATION AND GLYCOLLATE METABOLISM IN ALGAE

by

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ABSTRACT

Phaeodactylum tricornutum has a very low CO_2-compensation point like many other marine algae examined. A low activity of PEP Carboxylase coupled with the discovery of 3-PGA as the initial product of photosynthesis suggest that this is not due to a C_4 mechanism of photosynthesis. The 'Warburg effect' was shown by a number of algae including Phaeodactylum, but it was smaller than in tobacco and not reversible by increasing the CO_2 concentration. RuDP carboxylase from Ulva and spinach showed maximum CO_2 fixation in the absence of oxygen.

The release of $^{14}CO_2$ from the labelled photosynthate in CO_2- free air was much higher in Chlorella than in Phaeodactylum. In Chlorella it was also oxygen sensitive.

Phaeodactylum excreted little glycollate even in the presence of inhibitors of the glycollate pathway or on transfer from high to low CO_2. Cells photosynthesizing $^{14}CO_2$ under 100% oxygen failed to excrete glycollate.

Neither exogenous glycollate nor low concentrations of α-OHMS had any effect on the rate of photosynthesis in Phaeodactylum. Higher concentrations of α-OHMS proved toxic.

Phaeodactylum growing on glycollate as a sole carbon source showed maximum growth at 5 mM glycollate. The glycollate oxidising enzyme of Phaeodactylum was found to be a dehydrogenase although the rate of dark respiration was increased up to 2-fold by the addition of glycollate. It is suggested that

glycollate oxidation may couple to molecular oxygen *in vivo* but *in vitro* necessary components of the electron transport chain are lost so that the enzyme shows only dehydrogenase activity. The glycollic dehydrogenase of *Phaeodactylum* has about the same activity as that of *Chlorella* and appears to be constitutive. Other enzymes of the glycollate pathway were assayed and found to be present in *Phaeodactylum* but only low rates of $^{14}CO_2$ production were observed when glycollate-1-^{14}C or glycine-1-^{14}C were fed to the intact cells. Possible explanations for this are discussed.

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INTRODUCTION

It is not easy to define photorespiration in a sentence. Jackson & Volk (1970) realising the difficulties involved used the term photorespiration in a broad sense to describe all processes by which CO_2 is released and O_2 consumed by green tissues in the light.

It may be elaborated that the CO_2 evolution may take place during photosynthesis from current photosynthate (Jackson & Volk, 1970; Hatch, Osmond & Slatyer, 1971) or from the stored products of a previous period of photosynthesis (Goldsworthy, 1966; Holmgren & Jarvis, 1967; Chen & Colman, 1974). The latter conclusion was suggested by Goldsworthy (1966) from his experiments with ^{14}C-labelled tobacco leaf segments when he found $^{14}CO_2$ evolved in the light under conditions when no net photosynthesis was taking place. He also observed that the specific activity of $^{14}CO_2$ evolved in the light was much higher than that evolved in the dark, from which he concluded that the substrate for photorespiration was different from that for dark respiration.

Photorespiration in higher plants may be measured either by measuring O_2 uptake or CO_2 evolution. Both methods have their drawbacks because of the difficulty of making precise measurements in the presence of true respiration which also involves an O_2 uptake and CO_2 evolution and of photosynthesis which carries out the reverse overall reaction. The error, due to respiration, appears to be small, the rate of photores-

piration being several times higher than true respiration (Decker, 1957; Zelitch, 1969). The effect of photosynthesis may be minimized by putting the plant in a CO_2-free atmosphere and measuring its CO_2 output. However, this may result in an underestimate, because some of the CO_2 released may be refixed, although the error can be reduced by using fast air flow rates to sweep most of the CO_2 away from the leaf before it is refixed (Zelitch, 1971). Similarly, photorespiration measured as the rate of $^{18}O_2$ uptake could be underestimated because $^{16}O_2$ evolved during photosynthesis could also be taken up.

Another method of measuring photorespiration (Decker, 1955) is to switch off the light and measure the peak rate of CO_2 evolved immediately afterwards. This post-illumination CO_2 burst is considered to be a result of photorespiratory reactions carrying on in the dark period for a minute or so before being followed by a much lower level of CO_2 release due to true respiration. This method, however, is based on the assumption that photosynthesis stops immediately when the light is turned off, and the translocation of the photorespiratory substrate from the site of synthesis to the site of CO_2 production is unaltered. Clearly these assumptions may not be correct. Further details of measuring photorespiration and their drawbacks have been outlined by Goldsworthy (1970) and Jackson & Volk (1970) in their reviews.

Glycollate is believed to be the substrate for photores-

piration. It is metabolized via glyoxylate, glycine, serine and glycerate ultimately into carbohydrates in light, and malate in the dark (Tolbert, 1963). The CO_2 of photorespiration appears to be evolved during the conversion of two molecules of glycine into one of serine.

Low partial pressures of CO_2, high partial pressures of O_2 and high light intensity seem to favour glycollate biosynthesis in tobacco leaf discs after blocking the oxidation of glycollate in vivo with alpha-hydroxymethane sulphonate (αOHMS), a powerful inhibitor of glycollic oxidase (Zelitch, 1957). Glycollate formation was negligible in an atmosphere of nitrogen containing 300 ppm of CO_2 compared with normal air. Similar results have been reported by Fock & Egle (1967) in bean leaves.

Parallel results for Photorespiration were observed by Forrester, Krotkov & Nelson (1966) who found near zero photorespiration rates in soyabean leaves in the absence of O_2, which increased with increase in O_2 concentration over the full range of 0-100%, this increase also being reflected in an increase in the CO_2 compensation point.

An inhibitory effect of higher CO_2 concentration on glycollate synthesis was observed by Tolbert (1963) in sugar-beet leaves, where glycine and serine, early products of glycollate metabolism were strongly labelled in CO_2 concentration below 300 vpm, but this labelling was 80% in-

hibited at 0.3% $^{14}CO_2$. Zelitch & Walker (1964) and Zelitch (1965a) have reported that in tobacco glycollate biosynthesis in light was about 29% less in CO_2-free air than in normal air, about the same at 700 vpm CO_2 as in air and was 60% inhibited in 1.8% CO_2. Ellyard & Gibbs (1966) have reported a similar inhibitory effect of CO_2 on the rate of glycollate synthesis in isolated spinach chloroplasts.

High O_2 concentrations (20-100%) inhibit the rate of photosynthesis in many plants. This was first observed by Warburg (1920) in Chlorella and is known as the Warburg effect. He demonstrated that with increasing O_2 concentrations the rate of photosynthesis fell off in Chlorella. This effect was diminished by increasing the CO_2 concentration. Ellyard & Gibbs (1969) concluded that the Warburg effect is the result of an increased proportion of the total carbon fixed being channeled into glycollate in response to increased O_2 concentration. Turner & Brittain (1962) have provided some evidence that part of this phenomenon may also be caused by an inhibition by O_2 of enzymes of the photosynthetic carbon reduction cycle, photosynthetic electron transport or photophosphorylation.

Light appears to be essential for glycollate production and photorespiration in green tissues. The total rate of respiration is several times higher in light as compared to darkness (Decker, 1957; Zelitch, 1968) although the inhibition of the 'dark' component of respiration by light was reported by Benson & Calvin (1950) who found less incorporation of ^{14}C-labelled photosynthate into products of the citric acid cycle in

light. Holmgren & Jarvis (1967) observed that light at low intensity reduced the CO_2 efflux in Rumex acetosa, but higher light intensity promoted an efflux of CO_2 due to photorespiration. Goldsworthy (1970) interpreted it by saying that low light intensity inhibited the dark respiration, but higher light intensity was required for maximum rate of photorespiration.

It now seems probable that the effect of light in promoting photorespiration is due to its having an indirect activating influence on the carbon dioxide fixing enzyme RUDP carboxylase (Jensen & Bassham, 1968b). The effect of light on the enzyme appears to be mediated by changes in the magnesium concentration in the chloroplast stroma brought about in the light by chemiosmotic phenomena, the enzyme being activated by magnesium (Jensen, 1969). Since this enzyme is now believed also to synthesize the glycollate which is oxidized in photorespiration then light could clearly control the rate of photorespiration by acting at this point.

Although glycollate synthesis was discovered in the early experiments of Benson & Calvin (1950), its mechanism of formation remained obscure for a long time. Whittingham, Bermingham & Hiller (1963) proposed that the photosynthetic CO_2 acceptor was the source of glycollate when they demonstrated glycollate ^{14}C synthesis from glucose-^{14}C at low CO_2 concentration. They reasoned that at low CO_2 concentration, more CO_2 acceptor molecules would be available

which would break up to yield glycollate.

Tolbert (1963) claimed that phosophoglycollate is the immediate precursor of glycollate since it is a $^{14}CO_2$ fixation product and a highly specific phosphatase for its irreversible hydrolysis exists both in higher plants (Richardson & Tolbert, 1961) and algae (Yu, Tolbert & Orth, 1964). Its location in spinach chloroplast fractions (Thompson & Whittingham, 1967) gives further support to this theory, although objections have been raised to this work by Tolbert et al, (1968) who discovered phosphoglycollate phosphatase in both chloroplast and mitochondrial preparations. Zelitch (1965a) also has shown some doubt about the importance of phosphoglycollate in glycollate biosynthesis, because tobacco leaves photosynthesizing under $^{14}CO_2$ showed the specific activity of the carbon atoms of phosphoglycollate as only about one-tenth of the specific activity in glycollate.

Glycollate formation from the sugar phosphate intermediate of the Calvin cycle by the oxidation of a 2-carbon fragment was proposed by Wilson & Calvin (1955). Bradbeer & Racker (1961) supported this view when they demonstrated that isolated spinach chloroplasts produced glycollate when fed with fructose-6-phosphate apparently by the oxidation of the glycoaldehyde thiamine pyrophosphate addition complex from the transketolase reaction. However, all attempts to obtain high rates of glycollate synthesis from chloroplast preparations of spinach, beet and pea leaves have failed. The rates reported by Bradbeer & Anderson

(1967) are only 0.5% of the rates observed in intact leaves. Recently, Plaut & Gibbs (1970) and Shain & Gibbs (1971) elaborated on this mechanism by suggesting that the glycoaldehyde thiamine pyrophosphate complex is oxidised by an oxident generated in photosystem 2 or by H_2O_2 formed by photosystem 1 in a Mehler type reaction.

The work of Whittingham et al (1963) was extended by Ogren & Bowes (1971) who demonstrated a competition between O_2 and CO_2 for RUDP carboxylase. Bowes, Ogren & Hageman (1971) proposed that ribulose - 1,5 diphosphate is oxidised by RUDP carboxylase using molecular O_2 to yield one molecule of phosphoglycollate and a three carbon compound. Further evidence for this mechanism was provided by Andrews, Lorimer & Tolbert (1971) who were able to show the incorporation of $^{18}O_2$ into the carboxyl groups of the glycine and serine derived from glycollate during photosynthesis. Lorimer, Andrews & Tolbert (1973) identified the products of this reaction and showed them by a combination of gas chromatography and mass spectrometry to be phosphoglycollate and 3- phosphoglycerate. They described the enzyme responsible as RUDP oxygenase but were unable to separate it from RUDP carboxylase and they presumed the two proteins to be identical.

The site of glycollate synthesis is the chloroplast. Isolated spinach chloroplasts when fed with $^{14}CO_2$ in the light formed glycollate as a primary product (Arnon, Whatley

& Allen, 1959; Jensen & Bassham, 1966). It has been established that much of this glycollate then rapidly diffuses from the isolated chloroplasts into the suspending medium (Tolbert, 1958; Kearney & Tolbert, 1962). This suggests that the subsequent metabolism of glycollate may not be confined to the chloroplast.

A bulk of evidence has now accumulated using ^{14}C to show that glycollate is the substrate for photorespiration and glycine, serine and glycerate are intermediate products of glycollate metabolism. For example, glycollate was shown to be an early product of photosynthesis and became rapidly labelled when $^{14}CO_2$ was being photosynthesized in tobacco leaves (Hess & Tolbert, 1967). Serine and glycine were the only amino acids containing ^{14}C after short exposures (15 seconds) to $^{14}CO_2$ (Ongun & Stocking, 1965a). Rabson, Tolbert & Kearney (1962) demonstrated that when glycollate-2-^{14}C was supplied to wheat leaves in the light, it was incorporated equally into C_2 and C_3 of serine and glycerate. Infiltrating ^{14}C-labelled substrates revealed that the carboxyl carbon of glycollate was rapidly converted to $^{14}CO_2$ (Zelitch, 1966). Moss (1968) showed that the addition of glycollate increased the rate of CO_2 release into CO_2-free air by tobacco leaves.

Further evidence for the glycollate pathway was provided by the use of inhibitors. If glycollate is the main source of CO_2 produced in photorespiration, then blocking the glycine

to serine conversion or an earlier step in the pathway such as the oxidation of glycollate to glyoxylate should result in the accumulation of intermediates of the pathway. Experiments with glycollate analogues, the alpha-hydroxymethanesulphonates having the general structure R-CHOH-SO_3 have been carried out by Zelitch (1957, 1958). They cause an accumulation of glycollate in both higher plants and algae by inhibiting the oxidation of glycollate. Considerable glycollate accumulation with 10 mM alpha-hydroxy-methane-sulphonate (α-OHMS) was shown by Zelitch (1958) in tobacco leaves. When the inhibitor was added in the presence of $^{14}CO_2$ at 25°C, there was little effect on net $^{14}CO_2$ uptake and radioactivity in glycollate was 50% of the total ^{14}C assimilated as compared to 5% in the absence of the inhibitor (Zelitch, 1959). From this it was concluded that at least one half of the carbon fixed in photosynthesis in the normal leaf may be metabolized by the glycollate oxidation reaction, this being consistent with its major role in photorespiration. However these results should still be treated with caution, for α-OHMS has also been shown to close stomata under many conditions (Zelitch, 1961) and it may not be very specific for glycollate oxidase reaction, because it inhibits the activity of PEP carboxylase in a competitive manner and also slows malate-NAD^+ dehydrogenase (Osmond & Avadhani, 1970).

Once it has been formed glycollate is believed to leave the chloroplasts before being oxidised to glyoxylate. Evidence for this movement is provided by work on isolated spinach chloroplasts which produced large quantities of glycollate-^{14}C

from $^{14}CO_2$ in the supernatant fluid (Kearney & Tolbert, 1962). The glycollate oxidase of higher plants is present along with other enzymes of glycollate metabolism in sub-cellular particles called peroxisomes (Kisaki & Tolbert, 1969). This enzyme is not peculiar to green tissues and has been reported in fungi, bacteria, liver and castor-bean endosperm (Lord, 1971). However, one of its major functions seems to be related to photosynthesis, because when roots and potato tubers were allowed to green, glycollate oxidase activity developed within them (Tolbert & Burris, 1950; Mothes & Wagner, 1957).

Glycollate oxidase catalyses the irreversible oxidation of glycollate transferring its electrons to oxygen, H_2O_2 being produced as a byproduct (Zelitch, 1958). This enzyme appears to be an inducible enzyme at least in some organisms, because a several-fold increase in its activity was observed in etiolated leaves when they were sprayed with glycollate in the dark (Kuczamac & Tolbert, 1962). It oxidises L-lactate as an alternative substrate (Clagett, Tolbert & Burris, 1949; Zelitch & Ochoa, 1953). The enzyme is insensitive to inhibition by cyanide or sulphydryl reagents and is generally activated by adding FMN which is its prosthetic group.

It has been speculated that the oxidation of glycollate might be coupled to ATP production. Zelitch (1955) observed a high negative value of F^o (-41,000 cal/mole) in the oxidation of glycollate to glyoxylate coupled to molecular O_2. This would be sufficient to produce several molecules of

ATP if it were coupled to a phosphorylating mechanism. Evidence as to whether this actually occurs is conflicting and will be discussed later.

The glyoxylate produced as a result of glycollate oxidation can be photoreduced back to glycollate. This was observed by Kearney & Tolbert (1962) in isolated spinach chloroplasts. Added ^{14}C-glyoxylate rapidly moved into them and was converted into glycollate, whereas added ^{14}C-glycollate would not enter. This supported the existence of a glycollate-glyoxylate shuttle between the chloroplasts and the peroxisome. Its function is considered to consume excess photosynthetic reducing power and catalytic amounts of either glycollate or glyoxylate would result in the oxidation of NADPH without the release of CO_2 (Zelitch, 1971). CO_2 release would only occur if excess glyoxylate was further metabolized to serine. This hypothesis is supported by the observation that an NADPH-linked glycollate reductase occurred in the chloroplasts (Zelitch & Gotto, 1962). Another glycollate reductase occurs outside the chloroplasts in the peroxisomes. This is linked to NADH, and could, in theory, consume cytoplasmic NADH in a similar manner. Whether this reaction occurs to any significant extent is open to doubt since the enzyme has a very low affinity for glyoxylate. It has a much greater affinity for hydroxypyruvate and its true function may be to reduce this substance (Tolbert, 1971)

The conversion of glyoxylate to glycine can also occur in the peroxisomes and a glycine-glyoxylate aminotransferase

has been detected in them (Kisaki & Tolbert, 1969), though the activity of this enzyme is only a fraction of the glycollic oxidase activity. This suggests that a proportion of the glyoxylate may be secreted unmetabolised into the external cytoplasm. Since isolated peroxisomes do not release CO_2 when supplied with glycollate-^{14}C or glyoxylate-^{14}C (Kisaki & Tolbert, 1969) and apparently contain enzymes for the further metabolism of glycine, then the CO_2 producing stage of photorespiration almost certainly occurs outside the peroxisome from either glyoxylate or glycine.

Evidence for the exact nature of the CO_2-producing stage is conflicting. Zelitch (1966) suggested that the non-enzymic decarboxylation of glyoxylate brought about by the H_2O_2 generated in the glycollic oxidase reaction was responsible for the CO_2 evolution. But the presence of large amounts of catalase inside the peroxisomes (Kisaki & Tolbert, 1969) argues against any such reaction in the peroxisome at least, although its occurrence outside using an alternative oxidant cannot be ruled out. Recently, Zelitch (1972 b) compared the effectiveness of glycollic acid and glycine as substrates for photorespiration in the leaves of many species including tobacco. He supplied CO_2-free air to illuminated leaf discs floated on glycollate-1-^{14}C or glycine-1-^{14}C and collected the $^{14}CO_2$ evolved. He found that while α-OHMS severely blocked $^{14}CO_2$ production and labelling of intermediates of the glycollate pathway from glycollate-1-^{14}C, INH, an inhibitor of glycine to serine conversion had little effect on CO_2

production although the glycine to serine conversion was severly inhibited. From these results he concluded that not all the CO_2 evolved during photorespiration comes from the glycine to serine conversion, but a direct decarboxylation of glyoxylate to formic acid and CO_2 was the more likely source. By contrast, Kisaki & Tolbert (1970) reported a more rapid decarboxylation of carboxyl-labelled glycine than glyoxylate, glycollate or serine. In their experiments, INH blocked CO_2 release from both glycine and glycollate. Further support to this mechanism of CO_2 evolution was provided by the presence of two enzymes responsible for glycine metabolism in green leaves (Kisaki, Imai & Tolbert, 1971). Glycine decarboxylase was located in mitochondria and serine hydroxymethyltransferase was principally located in the mitochondria and partly in the chloroplasts.

Kisaki, Yoshida & Imai (1971) in their work on the mechanism involved in the glycine to serine conversion in higher plants found that glycine decarboxylase in leaf mitochondria catalysed the decomposition of glycine into CO_2, NH_3 and a C_1 unit. The level of the decarboxylase was also consistent with the amount of glycollate produced during photosynthesis. They also found that the serine synthesis was closely related to the decarboxylation of glycine from which they concluded that serine is formed from two molecules of glycine by the combined action of glycine decarboxylase and serine hydroxymethyltransferase. They also demonstrated a stimulation of glycine decarboxylase

by NAD and an inhibition by NADH, from which they proposed a control mechanism of photorespiration involving the mitochondrial electron transport chain. Despite this they observed that the mitochondrial suspensions did not show a significant increase in absorption at 340 nm due to NADH after the addition of glycine. From these results they inferred that the glycine decarboxylase system is coupled to NAD but may be specially oriented near a system which immediately oxidises the newly synthesized NADH.

Recently, Bird, Cornelius; Keys & Whittingham (1972) also reported the conversion of two molecules of glycine to one molecule of serine and one of CO_2 in isolated mitochondrial preparations from tobacco. They too postulated the involvement of the mitochondrial electron transport system because of the requirement for O_2 and the inhibition of glycine to serine conversion by inhibitors of mitochondrial electron transport chain. They further observed an increased rate of reaction in the presence of ADP which was converted to ATP, from which they concluded that photorespiration causes ATP to be synthesized in mitochondria during photosynthesis. In their experiments, however, the absence of NAD did not affect the rate of serine synthesis by the above mechanism and it was presumed not to be involved, the electrons from glycine being fed into the electron transport chain at a later stage than those from NADH.

Whatever the ultimate fate of the electrons, the first step in the synthesis of serine formed from the glycollate pathway is believed to be the decarboxylation of glycine, catalysed by glycine decarboxylase. In this reaction, CO_2 is produced from the carboxyl group of glycine, NH_3 is evolved, and the other carbon atom is carried by tetrahydrofolate as C_1-THFA. This C_1-unit is transferred to another molecule of glycine by a second enzyme called serine-hydroxymethyltransferase in the presence of O_2. Both these enzymes must work in conjunction to produce the CO_2 of photorespiration. Since some serine hydroxymethyltransferase has also been found in the chloroplasts, it is still not certain whether all serine synthesis takes place in the mitochondria or partly in the mitochondria and partly in the chloroplast.

It should be noted that glycollate metabolism is not the only source of serine to the plant. Chang & Tolbert (1965) demonstrated serine production from 3-PGA by isolated chloroplasts. Serine produced by these two routes can be distinguished by the labelling pattern during short term $^{14}CO_2$ fixation. That from PGA is predominantly carboxyl labelled like the PGA itself, whereas that from glycollate is uniformly labelled. It has been generally found from the pattern of labelling that the serine formed in photosynthesizing whole tissue, as opposed to chloroplasts is derived very largely from glycollate (Rabson, Tolbert & Kearney, 1962).

Part of the serine formed may be utilized for the synthesis of proteins and other metabolites but most of it seems to be converted to carbohydrates. This was demonstrated by Ongun & Stocking (1965a) who found the label from photosynthesized $^{14}CO_2$ passing first into glycine and serine and then into carbohydrate. Non-aqueous separation of the chloroplasts indicated that serine had entered them and it was thought that light was required for its conversion to carbohydrate. A similar conclusion was reached by Rabson, Tolbert & Kearney (1962) and Mifflin et al, (1966).

The enzymes for the conversion of serine into carbohydrate have been found in the higher plant leaves (Cheung, Rosenblum & Sallach, 1968). These workers detected an aminotransferase for the conversion of serine into hydroxypyruvate, and a glycerate dehydrogenase to convert hydroxypyruvate into D-glycerate. A D-glycerate kinase was also detected which would convert D-glycerate to 3-PGA, which is an intermediate of the Calvin cycle and could presumably be metabolised to carbohydrate by this route. They further emphasized the requirement for light by showing that dark-grown pea leaves had lower levels of glycerate dehydrogenase. Tolbert (1971) postulated the reduction of hydroxypyruvate to glycerate in the peroxisomes with reducing equivalents carried there as malate and transferred to NAD^+ via malate dehydrogenase. An NAD-linked malate dehydrogenase and a glutamate-oxalacetate aminotransferase have been reported by Tolbert (1971) in the

leaf peroxisomes which would convert the malate first to oxalacetate and then to aspartate. Some of the postulated reactions of glycollate metabolism occurring in the chloroplast, peroxisome and mitochondria are shown in Figure 1.

Unlike most higher plants, the majority of the algae are known to have low photorespiration rates as measured by CO_2 evolution in the light. This was observed by Brown & Tregunna (1967) in Chlorella, Scenedesmus, Fucus and Nitella using an infra-red CO_2 analyser. Cheng & Colman (1974) using the isotopic technique measured the photorespiration rates in 3 green and 4 blue-green algae and found the rate of loss of photosynthetically fixed $^{14}CO_2$ in light in CO_2-free air at 25°C to be considerably less than that in darkness, except for Chlamydomonas reinhardtii, which released slightly more CO_2 in the light. Light-dependent CO_2 release, nevertheless was shown by Zelitch & Day (1968 b) in Chlorella as well as Chlamydomonas, though these rates were still considerably less than those observed in higher plants.

Miller, Cheng & Colman (1971) reported the glycine to serine conversion in some blue-green algae to be of minor importance because an inhibitor of this reaction, INH had little effect on the assimilation of exogenous ^{14}C-glycollate at the concentration tested.

Goldsworthy (1970) suggested that the low photorespiration rate in Chlorella as measured by CO_2 evolution may be due to its complete refixation by the cup-shaped chloroplast which almost encircles the cell.

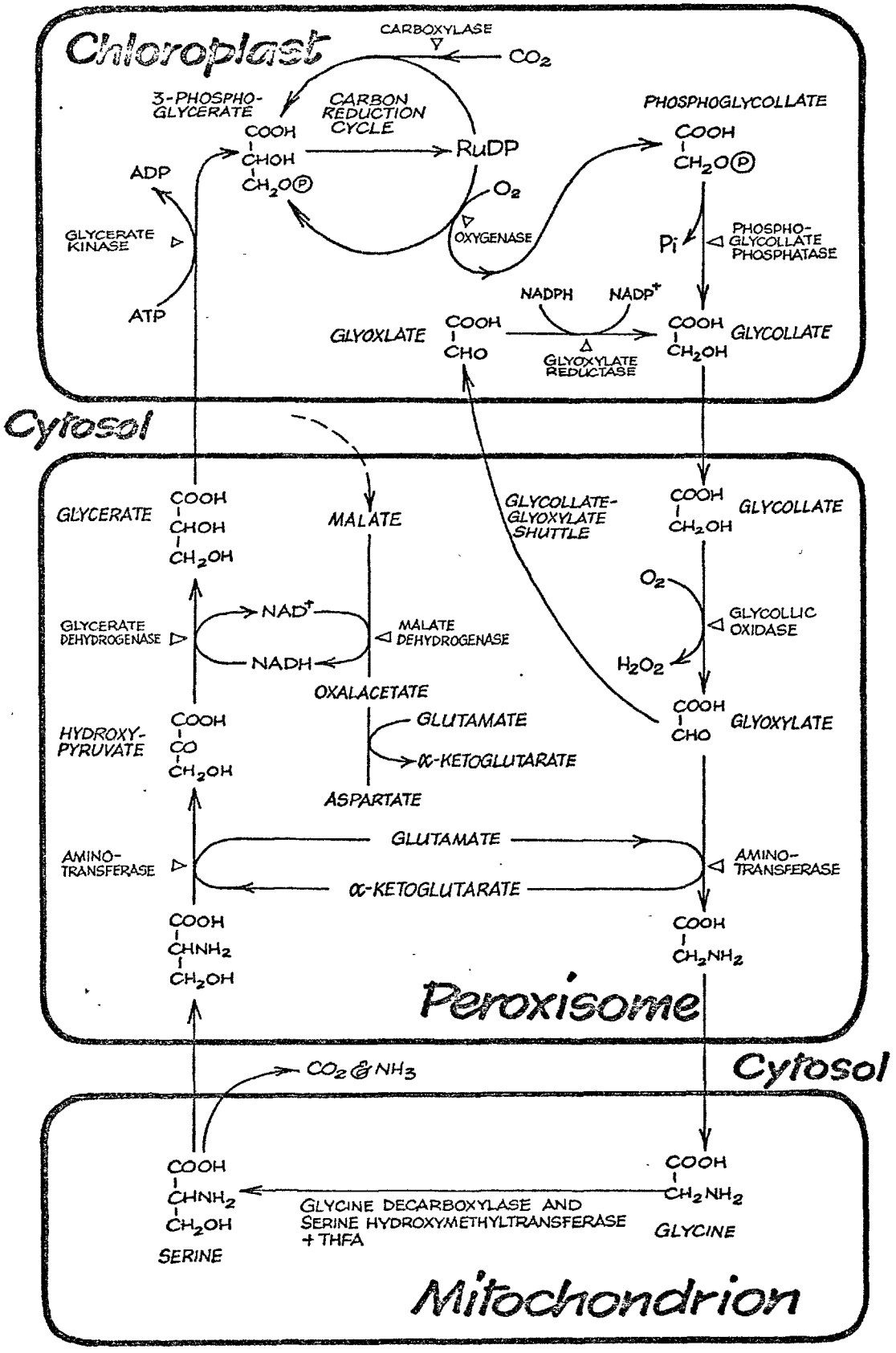


Fig 1 : The proposed sequence of the reactions of the Glycollate Pathway and their spatial location in the chloroplast, peroxisome and mitochondrion in higher plants. The scheme would be applicable generally with the exception that in most algae studied, glycollate oxidising enzyme is a dehydrogenase.

Photorespiration rates as measured by O_2 uptake have also given similar results. Although Lex, Silvester & Stewart (1972) reported light-dependent $^{18}O_2$ uptake in a blue-green alga, Anabena, this was thought not to be related to glycollate metabolism, because α-OHMS at 10^{-5} M concentration was without effect on the rate. Hoch et al, (1963), however, showed an initial decrease in $^{18}O_2$ uptake in Anacystis and Scenedesmus with increasing light intensity followed by 3-5 fold increase at the higher light intensities. This $^{18}O_2$ uptake was DCMU sensitive, which is an inhibitor of photosystem 2 in photosynthesis and also of photorespiration. (Downton & Tregunna, 1968 a).

Although photorespiration in algae has not been characterized (Merrett & Lord, 1973), they do synthesize and metabolize glycollate (Bruin, Nelsen & Tolbert, 1970; Lord & Merrett (1971 a). Goldsworthy (1970) attributed low photorespiration rates in algae at least in part to glycollate excretion, because the excreted glycollate would not be available for photorespiration.

Glycollate excretion was first reported by Tolbert & Zill (1956) in Chlorella. Since then it has been reported by various workers in many green algae such as Chlamydomonas, Euglena, Ankistrodesmus, Scenedesmus and Chlorella. Hellebust (1965) in an extensive study on glycollate excretion by unicellular organisms has found it of general occurrance and now well established in several classes of algae. Recently, Cheng, Miller & Colman (1972) reported glycollate excretion in 3 species of blue-green algae.

Glycollate excretion by mixed phytoplankton populations has been reported by Watt (1966) and Fogg & Nalewajko (1964). It has been detected in many natural waters (Fogg, Egle & Kinson, 1969). Its excretion appears to be quite widespread and it has been reported from the Eastern Indian Ocean (Jitts, 1966) to the Antarctic (Fogg & Horne, 1968) and in lakes in Britain (Fogg, Nalewajko & Watt, 1965), but glycollate has not been detected in all natural waters (Spear & Lee, 1968).

Glycollate excretion may have ecological significance in that it may be utilized for growth by bacteria and protozoa (Muscatine, 1965; Wright, 1970) or it may be taken up and reassimilated by algal populations under conditions unfavourable for photosynthesis (Nalewajko, Chowdhuri & Fogg, 1963). The importance of excreted glycollate as a carbon source for growth was emphasized by Fogg (1963) for phytoplankton populations under conditions unfavourable for photosynthesis. Sen & Fogg (1966) demonstrated that the lag phase during the growth of a culture of a planktonic strain of Chlorella was decreased by the addition of 1 mg l^{-1} of glycollate to the culture medium. However, Droop & McGill (1966) in an extensive study of the carbon nutrition of algae investigated 39 strains and found no convincing evidence that glycollate supported growth in the dark or enhanced growth in the light in the presence of CO_2 at physiological pH values.

Murray, Giovanelli & Smillie (1970) reported glycollate utilization by Euglena at pH 3.8 under a variety of atmospheric conditions, including CO_2-free air and nitrogen in the light,

but not in the dark. They also found that when the cells were grown in the dark on glucose, glycollate could be taken up. Similarly, cells grown in the light with glycollate as sole carbon source were still capable of glycollate uptake for up to 3 hours after transferring them to darkness. From these results they concluded that there was an energy requirement for glycollate utilization and this could be met either by light or by the aerobic metabolism of glucose in the dark. They also reported some inhibition of photosynthesis during the photoassimilation of glycollate or related compounds. Lord & Merrett (1971 a) also demonstrated the utilization of glycollate as a substrate for photoheterotrophic growth by a strain of Chlorella pyrenoidosa in the light but not in the dark.

The biosynthesis of glycollate in algae appears to resemble that of higher plants, for example, Warburg (1920) observed that the rate of photosynthesis in Chlorella was inhibited with increasing O_2 concentration and was only 65% of the maximum rate in 21% O_2 and 55% of the maximum in 100% O_2. However, Björkman (1967) reported that this 'Warburg effect' was not so pronounced at 21% O_2 in algae as in higher plants.

Conditions that favour glycollate biosynthesis also favour its excretion (Whittingham & Pritchard, 1963). Since optimal conditions for glycollate biosynthesis are the same in both higher plants and algae, e.g. low partial pressure of CO_2, high light intensity and high partial pressure of O_2, the

mechanism of glycollate synthesis may also be similar in both. The enhancement of glycollate excretion during photosynthesis by low CO_2 partial pressure was shown by Pritchard, Wendy, Griffin & Whittingham (1962) in Chlorella. Coombs & Whittingham (1966) reported an increase in glycollate excretion by increasing the partial pressure of O_2, and the effect of high light intensities was investigated by Tolbert & Zill (1956) and Pritchard et al, (1962). Whittingham & Pritchard (1963) in an extensive study of glycollate excretion by Chlorella found that the maximum rates of excretion occurred during photosynthesis with 0.1% CO_2 in the gas phase at light intensities of 1000 lum ft^{-2} or more. Hess & Tolbert (1967) found enhanced excretion in the presence of HCO_3 ions in the suspending medium and maximum enhancement occurred at 10 mM HCO_3. The excretion of glycollate was taken as evidence against a functional glycollate pathway in algae. This conclusion was supported by the results of $^{14}CO_2$ photosynthesis in Chlorella and Chlamydomonas where after 12 seconds of photosynthesis, 70-80% of the label was accumulated in the carboxyl carbon of serine which was similar to the distribution of label in 3-PGA from the same experiment. This result was different from that of higher plants, where after short term CO_2 fixation, uniformly labelled serine was formed from glycollate while 3-PGA was still carboxyl labelled (Rabson et al, 1962). Despite this, it is now clear that many algae, including Chlorella and Chlamydomonas, have at least some capacity to metabolize glycollate. Evidence for this comes from the fact that the Chlorella mutant (C. fusca variety vacuolate) used by Codd, Lord & Merrett (1969) did not excrete

glycollate irrespective of the growth conditions employed. The failure to excrete glycollate by this Chlorella strain was not due to its inability to synthesize glycollate, because when the cells were treated with the glycollate oxidase inhibitor, α-OHMS, they were forced to excrete glycollate which suggested that the cells were capable of metabolizing all the glycollate formed during photosynthesis. Similarly, air-grown cells not normally excreting glycollate were forced to do so by the use of INH, an inhibitor of the glycollate pathway at the glycine to serine conversion step (Dae & Writson, 1958). This was shown by Pritchard et al, (1962) in Chlorella and Nelson & Tolbert (1969) in Chlamydomonas reinhardtii. From these results it was concluded that the air-grown cells were synthesizing glycollate but in the absence of the inhibitors, did not excrete it.

If glycollate production does not always result in its excretion then it would be worthwhile to examine the factors which affect the balance between its excretion and its oxidation. Becker, Döhler & Egle (1968) proposed that light quality may affect this balance. They found glycollate excretion by Chlorella in white or red light but not in blue light. They suggested that the blue light enhances protein formation and so it results in rapid glycollate metabolism through the glycollate pathway to amino acids. Lord & Merrett (1970 a) using α-OHMS to inhibit glycollate oxidation in Chlorella, were also able to demonstrate glycollate excretion in red light but not in blue light, which suggested that the light effect was on glycollate biosynthesis rather than its

utilization, blue light alone not being sufficient for glycollate biosynthesis. Similarly, Döhler & Braun (1971) showed glycollate excretion by Anacystis nidulans in red or white light, but not in blue light.

Glycollate excretion seems to be regulated by the CO_2 availability in the culture medium prior to the experiment. This was shown by Nelson & Tolbert (1969) in Chlamydomonas which when grown phototrophically on air supplemented with 1% v/v CO_2, excreted glycollate into the medium on being transferred to ordinary air (0.03% CO_2) for the experiment. On the other hand, cells grown in air alone did not excrete glycollate under similar experimental conditions. Since Zelitch & Day (1968 a) had already reported a glycollate oxidising enzyme in Chlorella and Chlamydomonas grown phototrophically in air, it was concluded that the glycollate excretion by CO_2-grown cells was the result of a repression of this enzyme under high CO_2 concentrations. When the cells were transferred to air, the enzyme level was insufficient to oxidise all the glycollate synthesized, therefore most of it was excreted.

There is evidence that in many organisms the glycollate oxidizing enzyme is inducible. For example, Kornberg & Gotto (1961) have shown inducible synthesis of the glycollic oxidase in glycollate grown Pseudomonas cells which showed 50 times the activity of succinate grown cells. There is also evidence that in some algae, at least, the enzyme is also inducible, this

time by conditions of low CO_2. This has been observed by Codd, Lord & Merrett (1969) in Chlamydomonas and Euglena and by Lord & Merrett (1971 b) in Euglena. Cooksey (1971) confirmed this inducibility in Chlamydomonas but found a delay of about one cell generation time in the response of the enzyme synthesis to changes in CO_2 level.

In other cases it has been found that the glycollate oxidising enzyme is constitutive. Lord (1971) obtained a Chlorella fusca mutant which did not excrete glycollate on being transferred from high to low CO_2 and consistent with this, its glycollate oxidising enzyme appeared to be constitutive (Codd, Lord & Merrett, 1969). More recently, Colman, Miller & Grodzinski (1974) found evidence supporting this with Chlorella pyrenoidosa, when they observed that the rate of oxidation of exogenous ^{14}C-glycollate in the dark was similar in cells growing in both high and low CO_2 condition.

In contrast to higher plant glycollic oxidase, the corresponding algal enzyme is a dehydrogenase which does not link directly to oxygen, and can in vitro only transfer electrons to such artificial electron acceptors as 2,6-dichlorophenol-indophenol (DICPIP) and Phenazinemethosulphate (PMS),(Nelson & Tolbert, 1970). No H_2O_2 is produced in this reaction and no O_2 uptake occurs. It also differs from the higher plant enzyme in that it oxidises D-lactate

as an alternative substrate, is inhibited by cyanide and sulph-hydryl reagents and is not affected by adding FMN (Nelson & Tolbert, 1970; Grodzinski & Colman, 1972). The discovery of these basic differences between the algal and the higher plant enzyme solved a point of disagreement between Tolbert and Zelitch's groups, because Hess & Tolbert (1967) not realizing that the algal enzyme was a dehydrogenase, were trying to detect glycollate oxidation in their algae by using manometric, spectrophotometric and isotopic techniques, all requiring O_2 as the electron acceptor. As a result they concluded that these algae do not have the glycollate oxidising enzyme.

In earlier reports of glycollate oxidation in algae, the enzyme was not characterized (Downton & Tregunna, 1968; Lord & Merrett, 1968, Zelitch, 1968). Although now the algal enzyme has been designated as glycollate dehydrogenase to distinguish it from the higher plant enzyme (Nelson & Tolbert, 1969; Codd et al, 1969), Grodzinski & Colman (1970) have designated the glycollate oxidizing enzyme in two species of blue-green algae as glycollic oxidase, but no glycollate dependent O_2 uptake could be demonstrated. Nevertheless, there are reports of a typical glycollic oxidase which couples to O_2 in a colourless Chlorella mutant (Schmid & Schwarze, 1969) and a wild type Chlorella strain (Kowallik & Schmid, 1971).

The localization of glycollic dehydrogenase and other enzymes of glycollate metabolism is still obscure in algae, because peroxisomes have not been observed in electron micrographs or isolated on sucrose gradients in photosynthetically grown cells (Tolbert, 1971). Consistent with the absence of peroxisomes, no H_2O_2 is produced during the glycollic dehydrogenase reaction and algae only contain about one tenth as much catalase as leaves (Bruin, Nelson & Tolbert, 1970). Earlier work of Codd & Merrett (1970) had shown the glycollate dehydrogenase of Euglena to be located in the cytoplasm, which was later confirmed (Lord & Merrett, 1971 b), when it was demonstrated that the enzyme was associated with a particulate cytoplasmic structure distinct from the chloroplasts and mitochondria. The particulate nature of the Euglena enzyme and its possible localization in microbodies was also speculated by Graves, Trelease & Becker (1971). Recently, Grubber, Frederick & Tolbert (1974) have confirmed its particulate nature in Chlorella and Dunaliella, but they further observed that in Dunaliella cell homogenates, on sucrose density gradient separation, the enzyme gave a profile of activity closely similar to cytochrome C oxidase, the mitochondrial marker enzyme, from which they speculated the possible mitochondrial location of glycollate dehydrogenase. The location of glycollate dehydrogenase and other enzymes of glycollate metabolism outside the peroxisomes is consistent with the observation that Acetabularia chloroplast preparations which were free of

peroxisomes showed all the main features of photorespiration and glycollate metabolism (Bidwell, Levin & Shephard, 1969).

The presence of a glycollate-glyoxylate shuttle was speculated in Scenedesmus by Nelson & Tolbert (1969) when they demonstrated glycollate dependent O_2 evolution in the light. Since this evolution occurred in the absence of CO_2 and was CMU sensitive, they concluded that the glyoxylate shuttle served as an electron carrier oxidising the NADPH produced by photosynthesis. Similar conclusions were drawn by Butt & Peel (1963) in Chlorella, when they observed an anaerobic uptake of glucose in light, which they thought was a result of ATP synthesis by this mechanism, because α-OHMS inhibited this uptake and this inhibition could be reversed by the addition of glycollate.

Algae can metabolize part of the glycollate by the 'glycollate pathway' as established for higher plants. This was reported by Hess & Tolbert (1967) in 5 strains of algae by identifying the $^{14}CO_2$ fixation products, by feeding glycollate-^{14}C followed by the degradation of products, and by isolating the enzymes for each step. Lord & Merrett (1970a) reported the presence of a glycollate metabolizing system in a strain of Chlorella previously said to lack such a system. Similarly, Bruin, Nelson & Tolbert (1970) have also provided evidence for glycollate metabolism in Chlorella, Scenedesmus and Chlamydomonas. In these experiments, it was shown that when $^{14}CO_2$ was fed, both glycollate and glycine were uniformly

labelled as in higher plants, and when glycollate-1-^{14}C was supplied exogenously, glycine and serine were also labelled at the corresponding position, which showed a precursor-product relationship. Inhibitors of the glycollate pathway, α-OHMS and INH caused a reduction of the carbon flow through the glycollate pathway. During $^{14}CO_2$ photosynthesis, glycollate and glycine were uniformly labelled, but serine was predominantly carboxyl labelled like 3-PGA, glyceric acid and hydroxypyruvate. This showed that the glycine to serine conversion was slow and depicted two routes for serine formation, one from the glycollate pathway and the other from the photosynthetic carbon reduction cycle (Bruin, Nelson & Tolbert, 1970).

Blue-green algae may also have a low conversion rate from glycine to serine. Miller et al (1971) observed that α-OHMS severely inhibited ^{14}C-glycollate assimilation but INH had no effect. From this they concluded that the glycine to serine conversion was not very important in blue-green algae.

The stage of cell division may determine carbon flow in Euglena (Codd & Merrett, 1971 b, C). During or prior to cell division, division synchronised cultures of Euglena gracilis metabolised assimilated $^{14}CO_2$ through the glycollate pathway and glycine, serine and glycerate were uniformly labelled. At this stage, no label was found in glycollate and very little glycollate excretion was observed, indicating

its rapid metabolism. The cells at this stage had high levels of glycollate dehydrogenase. On the other hand, in non-dividing cells, most of the glycollate was excreted and the glycerate formed was carboxyl labelled like phosphoglycerate, maximum phosphoglycerate phosphatase activity being also observed at this stage.

From the foregoing it can be seen that the factors affecting the glycollate metabolism in algae are both complex and variable and it is not easy to explain their low rates of photorespiration on the basis of present knowledge. The reasons for the low rates of photorespiration in C_4 higher plants are better understood.

Like many algae, the C_4 plants have low CO_2 compensation points and appear to be without photorespiration (Hatch & Slack, 1966; Downton & Tregunna, 1968). Low O_2 levels do not stimulate CO_2 fixation in C_4 plants (Downes & Hesketh, 1968), nor does the photorespiratory inhibitor α-OHMS stimulate photosynthesis in maize, a C_4 plant (Zelitch, 1966). Forrester, Krotkov & Nelson (1966a) reported no post-illumination CO_2 bursts in maize and sugarcane.

Although C_4 plants have been classified as without photorespiration from experiments based on CO_2 exchange, they do show it with respect to oxygen exchange (Jackson & Volk, 1969; Fock et al, 1969b). Working with maize leaves, they observed an O_2 uptake in the light that was stimulated by increasing

the O_2 concentration in the atmosphere from 2 to 6%, but the measured rates were nevertheless quite low, being little different from the rate of dark respiration.

C_4 plants contain peroxisomal enzymes (Tolbert et al, 1969) and can oxidise exogenous glycollate in the dark to CO_2 at fairly high rates (Tolbert et al, 1969; Kisaki & Tolbert, 1970). When glycine-1-^{14}C was fed to corn leaf segments in the dark or with DCMU in the light, Kisaki & Tolbert (1970) found $^{14}CO_2$ released at $^2/_3$ the rate of that in normal tobacco leaf from which they concluded that corn also shows a significant rate of photorespiration. However, Goldsworthy (1970) claimed that this may not reflect the normal rate of photorespiration of the tissue, because it is more likely that the rate of glycollate synthesis rather than its rate of oxidation is the factor which limits the rate of photorespiration.

Low rates of photorespiration in C_4 plants have been attributed to their unusual mechanism of photosynthesis. This mechanism depends on their peculiar leaf anatomy. In the leaves of C_4 plants, the vascular bundles are surrounded by= a concentric layer of cells called the bundle sheath which has all the necessary enzymes for fixing CO_2 and metabolising it by the Calvin cycle. The layer of cells surrounding the bundle sheath is called the mesophyll, and is provided with another CO_2 fixing enzyme, PEP carboxylase (Hatch et al., 1967). Oxalacetate, the C_4 acid formed as a result of this reaction is converted into two other C_4 acids, aspartate and malate which are believed to be transported to the bundle-sheath where they lose this CO_2 again. The resulting pyruvate

is transported back to the mesophyll where it reacts with ATP and inorganic phosphate to form PEP and may then pick up another molecule of CO_2. The CO_2 released in the bundle-sheath is refixed by the enzymes of the carbon cycle (Johnson & Hatch, 1968). The apparent lack of photorespiration is probably due to a combination of two effects. Firstly CO_2 is generated from the C_4 acids at a very high concentration (Hatch, 1971). Such a high concentration of CO_2 in the bundle sheath would be expected to inhibit glycollate synthesis and hence photorespiration (Goldsworthy & Day, 1970). Furthermore should any glycollate still be formed and converted to CO_2 this would occur in the bundle sheath and would almost certainly be refixed by the PEP carboxylase of the mesophyll before it could reach the external atmosphere.

At first sight C_4 metabolism does not seem to be responsible for the low rates of photorespiration in algae. Short-term $^{14}CO_2$ fixation experiments give PGA as the initial fixation products (Calvin & Bassham, 1962), and, of course, unicellular algae cannot have the anatomical features characteristic of higher C_4 plants.

Despite this, at least some algae have relatively high levels of carboxylase. Codd & Merrett (1971 a) showed that the PEP carboxylase activity of Euglena was greater than its RUDP carboxylase activity although the labelling pattern from short term $^{14}CO_2$ photosynthesis still resembled that of a C_3

plant. Graham & Whittingham (1968) and Reed & Graham (1968) showed that in Chlorella, the major proportion of fixed $^{14}CO_2$ entered C_4 acid when the cells were transferred from 5% CO_2 to air. Again, under normal circumstances these algae show normal C_3 metabolism. Thus it would appear that they too possess substantial quantities of PEP carboxylase but the products of its activity, the C_4 acids, are not correspondingly apparent 'in vivo' under steady state conditions.

A possible explanation for the presence of PEP carboxylase may be that it functions as part of a CO_2 pump mechanism, similar to, but not necessarily identical with, that in the C_4 higher plants. PEP carboxylase outside the chloroplast may generate C_4 acids which could be translocated into the chloroplast and these release CO_2 at a higher concentration than outside. If the PEP carboxylase and CO_2 releasing enzyme were closely situated, say on opposite sides of the membrane then the formation of the C_4 acids would be so transitory that they would not be readily detected as intermediates in labelling experiments. Such plants would therefore still appear to possess a C_3 type of photosynthesis but would show an inhibited rate of photorespiration and glycollate synthesis.

The above, however, is not the only possible explanation for the low rates of photorespiration in algae. Another might be that the RUDP carboxylase of the algae is structurally different from that of higher plants so as to be inherently less affected by oxygen, again resulting in less glycollate synthesis and therefore less photorespiration.

A third explanation is that glycollate is synthesized in these algae but not metabolised to CO_2, either because it is excreted or because it is metabolised by an alternative pathway which does not involve significant CO_2 release.

A fourth explanation might be that glycollate is metabolized and does yield CO_2 but this happens in such a way that the CO_2 is refixed efficiently by photosynthesis before it is released from the cell.

It was to investigate these possibilities and to perhaps suggest others, that the present work was undertaken.

MATERIALS AND METHODS

ALGAL CULTURE METHODS

Most of the experiments were performed with Phaeodactylum tricornutum, a marine diatom and Chlorella pyrenoidosa. These algae were grown in 100 ml Erlenmeyer flasks for experiments requiring small amounts, whereas for enzyme extraction and other experiments requiring larger amounts 500 ml Dreschel bottles were used. The empty flasks and Dreschel bottles were stoppered with cotton wool plugs and autoclaved for 15 minutes at 15 lb/in^2. The medium was autoclaved in 1-liter flasks and 50 ml or 500 ml aliquots were dispensed aseptically into the flasks or Dreschel bottles using a Laminar-Flow bench.

For the purpose of raising an initial culture, a small amount of inoculum was added aseptically to a 100 ml flask having 50 ml of medium and the flask was kept in a culture room at 20°C and 600 lux warm white fluorescent light (the intensity determined with an EEL light meter).

The algae were subcultured by taking 1 ml or 10 ml from the initial culture and adding it to the flask or the Dreschel bottle containing the medium using the Laminar-Flow bench. The flasks and Dreschel bottles were transferred to the culture room at 20°C and 600 lux light intensity. Air or 4% CO_2 in air was bubbled through the Dreschel bottles, these gases being first humidified by passing through a Dreschel bottle of sterile distilled water. Most air borne contaminants were

removed by filtration through another Dreschel bottle containing cotton wool. All rubber tubing connecting the Dreschel bottles with the air-pump (Charles Austen, model MKI) and pipettes used for subculturing were plugged with cotton wool filters and autoclaved before use.

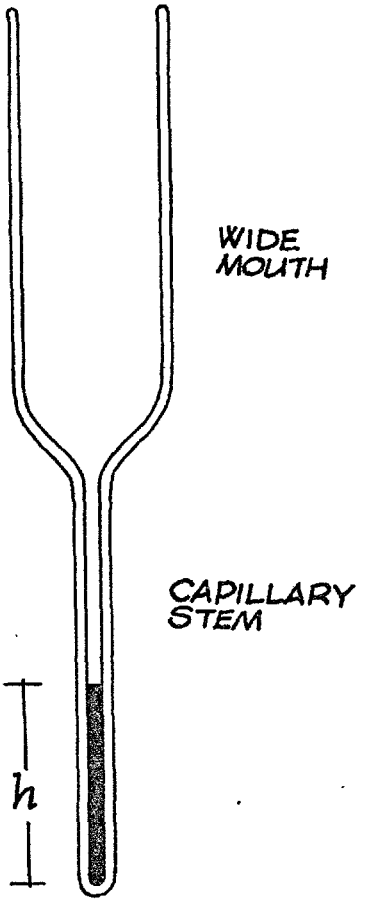
Various algal media were tested. In one, Erdschreiber's medium, one component, the soil extract, could vary from time to time depending upon the composition of the soil, so it was decided to grow Phaeodactylam in Mann and Myers' medium to obtain uniform culture conditions. Both Phaeodactylum and Chlorella were obtained from Cambridge Culture Collection.

Harvesting the cells for experimental use:

The cells were ready for use after about a week's growth. They were harvested by centrifugation at 5000 x g for 5 minutes at $4^{o}C$. The pellet was resuspended in the appropriate medium for the experiment.

Measurement of packed cell volume:

The packed cell volume was measured by taking 1 ml of suspension and centrifuging it down at 2000 x g for 5 minutes in a graduated centrifuge tube (Fig. 2).



Packed cell volume per ml of suspension $= \pi r^2 h$, where r is the radius of the tube and h the height of the packed column of algae.

Fig 2: Centrifuge tube for determination of packed cell volume
×2 magnification

COMPOSITION OF THE GROWTH MEDIA

Mann and Myers' Medium: (1968)

		g/litre
NaCl	-	5.00
$MgSO_4\ 7H_2O$	-	1.20
$NaNO_3$	-	1.00
KCl	-	0.60
$CaCl_2$	-	0.30
K_2HPO_4	-	0.10
Tris	-	1.00

Micronutrient stock (added at 10 ml/litre)

		g/litre
Na_2EDTA	-	3.00
H_3BO_3	-	0.60
$FeSO_4\ 7H_2O$	-	0.20
$MnCl_2\ 4H_2O$	-	0.14
$ZnSO_4\ 7H_2O$	-	0.033
$Co(NO_3)_2\ 6H_2O$	-	0.007
$CuSO_4\ 5H_2O$	-	0.0002

The medium was made up in distilled water. The pH of the autoclaved medium was 8.55.

Guillard and Ryther's Medium (1962) or 'medium f':

		mg/litre
$NaNO_3$	-	150.0
$NaH_2PO4\ 2H_2O$	-	10.0
Fe - Sequestrene	-	10.0
$Na_2SiO_3\ 9H_2O$	-	30 - 60.

Vitamins:

		mg/litre
Thiamine HCl	-	0.20
Biotin	-	1.00
B_{12}	-	1.00

Trace Metals:

		mg/litre
$CuSO_4\ 5H_2O$	-	0.196
$ZnSO_4\ 7H_2O$	-	0.044
$CoCl_2\ 4H_2O$	-	0.020
$MnCl_2\ 4H_2O$	-	0.360
$Na_2MoO_4\ 2H_2O$	-	0.0126

The medium was made up in sea water and vitamins were added after autoclaving.

Chlorella pyrenoidosa Medium:

		ml/litre
1M KNO_3	-	5.0
1M KH_2PO_4	-	0.5
1M $MgSO_4$	-	2.0
1M $Ca(NO_3)_2$	-	0.25
Hutner's Trace elements	-	2.00

Hutner's Trace elements:

		g/500ml
EDTA (Disodium Salt)	-	25.0
$ZnSO_4\ 7H_2O$	-	11.0
H_3BO_3	-	5.7
$MnSO_4\ H_2O$	-	2.2
$FeSO_4\ 7H_2O$	-	2.5
$Co\ (CH_3COO)_2\ 4H_2O$	-	0.84
$CuSO_4\ 5H_2O$	-	0.78
$(NH_4)_6\ Mo_7O_{24}\ 4H_2O$	-	0.55

GROWTH OF PHAEODACTYLUM ON GLYCOLLATE

250 ml Dreschel bottles were used, each containing 100 ml of Mann and Myers' medium. Both the bottles and the medium were autoclaved as described previously. Cultures were inoculated from photoautotrophically grown cultures to give an initial cell density at 500 cells/ml. Sodium glycollate dissolved in sterilised medium was added to give the desired concentration. The pH was determined with a pH meter (Pye Model 290), and was adjusted daily. The bottles were aerated with CO_2-free air by passing it through two, 500 ml Dreschel bottles filled with self-indicating soda-lime (carbosorb) and then through a 500 ml bottle filled with sterile distilled water to humidify it. The carbosorb was changed daily. The growth was measured after one week by counting the cell number with a hemocytometer (cavity volume 0.02 µl) and a telecounter.

In another experiment, where the effect of different pH values on growth was determined, 150 ml Erlenmeyer flasks were used, each having 50 ml of autoclaved medium with the desired glycollate concentration. These cultures were not aerated. The pH was adjusted daily and growth was measured after one week by counting the cell number.

MEASUREMENT OF CO_2 COMPENSATION POINTS

The infra-red gas analyser (IRGA) used was a Grubb-Parson Model SB2. This instrument was used to measure the CO_2 exchange of the plant materials. It had a sealed reference cell containing CO_2-free air and optical filters to suppress interference due to

water vapour. It was calibrated by allowing CO_2-free air to pass through the analysis tube for zero adjustment and a standard gas (285 vpm CO_2) for scale length adjustment. The instrument was coupled to read on a chart recorder.

1. For Microscopic Algae:

CO_2-compensation point measurements were made on a number of marine algae collected along the coast of South Wales in January, in large black polythene bags in which they were kept moist with sea water. In the laboratory, most of the algae were transferred to plastic buckets filled with sea water and stored for up to 3 days at 15° C. Pelvetia canaliculata was not submerged because it grows attached to the coastal rocks and is only wetted at high tide. These algae were allowed to equilibrate overnight with the temperature and light intensity of the culture room before measuring their compensation points.

The Mylar bag technique was used (Goldsworthy & Day, 1970). In this method, a small portion of the thallus was kept in each of two sealed Mylar bags, one containing CO_2-free air and the other ordinary air. The necks of these bags were sealed by twisting in clockwise direction and fastened with wooden clothes' pegs. Both bags were left overnight in a growth chamber for equilibration at 15° C and a light intensity of 10,000 lux. In most of the cases, however, a few hours equilibration was sufficient. If a plant shows photorespiration, then it removes CO_2

from the bag filled with air and liberates CO_2 into the CO_2-free bag before attaining the final equilibrium. When the two values coincide, that is the compensation point.

To sample the gas in the bags, a pipette connected to a polythene tube leading to the analysis tube of the IRGA was inserted into the neck of the Mylar bag. The neck was twisted around the pipette in an anticlockwise direction, that is, opposite to the first twist. This complementary twisting helped to open the bag so that when the clothes' peg was released, air could be forced out by the pressure of the hand and led into the analysis tube of the IRGA. The readings were recorded on the chart recorder, measurements being made at $15^{o}C$.

2. For Phaeodactylum tricornutum:

In this case, 100 ml of cell suspension were placed in a 500 ml flask provided with tight-fitting rubber bungs each having 2 holes fitted with polythene tubes. The flask was connected to the IRGA which was previously calibrated. The air from the flask was forced by a leak-proof Dymax pump into the IRGA and returned to the flask on a closed circuit till a steady level of CO_2 had been reached. The reading was recorded on a servoscribe chart recorder. The flask during the measurement was illuminated at 10,000 lux and kept at 19^{o} C temperature.

OXYGEN EXCHANGE MEASUREMENTS

These were made using an O_2 electrode (Rank Bros. Bottisham, Cambridge). This consists of a small perspex chamber (1 cm x 5 cm) separated from a tiny electrolytic cell by a Teflon membrane. The cell is fitted with a platinum cathode and a silver anode (Fig. 3). The electrolysis of a saturated solution of KCl put in the cell brings about the following reaction at the platinum cathode:

$$4H^+ + O_2 + 4e \longrightarrow 2H_2O$$

At low applied voltage (approximately 650 mV), the current flowing through the cell is proportional to the oxygen concentration in the region of the cathode. The solution to be analysed was put into the chamber and stirred by a small magnetic stirrer with a glass covered 'flea'. The chamber was closed with a cylindrical perspex plunger with a central capillary. Water at a controlled temperature was pumped from a thermostatically controlled bath through an outer perspex jacket. The electrode current was passed through a resistor so as to create a small voltage which after suitable amplification was recorded on a servoscribe chart recorder with a built in backing off system. The electrode was calibrated using air saturated water at 27°C which contains 0.25 μmole O_2 per ml, and full scale on the chart was made to read this value. The zero was adjusted after adding 2-3 mg of sodium dithionite. Calibration and zero adjustments were made alternately until both were correct. After calibration, the chamber was thoroughly rinsed with distilled water before making measurements with the cell suspension.

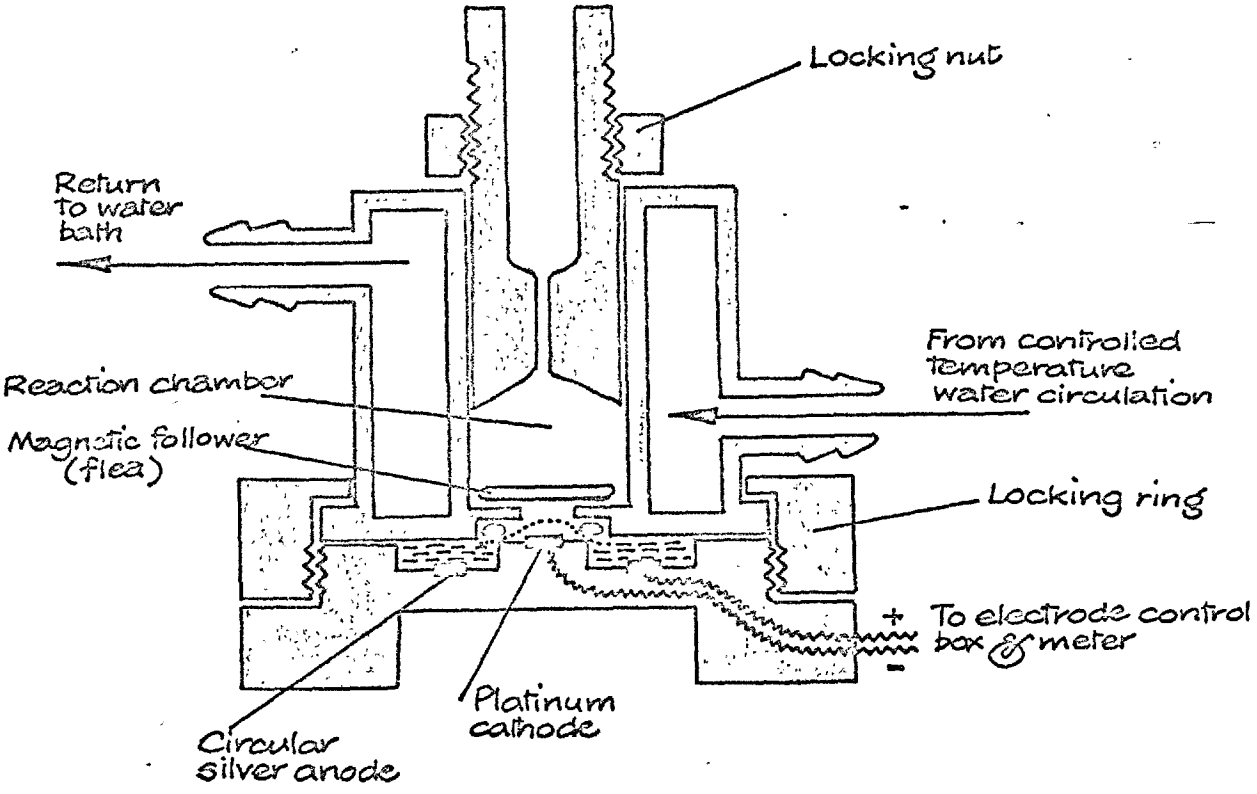


Fig 3 : The oxygen electrode
x 1·3 magnification

The Determination of Excreted Glycollate:

Glycollate was determined by the Calkin's (1943) method. In this method, a solution of glycollic acid reacts with a solution of 2-7, dihydroxynapthalene in conc. H_2SO_4, and a violet-red colour is produced on heating. The colour probably depends on the condensation of formaldehyde, split off from the glycollic acid with dihydroxynapthalene to form tetrahydroxynapthalene. This colourless product is then gradually oxidised to a deep red pigment.

Procedure

The algae were harvested by centrifugation at 5000 x g for 5 minutes and suspended in a known amount of medium in a flask. One ml from this suspension was normally removed for the determination of packed cell volume. The flask was kept in the culture room at 15^0C and 6000 lux light intensity. or in the laboratory, under specified light and temperature conditions.

Since the media used for growing algae gave a small amount of absorbance when read against a reagent blank, a control was always included using fresh medium and the glycollate excreted calculated by difference. The colour in all samples from the experiment and in glycollate standards was developed simultaneously. A small aliquot of the algal suspension to be analysed was removed from the flask and filtered through a Millipore filter disc (0.22-0.45 μm pore size). 0.1 or 0.2 ml of each

filtrate were made 2N with H_2SO_4, and put into separate 10 ml glass centrifuge tubes with a microsyringe. These tubes were then transferred to an ice-bucket before adding 2 ml of a freshly prepared 2 - 7, dihydroxynapthalene solution to each. The mixture was made homogenous by rotating the tube between hands. The tubes were covered with marbles and transferred to a boiling water bath for 20 minutes after which they were removed and allowed to cool to room temperature. After cooling, the solution was diluted with 4 ml of 2N H_2SO_4. The tubes were shaken lightly at first until the heat of the reaction had subsided, and then more vigorously. The absorbance was read on a Gallenkamp colorimeter using a blue filter, or on an Sp 800 spectrophotometer at 530 - 540 nm.

Treating known amounts of glycollate in the same way, a standard calibration curve was constructed (Fig. 4), showing it to be linear over the range 0 - 20 µg. With each series of subsequent determinations, two standard glycollate concentrations were included to account for any batch to batch variability in the development of the colour. The amount of glycollate produced was calculated by reference to these standards.

In some experiments involving glycollate pathway inhibitors, a-hydroxypyridinemethane sulphonate (α - OHMS) or Isonicotinylhydrazide (INH), the solution to be analysed was run through an anion exchange column. Blanks in these experiments also had the inhibitor in them.

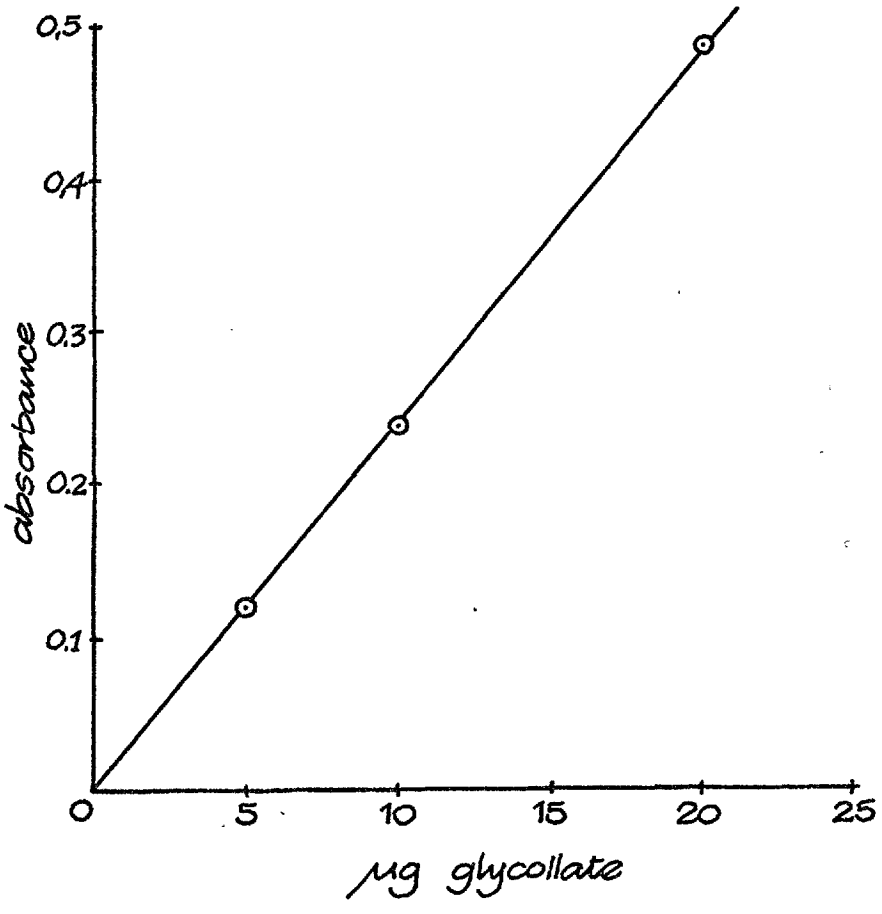


Fig 4 : Calibration curve for glycollate determination

Preparation of the Column:

Dowex-1 (400 mesh) anion exchange resin (chloride form) was made into a slurry with a 2N sodium acetate solution, packed into a 10 x 1 cm column to a height of 6 cm and washed with a little more of the sodium acetate solution. It was then washed with 10 ml of 6 N acetic acid to remove any impurities which give a colour with Calkin's reagent (Zelitch, 1958). The column was washed with 20 ml of water to remove acetic acid and then the solution to be analysed was loaded. The column was washed with 30 ml of water to remove the basic and neutral components. The elution was then carried out with 4 N acetic acid by applying a little pressure at the top with a pressure head and collecting the fractions at a rate of 1 ml per minute.

Glycollate Recovery Experiment:

An experiment was done to determine in which fraction glycollate was eluted and what was the percentage recovery. 10 μmoles of glycollate were dissolved in 10 ml of Mann & Myers' medium and loaded onto the column as above. Fractions collected during loading and washing were tested for glycollate, but no colour was given by either of these. Elution was started with 4N acetic acid and fractions of the eluate were collected at the rate of 1 ml per minute. The first 12 ml produced a little colour. The next 9 ml gave the glycollate colour and the following two, 2 ml fractions did not produce any colour. Recovery was 96% (Fig. 5).

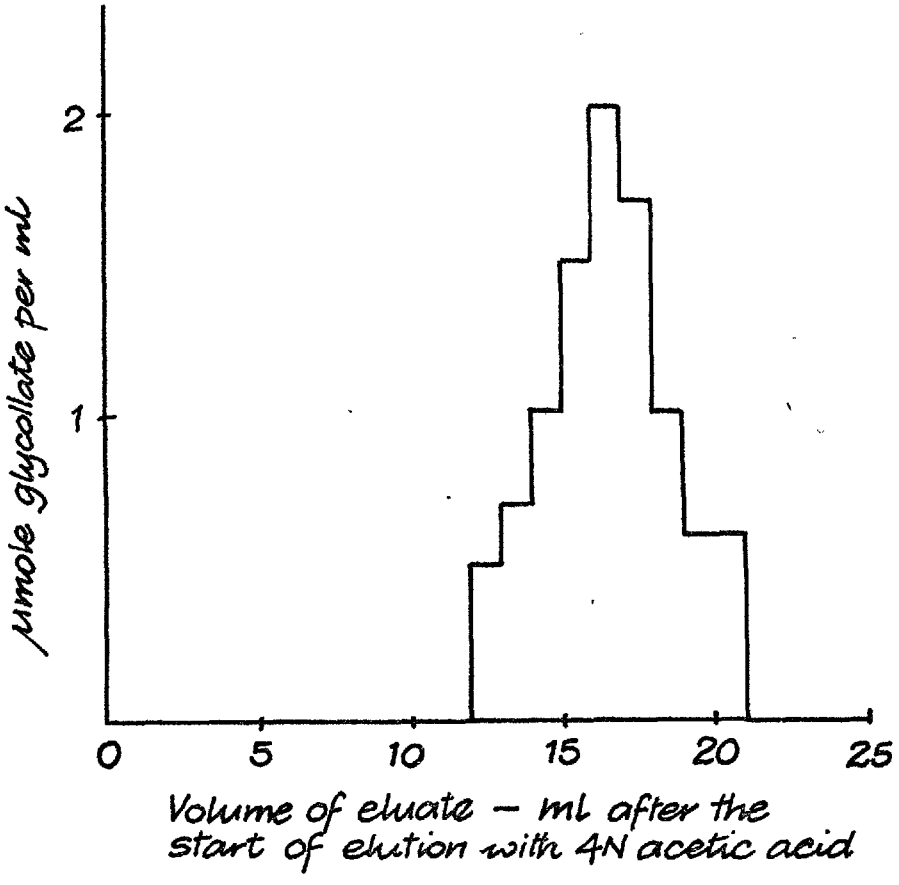


Fig 5 : Glycollate recovery from the column

For all fractions, appropriate controls were used i.e. fresh Mann & Myers' medium for the loaded test solution fraction, water for water fraction used for washing and 4N acetic acid for acetic acid fraction used for elution. The blank for all these was 2N H_2SO_4 treated in the same way. Since the acetic acid control (first 12 ml eluate supposed to contain no glycollate) gave a little colour, it was decided to use two exactly similar columns, one for running the test solution and the other to get the acetic acid eluate which was then used as the blank. This method would automatically cancel out any colour produced by the acetic acid.

In some experiments since no appreciable amount of glycollate could be detected in the medium, the cells were killed in water on a steam bath for 3 minutes and then the filtered cell extract was analysed. This also measured unexcreted glycollate.

In some other experiments, to accentuate the glycollate colour, the medium was evaporated down to a small known volume by vacuum evaporation at 30° C.

In still other experiments, along with the colourimeter, the test solution was also scanned through the entire light spectrum on an SP 800 spectrophotometer using 1 cm pathlength silica cells, each holding 3 ml of the solution.

Preparation of Cell Extracts:

For the enzymic assays, along with Phaeodactylum and Chlorella, some other marine algae such as Fucus and Ulva were also used. The latter two algae were ordered from Marine Biological Association, Plymouth and were sent by train in a box in sea water. They were stored in a glass trough in sea water at 400 lux light intensity and 20°C temperature for up to a week. In some cases, Fucus was stored in a deep freeze at -20° C.

Various methods were used for the preparation of cell extracts for enzyme assays. For Ulva, Fucus, Spinach and sugarcane, a Polytron homogeniser was used. However, it did not break a large proportion of cells in Ulva and Fucus and the residue had to be ground with a pestle and mortar.

For the unicellular algae, a French press was used in some experiments. Cells were suspended in an appropriate buffer and passed once through the press at 15,000 psi. This method was quite good for Phaeodactylum, but not for Chlorella.

Sonication was also tried, but heating of the suspension occurred quickly and degree of breakage was poor.

Grinding the frozen cells at -20° C in the appropriate buffer with a chilled pestle and mortar proved to be the best technique. In this, the cells after being centrifuged were suspended in 4 x their volume of buffer and put in the

mortar which was then kept in the deep freeze at -20° C along with the pestle till the cells had frozen completely. The frozen pellet was rapidly broken and ground, with a little acid washed sand. If the frozen mass thawed before being thoroughly ground, then the degree of breakage was poor, otherwise almost 100% breakage could be attained with Phaeodactylum, though Chlorella had to be frozen twice between the grinding attempts before attaining the same results. The details of the enzymic assays will be given along with the individual experiments.

RADIOACTIVE TRACER TECHNIQUES

1. Geiger Counting:

Samples having a reasonably high count rate but still losing no counts due to coincidence losses (doubling the amount of sample resulted in doubling the count rate) were counted by a bench counter, Ecko Automatic scaler Type N530G. The G-M tube, having about 5% efficiency, was fixed in a lead chamber. Aliquots were plated on aluminium planchets (2.45 cm diam.) over a prescribed area of 2.82 cm^2 that had been previously coated with an extremely fine layer of a spreader solution (0.1 % teepol in 50% EtOH) and dried. The planchets were dried under an infra-red lamp for 8-10 minutes and counted. A correction for self-absorption was made by counting sucrose-^{14}C of high specific activity with and without 0.1 ml of Mann & Myers' medium. A correction factor of 1.15 was obtained

which enabled the count rate for samples to be corrected to zero thickness.

The dead time of the counter was 200 μs and a correction was applied to correct the count rate by applying a formula

$$N = \frac{n}{1 - nt}$$

where

N = actual count rate

n = observed count rate

t = dead time

Some samples, having relatively low count rates were counted on a Nuclear Chicago gas flow counter fitted with a thin end window. This instrument had an efficiency of about 15%.

2. Scintillation Counting:

a) Sample preparation:

In most of the experiments, counting was done with a Nuclear Chicago Scintillation Counter. The scintillation fluid had the following composition:

Toluene (scintillation grade)	=	800 ml
Ethanol (A.R.)	=	200 ml
PPO	=	4.0 g
POPOP	=	0.05 g

All operations with this fluid were conducted away from direct sunlight. 15 ml of this solution were dispensed from an automatic dispenser into each of the scintillation vials containing 0.1 ml of the liquid sample to be counted. The vials were stoppered tightly and contents shaken thoroughly before counting. Algal cell residues were counted by suspending them in a known amount of water and counting 0.1 ml aliquots.

b) Determination of counting efficiency:

For all samples, the efficiency of the scintillation fluid was determined by using an internal standard and a correction factor was found to correct the count rate. The amount of quenching produced by the sample was determined by using ^{14}C-hexadecane which is a non-quenching form of ^{14}C. For this, the sample was counted before and after the addition of a known amount of hexadecane. Counting efficiency was determined as the difference in count rate compared to the disintegration rate of the added standard, i.e.

Original counts in the sample = x cpm

Counts after adding hexadecane = y cpm

Excess counts due to hexadecane = y-x cpm

Known disintegration rate of hexadecane = z dpm

$$\% \text{ efficiency} = \frac{Y - X}{z} \times 100$$

$$\text{Correction factor} = \frac{100}{\% \text{ efficiency}}$$

Chromatography

Paper chromatography was used for the separation and tentative identification of materials present in cell extracts and spent culture media.

The external medium of the cell or the cell extract was first concentrated to a small volume (1 ml) by vacuum distillation at 30° C and then spotted on Whatman No. 1 chromatography paper (18 x 22 cm) either for one-dimensional or two dimensional runs.

For one-dimensional chromatograms, 4-5 spots were applied about 6 cm apart using a microsyringe, spotting about 10 µl at each application and drying in between with a hair-drier blowing cold air. For two-dimensional chromatograms, a single spot was applied at the corner of the paper about 10 cm from each edge.

After thoroughly drying, the one-dimensional chromatogram was run in an appropriate solvent at 25° C in air-tight glass chromatography tank in a descending manner. After a specified time the chromatogram was removed and hung in a fume-cupboard to dry. Where required, the Rf value was determined by measuring the distance the solvent front had moved from origin. The ratio of the distance a compound had moved from origin to the distance the solvent front had moved gave the Rf value for that compound.

The two-dimensional chromatograms were first run in phenol : water (made by dissolving 500 g in 124 ml of water) along the long axis. After drying the paper thoroughly in a fume-cupboard, a second solvent was run at right angles to the

first. After drying it again, it was ready to be auto-radiographed.

Scanning of one-dimensional chromatograms

The chromatogram was cut lengthwise into 2.5 cm wide strips which were run under a thin end-window G - M tube fixed on a perspex chamber (Fig. 6). The chart recorder was zeroed by switching off the high voltage supply. One end of the chromatogram strip was fixed to the chart with sellotape, and the other end after passing under the G - M tube was held with a clip with a weight hanging on it. This arrangement caused the strip to remain taut and pass under the G - M tube at a constant speed. Two pencil marks were made, one on the chromatograph strip and the other on the chart simultaneously to align the detected compound on the chart with the actual radioactive area on the chromatogram. If the peak on the chart coincided with the radioactive area on the strip, then its identification was tentatively made by measuring its Rf value and confirmed by co-chromatography.

Autoradiography

Both one and two dimensional chromatograms were fixed with sellotape to card-board sheets of the size of the X-Ray film, turning the edges neatly in to facilitate easy introduction and extraction from the X-Ray film envelope. The film (Kodax, crystallax X-Ray) was removed from its envelope in a dark room provided with safe-light and stapled to the chromatogram at various points. The chromatogram was then returned to the same

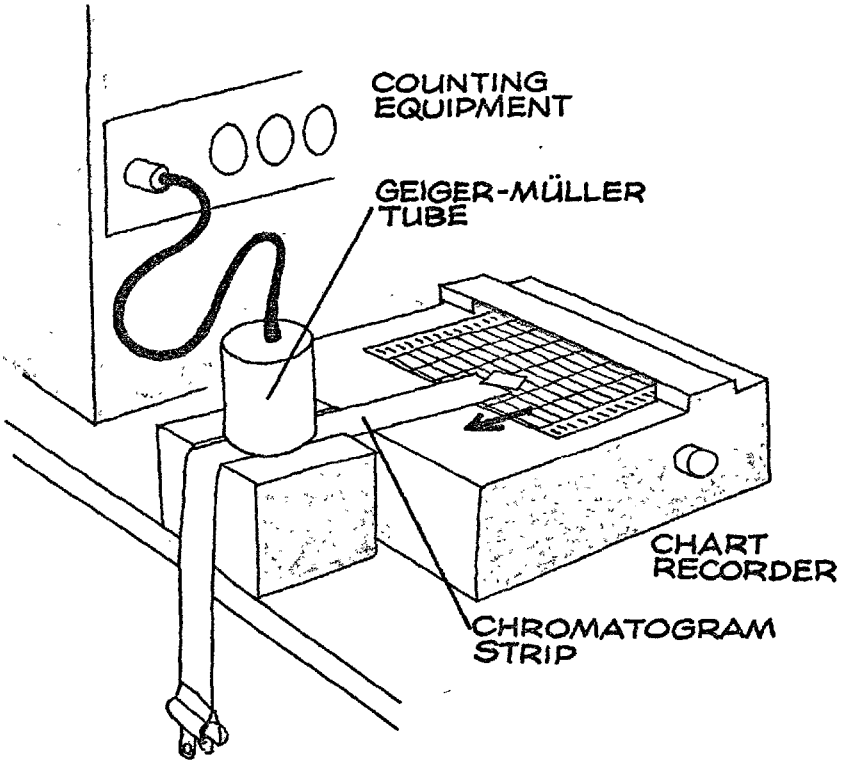


Fig 6 : The chromatogram scanning assembly

envelope, sealed with sellotape and placed in a dark room under a uniform heavy weight.

Chromatograms containing 1,000 cpm at the original spot were supposed to be ready for development in 1-2 days provided the radioactivity was expected to be present in only one compound.

Development of autoradiograms:

After exposure, the chromatogram was removed by pulling off the staples in the dark room and the autoradiogram was developed for 20 minutes in a tank filled with the developing fluid (Kodak D-19). It was then transferred to the second tank containing water and washed for 5 minutes. After that it was removed and transferred to the third tank containing the fixer (Kodak Unifix) until clear. After fixing it was transferred to the fourth tank filled with running water and washed for one hour. It was then removed and hung to dry.

The autoradiogram was aligned with the chromatogram with the help of the staple marks and radioactive areas marked with pencil on the chromatogram.

Co-chromatography:

If a compound was tentatively identified from its Rf value by scanning or by autoradiography its identity was further confirmed by co-chromatography. For this, the compound was eluted from the paper by the method of Dent (1947). The eluate was

evaporated to a small volume by keeping the beaker containing it in a vacuum desiccator. It was then mixed with about 10-20 µl of a 0.5% solution of the authentic compound and the mixture used for one-way chromatography. The chromatogram was run in another solvent, dried and scanned. It was then sprayed or dipped as required with a suitable location reagent, which reacted with the compound to produce a colour. If this coloured spot coincided with the scanned peak on the chart after chromatography in a number of solvents, then its identity was confirmed.

EXPERIMENTS, RESULTS

AND

CONCLUSIONS

Expt. 1

CO_2-Compensation Point Measurements in Various Algae.

Measurement of CO_2-compensation points can give an idea about the mechanism of photosynthesis present in a plant. Most of the higher plants having high compensation points fix CO_2 predominantly by the C_3 (Calvin) mechanism, whereas those showing low compensation points have a C_4 mechanism of CO_2 fixation.

Table 1. CO_2-compensation points of algae. The compensation-points are the means of 4-6 readings in each case. The plus and minus Co_2 treatments agreed within 4 vpm Co_2.

Equilibration time	Species	CO_2-compensation points at 15°C
	Phaeophyta	
Overnight	Ascophyllum nodosum	1.3
"	Pelvetia canaliculata	1.3
3 hours	Fucus vesiculosus	4.1
"	Fucus serratus	3.0
Overnight	Phaeodactylum tricornutum	4.0
	Chlorophyta	
4 hours	Ulva lactuca	3.8
"	Enteromorpha compressa	1.3
3 hours	Chara	48.0
"		
	Rhodophyta	
3 hours	Polysiphonia brodieae	9.1

As shown from the results (table 1), most of the algae gave low compensation points, which are of the same order of magnitude as observed by Brown & Tregunna (1967) in various algae, and by Moss (1962 a) and Tregunna & Downton (1967) in some C_4 plants.

Expt. 2

PEP Carboxylase Assay in Phaeodactylum and its Comparison with Sugarcane.

Since Phaeodactylum gave a very low compensation point, it suggested the presence of an unusual mechanism of photosynthesis such as the C_4 mechanism of higher plants. In order to test for a C_4 type metabolism in Phaeodactylum it was decided to assay the PEP carboxylase (which is a principal CO_2 fixing enzyme in C_4 plants) in Phaeodactylum and compare it with the enzyme from sugarcane, which is a C_4 plant.

Preparation of sugarcane cell extracts

The sugarcane used in this experiment was grown in the green-house. 4 g of sugarcane leaves without the central vein were homogenised at 0-4° C with a Polytron homogeniser for 90 seconds in 16 ml of the following buffer:

Tris HCl (pH 8.3)	-	100.0 mM
2-mercaptoethanol	-	5.0 "
$MgCl_2$	-	5.0 "
Na-pyruvate	-	2.5 "
Na-EDTA	-	1.0 "

Preparation of Phaeodactylum cell extracts

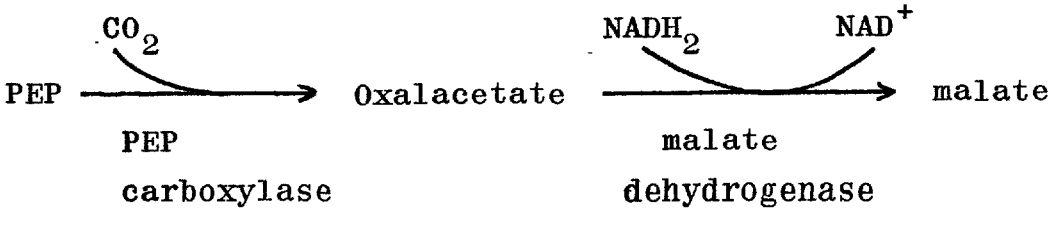
4 g of Phaeodactylum cells were centrifuged and resuspended in 16 ml of the buffer as used for the sugarcane extract and passed once through a French press at 15,000 psi. 95% of the cells were broken.

PEP carboxylase assay mixture

The following components of the assay mixture were present in a total volume of 3 ml:-

$NADH_2$	-	0.38 µmoles
PEP	-	0.40 "
Tris HCl (pH 7.6)	-	0.10 "
$NaHCO_3$	-	4.00 "
$MgSO_4$	-	10.00 "
Enzyme extract		

The oxidation of $NADH_2$ was determined with a spectrophotometer (SP 800) at 340 nm using a Servoscribe chart recorder to record the time-course of absorbance changes. PEP carboxylase activity was measured as the stimulation of $NADH_2$ oxidation following the addition of PEP. The chemical mechanism of the assay can be written as:-



In order to test for the presence of inhibitors of PEP carboxylase in Phaeodactylum cell extracts, extracts from both sugarcane and Phaeodactylum were mixed in equal proportions and the combined activity was assayed. The results are shown in table 2.

Table 2 Comparison of the PEP carboxylase activity of sugarcane and Phaeodactylum cell extracts. Rates were calculated just before and after adding PEP

Expt.No.	Extract	µmole $NADH_2$ Oxidised/m/g fresh wt.			
		before	after	Difference due to PEP carboxylase	
1	Sugarcane	0.07	0.94	0.87	Mean = 0.49
	Phaeodactylum	0.05	0.15	0.10	
	Sugarcane + Phaeodactylum	0.12	0.90	0.78	
2	Sugarcane	0.19	0.51	0.32	Mean = 0.19
	Phaeodactylum	0.08	0.13	0.05	
	Sugarcane + Phaeodactylum	0.08	0.33	0.27	

PEP carboxylase from Phaeodactylum showed a very low activity as compared to the sugarcane enzyme. $NADH_2$ was

oxidised by both extracts even without the addition of PEP, but this auto-oxidation stopped after a few minutes. When PEP was added, oxidation of $NADH_2$ by sugarcane extract was about eight times faster than that from Phaeodactylum.

When the two extracts were mixed together in equal proportions to determine whether there was any inhibitor in the Phaeodactylum extract, no inhibition was observed. In theory, if there was no inhibition, the rate for the combined extracts should equal the mean rate for the extracts assayed independently. In practice, the combined extracts gave rates, if anything, slightly above the mean (table 2) thus showing that Phaeodactylum extracts contain no significant inhibitors of PEP carboxylase.

Perhaps the somewhat higher than expected activity in the combined extracts might be due to an extra supply of malic dehydrogenase from Phaeodactylum permitting a more efficient coupling of the PEP carboxylase from the sugarcane component to NADH oxidation.

Expt. 3

Short-term ^{14}C-feeding Experiments with Phaeodactylum

Since the results of the previous experiment showed that CO_2 fixation by PEP carboxylase was not so important in Phaeodactylum as in sugarcane, it suggested the likelihood of a 'Calvin' type of CO_2 fixation mechanism. It was decided

to examine the initial products of photosynthesis using short-term $^{14}CO_2$ feeding experiments.

In order to minimize problems with chromatography due to the presence of large quantities of salt from the medium in the extracts, an unconventional method was devised which was a modification of Coombs & Baldry's (1970) work with isolated chloroplasts. In this, cells, after being centrifuged were resuspended in Mann & Myers' medium and filtered through Whatman no. 1 filter paper (9.0 cm in diameter) under a gentle suction to deposit an even layer of cells. The filter paper was then placed in a 200 ml pyrex dish and 2-3 ml of the medium were sprinkled over it to prevent desiccation. The dish was kept in the fume-cupboard for 15 minutes under tungsten illumination (800 lux) through a beaker filled with cold water to act as a heat filter.

80 μCi of $Na_2{}^{14}CO_3$ dissolved in distilled water was taken and diluted to 5 ml in a 50 ml beaker before use. After 15 minutes of steady state photosynthesis, the isotope solution was poured over the filter paper. After 5 seconds, 50 ml of boiling 80% ethanol were added to the dish which was then rapidly transferred to the hot-plate and boiling continued for 3 minutes. After this period, the extract was decanted and the filter paper with the cell residue washed again with two, 10 ml instalments of boiling 80% ethanol. The extracts were pooled, filtered and concentrated to about 1 ml by vacuum evaporation. The extract was spotted on Whatman no. 1 filter paper and both one-and two-dimensional chromatograms

were run in the butanol : acetic acid : water solvent whereas for two-way chromatograms the first solvent was phenol : water and the second was butanol : acetic acid : water (120 : 30 : 50).

The results of chromatography and scanning showed a single radioactive peak which from its Rf value was tentatively identified as 3-PGA. The results of co-chromatography confirmed this. For co-chromatography, two different solvents were used i.e. butanol : pyridine : water (80 : 80 : 40) or butanol : propionic acid : water (100 : 50 : 70). When the chromatograms were sprayed with ammonium molybdate reagent (Hanes & Isherwood, 1949), the blue spots coincided in each solvent with the radioactive peak.

For further confirmation, the partition coefficient was determined. In this the suspected PGA spot was eluted in a graduated centrifuge tube and the eluate dried completely with a fan. Then 2 ml of 0.1 N HCl were added to convert the PGA to glyceric acid. Seven ml of ether were put on top of this solution and mixed thoroughly with a thin glass rod, the mixture was then centrifuged at 500 x g for 5 minutes to separate the phases, 0.5 ml from each phase were plated on duplicate aluminium planchets, dried and counted. After subtracting the background, the ether layer had 2 cpm as compared to the aqueous layer which had 39. The distribution coefficient of glyceric acid was 0.051 which is close to that which Calvin et al (1950) obtained in their experiments (0.054) for glyceric acid.

Expt. 4

Measurement of Rates of Photosynthesis at Varying CO_2 and O_2 Concentrations

Inhibition in the rate of photosynthesis with increasing O_2 concentrations has been reported in both C_3 plants and algae and is called the 'Warburg effect'. This inhibition can be reversed by increasing the CO_2 concentration.

In some algae, the 'Warburg effect' is not as pronounced as in higher plants at 21% O_2 concentration (Bjorkman, 1967). Since recent researches have shown glycollate synthesis as a result of a competition between O_2 and CO_2 in higher plants, it was considered worthwhile to determine whether the same mechanism could explain glycollate synthesis in a selection of algae by showing a similar competition between O_2 and CO_2. For this, the rate of photosynthesis was measured in various algae and compared with that of tobacco in different low CO_2 concentrations and two levels of O_2, 21% and 0%. CO_2 uptake was measured with the IRGA and O_2 concentration with a paramagnetic O_2 analyser (Servomex Type OA 150).

Samples of either Phaeodactylum or Chlorella were put in a 500 ml flask in their respective media at a conc. of 88 µl pcv/ml. The flask was fitted with a rubber bung with 2 holes. Gas was drawn from the flask by a peristaltic pump (Watson & Marlow), passed into the analysis tube of the IRGA and returned to

the flask, thus establishing a closed circuit. The reading on the IRGA was recorded on a Servoscribe chart recorder. The O_2 analyser was connected into the circuit only when measuring the O_2 concentration at the start and finish of a run and was then disconnected. This was necessary, because the silica gel used to remove moisture adsorbs CO_2 and would cause inaccuracies in the CO_2 analysis. A schematic diagram of the flow circuit is given (Fig. 7).

The temperature was kept constant at 25^{o} C by immersing the flask in a thermostatic bath fitted with a refrigeration unit. The light intensity was 10,000 lux. The oxygen level was set by filling the apparatus from an appropriate cylinder with the taps set at 'B' until the oxygen analyser read the correct value. The cylinder and analyser were then disconnected by the two-way taps being set to 'A' so as to set up a closed circuit. A fraction of a ml of pure CO_2 from a cylinder was injected into a rubber tube forming part of the circuit, using a hypodermic syringe, so as to give a final concentration of 400 vpm CO_2 measured on the IRGA.

For tobacco, a rectangular perspex leaf chamber (10 x 20 cm) was used having an outer water jacket to circulate water from the thermostatically controlled water bath. The light intensity incident on the chamber was 10,000 lux. The circulating gas was introduced from an inlet at one end and was removed through an outlet near the other. The leaf was first thoroughly washed with distilled water, then cut into pieces avoiding the thicker

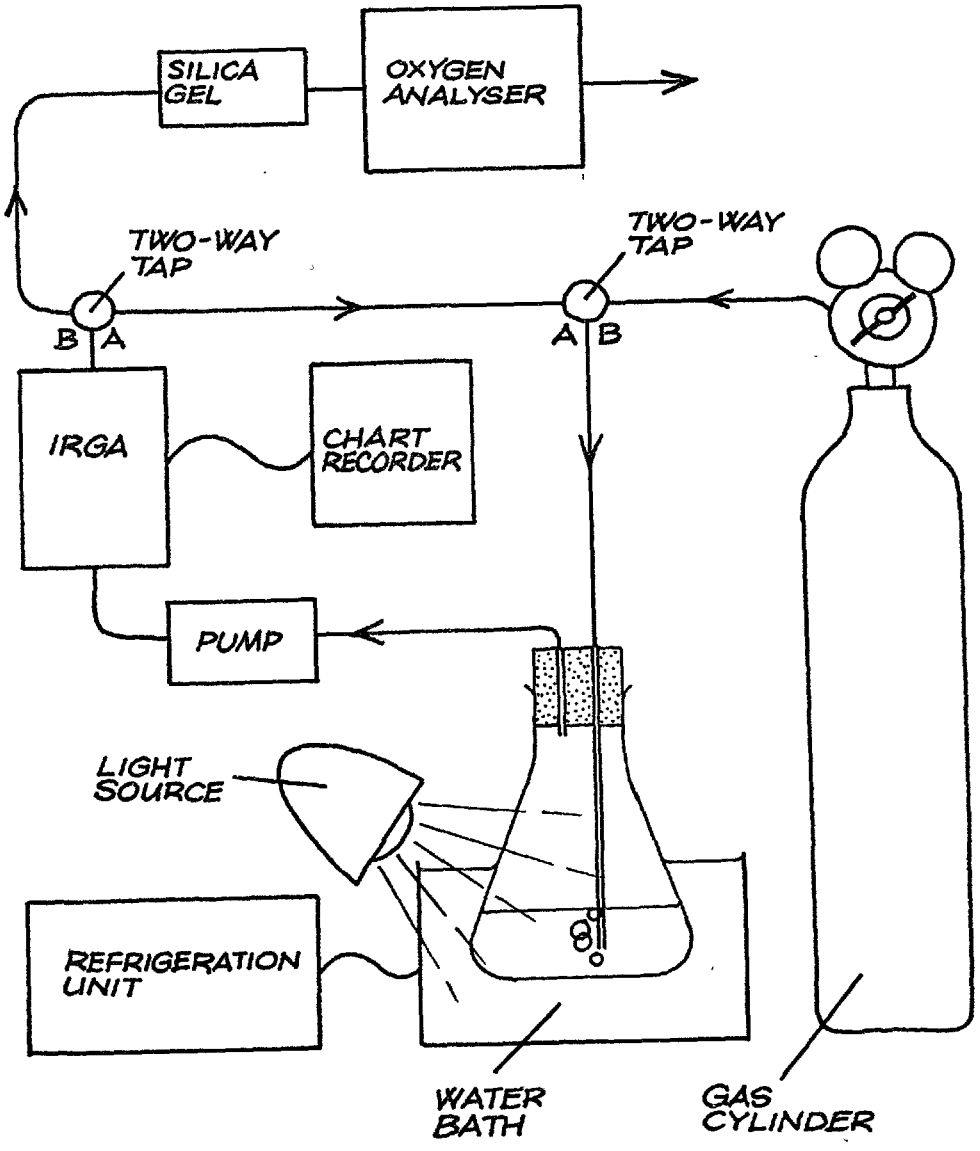


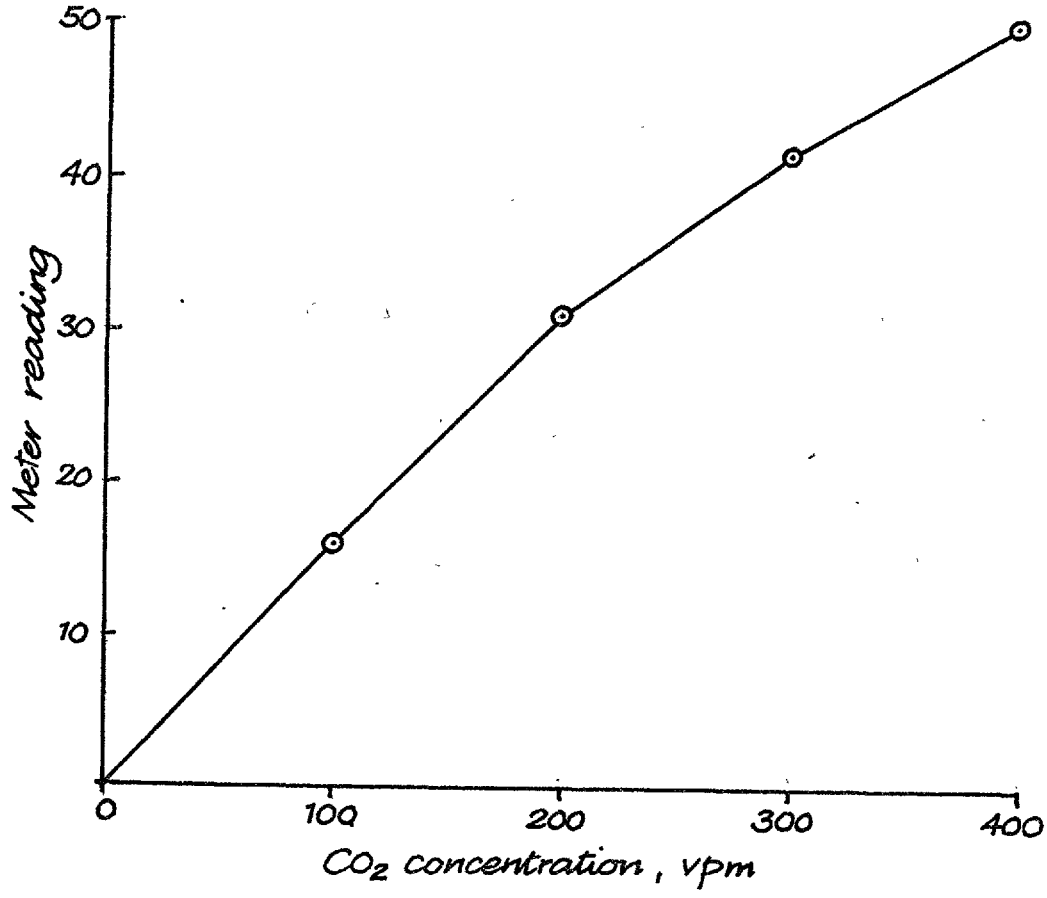
Fig 7: A schematic diagram of the apparatus used for measuring the rate of photosynthesis

veins, and floated lower side up in few ml of distilled water inside the leaf chamber. The door of the leaf chamber was sealed with lanoline and plasticine. The absolute rate of CO_2 uptake was calculated from the rate of change of CO_2 concentration and the total volume of the flow system. Calculations were made for uptake of CO_2 at three different points on the graph representing three different levels of CO_2. Measurements were made repeatedly on the same plant material at O_2 concentrations of 0 and 21%. The IRGA was nearly linear over the range of 0-285 vpm CO_2 (Fig. 8). Errors due to slight non-linearity would, of course, not affect the % inhibition by O_2. The results are shown in table 3, and figs. 9-11.

Table 3 A comparison of the % inhibition of photosynthesis between algae and tobacco in 21% O_2 at varying CO_2 concentrations. Inhibition was calculated from mean rates of photosynthesis.

Plant	CO_2 conc. vpm	Rate of photosyn. µl/g/h		% inhibition
		N_2	Air	
Phaeodactylum	80	15	12	18.2
	160	100	82	17.4
	280	311	257	17.4
Chlorella	80	16	14	7.7
	160	83	64	22.7
	280	294	226	23.0
Tobacco	80	630	330	47.6
	160	2900	2188	24.6
	280	3500	2700	22.9

Fig 8 : Calibration curve for the IRGA



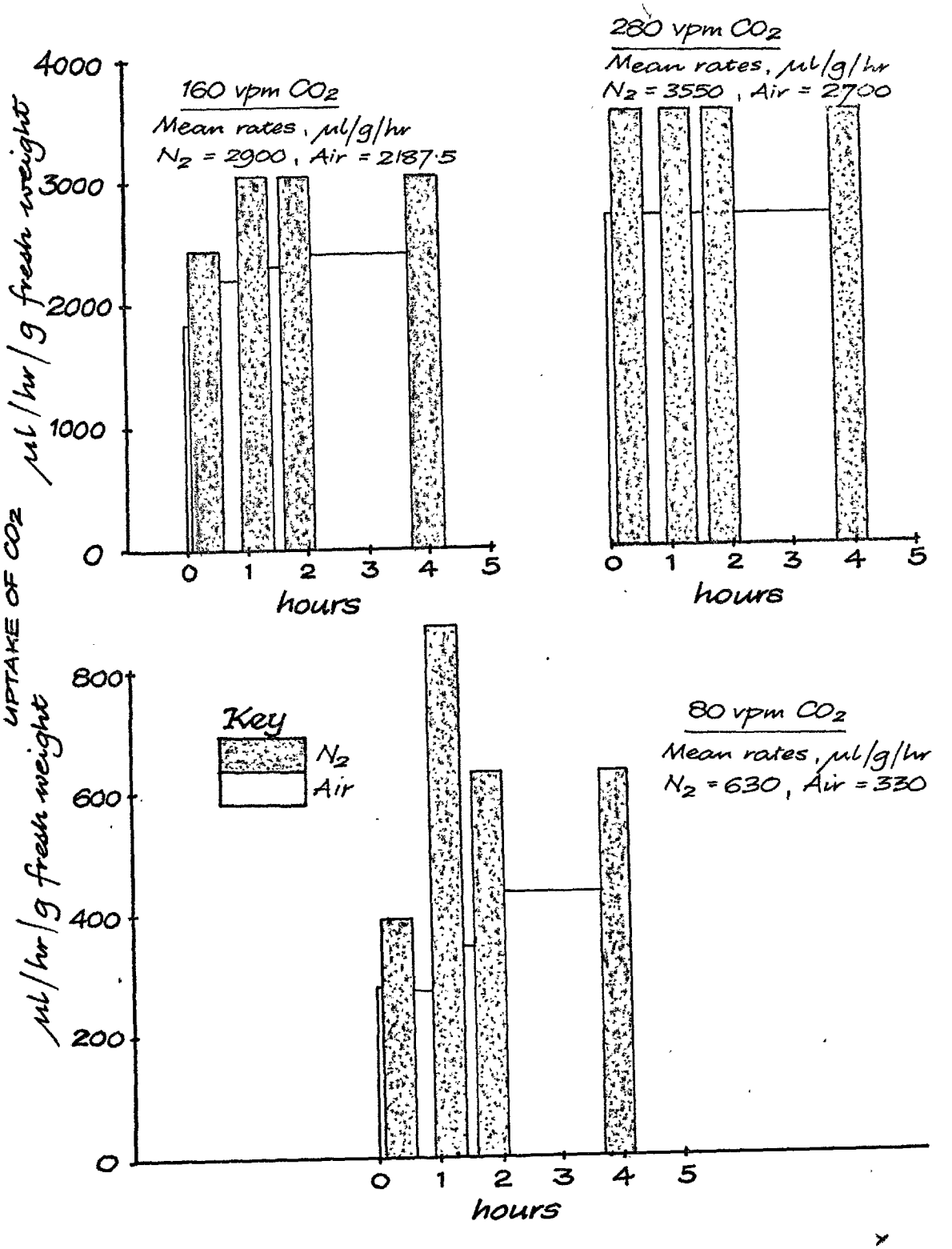


Fig 9: The rate of photosynthesis of Tobacco at varying concentrations of O_2 & CO_2

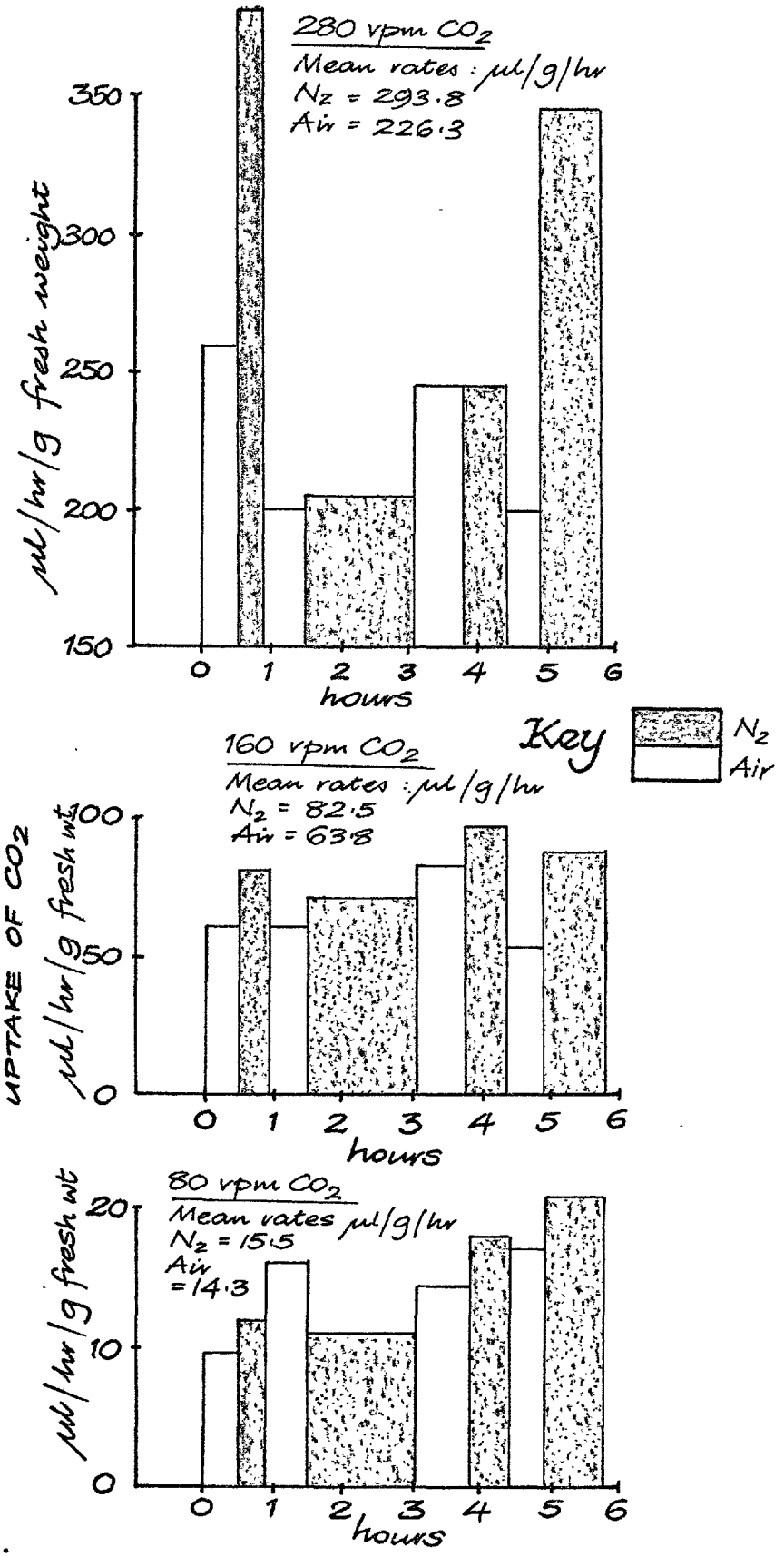
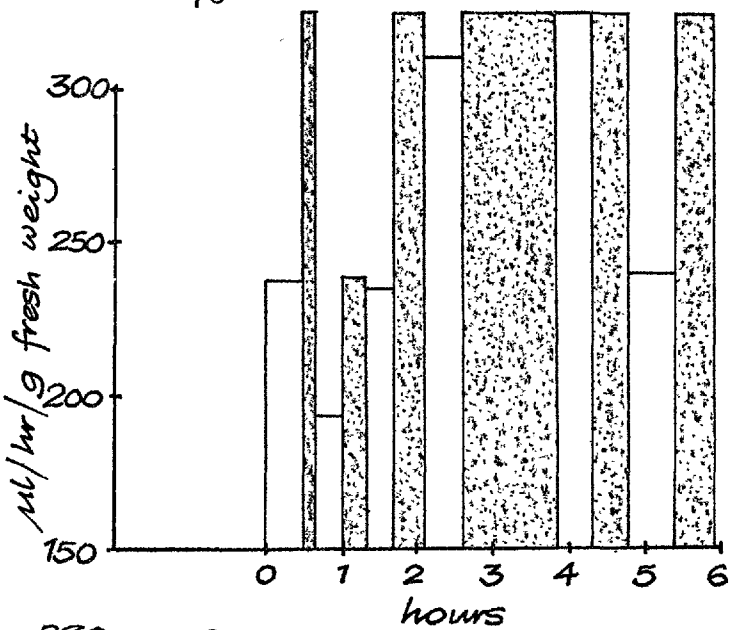
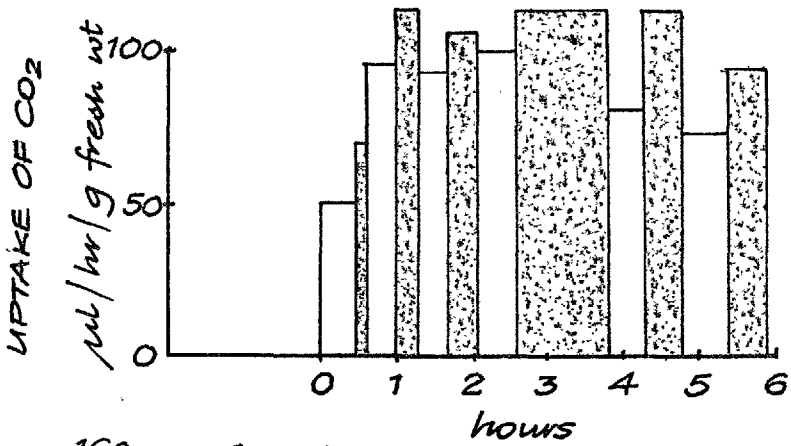


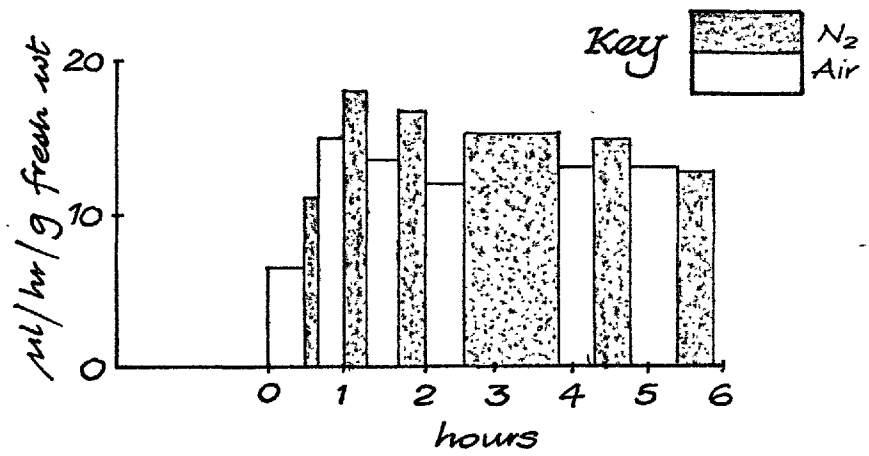
Fig 10: The rate of photosynthesis of Chlorella at varying concentrations of O_2 & CO_2



280 vpm CO_2 Mean rates µl/g/hr : N_2 = 311, Air = 257



160 vpm CO_2 Mean rates µl/g/hr : N_2 = 99.58, Air = 82.3



80 vpm CO_2 Mean rates µl/g/h : N_2 = 14.8, Air = 12.1

Fig 11: The rate of photosynthesis of *Phaeodactylum* at varying concentrations of O_2 & CO_2

Up to 18% inhibition of photosynthesis was observed in Phaeodactylum as compared to 23% inhibition in Chlorella at 21% O_2 concentration. Tobacco, however, showed up to 47% inhibition.

The results show a considerable variability even within a single experiment but the mean rates of photosynthesis were consistently higher at low oxygen for all organisms tested. This would imply that the apparently low rates of photorespiration in the algae are not due to a mechanism similar to C_4 metabolism in which O_2 concentrations up to 21% have no effect on the rate of photosynthesis (Downes & Hesketh, 1968).

Expt. 5

The Effect of Oxygen on Ribulose Diphosphate Carboxylase from Algae and Higher Plants

Since glycollate synthesis is believed to be an effect of O_2 on RuDP carboxylase in higher plants, it was decided to see whether this enzyme, when extracted from low compensation point algae, had similar properties.

Extraction of RuDP carboxylase from Fucus serratus

Fucus serratus has a low compensation point like Phaeodactylum (table 1) and it was decided to use it for enzyme assays because it would be easier to obtain in bulk.

The thallus was washed thoroughly with ice-cold distilled water to remove epiphytic algae and cut into small pieces with scissors. All operations were subsequently carried out at 0-4°C. 35 g were homogenised with 100 ml of buffer in a Polytron homogeniser. The buffer used was 10 mM Potassium phosphate pH 7.6 containing 0.1 mM EDTA and 10 mM mercaptoethanol. The cooling during homogenisation was provided by an external ice-jacket. Homogenisation was carried out in short bursts for 4-5 minutes, letting the homogenate cool down to 4° C in between.

The homogenate was filtered through 2 layers of destarched muslin and the pH of the filtrate adjusted to 7.0 with 5N NH_4OH. The filtrate was centrifuged at 26,000 x g for 30 minutes to remove the cell debris. One ml of this crude extract was passed

through a small Sephadex column G-25 (0.9 x 30 cm) and was eluted with 5 mM potassium phosphate buffer containing 0.1 mM EDTA and 10 mM 2-mercaptoethanol pH 7.6. This step was taken to remove any small molecules, free sugars, peptides, free amino-acids etc. It was hoped that tannins and even alginic acid would be removed by this column. The eluate was assayed for RuDP carboxylase (Paulsen & Lane, 1966), but no activity was observed.

It was thought that perhaps the enzyme was bound to the cell particles and could be freed and made soluble by adding detergent. Brandon (1963) and later Allen (1966) have successfully used the non-ionic surfactant Tween 40 (polyoxyethylene sorbitan monopalmitate) to liberate enzymes such as PEP carboxylase from particles in extracts of other tissues. The extraction technique for Fucus was modified accordingly. After homogenising the thallus, one part of 20% aqueous Tween 40 was added to nine parts of the extract, allowed to stand for 10 minutes and centrifuged at 26,000 x g for 5 minutes. A one ml portion of the supernatant was passed through a Sephadex column as described before and the eluate assayed. Again no activity was observed.

It was thought that the lack of activity might be due to the mucilagenous materials in the cell extract inhibiting the enzyme. In order to prevent this it was decided to prepare chloroplasts from the thallus, wash these free of mucilage and attempt to extract the enzyme from these. To do this, 0.3 M

sucrose was added to the extraction medium described before, whereas Tween 40 was excluded. The homogenate was filtered through 2 layers of muslin, centrifuged at 26,000 x g for 90 seconds and the chloroplast pellet washed three times and recentrifuged with the same buffer to remove any mucilage. After this, the chloroplasts were suspended in 10 mM potassium phosphate buffer minus the sucrose. A microscopic examination using an oil-immersion lens revealed that the chloroplast membranes were ruptured. The chloroplast debris was removed by centrifugation and the supernatant assayed for enzyme activity. Again no activity was observed. $(NH_4)_2\ SO_4$ fractionation with the supernatant produced a small precipitate at 37% saturation and no precipitate was obtained with further additions of $(NH_4)_2\ SO_4$.

The work done so far suggested four possibilities for the failure of the technique.

1. Inhibitory substances were still present in the extract and were inhibiting the enzyme action.
2. The presence of alginate and other mucilages retarded the extraction of proteins and RuDP carboxylase was not liberated.
3. There was a proteolytic enzyme which was causing the breakdown of RuDP carboxylase.
4. The plant contains no RuDP carboxylase.

The first possibility has been suggested by Jacobi (1958) and second by Smith and Young (1953) in Fucus vesiculosus where they could dissolve only 50% of the total nitrogen.

In the hope of testing the second possibility attempts were made to remove the alginate from the extract. McDowell (1955) observed that calcium alginate is extremely insoluble and could be precipitated by low concentrations of calcium ions. In accordance with this the extraction buffer was modified again and this time tris HCl buffer pH 7.8 was used instead to avoid precipitation of Potassium Phosphate buffer with $CaCl_2$. The extraction medium contained:-

Tris HCl pH 7.8	-	10 mM
$CaCl_2$	-	10 "
2-mercaptoethanol	-	10 "
sucrose	-	0.3 M

Isolated chloroplasts were suspended in the above buffer minus sucrose to break the chloroplasts. They were further ground with the pestle and mortar using a little acid washed sand for 3-4 minutes at 4^{o} C. The chloroplast debris and calcium alginate precipitate were removed by centrifugation and 1 ml portion of the supernatant was passed through Sephadex G-25 column and the eluate assayed. Again no activity was observed.

In another attempt, the amount of calcium chloride was doubled, again no activity was observed and no precipitate appeared even after the addition of 50% $(NH_4)_2SO_4$. This last observation suggested that the extract contained no protein with the same solubility in $(NH_4)_2SO_4$ as RuDP carboxylase. Although this could have been due to the genuine absence of RuDP carboxylase from the original plant it would seem unlikely that this would lead to a complete absence of protein in the 50% $(NH_4)_2SO_4$ fraction. A more likely explanation might be that the calcium treatment also precipitated the protein since Duncan, Manners and Ross (1956) observed a co-precipitation of protein with alginate from Laminaria extracts after $CaCl_2$ treatment.

In conclusion it might be stated that all attempts to detect RuDP carboxylase in Fucus extracts have failed. This may be due to an inhibitory effect of alginates or other substrates in the extract but present techniques do not permit the removal of the alginate from the protein fraction. We cannot therefore, conclude that RuDP carboxylase is absent, merely that the material is unsuitable for investigation with respect to this enzyme.

Extraction of RuDP Carboxylase from Ulva lactuca and Spinach

Ulva lactuca was chosen which also has a low CO_2 compensation point (table 1) and its RuDP carboxylase which proved amenable to extraction, was compared with the spinach

enzyme. Spinach was bought from the market. 35 g of Ulva thallus (or destalked spinach leaves) were washed thoroughly in ice-cold water and then homogenised in 100 ml of buffer in a Polytron homogeniser at 4^{o}C. The buffer contained

Potassium phosphate (pH 7.6)	-	10 mM
Na-EDTA	-	0.1 mM
2 - Mercaptoethanol	-	10.0 mM

The homogenate was filtered through 2 layers of de-starched muslin and the pH of the filtrate adjusted to 7.0 with 5N NH_4OH. After Polytron homogenisation, the Ulva residue showed a large number of intact cells under the microscope. It was therefore further homogenised with a pestle and mortar using about 1 g of washed sand. The filtrate was centrifuged at 26,000 x g for 30 minutes to remove the cell debris. The super-natant was brought to 37% saturation by adding 226 g/l of solid $(NH_4)_2SO_4$. The precipitate was removed by centrifugation at 9,000 x g for 30 minutes. The supernatant was brought to 50% saturation by adding 92.5 g/l of $(NH_4)_2SO_4$, and the precipitate collected by centrifugation at 9,000 x g. This precipitate was dissolved in 1.5 ml of 0.1 M potassium phosphate buffer (pH 7.6) containing 0.1 mM EDTA and 10 mM 2-mercaptoethanol. This fraction, called the $(NH_4)_2SO_4$ - 1 fraction was passed through a G-25 Sephadex column (0.9 x 30 cm) previously equilibrated with 5mM potassium phosphate buffer pH 7.6 containing 0.1 mM EDTA and 10 mM 2-mercaptoethanol. The enzyme was eluted with the same buffer at 4^{o} C. The first fraction which was light brown in colour was collected and the bright yellow fraction following it was discarded.

RuDP carboxylase assay at different oxygen concentrations

Each assay mixture contained in 1.5 ml

Tris HCl (pH 7.8)	-	300.0 µmoles	
RuDP (Na_4)	-	1.5 "	
$Na_2{}^{14}CO_3$	-	0.09 "	(6 µCi)
$MgCl_2$	-	15.00 "	
EDTA	-	0.09 "	

One such mixture, but minus the enzyme, RuDP, and $Na_2{}^{14}CO_3$, was placed in each of 3 test tubes which were then sealed with serum caps. Each tube was gassed with either 100% O_2, CO_2-free air (21% O_2) or N_2(0% O_2) for 20 minutes, by alternately evacuating, reintroducing the gas and shaking the tube to facilitate thorough mixing. The tubes were kept in a thermostatic water bath at 30°C.

Just before assay, the RuDP was injected through the serum cap, followed by 6 µCi of $Na_2{}^{14}CO_3$ and lastly the enzyme solution. The tubes were shaken and contents from each drawn into separate 1.0 ml syringes which were then left in their respective test tubes. Duplicate 0.1 ml samples were expelled at 3 minute intervals into scintillation vials, each containing 0.1 ml of glacial acetic acid to stop the reaction. After the experiment, all the vials were heated in an oven at 85° C to evaporate the acid and unused isotope. When the contents had dried completely, 0.1 ml of distilled water was added to each to redissolve the residue. Radio-activity was determined by scintillation counting.

Zero-time readings were taken by adding 0.2 ml of glacial acetic acid into the assay mixture before adding the substrate, isotope and enzyme solution.

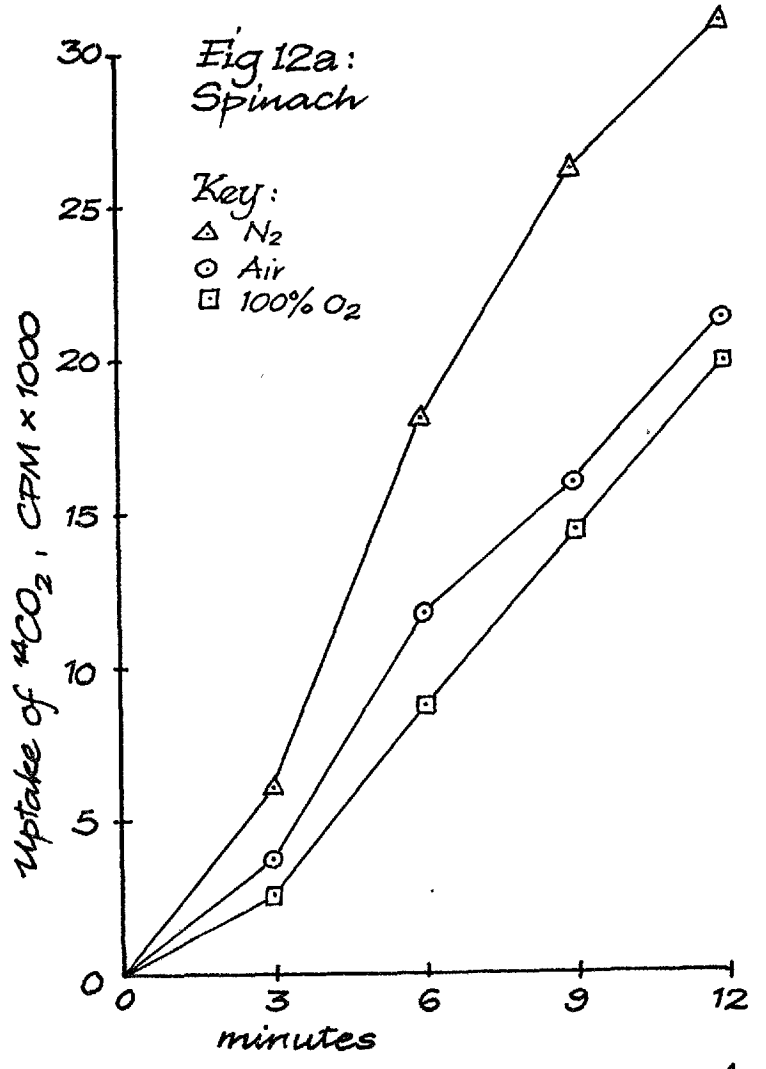
As shown from the graphs (Figs. 12a, b), the algal enzyme and the higher plant enzyme behave similarly, giving increasing inhibition with increasing oxygen concentration. If this reflects an increasing oxygenase activity at the higher O_2 concentration, then it would imply that the glycollate producing reactions are similar both in nature and magnitude in both organisms.

Expt. 6

$^{14}CO_2$ Release in Light and Darkness by Radio-activily Labelled Phaeodactylum and Chlorella Cells.

In order to test whether low compensation point algae had any capacity to release CO_2 by photorespiration, the algae were made radio-active by feeding $Na_2{}^{14}CO_3$ and the subsequent release of $^{14}CO_2$ in CO_2-free gas stream was measured. The oxygen sensitivity of this $^{14}CO_2$ release was taken as an indication as to whether it was due to photorespiration or dark respiration.

Phaeodactylum cells were harvested by centrifugation and resuspended in 50 ml of medium in a 1-litre flask kept in a thermostatic water bath at 15° C and illuminated at 7,000 lux. The cells were fed with 50 µCi of $Na_2{}^{14}CO_3$, the flask closed with a tight-fitting rubber bung and the cells allowed to photosynthesize for 4 hours. After this period, the cell suspension was centrifuged at 10,000 x g for 15 minutes, and washed



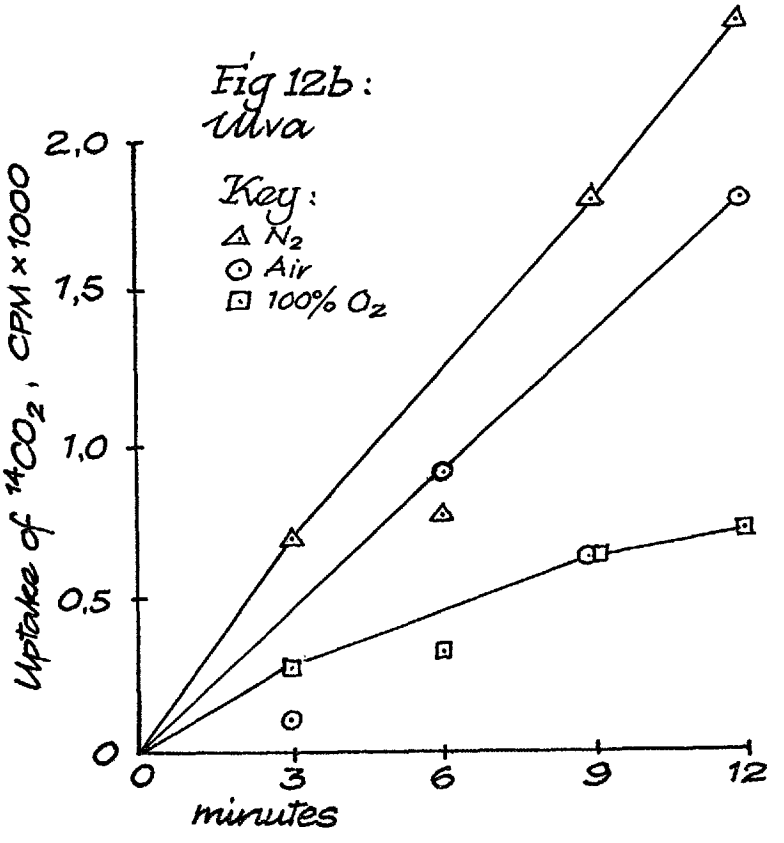


Fig 12 : The effect of O_2 concentration on the RuDP carboxylase activity of Ulva and Spinach

twice by shaking with fresh medium to remove the unused isotope. The cells were then suspended in about 11 ml of medium, out of which 3.0 ml were put into each of three, 20 ml Warburg flasks. Duplicate 0.1 ml samples of this suspension were placed in scintillation vials for counting and a further 1 ml was used to measure packed cell volume. Each flask had 0.9 ml pcv of algae. The Warburg flasks were connected to their manometers, placed in the water-bath of a Warburg apparatus at 15^{o} C and 7,000 lux and shaken.

The $^{14}CO_2$ produced by the algae was collected by passing CO_2-free air into one flask, CO_2-free nitrogen into the other flask and 100% oxygen into the third, the outlets of each of these flasks being connected to two, 250 ml bubblers connected in series, each containing 50 ml of 1.0 M ethanolamine. Duplicate 0.1 ml samples of the ethanolamine were taken at 15 minute intervals during an initial 1 hour period of light followed by 1 hour of darkness. For the dark period the lights were switched off and the flasks wrapped in aluminium foil. The gas flow during this procedure remained uninterrupted. At the end of the experiment, all the samples and replicates were counted by a scintillation counter as described previously. Counts were corrected by using an internal standard, ^{14}C- hexadecane.

The experiments was repeated with Chlorella pyrenoidosa for comparison. Here the cells were resuspended in Chlorella medium and had 0.7 ml pcv in each flask but the rest of the details were the same. The results are shown in table 4 and Fig. 13.

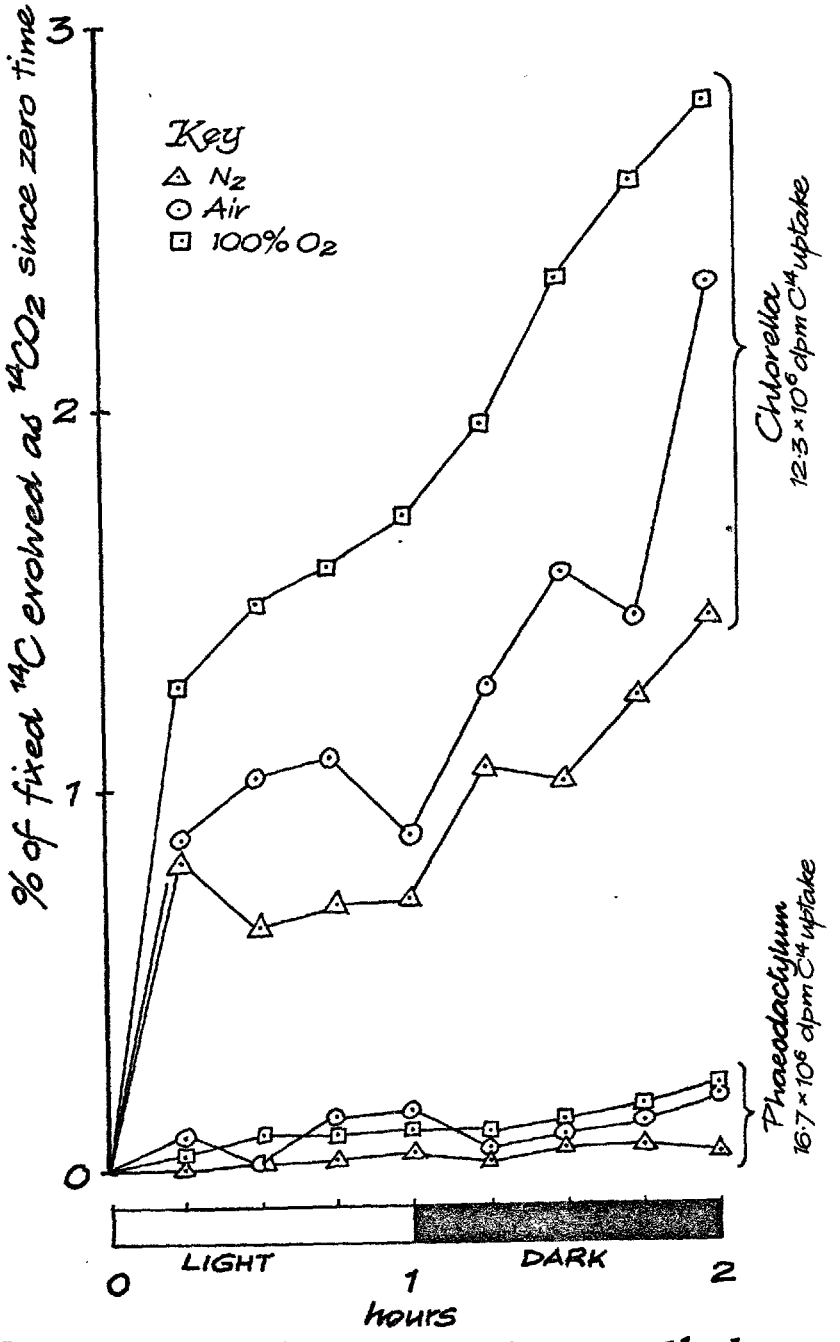


Fig 13 : The release of labelled photosynthate as CO_2 by *Phaeodactylum* and *Chlorella* at different oxygen concentrations

Table 4 Percentage of ^{14}C fixed evolved as $^{14}CO_2$/h. Data presented was derived from the slopes of regression lines calculated by the method of least squares from fig. 13. The standard deviations for each determination are also given.

Alga	Treatment	100% O_2	21% O_2	0% O_2
Phaeodactylum	Light	0.14(±0.02)	0.12(±0.04)	0.03(±0.01)
	Dark	0.12(±0.02)	0.06(±0.05)	0.00(±0.02)
Chlorella	Light	1.44(±0.42)	0.72(±0.40)	0.54(±0.29)
	Dark	1.20(±0.05)	1.20(±0.25)	0.60(±0.15)

When Chlorella or Phaeodactylum were allowed to accumulate radio-active photosynthate and were then put into a stream of CO_2-free air, both released radio-active CO_2. The rate of release when expressed as a % of the total radio-activity within the cells, was much higher in Chlorella than in Phaeodactylum in both light and dark. In Chlorella, in the light this $^{14}CO_2$ release was oxygen sensitive and might be attributed to photorespiration. The rate for Phaeodactylum was too low for its oxygen sensitivity to be accurately determined.

Expt. 7.1

Glycollate Excretion by Untreated Phaeodactylum Cultures

From the previous experiment it was seen that Phaeodactylum had an even lower rate of photorespiration than Chlorella, which is itself a low compensation point alga. If it achieved this by

excreting a greater proportion of its glycollate unmetabolised then it ought to be possible to detect appreciable glycollate concentrations in the spent medium.

The glycollate concentration of Mann & Myers' medium in which Phaeodactylum cultures had been grown was compared with that of fresh medium after culture periods ranging from 30 mins. to 48 hours while under illumination at 6,000 - 10,000 lux at 20° C. The results are shown in table 5.

Table 5

Culture Periods	Illumination (lux)	µmoles glycollate produced /ml pcv
30 minutes	10,000	not detectable
45 "	"	"
60 "	"	"
120 "	"	"
180 "	"	"
19 hours	6,000	"
24 "	"	0.13
48 "	"	0.05

Even after culture periods of 24-48 hours the level of glycollate production was scarcely detectable. The technique used in the above experiment gave somewhat high values for fresh medium (equivalent to about 0.11 µmoles of glycollate per ml) when measured against a reagent blank. This was found

to be largely due to the tris component of the medium. To increase the sensitivity of the measurements, the remaining experiments of the series using the Calkin method were conducted with medium f (Guillard and Ryther, 1962), which gave a 'glycollate' colour of only about one quarter of the Mann & Myers' medium.

Expt. 7.2

Glycollate Production by α-OHMS Treated Phaeodactylum Cultures

Lord & Merrett (1969) showed that for Chlorella pyrenoidosa, α-OHMS inhibits glycollate metabolism and forces its excretion. Accordingly α-OHMS was tested on cultures of Phaeodactylum to see whether any stimulation of glycollate accumulation was possible, either outside or inside the cells.

0.3 ml pcv of algae were suspended in each of two test tubes in 10 ml of medium f, one of which was made to 1 mM with α-OHMS. They were illuminated at 15,000 lux at 22° C for 60 minutes and then filtered through 0.45 μm Millipore filter discs. The filter discs were extracted for 3 minutes in 10 ml of water at 95° C and the extract centrifuged at 9,000 x g. The pellet was washed once with 5 ml of distilled water, recentrifuged and the washings added to the extract. Both the spent medium and the cell extract were purified by passage through anion exchange columns and the glycollate fraction estimated by the Calkin method. Neither the extract nor the spent medium showed any trace of glycollate either in the presence or absence of the inhibitor. A similar result was obtained in another experiment in which the incubation time was increased to 90 minutes.

It can be concluded that α-OHMS at this concentration does not cause glycollate accumulation, either inside or outside the cells of this organism.

Expt. 7.3.

Glycollate Excretion in the Presence of 20 mM α-OHMS by Paeodactylum Cultures

A higher concentration of α-OHMS was used in this experiment and no attempt was made to distinguish between excreted and un-excreted glycollate. 2 ml pcv of Phaeodactylum was suspended in 100 ml of medium f and aerated by bubbling air through the flask. The experiment was performed at 22° C and 15,000 lux. 10 ml of the cell suspension were removed at intervals from the flasks and heated on a steam bath for 3 minutes by which time the pigment was lost from the cells. After centrifugation at 5,000 x g for 10 minutes, the supernatant was concentrated by vacuum evaporation at 30° C.

As shown from the results (Fig. 14), the maximum amount of glycollate detected after 2 hours was 0.07 μmoles/ml pcv.

It will be noted that despite the fact that the extract was concentrated by evaporation before glycollate assay, little glycollate could be detected. To test the validity of the technique a sample of authentic glycollate was added to some medium and assayed exactly as above. After allowing for slight losses expected due to transfer from vessel to vessel it was found to give the correct absorbance at the correct wavelength as measured

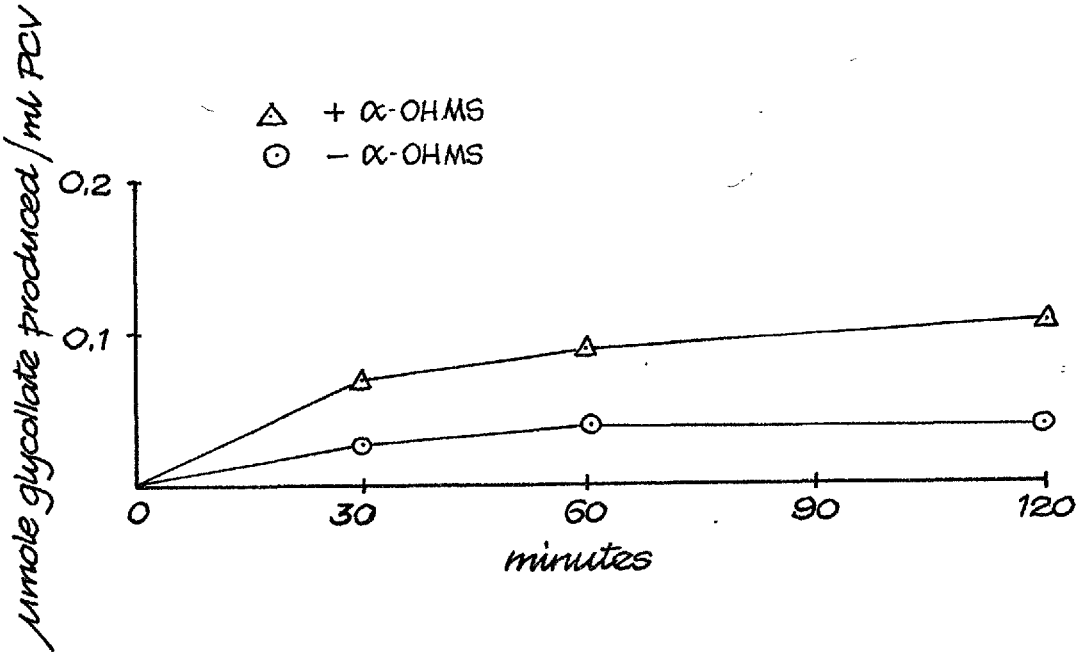


Fig 14 : Glycollate production by *Phaeodactylum* in the presence of α-OHMS

on an SP 800 spectrophotometer. This showed the technique to be valid and apparent low rate of glycollate production genuine.

Expt. 7.4

The Effect of INH on Glycollate Excretion in Chlorella and Phaeodactylum

Since the inhibitor α-OHMS caused little glycollate accumulation in Phaeodactylum it was decided to try to confirm this apparent lack of glycollate production using another inhibitor of the glycollate pathway, INH. For this purpose, Phaeodactylum was compared with Chlorella, an organism known to produce glycollate.

Chlorella pyrenoidosa:

Cells grown in 4% CO_2 in air were used. They were harvested by centrifugation and washed once with distilled water. 2 ml packed cell volume was resuspended in 100 ml of 0.5 mM KH_2PO_4 buffer (pH 4.0) containing 20 mM INH. The inhibitor was added one hour before illumination. The suspension was aerated at 20,000 lux and 22° C. A 20 ml sample at zero time was taken before switching on the lights. After that, 20 ml samples were taken after 15, 30 and 60 minutes. The samples were spun down at 3,000 x g and the supernatant was evaporated to dryness at 30° C. The residue was redissolved in 1 ml of 2N H_2SO_4 and assayed for glycollate. A control was also run in which no inhibitor was used.

Phaeodactylum tricornutum

A similar procedure was used except that the cells were resuspended in medium f. Again no inhibitor was used in the control. As shown in Fig. 15 no glycollate excretion could be detected in either Phaeodactylum or Chlorella in the absence of INH. When INH was present, only Chlorella excreted measurable amounts of glycollate. This is consistent with a hypothesis that Phaeodactylum does not synthesize appreciable quantities of glycollate.

Expt. 8

Excretion of Photo-assimilated Carbon (^{14}C) by Phaeodactylum tricornutum Under High and Low Oxygen Concentration

Because very little glycollate excretion could be detected in Phaeodactylum it was decided to test whether other substances were excreted instead, perhaps products of glycollate metabolism.

5 ml of medium were placed in two test tubes to which an inoculum was added under aseptic conditions to give a cell density of 2×10^7 cells/ml. The cells were counted using a hemocytometer and a telecounter. The mouth of each tube was covered with a serum cap. Nitrogen was bubbled through the medium for 10 minutes in one tube and 100% O_2 through the other, using hypodermic needles piercing the serum cap. The only carbon source was 50 µCi (0.89 µmoles) of $Na_2{}^{14}CO_3$, which was injected through the serum cap. The tubes were held in a tube-holder and the light (10,000 lux) was passed through a 1 litre glass beaker

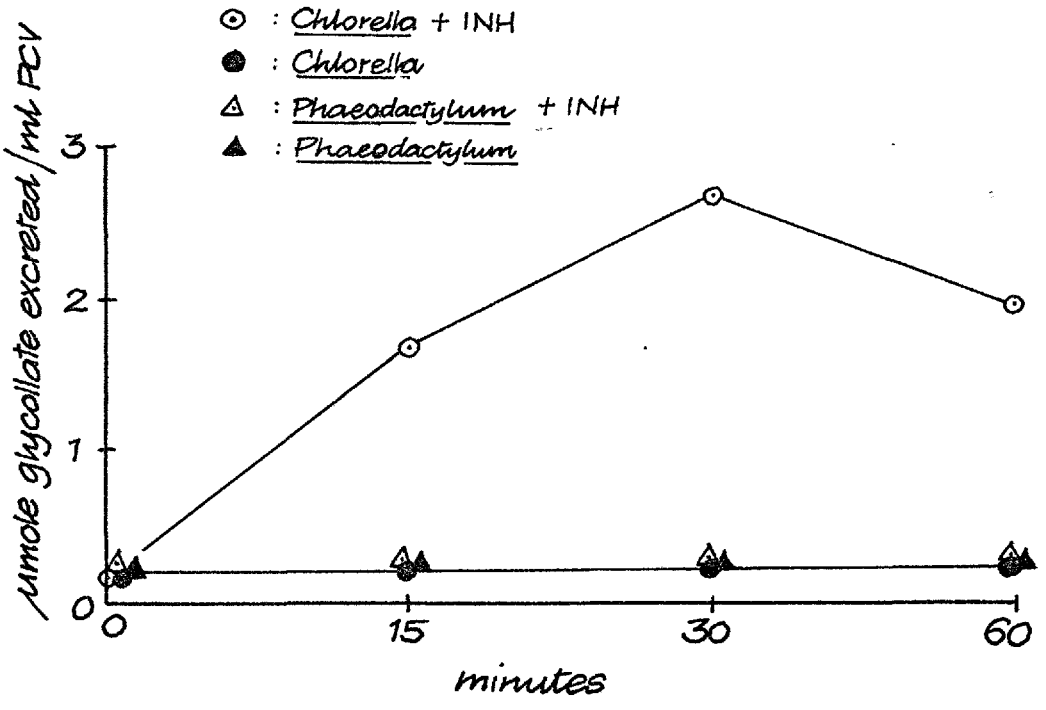


Fig 15 : Glycollate excretion by Chlorella & Phaeodactylum and the effect of INH

filled with running cold water to act as a heat filter. The algae were incubated with the isotope at 22° C for 23 hours which is one cell generation time for this diatom (Hellebust, 1965).

At the end of this period, duplicate 0.1 ml samples of the cultures were diluted with 10 ml of medium and filtered through a Millipore filter-disc (0.22 µm pore size) under vacuum. The filters were washed with 10 ml of medium, dried in a vacuum desiccator and counted on an automatic scaler (Ecko Type N 530G) having about 5% counting efficiency.

The remainder of the cultures were filtered through 0.22 µm pore size filter discs under vacuum without washing, the filtrates acidified with HCl to approximately pH 2.8 and air was bubbled vigorously through them for about 5 minutes to remove all inorganic carbonate.

Duplicate 0.1 ml samples of the filtrate were also spotted on Whatman no. 1 paper for one-way chromatography and run in a butanol : acetic acid : water solvent (4 : 1 : 1) and various excreted compounds located by scanning.

Three main radio-active substances were excreted. They were not identified but they had Rf values of 5.4, 10.8 and 24.3 respectively. None of these correspond to the published value of 56 for glycollate in this solvent (Smith, 1960).

Figure 16 shows the results of a large number of separate determinations of photosynthetic rate and glycollate excretion. There is a clear relationship between the percentage of photosynthate excreted and the rate of photosynthesis with a greater

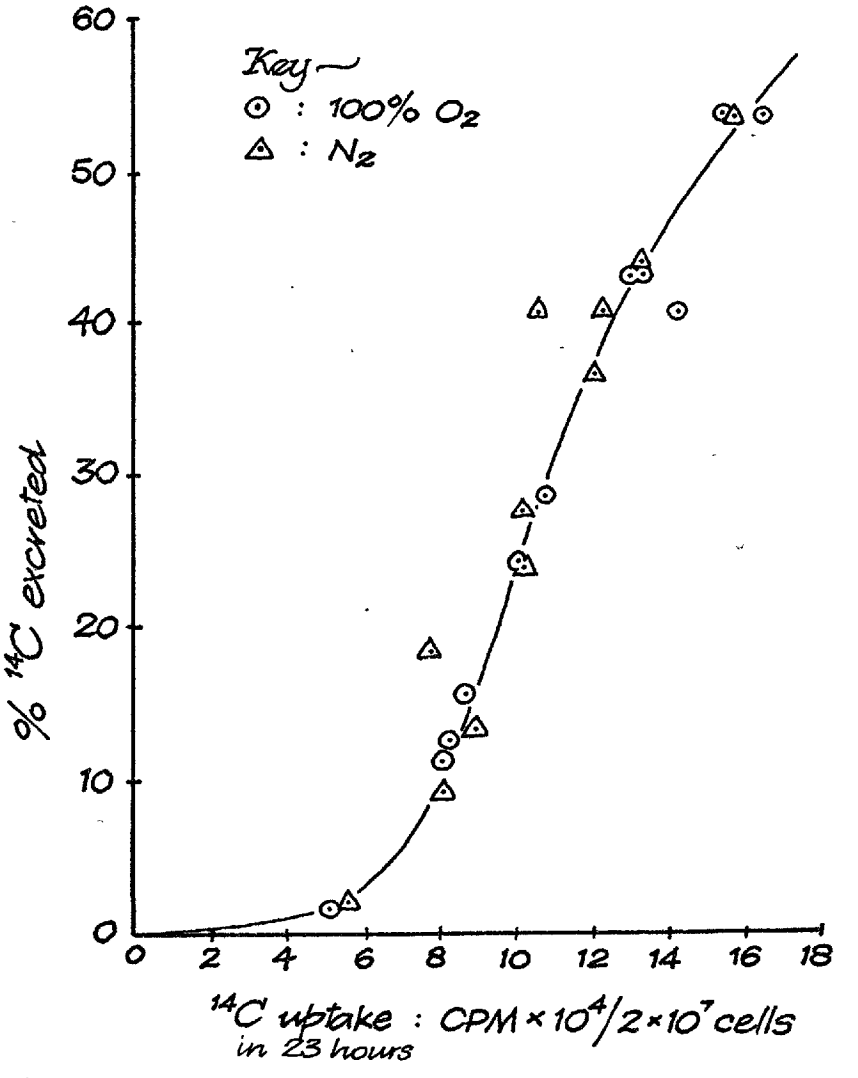


Fig 16 : The effect of O_2 concentration and photosynthetic rate on the % of ^{14}C-photosynthate excreted by *Phaeodactylum*

proportion of photosynthate being excreted at higher photosynthetic rates. This relationship, however, was unaffected by oxygen concentration which suggests that the substances excreted are probably not products of glycollate metabolism.

Expt. 9

Glycollate - Dependent Oxygen Exchange and the Effect of α-OHMS in Phaeodactylum

Since the results of the previous experiments suggested that glycollate is probably not synthesized in Phaeodactylum, it was decided to investigate whether this diatom can metabolise exogenous glycollate. It was also aimed to observe what effect the inhibitor α-OHMS would have on the rate of photosynthesis in the presence of added glycollate. For this cells were harvested by centrifugation, resuspended in fresh medium and the packed cell volume determined. 4 ml of algal suspension at 55 µl pcv/ml were placed in the reaction chamber of the oxygen electrode and the rate of photosynthesis observed after adding known amounts of first sodium glycollate and later α-OHMS solution through the capillary of the plunger. These additions were made during periods of active photosynthesis or respiration. Light intensity incident on the chamber was 10,000 lux. The effect of a particular metabolite was studied over a period ranging from 10-15 minutes. The pH of the stock glycollate and inhibitor solutions were adjusted to 8.2. Oxygen uptake in the dark was measured by covering the chamber with aluminium foil. Controls were run in which similar volumes of medium were added instead of the glycollate or the inhibitor to check whether an alteration in oxygen exchange rate was the result of a dilution effect on the cell suspension. Further

controls were also run to test the effect of the inhibitor in the absence of added glycollate. The rate of oxygen exchange was determined from the slope of the oxygen electrode graph over a period of 2-5 minutes after it had become linear either at the start of the experiment or following each addition. The results are presented in tables 6 and 7.

Table 6. The effect of glycollate and α-OHMS on O_2 evolution in light

Expt.No.	Details of Expts.	μmoles O_2/hour/ml pcv		
		before glycollate (or medium) addition	after glycollate (or medium) addition	after α-OHMS (or medium) addition
1	6 mM Glycollate 0.1 mM Inhibitor	+ 16	+ 16	+ 10
	Control	+ 14	+ 14	+ 15
2	17 mM Glycollate 0.094 mM Inhibitor	+ 13	+ 13	+ 13
	Control	+ 14	+ 15	+ 14
3	28 mM Glycollate 0.091 mM Inhibitor	+ 18	+ 20	+ 17
	Control	+ 18	+ 20	+ 20
4	37 mM Glycollate 0.087 mM Inhibitor	+ 18	+ 21	+ 18
	Control	+ 21	+ 25	+ 21
5	46 mM Glycollate 0.084 mM Inhibitor	+ 15	+ 17	+ 17
	Control	+ 20	+ 26	± 21
6	6 mM Glycollate 0.48 mM Inhibitor	+ 10	+ 9	- 8
	Control	+ 10	+ 9	+ 8

Table 7 Glycollate dependent O_2 uptake in the dark and the effect of α-OHMS on it.

Expt.No.	Details of Expts.	μmole O_2/hour/ml pcv		
		before glycollate (or medium) addition	after glycollate (or medium) addition	after α-OHMS (or medium) addition
1	6 mM Glycollate 0.1 mM Inhibitor	- 3	- 6	- 6
	Control	- 3	- 3	- 3
2	17 mM Glycollate 0.1 mM Inhibitor	- 5	-10	-10
	Control	- 3	- 3	- 3
3	28 mM Glycollate 0.091 mM Inhibitor	- 3	- 6	- 6
	Control	- 2	- 2	- 2
4	37 mM Glycollate 0.087 mM Inhibitor	- 2	- 5	- 5
	Control	- 2	- 3	- 3
5	46 mM Glycollate 0.084 mM Inhibitor	- 3	- 5	- 5
	Control	- 2	- 3	- 2

Minus glycollate controls:

Expt. No	Details of expts.	before α-OHMS addition	After α-OHMS addition
1	Algae+H_2O+0.08 mM Inhibitor	-2.5	-2.0
2	Algae+H_2O+0.45 mM Inhibitor	-2.5	-10.0

Table 6 shows that glycollate concentrations as high as 46 mM do not affect the rate of photosynthesis appreciably. An apparent increase in rate observed in some experiments was possibly a result of random variation because the controls also showed similar variations.

The inhibitor in concentrations of 0.084-0.1 mM did not affect the rate of photosynthesis, but at higher concentration (0.48 mM) the cells started taking up oxygen in the light. This inhibition, however, was reversible, because the cells after being washed were still capable of photosynthesis though at a lower rate.

The results in table 7 show that 6-46 mM concentrations of glycollate result in increasing the rate of respiration almost two-fold. One explanation is that oxygen acts as a natural ultimate electron acceptor for glycollate oxidising enzyme and the increased oxygen uptake is due to the oxidation of glycollate by this enzyme. Also even if the glycollate oxidising enzyme does not link to oxygen, a later stage in the pathway such as a conversion of glycine to serine still might. Another explanation is that this increased uptake of oxygen is a consequence of injury to the cells caused by glycollate and, ir order to repair the damage, the cells increase their respiration rate. The results in table 6 which show glycollate as having no harmful effect on photosynthesis tend to argue against the latter explanation.

One interesting observation was that the oxygen uptake always increased by a factor of two irrespective of the

glycollate concentration in the range tested. This may have been due to the existence of a certain maximum rate of uptake or metabolism of glycollate with a consequent proportional increase in oxygen uptake.

Concentrations of α-OHMS up to 0.084 mM did not inhibit oxygen uptake in the dark either in the presence or absence of glycollate. This suggests that the glycollate oxidising system may not be sensitive to this inhibitor or that the inhibitor fails to penetrate the cells in sufficient concentrations.

When the α-OHMS concentration was increased to 0.45 mM there was a stimulation of oxygen uptake in the absence of glycollate. Since at this concentration there was also a marked inhibition of photosynthesis (table 6) it indicates that the effect of the inhibitor at this concentration may not be specifically on glycollate oxidation. For this reason, higher concentrations of α-OHMS were not tested.

Expt. 10

Growth of Phaeodactylum at Various pH Values and Glycollate Concentrations

In order to confirm the results of the previous experiment in which glycollate appeared to be metabolised in the dark, it was decided to see whether it would support growth by acting as a carbon source.

Glycollate at a range of concentrations was tested at 3 pH values on the growth of cultures of Phaeodactylum. The cultures

were grown in 150 ml Erlenmeyer flasks at 600 lux and 20° C and the cell density determined after 7 days. The pH of the cultures was adjusted daily.

The results shown in figure 17a show that pH 6.5 gave the best growth. Glycollate appeared to stimulate growth and the lowest concentration tested (5 mM) was sufficient to provide the maximum stimulation. This result agrees with that of the previous experiment in which increasing the growth concentration above this value did not result in any further increase in the rate of respiration.

Although glycollate appeared to stimulate growth and respiration it was not clear whether it did this by functioning as a carbon source. This was because some CO_2 may have entered the cultures when the pH was adjusted and also bacterial contamination of the cultures might also result in CO_2 being produced. This CO_2, if it was photosynthesized, could support some growth not directly attributable to glycollate metabolism.

To test for this, a similar experiment was conducted, this time at only one pH, in which the cultures were maintained substantially CO_2-free by bubbling a stream of CO_2-free air through them continuously. The results are shown in figure 17b. Again a maximum rate of growth was obtained at 5 mM glycollate with extremely high concentrations being inhibitory.

We may therefore conclude that glycollate can be metabolized by Phaeodactylum and that it can act as a sole carbon source. The

Fig 17a:
The growth of Phaeodactylum at different pH values in varying glycollate concentrations.

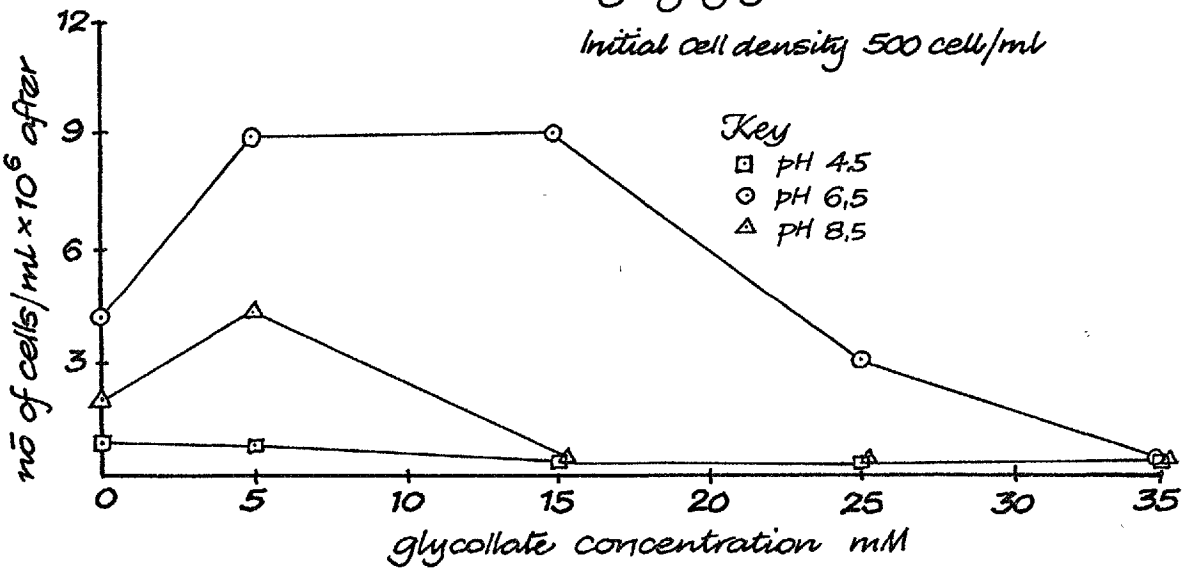
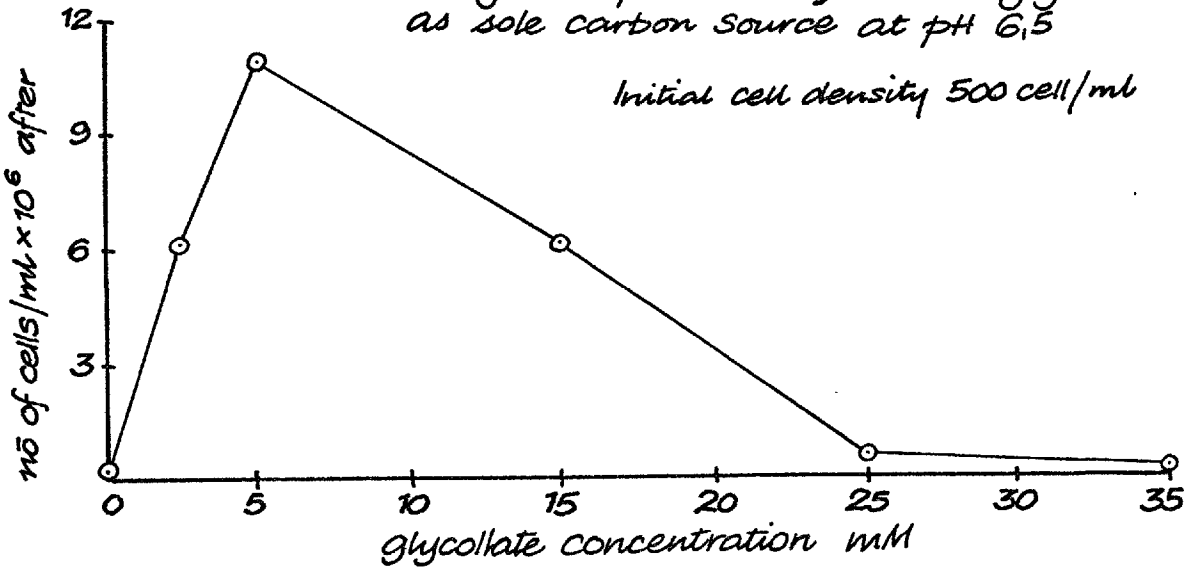


Fig 17b:
The growth of Phaeodactylum on glycollate as sole carbon source at pH 6,5



rate of uptake or metabolism of glycollate appears to be saturated at concentrations of 5 mM and concentrations of the order of 25 mM and above are inhibitory to growth.

Expt. 11

The Incorporation of Glycollate-1-^{14}C into Phaeodactylum and Chlorella.

In order to compare the rate of glycollate uptake and the rate of its metabolism to yield CO_2 glycollate-1-^{14}C was fed to both Phaeodactylum and Chlorella. Its uptake and conversion to CO_2 was measured in both light and dark and some of the metabolic products identified by chromatography.

The algae were harvested by centrifugation, resuspended in their respective media and the packed cell volume determined. 0.89 ml pcv for Phaeodactylum and 1.2 ml pcv for Chlorella in 2 ml of medium were put into the outer compartment of two, 20 ml Warburg flasks. The flasks were connected to their manometers and shaken in the constant temperature water bath (15^{o}C) of a Warburg apparatus at a light intensity of 7000 lux. An air-stream, humidified by passing it through water at room temperature, was passed through the flasks.

Just before adding the isotope, the flasks were removed briefly from their manometers and 10 µci Glycollate-1-^{14}C (17.3 µmoles) were added to each of the flasks which were rapidly connected to their manometers and returned to the water bath. The inlet of each flask was re-connected to its humidified air supply. The outlet from each flask was connected

to two, 250 ml of 1.0 M ethanolamine to trap $^{14}CO_2$ released. The flow of air was regulated to give a fine stream of bubbles at the inlet which was immersed to about 2.5 cm in the ethanolamine. Preliminary experiments had indicated that over 80% of the CO_2 released is trapped in the ethanolamine using this technique.

After one, two and three hours, duplicate 0.1 ml samples were removed from both sets of bubblers, put into previously labelled scintillation vials and stoppered tightly. At the end of the experiment, the algae from each flask were filtered through separate 0.45 μm pore-size Millipore filter discs under vacuum. The cells were washed with 50 ml of distilled water to remove unabsorbed ^{14}C-glycollate, the filter discs plunged separately into 50 ml of boiling 20% ethanol and boiling continued for 3 minutes. The extract was centrifuged at 10,000 x g for 10 minutes and the residue washed with 15 ml of distilled water, the washings being added to the cell extract. The cell residue was resuspended in 15 ml of distilled water. Duplicate 0.1 ml samples from both the extract and the residue suspension were removed and put into scintillation vials. All samples were counted in 15 ml of scintillation fluid as described before. In all cases, counts were corrected by using a ^{14}C-hexadecane internal standard. The experiment in the dark was done similarly but 1.49 ml pcv of Phaeodactylum and 1.67 ml pcv of Chlorella in 3 ml of medium were used. The lights of the Warburg apparatus were switched off and the flasks containing algae were completely

wrapped in aluminium foil. The extracts from both experiments were chromatographed in one-dimension in Phenol : Water. Glycollate, glycine and serine spots were tentatively identified from their Rf values and then co-chromatographed with authentic compounds in a Butanol : Pyridine : Water (60 : 60 : 60) solvent. The chromatograms were scanned and glycollate was located with Bromocresol green and glycine and serine with Ninhydrin (Smith, 1960).

Table 8 Fate of Glycollate-1-^{14}C after 3 hours as dpm x 10^6/ml pcv

Compound	Chlorella		Phaeodactylum	
	Light	Dark	Light	Dark
$^{14}CO_2$ released	0.045	0.006	0.06	0.009
Ethanol soluble fraction	2.00	0.567	0.33	0.369
Ethanol insoluble fraction	0.01	0.005	1.00	0.008
Total glycollate uptake	2.06	0.578	1.39	0.386

Table 8 shows that both Chlorella and Phaeodactylum took up glycollate at approximately similar rates and in each case there was a roughly 4-fold stimulation of uptake brought about by light. Both algae released $^{14}CO_2$ at about the same rate with an approximately 7-fold stimulation brought about by light. This suggests that the mechanism of the conversion of glycollate to

CO_2 may be similar in both organisms. The fact that light caused a greater stimulation of $^{14}CO_2$ release than it did of glycollate uptake, despite the fact that light should have caused refixation of some of this CO_2, suggests that the pathway from glycollate to CO_2 is light activated.

Only a few per cent of the label taken up (2-4%) was converted to CO_2, the remainder being incorporated into the cells. The distribution of counts in the ethanol soluble and insoluble fractions differed in the two organisms and was also affected by light. This possibly reflects differences in the nature of the food reserves in the two organisms.

Chromatography of the ethanol soluble fraction revealed small amounts of labelled glycine and serine in both algae. These were identified by co-chromatography. A number of other radioactive spots were present but not identified.

Expt. 12

Glycine-1-^{14}C Feeding to Phaeodactylum and Chlorella

The previous experiment had suggested that both of these algae took up glycollate and metabolised it via glycine and serine and that in the process CO_2 was produced. It was decided to see whether the CO_2 producing stage came after glycine by doing similar experiments but this time feeding radioactive glycine.

Two experiments were done. In the first the algae were incubated in the light with glycine-1-^{14}C, the liberated $^{14}CO_2$

collected and the cells killed and extracted whilst still in the presence of the labelled glycine. This enabled the immediate as well as the later products of glycine metabolism to be detected in the extract but did not permit a measurement of glycine uptake by the cells. In a second experiment again the $^{14}CO_2$ liberated in the light was collected but this time the cells were filtered and washed at the end of the incubation so that the amount of ^{14}C taken up could be determined. Although it was possible here also to extract the cells with ethanol and chromatograph the extract, the time taken for the filtration and washing would probably result in the loss due to further metabolism of the immediate products from glycine.

In the first experiment, both Phaeodactylum and Chlorella were harvested by centrifugation and resuspended in their respective media. 2.5 ml of this suspension (pH 7.7) having 0.7 ml pcv was put into the outer compartments of two, 15 ml Warburg flasks. The flasks were fitted with serum caps and supported on the shaker of a Warburg bath where they were illuminated at 7,000 lux and 18°C for 1 hour. After this period, 0.2 ml of 2N NaOH was put into the centre-well of each flask. 2 µci (8.05 µmoles) of glycine-1-^{14}C were added to each of the flasks, the serum caps replaced and they were returned to the water bath. The flasks were shaken during the experiment. After 1 hour incubation with the isotope the algae were killed by injecting 2.5 ml of 10% trichloroacetic acid (TCA) through the serum cap. They were shaken for 30 minutes after adding the acid and NaOH removed quantitatively with a microsyringe, and

0.1 ml added to each of two scintillation vials which were stoppered tightly and left for counting. The cell suspension was removed from the flask and centrifuged at 5000 x g for 10 minutes. The supernatant was extracted several times with equal volumes of ether to remove the TCA, purified on a cation exchange column and chromatographed by the method of Bird, Cornelius, Keys and Whittingham (1972). The finished chromatograms were auto-radiographed. Serine was identified by its Rf value and confirmed by co-chromatography.

The second experiment was designed to measure the rate of glycine uptake. Here the algae were removed with a syringe after one hour incubation with the isotope and first filtered through Whatman no. 1 filter disc under suction and then filtered through a 0.45 μm pore size Millipore filter disc. During this procedure, algae were kept illuminated with a spot-lamp. After washing the algae on the filter discs with about 20 ml of distilled water, the discs were plunged into 100 ml of boiling 80% ethanol, and boiling continued for 3 minutes. The filter discs were removed, and the adhering algae washed into the extract with 80% ethanol. The extract was centrifuged at 10,000 x g for 10 minutes. The residue was resuspended in a known amount of distilled water. Duplicate 0.1 ml samples were removed from both the extract and the residue suspension for counting. The rest of the extract was reduced to 1 ml by vacuum distillation and chromatographed.

Table 9 Glycine-1-^{14}C incorporation into various fractions by Phaeodactylum

Expt.	dpm x 10^6/ml pcv/h			
	$^{14}CO_2$ released	Ethanol soluble	Ethanol insoluble	Total
1	0.004	-	-	-
2	0.003	0.340	0.134	0.477

Table 10 Glycine-1-^{14}C incorporation into various fractions by Chlorella

Expt.	dpm x 10^6/ml pcv/h			
	$^{14}CO_2$ released	Ethanol soluble	Ethanol insoluble	Total
1	0.007	-	-	-
2	0.006	1.36	1.62	2.98

It can be seen from tables 9 and 10 that both Phaeodactylum and Chlorella took up and metabolised glycine with the rate being greater in Chlorella. In either case, less than 1% of the counts taken up were released as CO_2. Chromatography of the ethanol soluble fraction of both algae revealed a large number of radioactive spots thus suggesting an extensive metabolism of the

isotope. Although small quantities of serine were detected by Rf on the chromatograms and confirmed by co-chromatography, the presence of such a large number of other radio-active products makes it difficult to assign even this small amount of $^{14}CO_2$ to the glycine to serine conversion.

Expt. 13

Assay of the Glycollate Oxidising Enzyme in Phaeodactylum and Chlorella

Since the previous experiments had shown that Phaeodactylum was capable of taking up glycollate and using it as a substrate for growth, it was hoped to discover whether this was by the same metabolic pathways as in other algae and higher plants. To do this the various enzymes known to be necessary for glycollate metabolism in other organisms were assayed in extracts of Phaeodactylum. The first of these enzymes is the glycollate oxidising enzyme which converts glycollate to glyoxylate. In higher plants this is an oxidase but in most algae it is a dehydrogenase. However, both enzymes can transfer electrons from glycollate to dichlorophenolindophenol (DICPIP) and can be assayed by following the rate of DICPIP reduction. They can also both be assayed by following the rate of glyoxylate formation. In this experiment, the glycollate oxidising enzyme was extracted from Phaeodactylum, its activity measured with both assay methods and the results compared with those from Chlorella which is an organism known to have an active glycollate oxidising system (Zelitch & Day, 1968a).

Initially the enzyme extracts were prepared using a French press, but few Chlorella cells could be broken with this method. In later experiments, grinding the frozen cells with a little sand in a pestle and mortar proved to be the best technique, and quite active cell extracts were prepared. Here the cells were frozen at -20°C in the suspending buffer in a mortar and ground, while still frozen. The buffer used was 0.1 M tris HCl, pH 6.4. Absorbance was read on an SP 800 spectrophotometer connected to a chart recorder. Two different assay methods were used. Controls in each case either contained boiled enzyme or the substrate was omitted or both. The initial rates of reaction were determined by drawing a tangent to the curve immediately after the addition of substrate.

DICPIP method

This was based on the method of Lord (1970). Here a dye (Dichlorophenol-indophenol) is reduced to a colourless form by the electrons produced during the oxidation of glycollate to glyoxylate. The components of the assay mixture in a total volume of 3 ml were as follows:-

Sodium cacodylate buffer (pH 6.3)	- 100.0 µmoles
DICPIP	- 0.3 "
Sodium Glycollate	- 12.5 "
Cell extract	

The reaction was started by adding the 12.5 µmoles of sodium glycollate and the initial rate of dye reduction was measured at 600 nm on an Sp 800 spectrophotometer. However, in the assay mixture, 200 µmoles of sodium pyrophosphate buffer

(pH 8.7) as used by Lord (1970) inhibited the reaction, but when it was replaced by 100 μmoles of sodium cacodylate buffer (pH 6.3), the reaction proceeded much faster. A calibration curve was prepared taking known amounts of DICPIP and measuring the absorbance at the above wavelength (Fig. 18a).

Phenylhydrazine method:

Here phenyl hydrazine forms a U.V. absorbing addition compound with the glyoxylate produced by the oxidation of glycollate. The assay mixture contained in a total volume of 3ml:-

Phenyl hydrazine HCl (pH 6.8)	-	10 μmoles
Cysteine HCl (pH 6.8)	-	10 μmoles
Sodium Glycollate	-	30 μmoles
Cell extract	-	

The reaction was started by adding the 30 μmoles of sodium glycollate and the absorbance read at 324 nm on an SP 800 spectrophotometer. A calibration curve was prepared by adding known amounts of glyoxylate to the reaction mixture and measuring the absorbance at the above wavelength (Fig. 18b).

Table 11 The activity of the glycollate oxidising enzyme in Phaeodactylum and Chlorella

Assay	Initial rates of enzyme activity (μmoles glycollate presumed oxidised/h/g f.w.	
	Phaeodactylum	Chlorella
Phenyl Hydrazine	1.80, 0.60	0.24
DICPIP	0.13	0.03

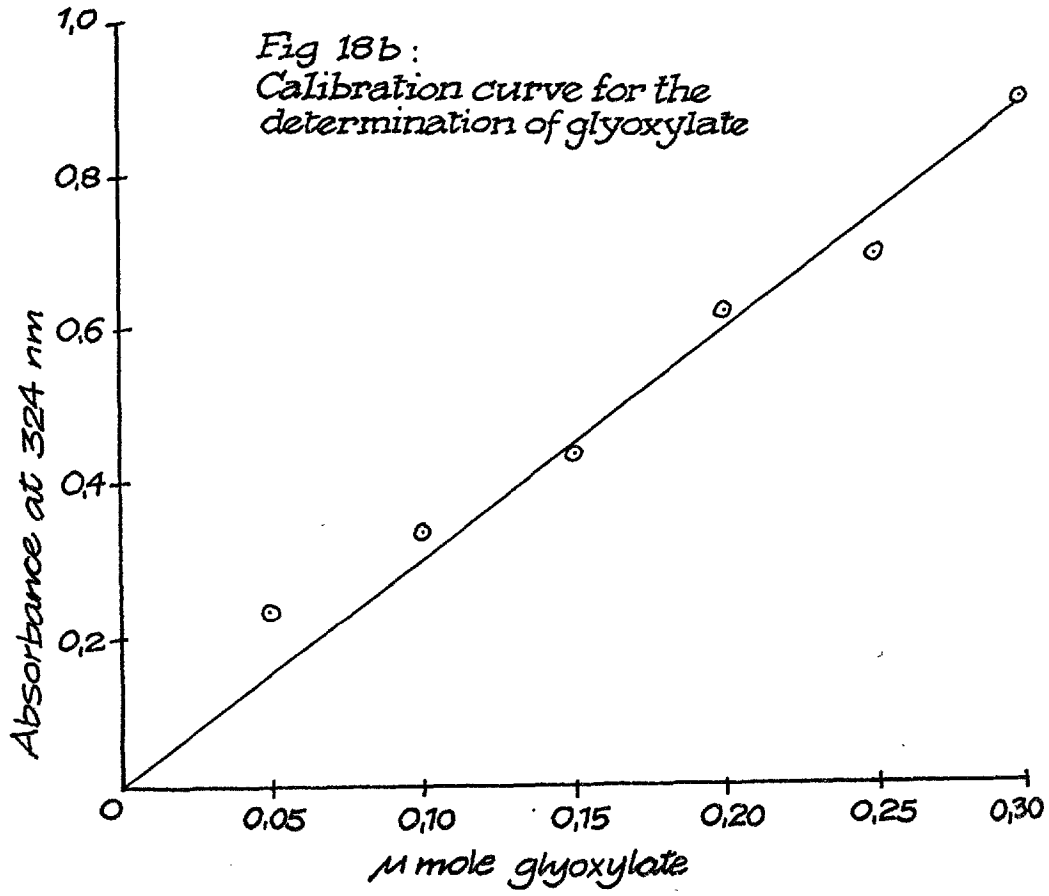


Fig 18b :
Calibration curve for the determination of glyoxylate

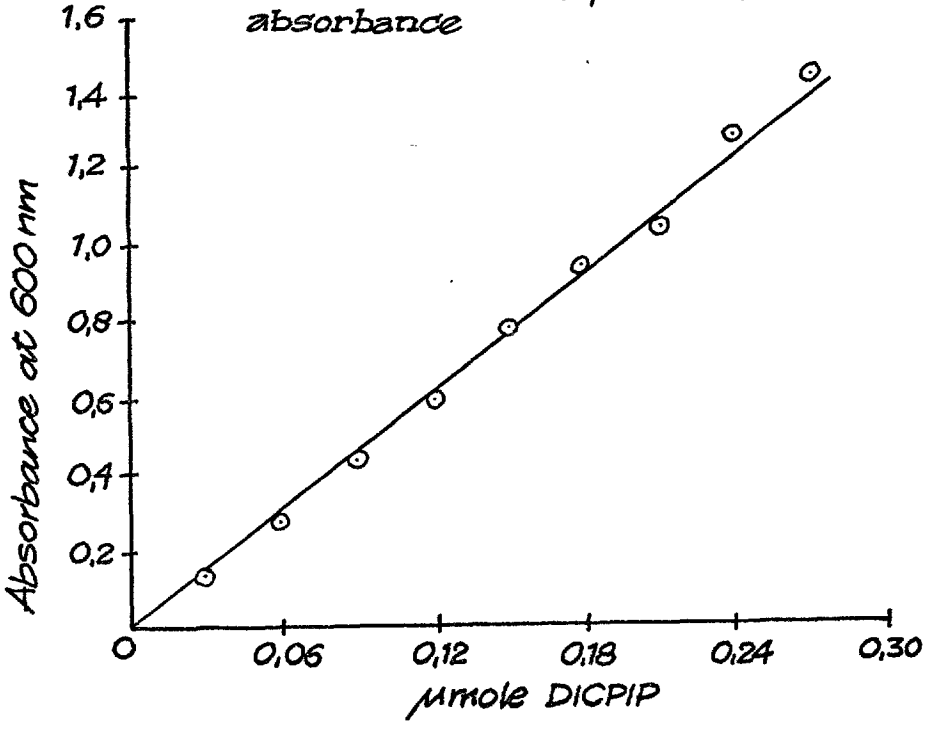
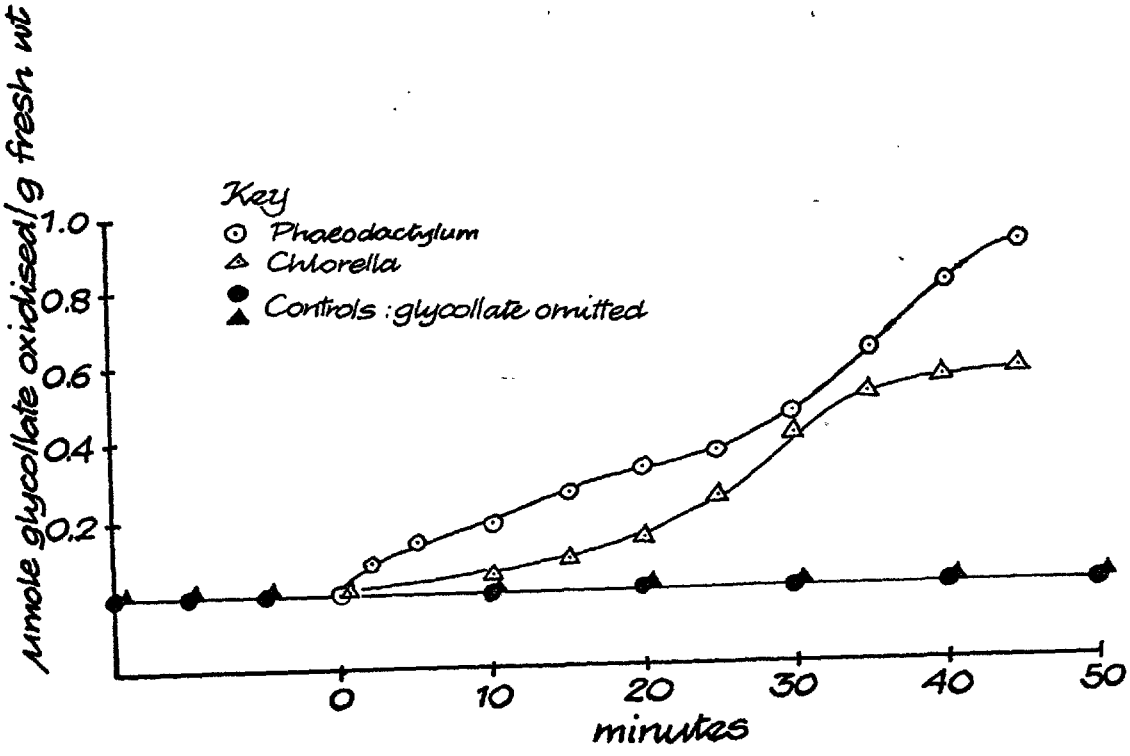


Fig 18a :
Calibration curve for DICPIP absorbance

Fig 19 :
A comparison of the glycollic dehydrogenase activity of Phaeodactylum and Chlorella by the phenylhydrazine method of assay



As shown from the figure 19 and table 11, a higher activity was present in the Phaeodactylum cell extracts than in those from Chlorella. This may be due to the manner in which the extract was prepared. Since Chlorella cells were more resistant to breakage, a lower activity may be due to that factor. This is further supported by the observation that different extracts from Phaeodactylum did not always give reproducible results.

Expt. 14

The Characterisation of the Glycollate Oxidising Enzyme in Phaeodactylum

Glycollate-dependent oxygen uptake in the dark by intact Phaeodactylum cells (table 7) suggested that the glycollate oxidising enzyme may be an oxidase as found in higher plants. It was decided to assay this enzyme in the cell-free extracts to determine whether its oxidation is coupled directly to oxygen. For this, glycollate oxidase activity was measured by the oxygen electrode and compared with that from tobacco. The glycollate dehydrogenase activity was assayed by the DICPIP method in the same extract on an SP 800 spectrophotometer.

The tobacco extract was prepared by homogenizing the leaves in a Polytron homogeniser at 0-4^{o}C in a 5 mM potassium phosphate buffer pH 7.8 (1:4) whereas the Phaeodactylum extract was prepared in the same buffer by freezing and then grinding the cells with a pestle and mortar. In both cases, the cell residue was first removed by filtration through 4 layers of destarched muslin and then by centrifugation at 4^{o}C at 5000 x g for 10 minutes. The glycollic oxidase activity of tobacco was assayed at 27^{o}C at

which the full scale on the chart recorder was equivalent to 1 μmole O_2. There was a considerable amount of O_2 uptake even without glycollate. The amount of glycollate oxidised was calculated from the rates just before and after adding 60 μmoles of glycollate. In the control, water was added instead of glycollate. The results are shown in table 12. The slight reduction in O_2 uptake in the control was probably due to the displacement of some of the active extract by the water.

Table 12 μmoles O_2 uptake/h/g fresh weight

Expt.	before adding glycollate	after adding glycollate
1	5.0	7.6
2	5.3	7.6
Control	5.4	5.0

The Phaeodactylum extract was assayed in the same way. Although a little O_2 uptake was observed before adding glycollate, there was no difference in slope after the addition of glycollate. When the same extract was assayed by the DICPIP method on an SP 800 spectrophotometer at 600 nm, considerable dye reduction was observed on adding the glycollate. The assay mixture contained in a total volume of 3 ml

Potassium Phosphate buffer (pH 7.8) - 5 μmoles
DICPIP - 0.3 "
Sodium Glycollate - 60.0 "
Cell extract

Table 13 μmole glycollate oxidised/h/g fresh weight

Expt.	Glycollate Oxidase	Glycollate dehydrogenase
1	0	0.96
2	0	-
3	0	-

From these results it can be seen that the glycollate oxidising enzyme in Phaeodactylum is a dehydrogenase which does not link to atmospheric oxygen directly as in tobacco. The possibility of O_2 acting as an ultimate electron acceptor cannot be ruled out, however, because O_2 uptake was observed by the intact cells in the dark on adding glycollate, perhaps there are natural electron acceptors which act as links to carry electrons between glycollate and atmospheric oxygen and these are lost during the preparation of the algal extracts.

Expt. 15

A Comparison of the Glycollate Dehydrogenase of Phaeodactylum Grown in the Presence or Absence of Glycollate

There are conflicting reports about the CO_2 control on the synthesis of glycollate oxidising enzyme in algae. Since this CO_2 control may be exerted through the synthesis of glycollate (Nelson & Tolbert, 1969; Codd et al, 1969) it was decided to grow Phaeodactylum in the presence or absence of glycollate and then compare the difference in the enzyme activity. For

this, cells were grown for one week in a Dreschel bottle in Mann & Myers' medium containing 5 mM glycollate (pH 6.5) and were aerated with humidified air. Another bottle contained a similar culture without the glycollate.

The extract was assayed both by the DICPIP and the phenylhydrazine assays. The glycollate oxidised was presumed to be equivalent in molar quantities to the glyoxylate formed or the DICPIP reduced. The results were represented either on a fresh weight basis or on a chlorophyll basis. Chlorophyll was determined by taking 0.1 ml of the extract in 20 ml of 80% acetone and the absorbance read at 625 nm on an SP 800 spectrophotometer. This figure was multiplied by a factor of 4.4 to obtain number of micromoles of chlorophyll per ml of cell extract. The results are shown in figures 20a and 20b and table 14.

Table 14 Comparison of the glycollate dehydrogenase activity of Phaeodactylum after growth in the presence or absence of glycollate

Assay	Initial rates of enzyme activity	
	µmole glycollate presumed oxidised/h/g fresh weight	
	plus glycollate	minus glycollate
Phenyl hydrazine	0.42	0.60
	0.18	0.18
	µmoles glycollate presumed oxidised/h/g µmole chlorophyll	
	plus glycollate	minus glycollate
DICPIP	0.48	0.60

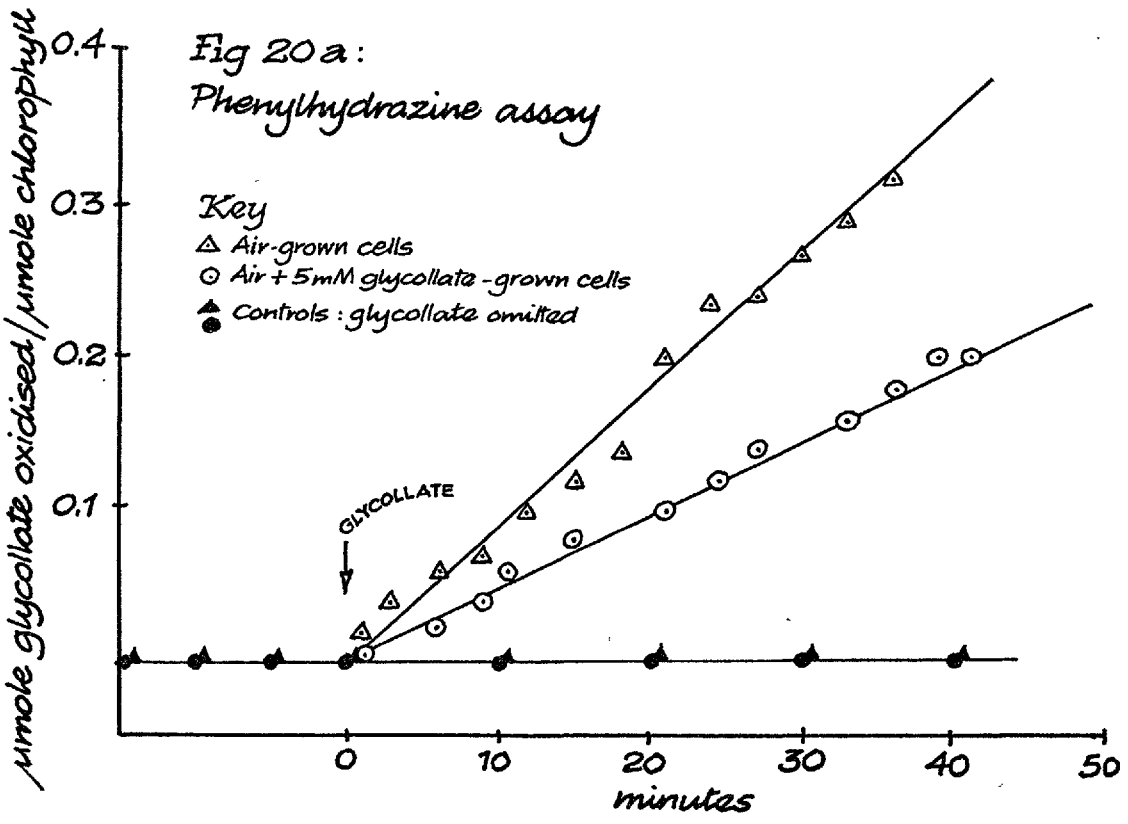


Fig 20a:
Phenylhydrazine assay
Key
Air-grown cells
Air + 5mM glycollate-grown cells
Controls: glycollate omitted
GLYCOLLATE
μmole glycollate oxidised/μmole chlorophyll
0.4
0.3
0.2
0.1
0
10
20
30
40
50
minutes

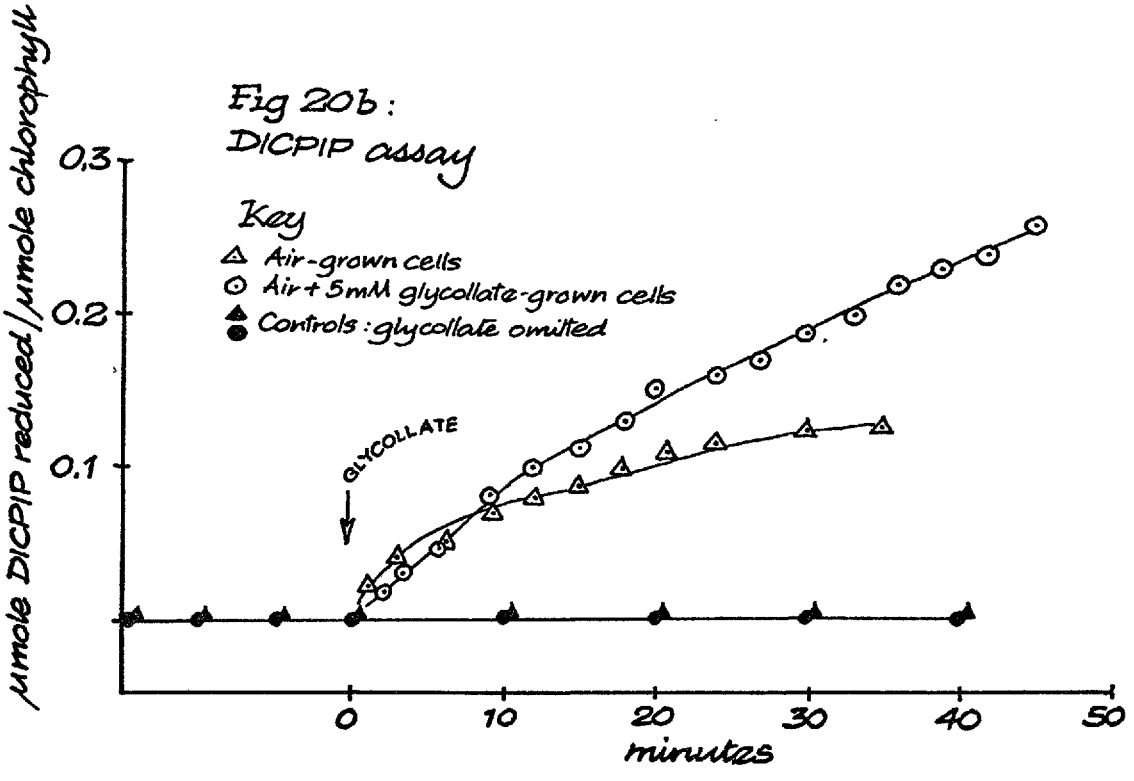


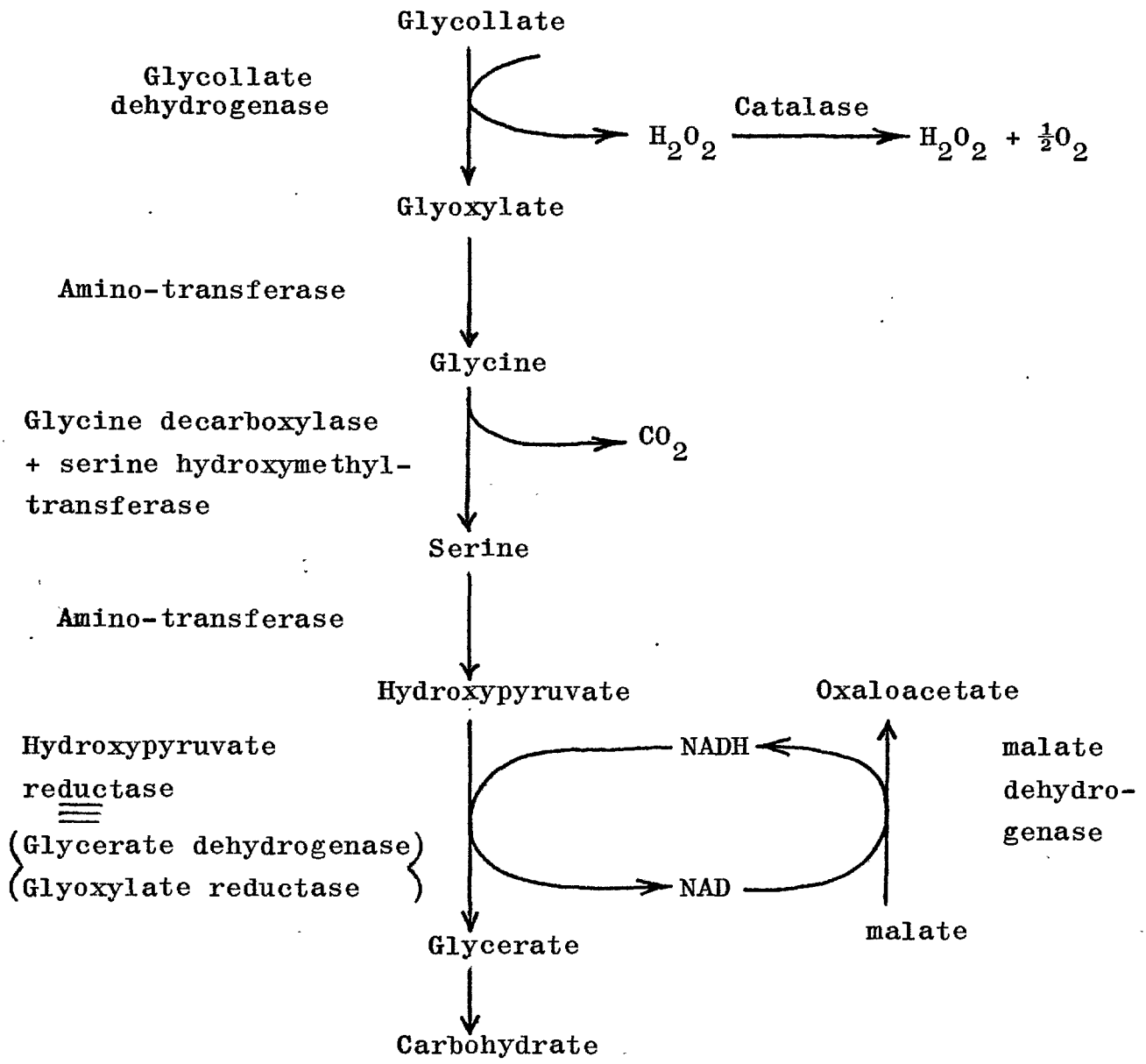
Fig 20b:
DICPIP assay
Key
Air-grown cells
Air + 5mM glycollate-grown cells
Controls: glycollate omitted
GLYCOLLATE
μmole DICPIP reduced/μmole chlorophyll
0.3
0.2
0.1
0
10
20
30
40
50
minutes

It is quite clearly shown by these results that the rate of glycollate oxidation by Phaeodactylum extracts as assayed by the two assay methods is approximately the same. However, they did not always agree exactly even when the identical extract was used for both procedures. This may be because the phenylhydrazine assay is limited by the amount of natural electron acceptor being present but the DICPIP assay is not. The DICPIP method should give a more reliable comparison, because there the dye itself acts as the electron acceptor. However, there does not seem to be a great difference in the enzyme activity between the plus glycollate and minus glycollate treatments using either method. It may therefore be concluded that the glycollate oxidising enzyme of Phaeodactylum is probably constitutive.

Expt. 16

The Enzymes for the Remainder of the Glycollate Pathway

The discovery of an active glycollate oxidising enzyme in Phaeodactylum suggested that this alga may also possess enzymes for the further metabolism of the glyoxylate produced. If this metabolism were to resemble that of higher plants the enzymes for the following pathway would be present (Tolbert, 1971).



The glycollate oxidising enzyme has already been assayed and the activity of the glycine-glyoxylate aminotransferase demonstrated *in vivo* by the formation of radio-active glycine from glycollate-1-^{14}C. Other enzymes of the pathway were assayed *in vitro* as follows:-

Cell-free extracts were prepared as described in 'materials and methods'. The time course of absorbance changes was measured

on an SP 800 Spectrophotometer coupled to a servoscribe chart recorder at 230 nm for H_2O_2 destruction and 340 nm for NADH/NAD conversions. All incubations were at room temperature (20^oC) and the initial rates of the reactions determined. This was done by measuring the difference in the initial rate between the experimental assay mixture and that of a control with the substrate omitted. In some cases a boiled enzyme control was also carried out.

Catalase

This enzyme brings about the catalytic destruction of H_2O_2. In higher plants catalase is present in the peroxisomes and H_2O_2 produced as a result of glycollic oxidase reaction is removed by its activity. In most of the algae, its activity is reported to be considerably less than that found in higher plants. The components of the assay mixture in a final volume of 3 ml were:-

Potassium Phosphate buffer (pH 7.0)	-	200 µmoles
H_2O_2	-	5 µmoles
Cell extract (diluted)		

the control consisted of the boiled enzyme.

Hydroxypyruvate Reductase

This enzyme carries out the reversible interconversions of hydroxypyruvate and glycerate. It has therefore also been given the name glycerate dehydrogenase. According to Tolbert (1971) it has a secondary activity in that it reduces glyoxylate to

glycollate. It has therefore also been called glyoxylate reductase. Its activity in all these modes has been assayed in Phaeodactylum extracts.

(1). Hydroxypyruvate as substrate:

This reaction brings about the reduction of hydroxypyruvate to glycerate. The reaction mixture contained in a final volume of 3 ml:-

Potassium Phosphate buffer (pH 6.3)	-	100 µmoles
Sodium Hydroxypyruvate	-	50 µmoles
$NADH_2$	-	2 µmoles
Cell extract		

The reaction was started by adding 2 µmoles of $NADH_2$. The control did not have the substrate and $NADH_2$ oxidation was calculated from the difference between the plus and minus hydroxypyruvate treatments. The boiled enzyme did not show any activity.

(2). D-glycerate as substrate:

This reaction is the same as described in (1) but in the opposite direction. The reaction mixture contained in a final volume of 3 ml:-

Potassium Phosphate buffer (pH 6.5)	-	100 µmoles
D-glycerate	-	50 "
NAD	-	2 "
Cell extract		

The reaction was started by adding 2 µmoles of NAD. Here also, the control did not contain the D-glycerate

and the reduction of NAD was determined after substracting the auto-reduction of NAD by the cell extract.

(3). Glyoxylate as substrate:-

This reaction brings about the reduction of glyoxylate to glycollate. The reaction mixture contained in a final volume of 3 ml:-

Potassium Phosphate buffer (pH 6.5)	-	100 µmoles
Sodium Glyoxylate	-	50 "
$NADH_2$	-	1 "
Cell extract		

The reaction was started by adding 1 µmole of $NADH_2$. In the control, glyoxylate was omitted and the oxidation of $NADH_2$ was calculated from the difference between plus and minus substrate treatments.

Conversion of Serine to Glycerate:-

This is a linked reaction in which serine is first converted into hydroxypyruvate by the enzyme serine-hydroxypyruvate amino-transferase and then the hydroxypyruvate is reduced to glycerate by hydroxypyruvate reductase. Assay is by measuring the rate of $NADH_2$ consumption in the second reaction. The components of the assay mixture in a total volume of 3 ml were:-

Potassium Phosphate buffer (pH 8.3)	-	200 µmoles
Sodium Pyruvate	-	20 "
Pyridoxal Phosphate	-	0.1 "
D, L-Serine	-	20.0 "
$NADH_2$	-	1.0 "
Cell extract		

Malate Dehydrogenase

In higher plants this occurs along with hydroxypyruvate reductase in the peroxisomes where its formation is believed to be the provision of the necessary $NADH_2$. It was therefore assayed in a Phaeodactylum extract. The reaction mixture contained in a final volume of 3 ml

Potassium phosphate buffer (pH 6.5)	- 100 µmoles
Oxalacetate	- 50 "
$NADH_2$	- 1 "
Cell extract	

The reaction was started by adding 1 µmole of $NADH_2$. Oxalacetate was omitted from the control and $NADH_2$ oxidation was calculated by difference. The boiled enzyme did not show any activity.

Table 15 The assay of different enzymes of the glycollate pathway in Phaeodactylum cell extracts. Figures with asterisk are quoted from table 11 and 14.

	Enzyme	Initial rates of reaction µmoles/ h/g fresh weight	Mean rates
	Glycollate Dehydrogenase	(1.8, 0.60, 0.13, 0.60, 0.18, 0.60)*	0.65
	Hydroxypytuvate reductase		
(1)	Hydroxypyruvate as substrate	78, 120, 50	83
(2)	Glycerate as substrate	47, 70	59
(3)	Glyoxylate as substrate	34, 42	45
	Conversion of serine to glycerate	75	
	Malate Dehydrogenase	120, 105, 90	105
	Catalase	54, 375	215

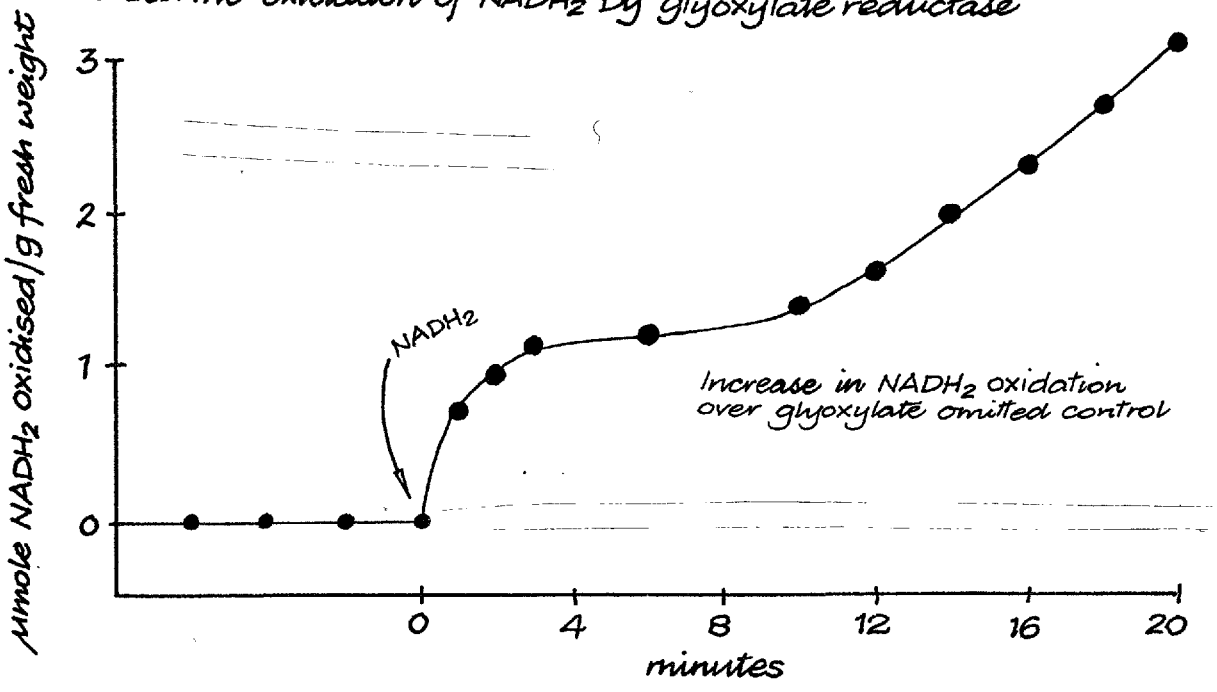
An examination of table 15 shows that Phaeodactylum possesses at least some of the necessary enzymes for a functional glycollate pathway. Of the enzymes assayed, glycollic dehydrogenase appeared to limit the rate of the whole pathway. Hydroxypyruvate reductase was assayed by using either hydroxypyruvate or glycerate (Fig. 21b) as the substrates for the reversible conversion of hydroxypyruvate to glycerate and vice-versa. Hydroxypyruvate proved to be the better substrate than either of the other two.

The linked reaction for the conversion of serine into glycerate (Fig. 22a) showed an activity almost equal to that of hydroxypyruvate reductase indicating that the reaction was not limited by the amino transferase reaction.

Malate dehydrogenase (Fig. 22b) gave one of the highest activities of all the enzymes assayed which was consistent with the findings of other workers in the higher plants. Catalase (Fig. 22c) however, gave a much lower activity than that found in the higher plants.

Fig 21

21a: The oxidation of $NADH_2$ by "glyoxylate reductase"



21b:

An assay of hydroxypyruvate reductase using

● hydroxypyruvate as the substrate

▲ glycerate as the substrate

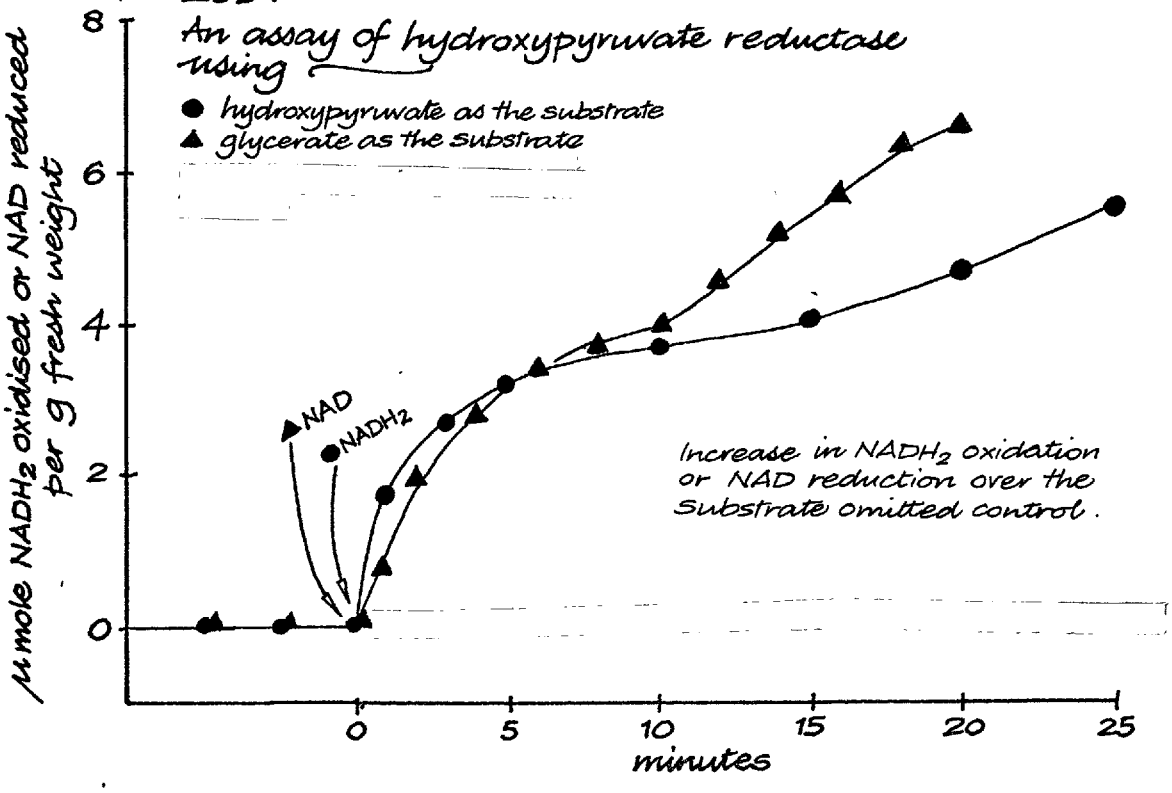
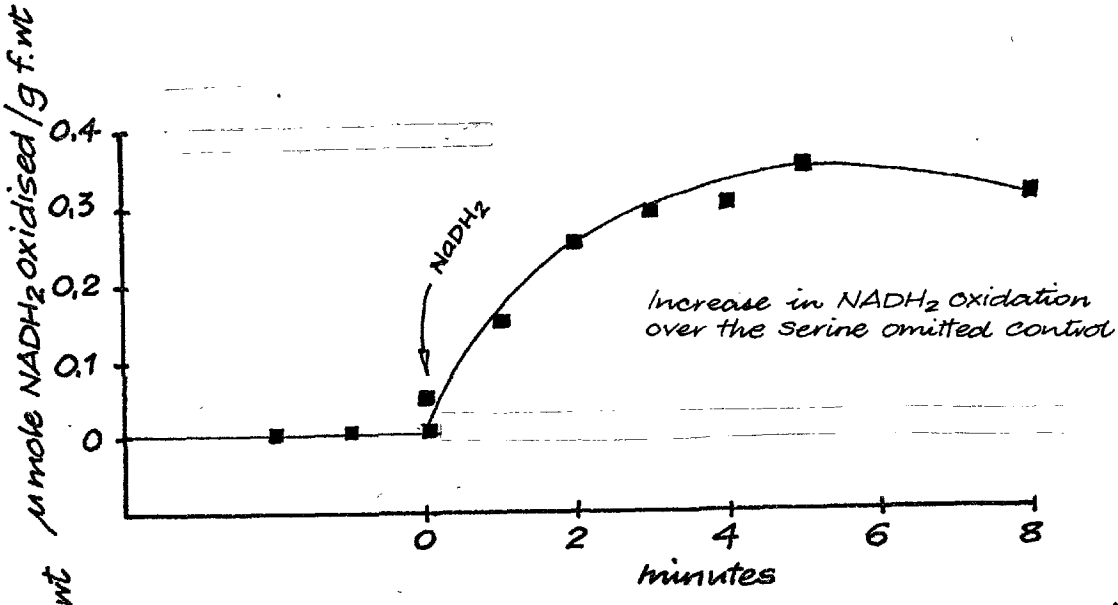
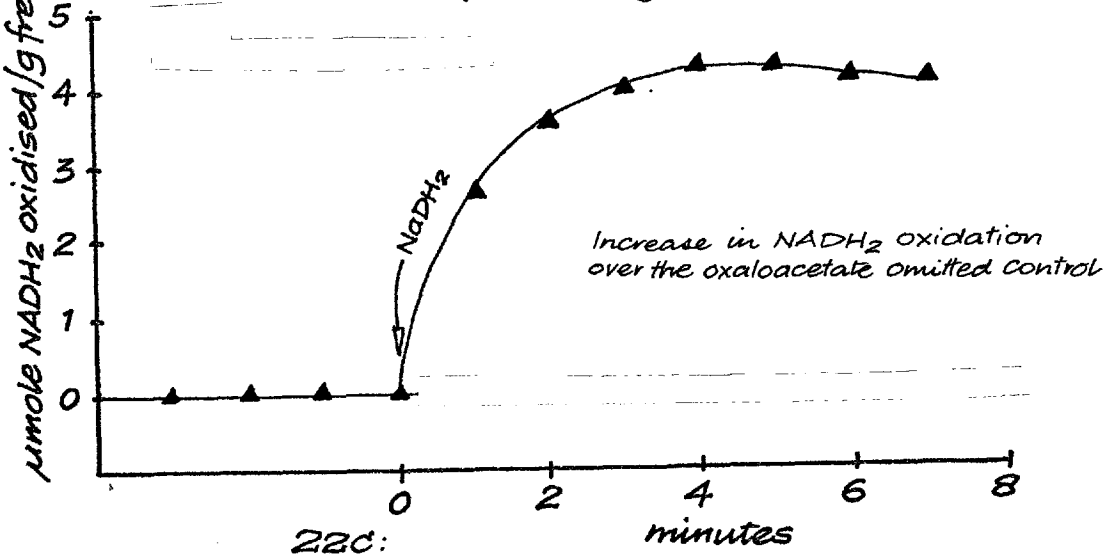


Fig 22 : Enzyme activities of Phaeodactylum extracts

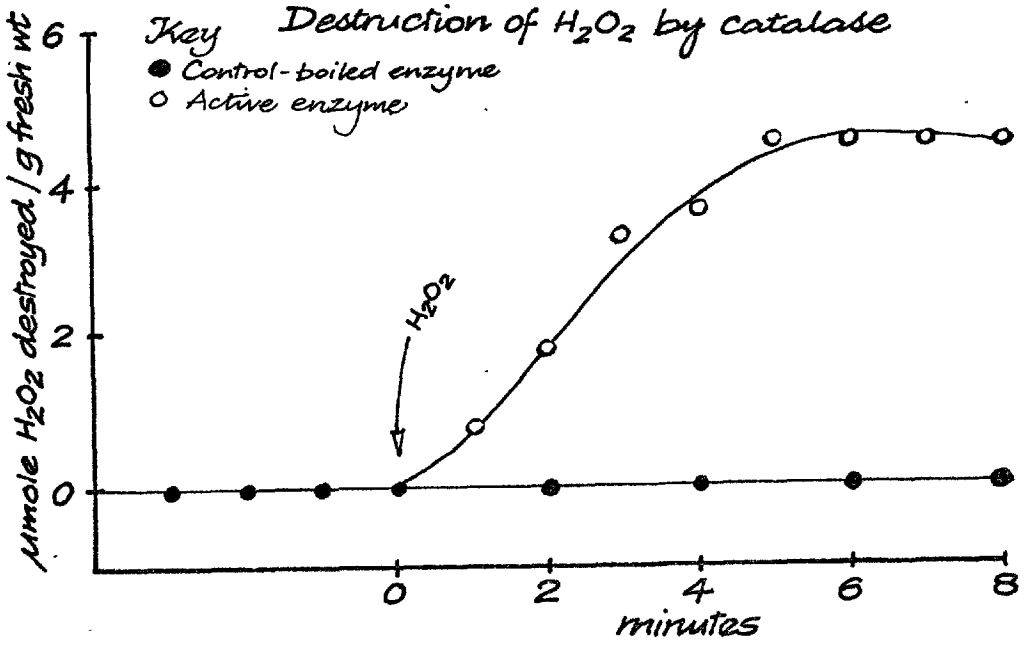
22a: Conversion of serine into glycerate



22 b : Oxidation of $NADH_2$ by malate dehydrogenase



22c: Destruction of H_2O_2 by catalase



DISCUSSION

Measurements of CO_2 compensation points in algae have not been made as extensively as in higher plants, but from the available data it appears that the algae as a rule show lower compensation points. This makes them comparable with the C_4 plants, which are believed to be more efficient photosynthetically than the C_3 plants, because photorespiratory losses are minimized in them. For this reason, a study of photorespiration and glycollate metabolism could throw some light on how these algae may have managed to escape photorespiration and whether it helps them to increase their photosynthetic efficiency.

As shown in table 1, CO_2 compensation points were measured for a number of marine algae. Out of 10 algae investigated, eight including Phaeodactylum tricornutum showed very low compensation points, which were comparable with those shown by typical C_4 plants (Moss, 1962a; Tregunna & Downton, 1967). Although these measurements were made at 15°C, which is the natural growing temperature for these algae, the low compensation points were still in agreement with those observed by Brown & Tregunna (1967) in some marine algae at 25°C.

The low CO_2 compensation points in these algae at low temperature suggests the possibility of refixation of respired CO_2 as suggested by Goldsworthy (1970) for Chlorella. The presence of photosynthetic mechanism very similar to C_4 plants could also result in low compensation points. Goldsworthy's suggestion may have some merit for Chlorella or other algae

where the chloroplast almost completely surrounds the cell, and photorespiratory CO_2 must first cross the chloroplast to be able to escape, and in so doing, an appreciable amount may be refixed. But this explanation would not hold for Phaeodactylum and many other algae which do not have the same chloroplast structure as Chlorella and yet photorespiration rates are very low.

Phaeodactylum tricornutum, a pennate diatom, exists in 3 forms, oval, fusiform and tri-radiate (Hendey, 1963). The oval cells, under certain conditions develop into elongated or fusiform cells. The central part remains elliptical and two polar extensions develop. The experimental material consisted of a mixture of oval and fusiform cells, the oval cells being 3-4 x 8μ and the fusiform cells 3-5 x 25-35μ in size, each cell having one or two slightly oblong chloroplasts. Although Phaeodactylum does not have the same chloroplast structure as that of Chlorella, it shows along with many other algae, some peculiarities of the chloroplast structure which might be significant in determining its photosynthetic behaviour. The chloroplast, in members of Phaeophyta, to which this diatom belongs, possesses, besides the basic 2-membrane envelope, a sheath of endoplasmic reticulum which is usually continuous with the nuclear membrane (Gibbs, 1962b). Stoermer et al (1965) also observed in some diatoms tubular structures forming a branched net-work in the space between the nuclear envelope and the true chloroplast envelope. This could perhaps perform some function, similar to that of the peripheral reticulum

inside the chloroplast membrane of C_4 plants (Osmond et al, 1969; Laetsch, 1970). Although these algae are very different in many ways from the C_4 plants, it was thought worthwhile to compare them at the biochemical level to see if they had any similarities which might explain their low compensation points.

The C_4 plants have a different morphology of the leaf to the C_3 plants in having two concentric layers of cells, the outer mesophyll and the inner bundle-sheath lying in close proximity to one another, and having well developed plasmodesmata connecting both types of cells. Moreover, both layers have been shown to have different enzyme complements (Björkman & Gauhl, 1969; Slack, Hatch & Goodchild, 1969; Berry, Downton and Tregunna, 1970; Kisaki & Tolbert, 1970), the mesophyll contains one CO_2 fixing enzyme, called phosphoenolpyruvate (PEP) carboxylase and the bundle-sheath contains another CO_2 fixing enzyme ribulose -1,5 diphosphate carboxylase (RuDP) which is the principal CO_2 fixing enzyme for the C_3 plants. The PEP carboxylase fixes CO_2 and converts PEP into a C_4 acid, oxaloacetate. This is then converted into other C_4 acids, malate and aspartate. Malic acid is believed to dissociate into pyruvate and CO_2 by the action of another enzyme called the malic enzyme (1.1.1.40). It was demonstrated by Berry, Downton & Tregunna (1970) that these C_4 acids are translocated from the mesophyll to the bundle-sheath cells via plasmodesmata, where malate generates CO_2 at the site of RuDP carboxylase which then fixes it in a normal 'Calvin type' fashion, without apparent loss of CO_2 into the intercellular spaces (Johnson & Hatch, 1968; Slack et al, 1969). The pyruvate formed by the breakdown of malic

acid is believed to migrate back to the mesophyll where it is again converted into PEP by the enzyme pyruvate phosphate dikinase using ATP and inorganic phosphate thus enabling it to fix another molecule of CO_2.

Along this peculiar leaf anatomy, the detection of glycollate in C_4 plants (Zelitch, 1958) and the presence of glycollate oxidase (1.1.3.1) and peroxisomes in the bundle-sheath cells (Kisaki & Tolbert, 1970) led these workers to believe that C_4 plants also photorespire and the apparent absence of photorespiration is due to the refixation of photorespired CO_2 by the PEP carboxylase situated in the mesophyll.

A second school of thought does not consider refixation of respired CO_2 as a sufficient explanation for low apparent photorespiration rates in C_4 plants. If it were so, then the C_4 plants which are more efficient photosynthetically and show approximately twice the rate of CO_2 fixation as the C_3 plants, should also have a carboxylase with a very low Km for CO_2. This was not the case as shown by Goldsworthy (1968) who measured the Km values for the photosynthetic CO_2 uptake in tobacco, maize and sugar-cane and found similar values for the Michaelis constant in both C_3 and C_4 plants which led him to conclude that the higher efficiency of the C_4 plants is not due to their having a higher affinity for CO_2.

Further support to the above explanation was provided by Downes & Hesketh (1968) who did not find any enhancement in the photosynthetic rate in C_4 species at low O_2 concentration when photorespiration was supposed to be inhibited. Whereas, Forrester,

Krotkov & Nelson (1966a) showed at least part of stimulation of photosynthesis at low O_2 concentration in C_3 plants to be due to the inhibition of photorespiration. Similarly, Zelitch (1966) did not observe a stimulation of net photosynthesis rate in maize when treated with α-OHMS. From these data, Goldsworthy (1970) concluded that the failure to obtain a stimulation of net photosynthesis rate in C_4 plants may be because there is little photorespiration to inhibit.

Goldsworthy & Day (1970) put forward a hypothesis that the low photorespiration rates in C_4 plants may be a result of very low rates of glycollate synthesis. They proposed that the malate formation in these plants is part of a CO_2-pump generating a high CO_2 concentration at the site of the RuDP carboxylase so as to inhibit glycollate synthesis. Goldsworthy (in press) suggested that so long as this pump is working, glycollate synthesis would remain suppressed, but if in adverse conditions e.g. dry weather, the stomata close, then the CO_2 fixation by PEP carboxylase would stop and with this, the CO_2 pump would stop too. This would explain the presence of peroxisomes and requisite enzymes for glycollate metabolism in C_4 plants. Although they would not normally be required, they would still be necessary to dispose of the glycollate produced when the CO_2 pump failed under adverse conditions. Goldsworthy's hypothesis is therefore consistant with the work of Zelitch (1958) who detected glycollate in C_4 leaves in the presence of α-OHMS which is known to cause stomatal closure (Zelitch & Walker, 1964). Hatch (1971) provided some evidence for this hypothesis when he measured the size of

the CO_2 pool generated in the mesophyll from C_4 acids and he estimated that its concentration must be about one hundred times greater than that of a tissue in direct equilibrium with the atmosphere. If this is correct, then an almost complete inhibition of glycollate synthesis should occur with no further reduction being possible by altering the O_2 concentration of the air. (Goldsworthy, in press).

If low photorespiration rates in algae are to be explained by a mechanism similar to C_4 plants, this would require the presence of PEP carboxylase in a region surrounding the RuDP carboxylase. Since algal cells such as Phaeodactylum cannot possess the anatomy of a C_4 leaf, an intracellular version of the C_4 pathway must be sought. Perhaps the extra membranous envelope around the chloroplast could be a likely place for the presence of PEP carboxylase. Alternatively, this membrane may facilitate the transport of CO_2 by some other mechanism. The former explanation does not seem likely because the activity of PEP carboxylase was very low in Phaeodactylum when compared to C_4 plants. This observation was not the result of any powerful inhibitors of this enzyme being present in the extract, because the Phaodactylum extract did not inhibit the PEP carboxylase of a sugar-cane extract. As shown in the result's section in table 2, the combined extract gave rates somewhat higher than the mean rate of the individual extracts. This may have been due to the presence of large amounts of malate dehydrogenase in the Phaeodactylum extract (table 9) which would have permitted a more efficient coupling of the PEP carboxylase of the sugar-cane

extract to NADH oxidation. An eight times lower activity of PEP carboxylase in the Phaeodactylum extracts as compared to the sugar-cane extracts suggested that the C_4 pathway is not the dominant route, and probably this diatom fixes CO_2 predominantly by the Calvin mechanism. This was confirmed to be so when the first product of fixation after 5 seconds photosynthesis was found to be phosphoglycerate. The operation of the Calvin cycle has also been found in Laminaria, another brown alga by Kremer & Willenbrink (1972).

Although a mechanism of glycollate synthesis has now been established in higher plants, its operation in algae can be inferred from circumstantial evidence. Whittingham et al (1963) pioneered the way when they showed glycollate-^{14}C synthesis from glucose-^{14}C in Chlorella in the light. Marker & Whittingham (1966) extended this when they showed that it was carbon atoms 1 & 2 of the glucose molecule which produced the glycollate. This mechanism of glycollate synthesis was CO_2 sensitive, being inhibited at higher concentrations.

Coombs & Whittingham (1966) showed that glycollate synthesis in Chlorella was stimulated by high oxygen and low CO_2 levels. This suggests a competition between oxygen and CO_2 similar to that proposed by Ogren & Bowes (1971) which leads to the production of glycollate in higher plants.

Ogren and Bowes (1971) and Bowes, Ogren & Hageman (1971) showed a competition between oxygen and CO_2 for the RuDP carboxylase reaction with a high oxygen to CO_2 ratio resulting in glycollate production at the expense of CO_2 fixation. A similar observation was made by Ellyard and San Pietro (1969) in extracts

of Euglena which when provided with ribose-5-phosphate, ATP and NADPH fixed $^{14}CO_2$, this was stimulated by high CO_2 and low O_2 levels with a high oxygen partial pressure resulting in an increase in the synthesis of C_2 compounds.

The participation of O_2 in glycollate synthesis was established by Andrews, Lorimer & Tolbert (1971) who showed incorporation of $^{18}O_2$ into the carboxyl group of glycine and serine derived from glycollate synthesized during photosynthesis in higher plants. The immediate products of this reaction were isolated recently from spinach leaves by Lorimer, Andrews & Tolbert (1973) and found to be 3-phosphoglycerate and phosphoglycollate. Both products appeared to be formed by the catalytic activity of the same protein, because the 'carboxylase' and 'oxygenase' activities could not be separated from each other by any of the isolation procedures used. The phosphoglycollate is believed to be hydrolysed by the action of phosphoglycollate phosphatase (3.1.3.18) present in the chloroplasts (Richardson & Tolbert, 1961). This mechanism of glycollate synthesis provides a good explanation of the Warburg effect hitherto unexplained. At higher O_2 concentrations (21-100%), the oxygenase reaction would become increasingly important compared with the carboxylase reaction, and at lower O_2 concentrations (below 21%), the carboxylase reaction would be favoured. The effect of high O_2 concentration would be diminished by increasing the CO_2 concentration.

The results given in table 3 showed an inhibition of photosynthesis by increasing the O_2 concentration to 21% under low CO_2 conditions (80-280 vpm) in both tobacco and the algae. Tobacco,

however, showed a much greater inhibition at all CO_2 levels as compared to Phaeodactylum. While inhibition of photosynthesis significantly decreased with increase in CO_2 concentration in tobacco, it was not much affected in any of the algae examined.

Although the main feature of the Warburg effect i.e. the inhibition of photosynthesis at higher O_2 concentration was realized in these algae, the insensitivity towards increasing CO_2 concentration still needed an explanation and suggested that this inhibition was caused by reactions not directly concerned with the synthesis of glycollate. Turner & Brittain (1962) proposed that this oxygen inhibition may have a variety of causes such as the inhibition of enzymes of the photosynthetic carbon reduction cycle, photosynthetic electron transport and photophosphorylation. In the present work it was shown that the inhibition of photosynthesis was not completely reversible in either tobacco or algae after treatment with 100% oxygen. This suggests at least one additional site for the oxygen inhibition of photosynthesis other than the simple competition with CO_2 leading to glycollate synthesis. We may therefore conclude that even if an organism does show an inhibition of its photosynthesis by oxygen it is by no means certain that this is because it synthesizes glycollate.

C_4 plants are even better than algae in not showing inhibition of photosynthesis at 21% O_2. This was shown by Forrester et al (1966a) who could not see any effect up to 40% O_2, although at 80% O_2, 50% inhibition and at 100% O_2, 70% inhibition was observed. From these data it appears that algae and the C_4 plants behave differently from the higher C_3 plants in that

they show much less competition between CO_2 and oxygen. This raises the possibility that algae like C_4 plants have a mechanism by which they can concentrate the CO_2 in their chloroplasts which would make them relatively independent of ambient CO_2 concentration.

Further investigations into the nature of the Warburg effect were made to confirm this last point. For this, the effect of various O_2 concentrations on RuDP carboxylase was studied in cell-free extracts when it would be free from any CO_2 concentrating mechanism. RuDP carboxylase was extracted from Ulva, a green alga, which like Phaeodactylum has a low CO_2 compensation point. Its chloroplast, however, is not cup-shaped like that of Chlorella but is central like that of Phaeodactylum. The algal enzyme was compared with the spinach enzyme (Figs. 12a & 12b). Maximum CO_2 fixation occurred in N_2 (0% O_2), intermediate in air (21% O_2) and least in 100% O_2. The only difference was in the activity of the enzyme. The spinach enzyme being much more active than the Ulva enzyme on a fresh weight basis. This experiment clearly showed that the isolated enzyme from this alga behaves similarly to that of higher plants at all O_2 levels. The CO_2 reversibility of O_2 inhibition of photosynthesis in higher C_3 plants but not in algae may be due to some other factors which differ between these organisms, such as the occurrence of a CO_2 concentrating mechanism in the algae. The presence of an extra membrane system associated with the chloroplasts in some algae could be the site of such a concentrating mechanism e.g., in Phaeodactylum

but not in Ulva which does not have these extra membranes. Despite this, a CO_2 pump as suggested by Goldsworthy & Day (1970) in C_4 plants does not seem likely in Phaeodactylum, because about eight times less PEP carboxylase was detected as compared to sugar-cane. However its activity was still higher than that of C_3 plants since Hatch (1971) reports a 60-fold difference for this enzyme between C_3 and C_4 plants.

Perhaps, other carboxylation reactions occurring in the cell might provide the basis for a CO_2 pump. Weidner & Küpper (1973) found high activities of PEP carboxykinase (EC 4.1.1.32) in fronds of Laminaria, a brown alga, which was even more active than the RuDP carboxylase. However, even here it may be doubtful whether this forms the basis of a CO_2 pump similar to C_4 plants since Kremer & Willenbrink (1972) have reported a normal Calvin cycle in this alga. However, the failure to detect anything other than normal C_3 intermediates during short term photosynthesis does not necessarily preclude the operation of a CO_2 pump, because the intermediates of the pump mechanism may only have a very transitory existence and would not be detected by conventional techniques. This might particularly be the case if the entire pump mechanism occurred within a single cell or across a single membrane, as would have to be the case in the unicellular algae.

It also seems possible that the low compensation points of these algae may not depend on a C_4 type of metabolism. It must be borne in mind that even amongst the algae, different species may achieve their low compensation points by different mechanisms. Evidence supporting this comes from the experiments in which the

$^{14}CO_2$ released by Chlorella and Phaeodactylum were compared under different environmental conditions. The cells were labelled by photosynthesis in $^{14}CO_2$ and the $^{14}CO_2$ evolved in light was collected by passing CO_2-free air having different O_2 concentrations over them. The results (table 4) show that the Chlorella cells evolved much more $^{14}CO_2$ than the Phaeodactylum cells and the CO_2 evolution was O_2 sensitive in Chlorella but not in Phaeodactylum (Fig. 13). The CO_2 evolved in Chlorella could be attributed to photorespiration. The results of this experiment suggest that the oxygen inhibition of photosynthesis observed in Chlorella at 21% O_2 (table 3) was at least, in part, a consequence of glycollate production. This is consistent with the finding of Pritchard et al (1962) who detected glycollate in Chlorella at 21% O_2. These differences in photorespiration between Phaeodactylum and Chlorella may be due to a failure of Phaeodactylum to produce glycollate to the same degree as Chlorella from stored photosynthate. This may be an effect on either glycollate synthesis itself, the further metabolism of any glycollate formed, or on the biochemical pathway leading from stored photosynthate to glycollate. This last possibility is suggested by the fact that the storage products in these two algae are different and may be differently located. Starch and similar polysaccharides are characteristic of the Chlorophyta whereas leucosin is characteristic of diatoms (Beattie et al, 1961). Furthermore reserve products are typically located within the chloroplast in the Chlorophyta but are typically external in other algae

(Kirk & Tilney-Bassett, 1966). We would therefore expect a greater feedback of labelled materials from reserve products into the Calvin cycle and hence to glycollate in Chlorella.

Low photorespiration rates in Phaeodactylum may also be a result of a large amount of glycollate excretion as suggested by Goldsworthy (1970) in Chlorella. If so, then Phaeodactylum which has a much lower photorespiration rate than that of Chlorella should excrete an even larger proportion of its fixed carbon as glycollate. As shown from the results (table 5), Phaeodactylum excreted only negligible amount of glycollate at low CO_2 and high light intensity conditions reported to stimulate glycollate excretion in green algae by Watt & Fogg (1966). Even if glycollate excretion is not observed in normal culture conditions, large amount of glycollate production and excretion can often be shown by the use of inhibitors, INH (Pritchard et al, 1961, 62; Whittingham & Pritchard, 1963), and α-OHMS (Lord, 1971). α-hydroxymethanesulphonates are inhibitors of the glycollic oxidase of higher plants (Zelitch, 1957) and have also been shown to inhibit glycollate oxidation in green algae (Lord & Merrett, 1969), blue-green algae (Gimmler et al 1969; Miller et al 1971) and cell-free extracts of blue-green algae (Grodzinski & Colman, 1970).

When Phaeodactylum cells were treated with 1 mM α-OHMS, glycollate could not be detected in the medium or in the cell extract. This showed that it was perhaps not being synthesized

under the conditions of the experiment. When the inhibitor concentration was increased to 20 mM, glycollate excretion was only barely detectable (Fig. 14). The 0.07 μmole glycollate/ml p.c.v. excreted in 2 hours was orders of magnitude less than observed by Lord (1971) in Chlorella.

Other factors reported to stimulate glycollate production and excretion are the transferance from high to low CO_2 (Watt & Fogg, 1966; Nelson & Tolbert, 1969), and treatment with INH, an inhibitor of the glycine to serine conversion (Whittingham & Pritchard, 1963). A comparison of glycollate excretion rates between Chlorella and Phaeodactylum was made using the above 2 techniques simultaneously. Cells grown in 4% CO_2 in air were treated with 20 mM INH and glycollate excretion determined at 15 minute intervals. As shown from the results (Fig. 15), no glycollate could be detected in Phaeodactylum whereas Chlorella excreted 2.7 μmoles/ml p.c.v in 30 minutes. The low glycollate excretion in the diatom resembles that in the blue-green algae. Cheng et al (1972) using Anabena and Oscillatoria could not find an appreciable amount of glycollate excreted even in the presence of inhibitors of the glycollate pathway. Cheng & Colman (1974) found low photorespiration rates in some green and blue-green algae, the blue-greens having lower rates than the green algae, from which they concluded that these blue-green algae have a low rate of glycollate synthesis.

Since in the excretion experiments, glycollate was not detected by Calkin's (1943) method, it was decided to use a radio-active assay to make its detection more sensitive. In this experiment, Phaeodactylum cells were allowed to excrete photo-assimilated ^{14}C under low CO_2 and varying O_2 concentrations. As shown from the results (Fig. 16) the rate of excretion was unaffected by the high (100%) or low (0%) O_2 concentration. The excretion rate, however, was dependent upon the photosynthetic rate. The absence of the O_2 effect on the excretion rate suggested that glycollate was not among the excreted compounds. This was confirmed by chromatography, although three other compounds were excreted, whose identity could not be determined. These results confirm Hellebust's (1965) who found no glycollate excretion in Phaeodactylum under bright sun-light. These results support the previous suggestion that this diatom has a mechanism for inhibiting glycollate synthesis even at 100% oxygen concentration.

Another possibility is that glycollate is produced but its metabolism is very fast and this results in negligible excretion rates. This was tested by feeding glycollate to intact cells and following the rate of oxygen exchange. If the lack of glycollate excretion was a result of its rapid oxidation, then the added glycollate should stimulate the rate of oxygen uptake. No effect on oxygen evolution was observed when glycollate was supplied to Phaeodactylum in the light (table 6). Although at first sight we might expect the oxidation of glycollate to depress the rate of oxygen

evolution this need not be the case. If glycollate was being metabolised right through to carbohydrate as in higher plants then the NADH and NADPH required for the later stages in the pathway would, if derived from photosynthesis, result in extra O_2 evolution. This would largely offset the oxygen consumption by the initial stages so that there would be little overall effect of added glycollate on net oxygen exchange. For the same reason the addition of the inhibitor α-OHMS should also have little effect on oxygen exchange in the light. For the above reasons the effect of glycollate on oxygen exchange is best measured in the dark when the reductive stages in the glycollate pathway would not be expected to operate (Ongun & Stocking, 1965 a). Table 7 shows the results of such an experiment. It can be clearly seen that even the lowest concentration of glycollate tested caused a 2-fold increase in oxygen uptake. This suggests that the cells have a capacity to oxidise glycollate at a rate approximately equivalent to the normal rate of respiration. The rate, however, did seem to be limited to this figure, for increasing the external glycollate concentration had no further effect. Low concentrations of α-OHMS did not inhibit the glycollate-dependent oxygen uptake. This may be because the enzymes responsible are insensitive to the inhibitor or because it failed to penetrate the cells. It seems likely, however, that α-OHMS does penetrate because higher concentrations caused non-specific effects such as the stimulation of oxygen uptake both in the presence and absence of glycollate and also the inhibition of photosynthesis (tables 6 & 7). A similar inhibitory effect of α-OHMS on photosynthesis has been observed in higher plants (Moss, 1968).

A tentative conclusion from these results is that Phaeodactylum has a significant capacity to oxidise glycollate but that the enzyme system responsible is less sensitive to α-OHMS than is photosynthesis. This suggests that the use of this inhibitor to demonstrate glycollate synthesis in this alga is questionable.

In order to confirm these findings and eliminate the possibility that the effect of glycollate on oxygen uptake was a result of a stimulation of respiration due to an injury effect rather than an oxidation of the glycollate itself, a number of experiments were done. These included testing whether glycollate could function as a sole carbon source for growth, measuring the uptake and metabolism of radio-active glycollate by the alga and the biochemical assay of the enzymes of glycollate metabolism in cell extracts.

When glycollate was tested as a sole carbon source it was found to be capable of supporting good growth (Fig. 17). As the results show, glycollate utilization in the light was more efficient at the slightly acidic pH of 6.5 (Fig. 17a) and the maximum growth rate occurred at a 5 mM concentration. These results confirmed the results discussed in the previous experiment that this diatom can take up exogenous glycollate not only in the dark but also in the light and can metabolise it to support growth when no other carbon source is available. Above 25 mM glycollate became toxic and growth did not occur. These results seem to be contradictory to the results discussed previously where the 6-46 mM glycollate concentrations used did

not affect the rate of photosynthesis. This may be a result of a limited uptake of glycollate in the short term experiments described in tables 6 and 7, whereas the results of glycollate-dependent growth of Phaeodactylum were based on a period of 7 days, when enough glycollate may have entered the cells to show its toxic effect.

The ability of Phaeodactylum to use glycollate as a carbon source has been confirmed using radio-active glycollate. The rate of uptake and metabolism of glycollate-1-^{14}C by Phaeodactylum was compared with that of Chlorella, an alga known to metabolise glycollate. The results (table 8) show that the rates of glycollate uptake differed by only a factor of 2 in the two organisms and that the rates of its metabolism as measured by $^{14}CO_2$ release were also similar. In both organisms the uptake of glycollate and the release of $^{14}CO_2$ was stimulated several-fold by light and the products of glycollate metabolism included both glycine and serine. The results indicate that Phaeodactylum and Chlorella have the capacity to take up and metabolise glycollate at similar rates and that the mechanism of this may be similar in both organisms.

The discovery of glycine and serine as products of glycollate metabolism in Phaeodactylum suggested that the mechanism of its metabolism was similar to that of higher plants. However, the distribution of counts in the ethanol soluble and insoluble fractions was different in the two organisms which probably was due to differences in the nature of food reserves.

The similarity between Chlorella and Phaeodactylum with respect to glycollate-^{14}C metabolism suggested that their glycollate oxidising enzyme would also be similar. As shown from the results (table 11), this seemed to be the case. Although Phaeodactylum gave higher rates of glycollate oxidation as compared to Chlorella by both phenylhydrazine and DICPIP methods of assay, this was not considered due to a real difference in the enzyme activity. There was considerable variability in the enzyme activity of different extracts from even the same algae and this was thought to be due to different degrees of cell breakage. Microscopic observation indicated that the breakage in Chlorella was typically much lower than in Phaeodactylum.

Although the glycollate oxidising enzyme has been found in all of the algae investigated so far, it is yet not clear whether its synthesis is controlled by the availability of the substrate.

The CO_2 control of the enzyme synthesis reported by Codd, Lord & Merrett (1969) in algae was supported by the work of Lord & Merret (1970 b) in Chlamydomonas and Lord & Merrett (1971 b) in Euglena in which it seems to be a result of substrate availability. At higher CO_2 concentrations, little glycollate is synthesized, therefore no enzyme is required for its oxidation and little or no enzyme is produced. At lower CO_2 concentration, glycollate synthesis is enhanced, the enzyme is needed and is synthesised as a result of substrate induction. Cooksey (1971) found similar results in Chlamydomonas, though he did not find

enzyme synthesis under the immediate control of CO_2, which was perhaps due to some enzyme synthesis still occurring from the mRNA of the cytoplasm and it took about one cell generation time before the CO_2 control of the enzyme synthesis could be realized. However, different conclusions were reached by Colman, Miller & Grodzinski (1974) in Chlorella where glycollate dehydrogenase level was not affected by the availability of CO_2.

Whether Phaeodactylum, which had a very low rate of glycollate synthesis would have an inducible or a constitutive enzyme was clarified by growing Phaeodactylum in the presence or absence of glycollate and then assaying the enzyme by both Phenylhydrazine and DICPIP methods of assay. As the results show (table 14), there were no large differences in the enzyme activity in either of these two treatments thus suggesting that the glycollate oxidising enzyme was probably constitutive in this organism.

The glycollate oxidising enzyme has been reported to be a dehydrogenase in Chlorella (Lord, 1971, Tolbert, 1971). In Phaeodactylum, since there was evidence of oxygen acting as an ultimate electron acceptor in vivo from glycollate in the dark, it suggested that it might also be so in vitro. The results, however, showed (table 13), that the Phaeodactylum enzyme was also a dehydrogenase like most other algae investigated. Oxygen might be acting as an ultimate electron acceptor in intact cells through some natural acceptor(s) which may be lost on preparing the cell extracts.

The possibility of an intermediate electron acceptor is also suggested in Grubber et al's (1974) work in Dunaliella, where the enzyme gave a profile of activity closely similar to cytochrome oxidase, the mitochondrial marker enzyme, suggesting the possible localization of glycollic dehydrogenase in the mitochondria. If it were so, then glycollate electrons might use one or more electron acceptors of the mitochondrial electron transport chain for the transfer of electrons during glycollate oxidation.

The particulate nature of the glycollate dehydrogenase has also been suggested by Lord and Merrett (1971 b) and Graves et al (1971) in Euglena, though in their work its mitochondrial location could not be ascertained and it appeared to be present in peroxisome like structures as in higher plants.

If the glycollic dehydrogenase either occurs in the mitochondria or uses a natural electron acceptor which can penetrate the mitochondria then the electrons from glycollate oxidation could pass through the mitochondrial electron transport chain so as to generate ATP. In theory, there should be enough energy from glycollate oxidation (-41,000 cal/mole) to generate 2 or 3 ATP molecules (Zelitch, 1955).

As yet there is no evidence of ATP synthesis from glycollate oxidation in algae but there is a little evidence that this may occur in higher plants. Despite the fact that the glycollate oxidising enzyme in higher plants has been called an oxidase there is evidence that it can also function as a facultative

dehydrogenase, feeding its electrons to suitable acceptor molecules. One such acceptor is of course the artificial dye DICPIP. Codd and Schmid (1971) found that the enzyme from tobacco worked equally well with either DICPIP or air as the electron acceptor. In either case the addition of PMS (phenazine-methosulphate) stimulated the rate of glycollate oxidation. They took this to suggest that PMS substituted for a natural acceptor which could transfer electrons from glycollate to either DICPIP or oxygen. They did not identify the natural acceptor but suggested it might be a quinoid compound. If this compound could also feed its electrons into the mitochondrial electron transport chain then ATP synthesis could result.

A comment should be made at this point that it might be an advantage for the plant to have two alternative means to dispose of glycollate, one a rapid irreversible reaction (the oxidase) which could effectively remove glycollate under conditions of rapid synthesis so as to prevent its accumulation in toxic quantities, and second, a reaction which recovered some of the energy of oxidation perhaps as ATP. This would be of a lower overall free energy change and therefore less rapid and suitable only for conditions of low glycollate synthesis.

It might be speculated that glycollic 'oxidase' is an allosteric enzyme which tends to function as an oxidase under conditions of high glycollate synthesis but as a dehydrogenase linked to ATP production under conditions of low glycollate

synthesis. Evidence for this comes from the work of Omata & Tsukamoto (1970) who, working with unwashed spinach chloroplasts found that small additions of glycollate added at 5 minute intervals caused 9 times more phosphorylation than one initial addition of the same total amount.

Apart from this there is little other evidence to link glycollate oxidation to ATP production. Zelitch & Barber (1960) showed only very low rates of ATP synthesis in response to glycollate in isolated spinach mitochondria , giving P/O ratios of only 0.1 for glycollate, whereas they were in the region of 2-3 for the various citric acid cycle acids. However, the fact that they did get some ATP synthesis with glycollate may be significant, and the low rate may be due to the loss from their preparation of an electron carrier which could carry electrons from the glycollic oxidase in the peroxisomes to the mitochondria.

Glycollate has been considered as the main source for the origin of the CO_2 of photorespiration (Goldsworthy, 1966, 1968; Zelitch, 1958, 1966-68; Zelitch & Day, 1968; Bjorkman, 1966; Moss, 1968), but the exact point in the glycollate pathway at which the CO_2 is released is less clear. There is convincing evidence that glycine decarboxylation during serine formation is one source for CO_2 evolution during photorespiration (Kisaki & Tolbert, 1970; Kisaki et al, 1971; Bird et al, 1972).

The results of glycine-1-^{14}C feeding to both Phaeodactylum and Chlorella showed its uptake and metabolism to a number of compounds including serine. Although there was only a relatively

low proportion of the total counts in serine it does not follow that there is little conversion to serine because in vivo there is nothing to prevent its further metabolism to other compounds. However, the finding that less than 1% of the total radioactivity taken up by the cells was released as CO_2 suggests that the glycine to serine conversion only occurs at a very low rate or that it occurs without the production of CO_2. Since 1966, Zelitch has been advocating the non-enzymatic decarboxylation of glyoxylate brought about by the H_2O_2 generated in the glycollic oxidase reaction as the source of most CO_2 evolution during photorespiration. When Zelitch (1972 b) compared glycollate-1-^{14}C or glycine-1-^{14}C as a substrate for CO_2 evolution in leaf discs of tobacco he found that glycine and glycollate were metabolised to CO_2 at approximately similar rates, and interestingly, INH inhibited the conversion of glycine to serine but not the release of $^{14}CO_2$. He concluded that the bulk of the CO_2 released in photorespiration was not, therefore, coming from the glycine to serine conversion. He suggested instead that it arises from the decarboxylation of glyoxylate. If it is so, then the site of this CO_2 evolution could not be the peroxisomes which have a large amount of catalase (Kisaki & Tolbert, 1969) which would destroy H_2O_2, the oxidising agent of Zelitch's reaction. Also, isolated peroxisomes do not release $^{14}CO_2$ when supplied with glycollate-^{14}C or glyoxylate-^{14}C (Kisaki & Tolbert, 1969). CO_2 release from glyoxylate if it occurs would therefore, be more likely to occur after its excretion into the cytoplasm. In that case, CO_2 evolution from glyoxylate could occur in the cytosol, or in the chloroplast, if glyoxylate can enter them. Such a trans-

location of glyoxylate into the chloroplast was shown by Kearney & Tolbert (1962) in isolated spinach chloroplasts where added ^{14}C-glyoxylate rapidly moved into them.

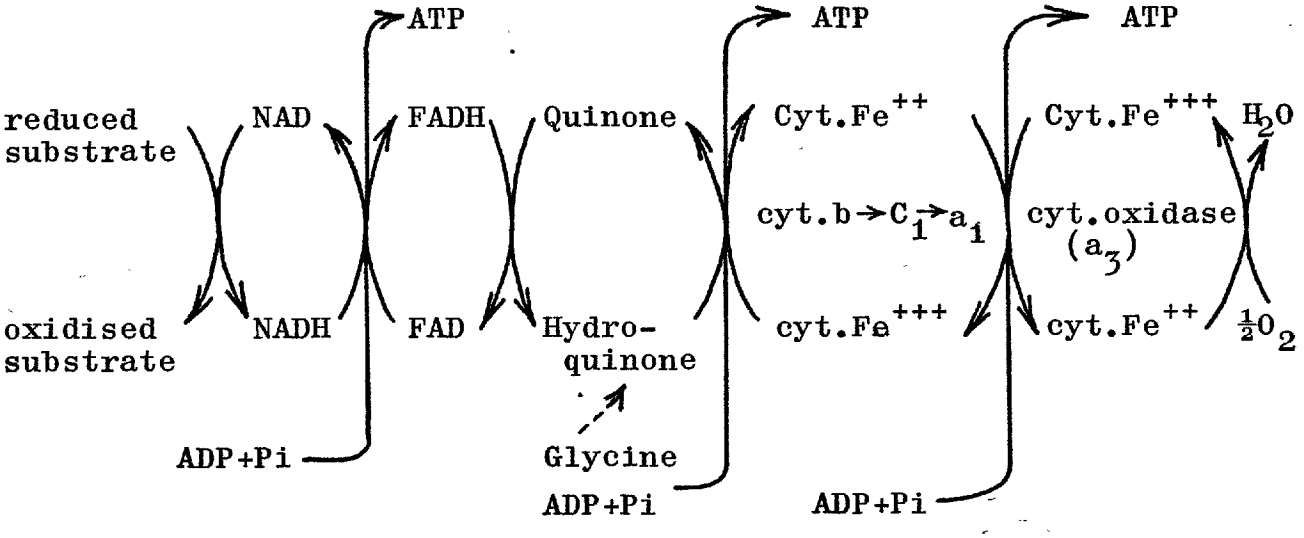
In contrast to Zelitch's work, Kisaki & Tolbert (1970) when they fed carboxyl labelled glycine or glycollate to tobacco and corn leaf segments, found that INH blocked CO_2 release from glycine and glycollate. From these results they concluded that CO_2 release during photorespiration comes from the conversion of two molecules of glycine to one serine and one CO_2.

The enzyme system responsible for the glycine to serine conversion is fairly complex. It is considered that the decarboxylation of glycine is brought about by an enzyme called glycine decarboxylase which decomposes the glycine into CO_2, NH_3 and a C_1 unit. The C_1 unit is supposed to be carried by tetrahydrofolate and transferred to another glycine molecule to produce serine in the presence of a second enzyme called serine hydroxymethyltransferase. By the combined action of these two enzymes, two molecules of glycine are converted to one molecule of serine and one molecule of CO_2. Evidence for this mechanism was provided by Kisaki et al (1971) and Bird et al (1972) who found the decarboxylation of glycine always accompanied by the synthesis of serine in their preparations. Serine hydroxymethytransferase was found principally in the mitochondria and partly in the chloroplasts, while glycine decarboxylase was located in the mitochondria (Kisaki, Imai & Tolbert, 1971).

Kisaki et al (1971) found that the treatments which disrupt mitochondria destroy the activity of these enzymes from which they suggested the involvement of the mitochondrial electron transport chain. From these results they proposed a scheme in which NAD functioned as on electron carrier for the oxidation involved in the reaction and that the NAD/NADH ratio controlled the rate of the reaction and therefore also the rate of photorespiratory CO_2 loss. However, they did not observe a significant increase in absorption at 340 nm due to NADH after the addition of glycine and they inferred that the glycine decarboxylase system was specially oriented near a system which rapidly oxidised the newly synthesized NADH.

Bird et al (1972) have presented data which do not confirm some of the conclusions presented by Kisaki et al (1971). They too found the glycine to serine conversion in mitochondrial preparations from tobacco, but NAD was not required and aerobic conditions were necessary for the reaction to proceed at a significant rate. The reaction was inhibited by inhibitors of the mitochondrial electron transport and the glycine to serine conversion was coupled to ATP synthesis from ADP. This was concluded because glycine stimulated ATP synthesis and INH inhibited this glycine-stimulated phosphorylation but not phosphorylation which occurred in the absence of glycine. Semicarbazide and Arsenite, inhibitors of phosphorylation also inhibited all additional ATP synthesis caused by adding glycine. From these results they concluded that an electron acceptor in mitochondria that transfers electrons to the mitochondrial electron transport

chain must be an essential component of the catalytic system converting glycine to serine. Their finding that only two ATP molecules were formed per serine molecule argues that the electrons are fed into the chain beyond the NAD stage. If so, this would explain the lack of apparent NAD reduction observed by Kisaki et al (1971) and the apparent control of the glycine decarboxylase reaction by NAD and NADH must have some other explanation not involving their direct participation in the electron transport chain from glycine. A schematic representation of such a system is given below which is modified from Conn & Stumpf (1972).



There seems to be no published work on isolated enzyme systems from algae for the conversion of glycine to serine so that little can be said about the necessity for the reaction to be linked to CO_2 production. It is possible for example that the algae may possess the serine hydroxymethyltransferase reaction but not glycine decarboxylase. If they used an alternative

source of C_1 units then the glycine to serine conversion could occur without loss of CO_2. Alternatively the glycine decarboxylase reaction could be coupled to a carboxylation reaction, photosynthetic or otherwise, which would refix this CO_2 before it could be released. Such a secondary carboxylation reaction might be operative side by side with the principle CO_2 fixation reaction. Apart from the already described PEP and RuDP carboxylation reactions, certain other carboxylations can be described in this context. Two ferredoxin-linked carboxylations have been described in photosynthetic bacteria like Chlorobium and Rhodospirillum. These bacteria fix CO_2 at the expense of reduced ferredoxin (Evans et al, 1966; Buchanan & Evans, 1965) by the following reactions, although these bacteria also possess the enzymes of the photosynthetic carbon reduction cycle and hence are capable of fixing CO_2 via the Calvin cycle.

1. Acetyl CoA + CO_2 + Fd_{red} $\xrightarrow[\text{Synthetase}]{\text{Pyruvate}}$ Pyruvate + Fd_{ox} + CoA

2. Succinyl CoA + Co_2 + Fd_{red} $\xrightarrow[\text{Synthetase}]{\alpha\text{-Ketoglutarate}}$ α-Ketoglutarate + Fd_{ox} + CoA

Some NADPH-linked carboxylations are also known. In one, pyruvate is converted to malate by an enzyme called the 'malic enzyme' (1.1.1.40). This reaction was studied by Ochoa, but the equilibrium of the reaction favours oxidative decarboxylation rather than CO_2 fixation (Walker, 1962). Another NADPH-linked

carboxylation in which α-ketoglutarate is converted into isocitrate has also been described by Dalziel & Londesborough (1968). Although the equilibrium of this reaction also favours a decarboxylation, the reaction can be used to synthesize isocitrate using high concentrations of the reactants. A pyruvic carboxylase is also known to exist which converts pyruvate into oxaloacetate in the presence of ATP.

$$\text{Pyruvate} + CO_2 + \text{ATP} \xrightleftharpoons[Mg^{2+}]{\text{Acetyl CoA}} \text{Oxaloacetate} + \text{ADP} + H_3PO_3$$

This reaction is readily reversible due to quite small $\Delta G'$ (Conn & Stumpf, 1962). Another reversible reaction is catalysed by PEP carboxykinase which produces oxaloacetate and guanosine triphosphate from PEP and CO_2.

$$\text{PEP} + CO_2 + \text{GDP} \xrightleftharpoons{Mg^{2+}} \text{Oxaloacetate} + \text{GTP}$$

However, these enzymes are either freely reversible, and so would not be efficient scavengers for photorespired CO_2, or their presence has not been detected in algae.

Another possibility worth investigating is that the CO_2 releasing stage of photorespiration occurs in the chloroplast in algae so that its refixation could occur by normal photosynthesis. Evidence for this comes from the finding by Bidwell et al (1969) that chloroplasts from Acetabularia showed an oxygen sensitive CO_2 release in the dark. Unfortunately,

their chloroplast preparations contained some mitochondrial contamination so it is impossible to say with certainty whether this CO_2 release was from the chloroplasts themselves. It would be valuable to continue this work, and to use preparations free of mitochondria to test glycine-1-^{14}C or glycollate-1-^{14}C as a source for this CO_2.

From the above it would seem likely that two possibilities exist for the low rates of photorespiration in algae. Either the glycine to serine conversion does not exist, or that if it does, it does not release CO_2. In an attempt to distinguish between these possibilities it was decided to see whether the remainder of the pathway leading from serine to carbohydrate was present. To do this certain selected enzymes from this pathway were assayed.

Apart from the glycollate oxidising enzyme, a number of other enzymes of the glycollate pathway were detected in Phaeodactylum (table 15). An NADH-glyoxylate reductase was detected (Fig. 21a) whose activity was far in excess of that of glycollate dehydrogenase. Glyooxylate reductase could convert glyoxylate to glycollate, but it has been found to function more efficiently when hydroxypyruvate is the substrate. The Km for hydroxypyruvate is 0.5 or 1.2 x 10^{-4}M(Kohn & Warren, 1970; Tolbert et al, 1970) and 4-5 times faster reduction was observed with hydroxypyruvate as the substrate than glyoxylate. In Phaeodactylum, the activity of hydroxypyruvate reductase was more than twice the glyoxylate reductase (Figs. 21a, b). Since

probably, glyoxylate never reaches the saturating substrate concentration in vivo this enzyme may be preferentially called a hydroxypyruvate reductase (Tolbert, 1971).

The linked reaction of the conversion of serine into glycerate had an initial rate equal to that of hydroxypyruvate reductase alone (table 15) which was indicative that the reaction was not limited by the aminotransferase.

Hydroxypyruvate was found to be a better substrate than either glyoxylate or glycerate for the enzyme hydroxypyruvate reductase. This would tend to favour the glycollate metabolism towards glycerate formation. In leaves also, the pH optimum and kinetics of hydroxypyruvate reductase are unfavourable for glycerate oxidation (Tolbert, Yamazaki & Oeser, 1970). This shows that in both higher plants and algae, the flow of carbon in glycollate pathway should be towards carbohydrate formation.

In Phaeodactylum cell extracts, the activity of NAD^+-Malate dehydrogenase was found to be the highest of all the enzymes assayed (Fig. 22b). In higher plants also, after catalase, this enzyme has the greatest activity (Tolbert, 1971) and is probably involved for the shuttling in of the reducing power from the malate pools to hydroxypyruvate (Yamazaki & Tolbert, 1969). However, much of this enzyme could also be involved in other functions e.g. the citric acid cycle. Isoenzymes of NAD^+-malate dehydrogenase are present in the microbody, mitochondria and cytosol (Yamazaki & Tolbert, 1969, Rocha & Ting, 1970 a, b), and differences in their kinetic properties may relate to the

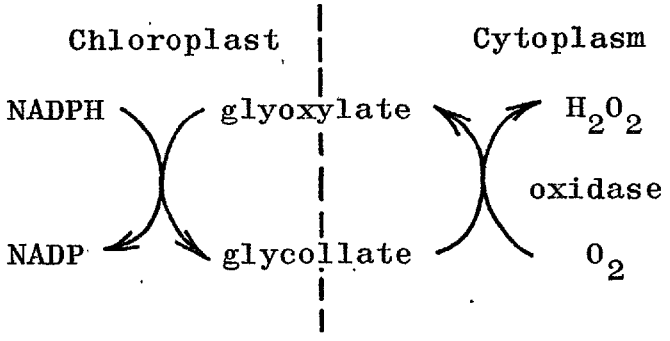
direction of the reaction at a given site and its regulation (Tolbert, 1971).

Another enzyme assayed was catalase, which is a major protein in leaf peroxisomes where its specific activity is 100-1000 fold greater than that of other enzymes of the glycollate pathway (Tolbert, 1971). In Phaeodactylum its activity was lower than many of the other enzymes assayed (Fig. 22C). In higher plants, a higher enzyme activity is associated with the presence of glycollic oxidase which produces H_2O_2 during glycollate oxidation. Catalase would be needed to remove this H_2O_2. Lower activity in algae is considered to be due to the algal enzyme being a dehydrogenase, which does not link to O_2, so no H_2O_2 is produced and no catalase is required for its destruction. From these findings it seems possible that the full photorespiratory pathway is present in Phaeodactylum and that glycine to serine conversion occurs. This could be confirmed by the extraction and assay of the necessary enzymes in Phaeodactylum, although there may be technical difficulties if their activity depends on the production of intact mitochondria.

Another enzyme worth examining in more detail is the RuDP carboxylase from Phaeodactylum, in particular to supply it with RuDP-^{14}C at various O_2 and CO_2 levels to test for the formation of radio-active glycollate since it has not yet been demonstrated that this alga synthesises large amounts of glycollate by the mechanism proposed by Lorimer et al (1973). Other mechanisms of glycollate synthesis should also be investigated, e.g. its possible production from intermediates of glyoxylate cycle as

proposed by Harrop & Kornberg (1966). Perhaps at this point it is worth discussing the possible significance of photorespiration in the context of this and other work and in particular what could be the possible functions of glycollate metabolism:-

1. To dispose of surplus chloroplast reducing power using glyoxylate shuttle:



This does not require a continuing synthesis of glycollate since it performs only a 'catalytic function' as an electron carrier, nor does it give rise to CO_2.

It may cause an increase in ATP synthesis in the chloroplast due to a stimulation of non-cyclic electron flow.

2. ATP synthesis outside the chloroplast:

This is more speculative but if e.g. as in algae the glycollate oxidising enzyme is a dehydrogenase, and if it is coupled to the mitochondrial electron transport chain, then the glyoxylate shuttle could generate ATP in the mitochondria at the expense of NADPH in the chloroplast. Again, only catalytic amounts of glycollate are needed and no CO_2 is generated. This might also occur in higher plants if the 'glycollic oxidase' is also a facultative dehydrogenase.

3. Translocation of carbon from the chloroplast:

The transfer of glycollate from the chloroplasts to the cytoplasm and its subsequent metabolism to carbohydrate outside may be a major pathway for the transfer of photosynthate to the cytoplasm. However, translocation from the chloroplast can also occur without glycollate synthesis since leaves can be destarched by placing the plant in the dark. Since glycollate synthesis does not occur in the dark then the loss of the starch from the plastids cannot be by this means. Also chloroplasts are known to be readily permeable to other materials e.g. triose phosphates.

4. Provision of Carbon skeletons:

Glycine and serine are precursors of many substances including protein. This could provide a use for some of the carbon going through the glycollate pathway but all the intermediates of the pathway can be synthesized by other means too; so the glycollate pathway is not essential. The fact that non-green tissues (which do not make glycollate) survive at all is evidence for this.

Whether or not the use of C skeletons from the glycollate pathway results in CO_2 production will depend upon which stage in the pathway they come from (i.e. before or after the CO_2 producing stage).

5. Accident theory:

This suggests that glycollate production had no initial function but arose during evolution as a consequence of the

reduction in the CO_2 concentration of the Earth's atmosphere and the effect of this on RuDP carboxylase. If this is so, we would expect evolution to occur so as to minimize the effects. This may occur in many ways.

1) Excretion mechanisms develop to remove glycollate and prevent it reaching toxic levels.

2) Remetabolism of the glycollate to yield useful products. This must inevitably use energy but would be less wasteful than excretion. It is possible that more than one mechanism exists for this. Systems involving glycollic oxidase would be very wasteful of energy but those involving a dehydrogenase coupled to ATP synthesis would be less wasteful. There is now a suggestion that the higher plant enzyme may be able to function in both ways (a) as an oxidase with a big free energy loss to get rid of large (toxic) quantities of glycollate rapidly, and (b) as a dehydrogenase in which some of the energy is recovered as ATP from the oxidation of lower concentrations of glycollate. Evidence for this is that low concentrations of glycollate stimulate ATP synthesis (Omata & Tsukamoto, 1970). If so we may speculate that glycollic oxidase in higher plants is an allosteric enzyme which functions as a dehydrogenase at low glycollate concentration and as an oxidase at high glycollate. This needs further investigation.

3. <u>The prevention of glycollate synthesis</u>:

(a) If glycollate synthesis is wasteful, we would expect RuDP carboxylase to have evolved in structure so as to minimize

this. If so, we might expect different evolutionary groups to have different capacities for glycollate synthesis by this enzyme. Some in vitro enzyme assays throughout the plant kingdom might be worthwhile. Perhaps the apparent low rates of glycollate synthesis by some algae (perhaps Phaeodactylum) may be due to this cause.

(b) An alternative approach might be for the plant to adapt the environment of its RuDP carboxylase to minimize glycollate synthesis. This appears to be the case in C_4 plants where glycollate synthesis is inhibited by a high CO_2 concentration. However, it involves the operation of a CO_2 pump and this requires energy in the form of ATP. It is therefore a 'second best' solution i.e. wasteful but less wastefull than photorespiration. There may be other CO_2 pump mechanisms perhaps using enzymes other than PEP carboxylase. This should also be investigated in different groups. There may therefore be many ways in which organisms can suppress photorespiration or minimize its effects, the C_4 mechanism being perhaps the best known. However, present work suggests that the algae may possess similar mechanisms. The fact thay they are primitive organisms taxonomically does not prove that they are primitive at the biochemical level. In fact, the reverse could be true, if they are to survive in competition with the 'so-called' higher plants they must have some advantage which makes up for their deficiencies elsewhere. Such an advantage could be more efficient photosynthesis, or a comparative

lack of photorespiration. Perhaps it is not an accident that the most primitive of the algae, the blue-green algae, have been reported to have the lowest photorespiration rates (Cheng & Colman, 1974) and that these algae become dominant in natural populations under conditions of restricted CO_2 supply (King, 1970 & Shapiro, 1973)

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