CARBON METABOLISM IN CHLORELLA IN LOW CONCENTRATIONS OF CARBON DIOXIDE

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ABSTRACT

The photometabolism of carbon by Chlorella was investigated using radioactive carbon compounds and paper chromatography.

During periods of photofixation of carbon-14-dioxide, up to one hour at varying concentrations of carbon dioxide, the major end products were sucrose, poly-glucan and lipid. At low concentrations of carbon dioxide the addition of isoniazide stimulated the formation of glycollate and glycine but did not affect the total fixation. The addition of iodoacetamide or high oxygen p.p. increased the production of glycollate but decreased the total fixation. As the carbon dioxide concentration was raised the inhibitory effect of oxygen and the stimulation of glycollate decreased but the inhibition of fixation by iodoacetamide increased.

Short term experiments showed that if carbon-14 was incorporated into the sugar phosphates and the further metabolism of this carbon studied, in the absence of carbon-14, the main products in air were sucrose and poly-glucan, and in the presence of isoniazide or high oxygen p.p. were glycollate and glycine.

Following the transition from air to oxygen the drop in rate of photofixation of carbon-14-dioxide was associated with a decrease in the size of the sugar phosphate pools, and an increase in the production of glycollate. The inhibition of photosynthesis by high oxygen p.p. increased as the light intensity was raised.

Various intermediates were fed to intact cells. Both glucose and serine were metabolised in carbon dioxide free conditions. In low light glucose was converted directly to poly-glucan. However as the light intensity or the oxygen p.p. was raised sucrose, glycollate and carbon dioxide became the main products. The main products of the photometabolism of serine were sugar phosphates and glycollate.

When glycollate was degraded the labelling was found to be consistent with the direct formation from the compound fed.

The importance of glycollate production and metabolism in the photometabolism of carbon at physiological concentrations of carbon dioxide is discussed.

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Abbreviations

Adenosine diphosphate ADP

Adenosine triphosphate ATP

Diphosphopyridine nucleotide, oxidised DPN, reduced DPN.H

Triphosphopyridine nucleotide, oxidised TPN, reduced TPN.H

P.G.A., phosphoglyceric acid. D.H.A.P.,dihydroxyacetone-phosphate.,U.D.P.G.,uridine diphosphate glucose., P.E.P., phospho enol pyruvate.

I.N.H., isoniazide.,I.O.D., iodoacetamide.

Other abbreviations used in the tables and figures are given on pages 39 and 48.

INTRODUCTION

1) The Pathway of Carbon in Photosynthesis

In 1954 Calvin et al proposed a mechanism for the uptake and further metabolism of carbon dioxide, in which carbon dioxide reacts with a molecule of ribulose-1-5-di-phosphate to produce two molecules of phosphoglyceric acid. The phosphoglyceric acid is then reduced to triose phosphate by reduced pyridine nucleotide in reactions which are basically the reverse of those found in the glycolytic breakdown of hexose. Part of the triose then undergoes aldolase condensation forming fructose-1-6-di-phosphate, which may be further metabolised to end products. The rest of the triose is used in the autocatalytic regeneration of the carbon dioxide acceptor, through a series of reactions involving the metabolism of C-7 C-5 and C-4 sugar phosphates. From these reactions ribulose-5-phosphate is produced, which after phosphorylation by ATP is available once more as the carbon dioxide acceptor. The overall mechanism for the cyclic regeneration of ribulose-di-phosphate is shown in Fig.I.1.

The evidence for this cyclic mechanism and the relevant literature are detailed in Calvin and Bassham 1957.

The carboxylating enzyme, carboxydismutase, has been demonstrated in cell free systems from spinach and other higher plants. (Quayle 1954. Weissbach 1954, 1955b, 1956. Jakoby 1956. Mayaudon 1957. Pon 1963. Rabin 1964.)

The work of Calvin et al. was almost entirely confined to experiments using Chlorella and other algae grown in conditions

FIG 1.

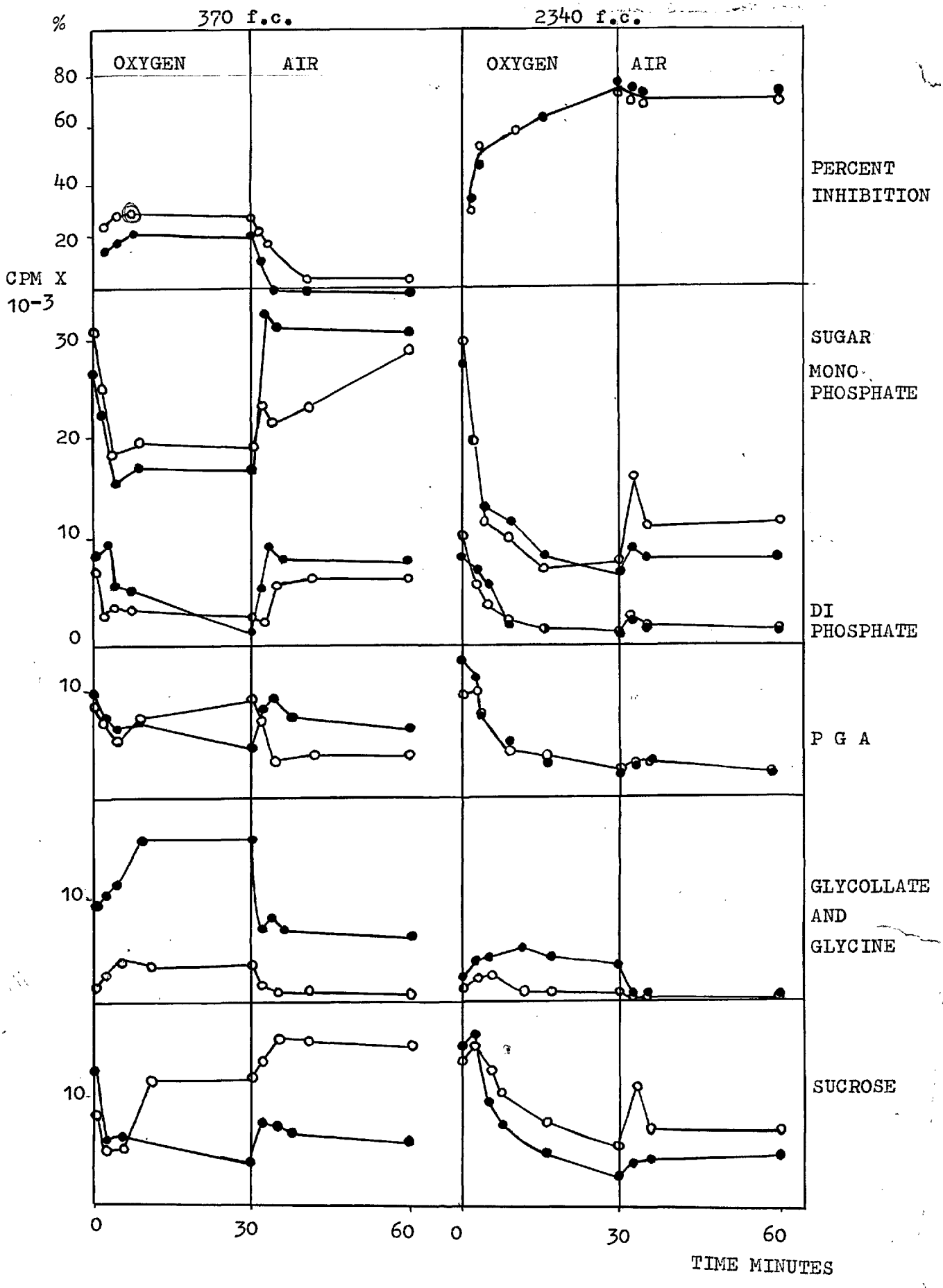
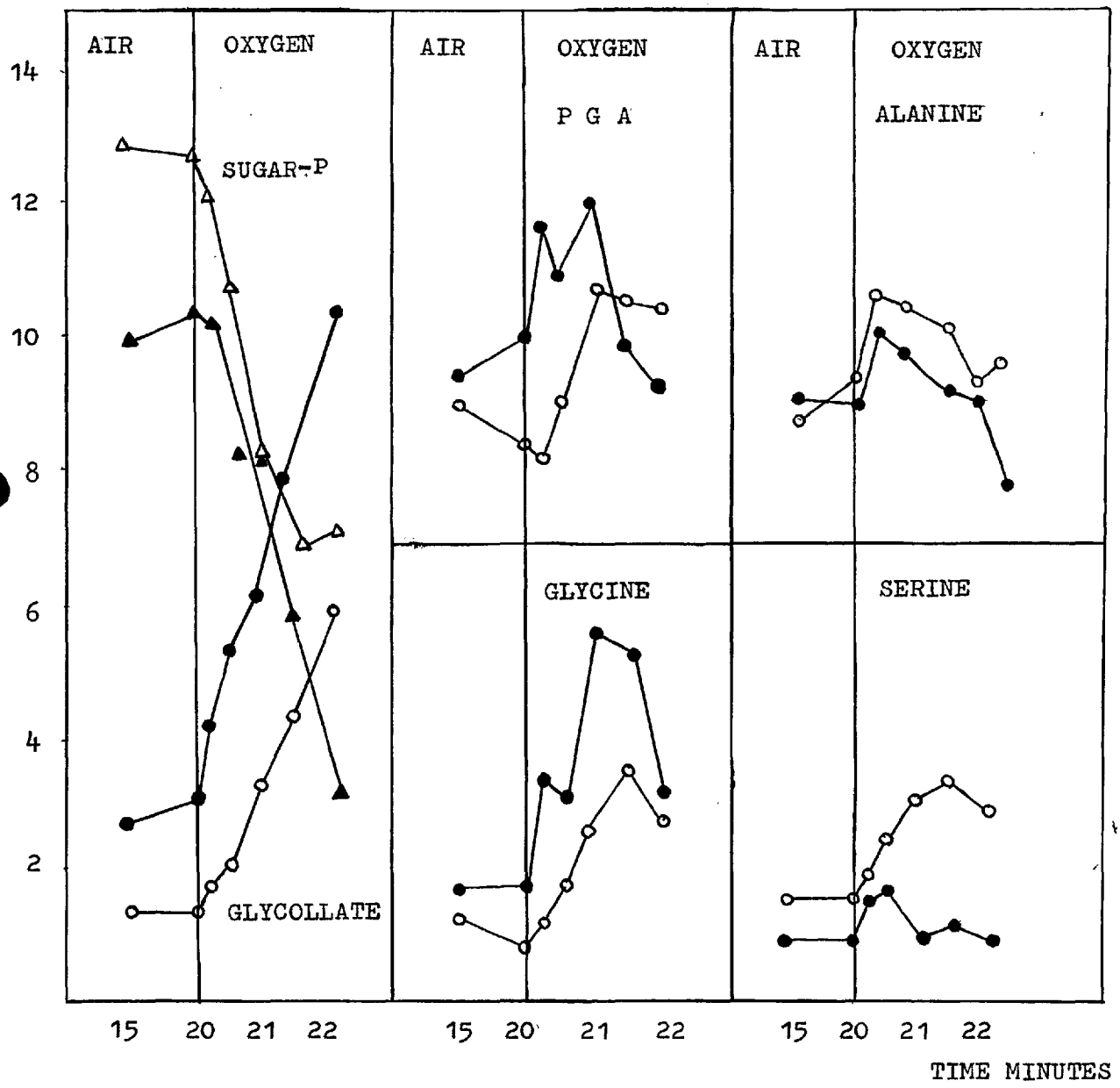

%
370 f.c.
2340 f.c.
OXYGEN
AIR
OXYGEN
AIR
80
60
40
20
PERCENT INHIBITION
CPM X 10-3
30
20
10
0
SUGAR MONO-PHOSPHATE
DI PHOSPHATE
10
P G A
10
GLYCOLLATE AND GLYCINE
10
SUCROSE
0
30
60
0
30
60
TIME MINUTES

FIG 2

POOL SIZE RELATIVE UNITS (Based on c.p.m. recovered on paper chromatograms)



OPEN SYMBOLS = CONTROL

CLOSED SYMBOLS= + I.N.H.

FIG 3

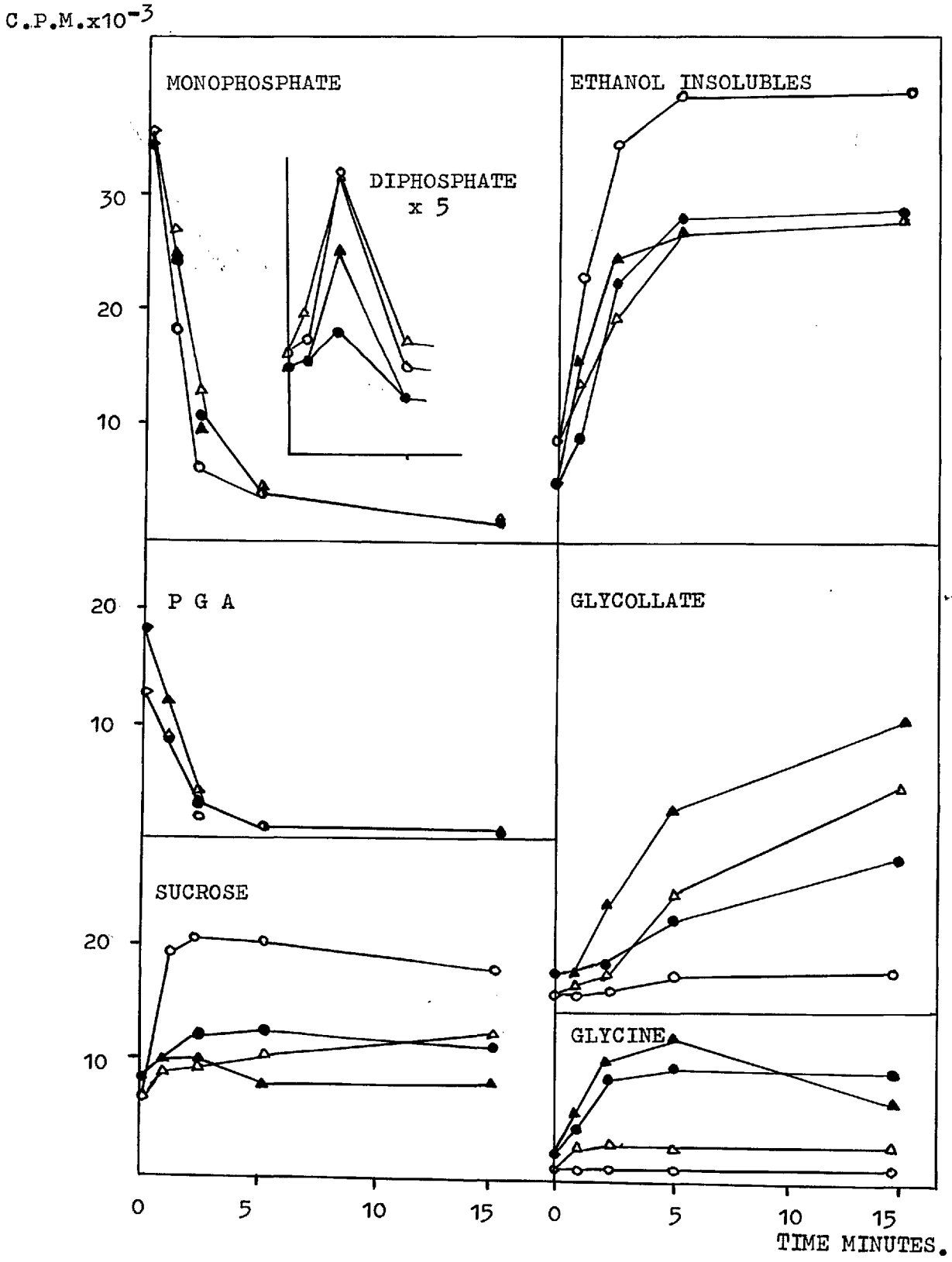
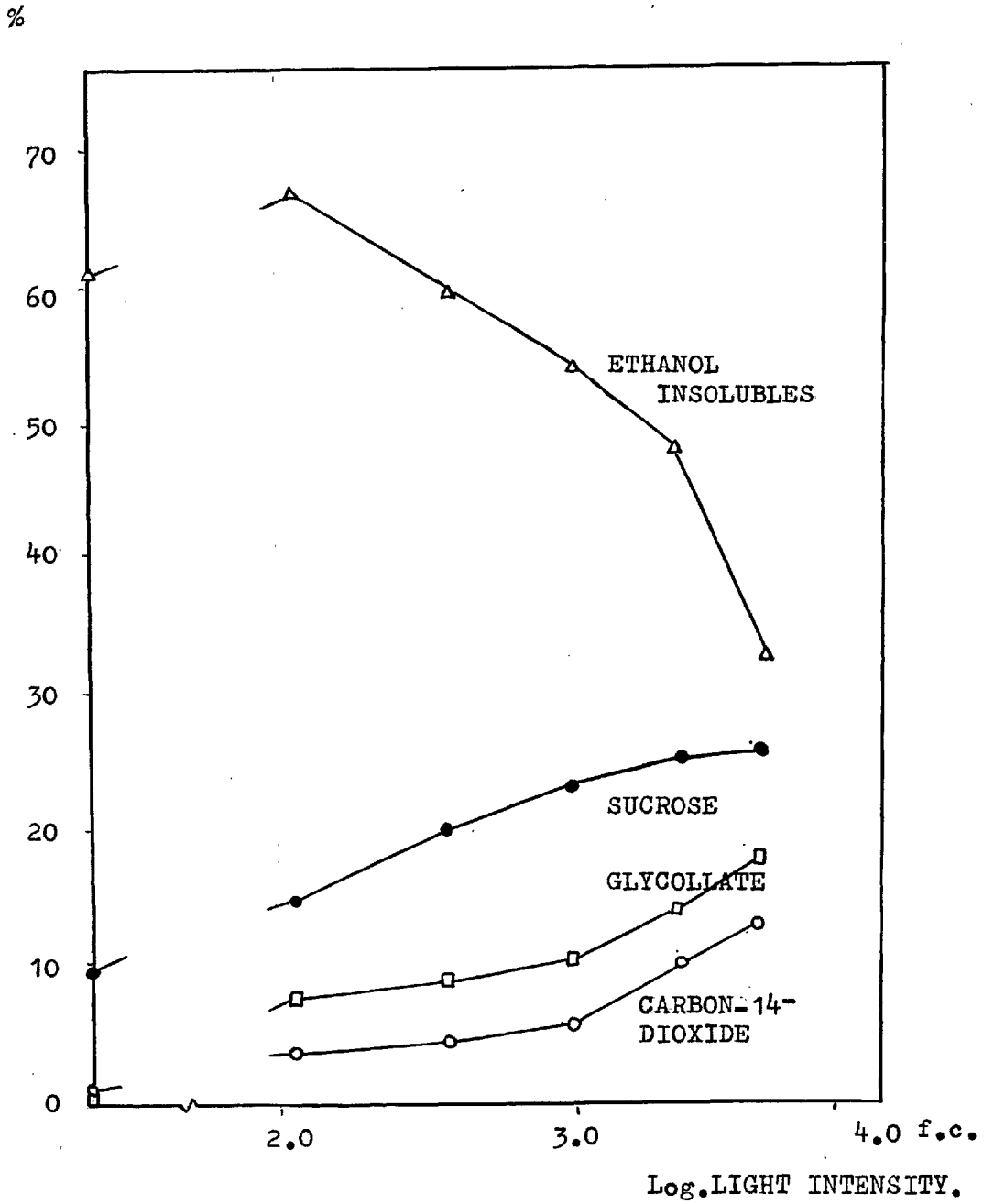

C.P.M.x10⁻³
MONOPHOSPHATE
DIPHOSPHATE
x 5
ETHANOL INSOLUBLES
P G A
GLYCOLLATE
SUCROSE
GLYCINE
30
20
10
0
5
10
15
TIME MINUTES.
AIR
OXYGEN
AIR/I.N.H.
OXYGEN/I.N.H.

FIG 4


%
70
60
50
40
30
20
10
0
ETHANOL
INSOLUBLES
SUCROSE
GLYCOLLATE
CARBON-14-
DIOXIDE
2.0
3.0
4.0 f.c.
Log.LIGHT INTENSITY.

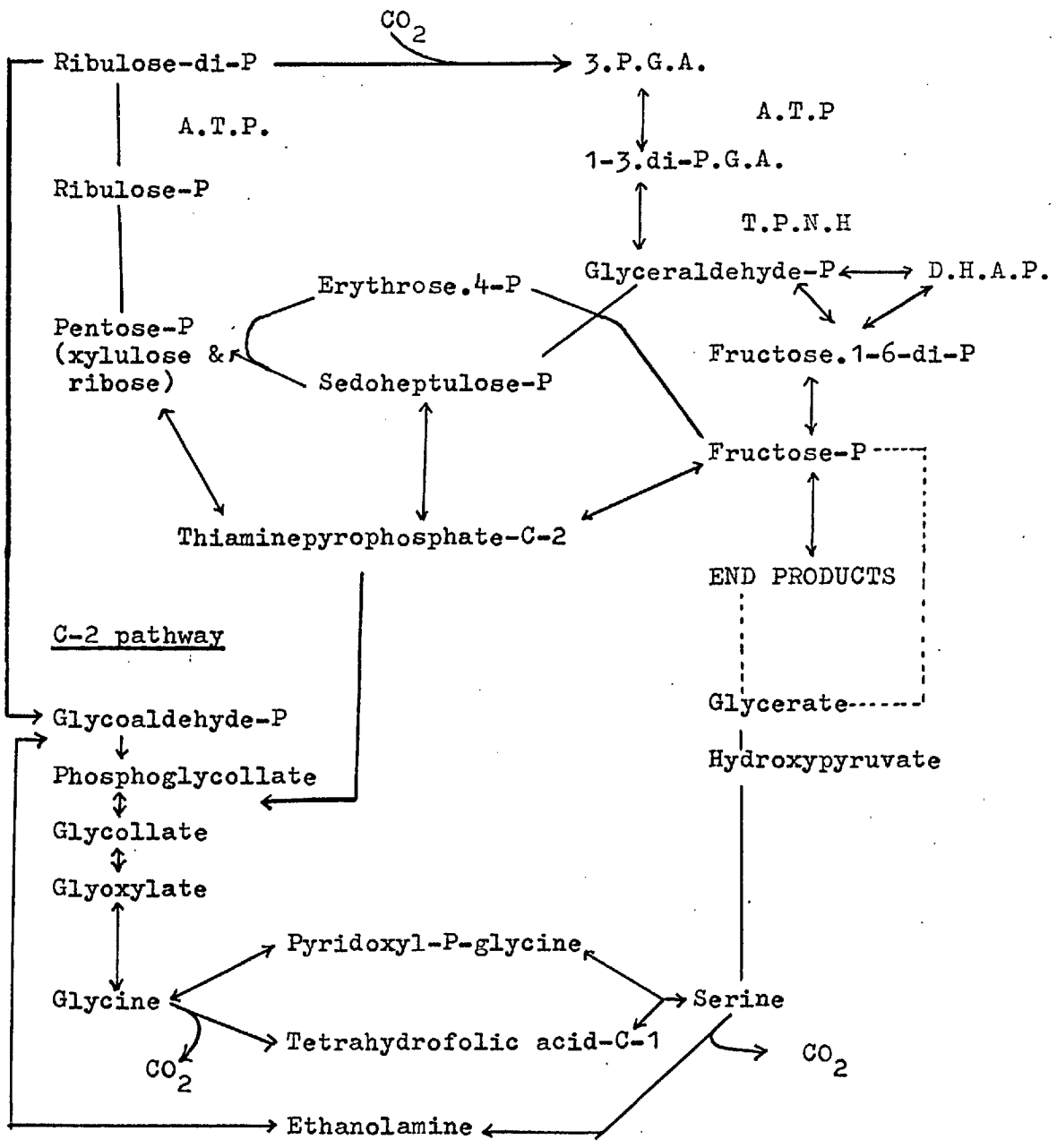


Fig I 1. The photosynthetic carbon reduction cycle, and the C-2 pathway.

of high carbon dioxide concentration, and then supplied with a high concentration of carbon dioxide during the experimental period. Wilson and Calvin (1955) investigated the transient changes in carbon metabolism when Chlorella photosynthesising in 1% carbon dioxide containing C^{14} was transferred to 0.003% carbon dioxide containing the same level of radio activity. It was found that an accumulation of glycollic acid occurred under these conditions. Earlier work by Calvin (1951 1952) and Benson (1950) showed that C-2 compounds were produced as early products of photosynthesis.

It has been suggested by several authors that other photochemical mechanisms for the uptake of carbon dioxide exist (Newburg 1954. Zeehuisen 1957. Warburg 1958. Tanner 1960. Stiller 1962) However, little experimental or enzymic evidence is available to support these suggestions.

Dark carboxylation mechanisms in green plants have been reviewed by Walker (1962). Probably the only reaction which is of any significance in the dark fixation of carbon-14-dioxide by Chlorella is the phospho enol pyruvate carboxylase reaction (Bandurski 1953)

PEP + CO_2 + H_2O → Oxaloacetic acid + Inorganic phosphate.

2) <u>Light Stimulated Dark Fixation</u>

The early work of Calvin et al and of Gaffron et al (see Photosynthesis Vol II.2-Rabinowitch) showed that dark fixation of carbon-14-dioxide by various plants could be greatly stimulated by a preceding period of illumination. The main products of this

fixation were PGA, PEP, malic acid and alanine.

The Japanese group, under Tamiya, have extensively investigated this phenomenon, which they have termed the 'R' effect (Miyachi 1955. 1959. 1960. Oh-Hama 1959. 1960). From studies of the effects of oxidants, sulfhydral reagents and light/dark transitions on the level of 'R' and of pyridine nucleotides, they have concluded that in part 'R' consists of a reduced (-SH?) grouping which is not reduced pyridine nucleotide. Bassham 1963 b and c has suggested that an enzyme disulphide is reduced directly to the disulfhydral by ferredoxin and that the sulfhydral groups function directly in the reductive carboxylation mechanism. Such a mechanism involving reduced -SH groups has been suggested (Rabin 1964).

Bassham (1963 d) found that if the light was turned off after Chlorella cells had reached a steady state of photosynthesis, the subsequent increase in PGA was too great to be accounted for by the carboxylation of the ribulose diphosphate present. However, it was found that the total drop in all diphosphates (ribulose, fructose and sedoheptulose) was sufficient to account for the rise in PGA.

There is evidence that the substrate for the light stimulated fixation consists of 1. Ribulose di-P. plus ATP, plus sugar phosphate precursors of ribulose di-P.

2. A reduced enzyme group.

3) Glycollate Production

The enhanced production of glycollic acid by Chlorella and other green plants has been investigated by a number of workers as summarised in the following table.

Condition	Authors
High Light	1, 4, 5.
High oxygen p.p.	1, 4, 6, 7, 8.
Low Carbon dioxide	1, 4, 5, 6, 7, 8.
High Bicarbonate	1
Low Phosphate	1, 10.
Blue Light	2, 10.
Isoniazide	5, 6.
α-hydroxysulphonates	3.
8-Methyl Lipoic acid	9.
Glucose feeding	6.

1. Tolbert 1956. 2. Emerson 1957. 3. Zelitch 1958. 4. Warburg 1960
5. Pritchard 1962. 6. Whittingham 1963. 7. Miller 1963.
8. Bassham 1962. 9. Bassham 1963a. 10 Tolbert 1963.

4) The Metabolism of C-2 Compounds. (See Tolbert 1963. Zelitch 1964)

Considerable evidence has now accumulated that the metabolism of glycollate, glyoxylate, glycine and serine, in both animal and plant tissues, are closely interrelated. The metabolism of a C-2 unit via these compounds to a C-3 sugar which may then be utilised to form sucrose, starch and other end products, has been termed the C-2 pathway.

Although it has been suggested (Tanner 1960. Stiller 1962) that the initial C-2 compound arises from a carboxylation other than that catalysed by carboxdismutase, it seems more probable that it arises from the sugar phosphates of the photosynthetic carbon reduction cycle. (Bassham 1962 1963 1964. Delavan 1957. Wilson 1955).

The glycollic acid formed during photosynthesis in Chlorella is uniformly labelled (Bassham 1954) as would be expected if it was derived from the carbon atoms 1 and 2 of a pentose sugar phospate of the P.C.R. cycle. However, Schou 1950, has shown that if specifically labelled glycollate is fed to Chlorella in the light it becomes randomised. Ullrich 1958 and Kearney 1962 have shown that phosphoglycollate is produced in large amounts, from sugar phosphates, in chloroplast preparations. Weisbach 1955a, Griffith 1959 and Zelitch 1960 have studied the formation of C-2 compounds from labelled pentose sugars. Similar results have been obtained by Goldberg 1962 when labelled glucose was fed to Lactobacillus.

Horecker 1953 showed that the active intermediate of the

transketolase reaction is a thiamine pyrophosphate glycoaldehyde complex. Bradbeer 1961 found that crystalline transketolase, in the presence of ferricyanide, would catalyse the formation of glycollate from fructose-6-phosphate, a similar oxidation of the C-2 fragment from the carrier using 2.6.dichlorphenolindophenol was shown by da Fonseca-Wollheim 1962. Goldberg 1962 proposed a scheme whereby glycoaldehyde, acetate, acetyl phosphate and glycollate could be formed from the transketolase intermediate.

Orth 1962 has reported a glycoaldehyde phosphate dehydrogenase.

An aldolase which catalyses the following reaction has been found by Byrne 1954:- Glycoaldehyde-P + DHAP Xylulose-di-P. The reverse of this reaction would produce glycoaldehyde from the C-1 and C-2 of pentose. The two carbons would initially be uniformly labelled if the source of the C-2 fragment was a pentose phosphate labelled with carbon-14 fixed by the P.C.R. cycle.

Ribulose-di-phosphate is very slowly cleaved by crystalline aldolase (Hurwitz 1956). It will also decompose nonenzymatically.

If the initial C-2 fragment was derived from a diphosphate it would, on oxidation, give phosphoglycollate. However very active phosphatases are present in plant cells which will dephosphorylate this compound to free glycollate. These phosphatases have been studied by Richardson 1961, Ulrich 1963 and Yu 1964, and shown to be stable in various organic solvents used for extraction.

Glycollic oxidases and glyoxalate reductases have been studied by Zelitch (1953. 1960b. 1962) and Tolbert (1953. 1958) Tolbert 1953 and Kuczmak 1962 have studied the formation of glycollic oxidase during greening of etiolated material (Monocotyledons).

Both a DPN linked glyoxylate reductase and a TPN linked glyoxylate reductase have been shown by Zelitch 1953 and 1962.

Tolbert 1962 has suggested that the glycollic(C-2) pathway could constitute a link between the chloroplasts and the cytoplasm. Phosphoglycollate can cross the organelle membranes but not glycollate. By contrast, glyoxylate can enter the chloroplast. Hence the scheme depicted in fig. I.2 could operate, in which the C-2 interconversions also serve as a hydrogen carrier and an ATP sink.

Zelitch 1958, 1959, 1964, has shown that the interconversion of glycollate and glyoxylate is inhibited by α-hydroxsulphonates. The addition of this inhibitor to Tobacco Leaves causes a marked increase in the formation of glycollic acid. As a result of these observations, he suggests that oxygen consumption in the glycollic oxidase reaction may account for a large part of the oxygen consumed in the light.

A large part of the detailed work on the further metabolism of glyoxylate via glycine to serine has been performed with animal tissues. However, it seems probable that similar mechanisms exist in plant cells.

The synthesis of glycine by the amination of glyoxylate has been shown in the rat by Weinhouse 1951, 1956, and Weissbach 1953. Tolbert 1953, has shown the formation of glycine from glyoxylate in

higher plants, and Campbell 1956, a similar reaction in Pseudomonas.

In 1946 Shemin showed that the principal source of glycine in animal tissues was serine:-

Serine $\longrightarrow$ Glycine + C-1

Sakami 1948 demonstrated the incorporation of formate into the β carbon of serine, and in 1955 Kisliuk showed that this reaction was, in fact, reversible. The incorporation of formate into serine in plant tissues was demonstrated by Tolbert 1955b.

The work of Lascelles 1954 showed that the vitamin Pteroyl-L-glutamic acid (Folic acid) is involved in the serine/glycine interconversions. The exact functional form of the vitamin is uncertain; however, the combination of formate with folic acid or tetrahydrofolic acid has been shown. (Zakrzewski 1955. Jaenicke 1955. Doctor 1956)

Kisliuk 1955 has shown that in the biosynthesis of serine the folic acid co-factor transfers a C-1 unit at the oxidation level of a Hydroxymethyl group ($-CH_2OH$). Blakely 1955 has shown that pyridoxal phosphate is also required as a co-factor in this conversion.

A series of papers by Whittingham and co-workers (Pritchard 1961, 1962, 1963, Whittingham 1963) report the effect of isoniazide (I.N.H.) on carbon metabolism in Chlorella, when fed both with carbon-14-dioxide and radioactive glucose-C-14. The main effects reported are an increase in the formation of glycollic acid and glycine, with a decrease in serine formation. In short term experiments no inhibition

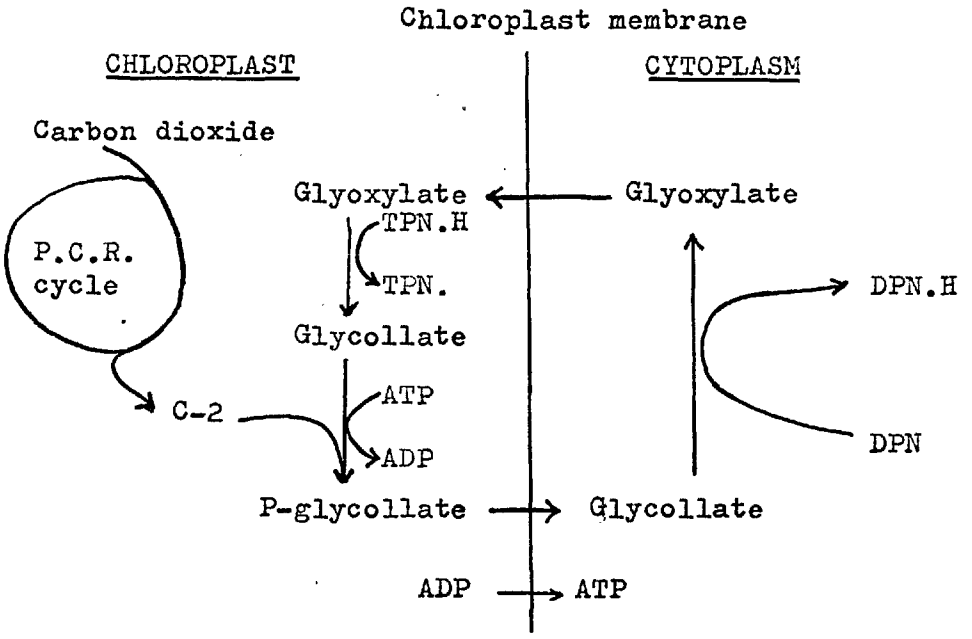


Fig I 2. Interconversion of DPN.H and TPN.H by a glycollate glyoxylate cycle working between the chloroplast and the cytoplasm. Based on Tolbert et al 1962.1963 and Butt 1963.

of incorporation of activity into the ethanol insoluble fraction was found, but the incorporation of activity into sucrose showed an initial stimulation. No change in (or a slight stimulation of) photosynthetic incorporation of carbon-14-dioxide was found.

In longer term experiments Khurdak 1962 found that I.N.H. had an inhibiting effect on photosynthesis in Chlorella, after periods of about one hour incubation.

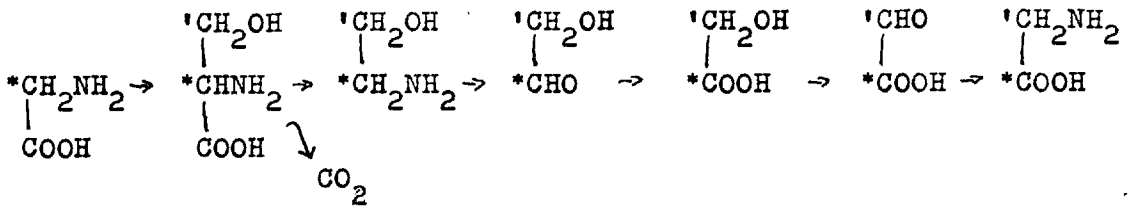
Whittingham has suggested that the effects of I.N.H. on the carbon metabolism of Chlorella are consistent with the inhibition of the conversion of glycine to serine. Such a view is supported by experimental evidence from studies of the effects of I.N.H. on enzyme systems from animals and bacteria. Dac et al (1958) have shown that in animal tissues I.N.H. inhibits the formation of serine by the inactivation of pyridoxal phosphate required for the activation of glycine in this reaction.

Zatman 1953, 1954 have shown that enzymes from some animal tissues catalyse the replacement of the nicotinamide group of D.P.N. by structurally related pyridine derivatives such as 3-acetylpyridine and I.N.H.

A reduction in transaminase activity (Youatt 1955) and of amino acid decarboxylase activity (Davidson 1956) has been attributed to an anti-vitamin B_6 (pyridoxal-phosphate) activity of I.N.H.

The formation of ethanolamine from glycine involves the intermediate formation of serine, which is then decarboxylated. The ethanolamine can then be reconverted to glycine with a reversed

labelling pattern (Elwyn 1955. Weisbach 1953)



Serine can be converted to pyruvic acid by the enzyme serine dehydrase (Chargaff 1943. Sayre 1956). The deamination of serine will give hydroxypyruvate. Stafford 1954 has reported the formation of glyceric acid from hydroxypyruvate, and Zelitch 1955 has shown that this reaction is catalysed slowly by glycollic oxidase. Dickens 1956 found that transketolase would also catalyse the decarboxylation of hydroxypyruvate to erythrulose and carbon dioxide.

Tolbert 1962 found that after four seconds uniformly labelled serine could be obtained from carbon-14-dioxide feeding experiments even though the P.G.A. formed in the same time had considerably more activity in the carboxyl carbon than in the α and β carbons.

Most investigations have shown that free glyceric acid formed in plant tissues is usually predominantly labelled in the carboxyl group (e.g., Aronoff 1951). However, if the actively metabolised form of serine was phosphoserine, the product would be P.G.A. and not free glycerate.

In 1948 Sakami showed that in animal tissues glycine could be converted to glycogen via serine, more recently Weinhouse (1951, 1956) obtained a similar production of glycogen in rats fed glycoaldehyde-2-C^{14} Similarly, glycollic acid, glyoxylic acid or glycine have been fed to

algae or higher plants and the products determined and in some cases degraded. (Schou 1950. Jiminez 1962. Rabson 1962. Wang 1962, 1963)

A series of papers (Tolbert 1953. 1955., Kuczmak 1962) indicate that in monocotyledons glycollic oxidase and a glyoxylate-serine transaminase system are absent in etiolated leaves, but form during greening. In wheat allowed to fix carbon-14 dioxide during greening activity did not appear in serine until after the period for activation of glycollic oxidase.

5) The effect of high partial pressures of oxygen on photosynthesis.

The literature on the effect of high partial pressures of oxygen on photosynthesis has been reviewed by Brittain (1962).

The effect of oxygen on photosynthesis was discovered by Warburg in the unicellular algae Chlorella. Confirmatory data came from various authors between 1925 and 1949 when Tamiya produced the first thorough study of the problem. Tamiya (1949) showed that the Warburg effect was reversible. This has been confirmed by Turner 1956, who has also shown it to be widespread throughout the plant kingdom.

Although Gaffron (1940) found that the assimilatory quotient in Scenedesmus was unchanged upon the transfer from air to oxygen, Brittain (1957) found that in Chlorella this quotient dropped from 0.93 in air to 0.63 in oxygen, i.e., carbon dioxide fixation was inhibited to a greater extent than oxygen evolution.

Tamiya (1949) and Miyachi (1955) found that their data for the inhibition of photosynthesis by high p.p. of oxygen fitted the

following equation of the second order:-

$$\text{Degree of inhibition} = \frac{(O_2)^2}{K^2 + (O_2)^2}$$

where K is a constant representing the p.p. of oxygen causing 50% inhibition of photosynthesis.

The work of Tamiya (1949), Briggs (1952) and Turner (1956) indicates that the inhibition of photosynthesis by high p.p. of oxygen increases as the carbon dioxide concentration is decreased.

The evidence for an influence of light intensity on the Warburg effect is conflicting, and has been summarised by Brittain 1962:-

> "All that one can say at present is that the effects suggest that inhibition by oxygen is likely to be highest in saturating light, and especially at low carbon dioxide concentrations."

A light catalysed back reaction between molecular oxygen and the primary photochemical product has been suggested by Mehler 1951 i.e.,

$$\left.\begin{array}{l} 4\ H_2O \rightarrow 4\ H + 4\ OH \\ 4\ OH \rightarrow 2\ H_2O + O_2 \end{array}\right\} \text{Photosynthesis}$$

$$4\ H + 2\ O_2 \rightarrow 2H_2O_2 \qquad \text{Mehler Reaction}$$

The Warburg effect could be explained in terms of such a reaction lowering the apparent rate of photosynthesis.

However, Good 1955 suggests that oxygen is not normally reduced in this way. Brittain 1962 was unable to detect any oxygen effect on the rate of the hill reaction with ferricyanide as the hydrogen acceptor.

Under certain conditions (High light, high oxygen p.p., low carbon dioxide concentration, and in the presence of various narcotics (Rabinowitch 1945. 1956) carbon which has already been fixed by normal photosynthesis may be oxidised to carbon dioxide with the consumption of oxygen in a light/chlorophyll dependent reaction. Alternatively, oxygen may be consumed without the production of carbon dioxide in reactions such as that catalysed by glycollic oxidase.

These processes have been variously termed photautoxidation, photorespiration, photo-oxidation or photoxidation. The last term will be used here to describe all light dependent oxidation reactions.

Frank and French 1941 have shown that photoxidation shows the same qualitative dependence on oxygen p.p. as the inhibition of oxygen production, but the latter phenomenon is ten or more times larger. The Warburg effect cannot, therefore, be attributed solely to the observed rate of photoxidation. These authors have shown further that the damage which results to the photosynthetic apparatus from prolonged photoxidation can be postponed if the plant is allowed to take up a sugar solution through the cut stem.

A light stimulated production of carbon dioxide and/or uptake of oxygen has been reported by numerous authors, e.g.,

Fockler 1938. Myers 1940. Frank 1941. Weis 1956. Krotkov 1958 1963. Decker 1959. Brown 1959. Forrester 1963. Hoch 1963.

The main effect of the conditions specified on carbon metabolism in most plants is an increased production of C-2 compounds. Whittingham 1963 has shown that in carbon dioxide free conditions Chlorella can metabolise glucose to glycollate. Delavan 1957, 1958 has demonstrated a slow light stimulated oxidation of glycollate to carbon dioxide by chloroplasts, and Zelitch (see previously) has suggested that the uptake of oxygen by glycollic oxidase may account for a large part of the oxygen consumed in the light.

Franck (1941b 1951 1960) favours the hypothesis that a narcotic substance is produced under conditions of high oxygen p.p., capable of inhibiting photosynthesis. Good 1963 has shown that the reduction of dichlorphenolindophenol in vitro is inhibited when glycollic acid is added to the reaction mixture.

Turner 1958 has considered the possibilities that high p.p. of oxygen inhibit photosynthesis by inhibiting the enzyme glyceraldehyde phosphate dehydrogenase. It is possible that high p.p. of oxygen would inhibit all reactions involving -SH groups or -SH dependent enzymes. However, the sensitivity of other enzymes of the photosynthetic carbon reduction cycle to oxygen has not been investigated, but considerable work has been done into the effects of the iodoacetyl radicle on the various constituent processes of photosynthesis.

The iodoacetyl radicle was shown by Dickens 1933 to complex with -SH groups.

Kohn 1935 showed that the iodoacetyl radicle was a specific inhibitor of photosynthesis in Chlorella pyrenoidosa, at a concentration of 10^{-4} M, whereas respiration was not inhibited until a concentration of 10^{-3} M was reached. Although the overall process of photosynthesis is inhibited at low concentrations, the Hill reaction, photophorylation and photolysis of water is not inhibited until concentrations of the order of 10^{-2} M are reached. (Frazer 1954. Macdowell 1949. Clendenning 1951. Arnon 1956) This suggests that the primary effect is on the carbon dioxide fixation system. Gibbs 1960 has made a study of the effects of the iodoacetyl radicle on the enzymes of the photosynthetic carbon reduction cycle. He found that the fixation of radioactive carbon dioxide was inhibited in chloroplasts at 5×10^{-5} M, but not until 5×10^{-3} M did it affect the production of ATP, TPNH or oxygen. In vitro 5×10^{-5} M iodoacetyl did not affect G-3-P dehydrogenase, transketolase, trans aldolase, aldolase or ribose-5-P isomerase. It was concluded that the inhibition of photosynthesis by low concentrations of iodoacetyl radicle was due to the inhibition of phospho ribulokinase. However, it was noted that ribulose-5-P did not accumulate. Similarly, Losada et al 1960 concluded that phosphoribulokinase was preferentially inhibited by low concentrations of iodoacetyl; since they found ribulose-di-P would reverse the inhibition in chloroplasts, whereas ribulose-5-P would not.

They reported little effect of iodoacetyl radicles on the conversion of P.G.A. to sugar phosphate until a concentration of 10^{-2} was reached.

Summary

It is clear that although the photosynthetic carbon reduction cycle of Calvin et al accounts for the photofixation and further metabolism of carbon dioxide at high concentrations of carbon dioxide and saturating light intensity, the metabolism of carbon is changed as the carbon dioxide p.p. is lowered or the oxygen p.p. or light intensity raised. This change in carbon metabolism is reflected in the production of glycollic acid, the onset of photoxidation and the inhibition of photosynthesis.

The further metabolixm of glycollic acid by a C-2 pathway has been proposed, and the necessary partial reactions which constitute it have all been demonstrated. However, the significance of this pathway has not been established.

It has been suggested that photoxidation entails the oxidation of carbohydrate to carbon dioxide. The mechanism of this reaction is not known.

The work reported here represents an attempt to investigate the significance of glycollate production and its further metabolism with regard to:-

1. The inhibition of photosynthesis by high p.p. of oxygen, and the mechanism of photoxidation.
2. The importance of glycollic acid as an intermediate in a C-2 pathway from the sugar-phosphates of the photosynthetic carbon reduction cycle to end products.

EXPERIMENTAL METHODS

1) Chlorella Culture

Chlorella pyrenoidosa (Emerson strain) was used.

Stock cultures were kept on agar slopes at about 3°C. These slopes were used to innoculate liquid cultures which were grown on four percent carbon dioxide in air for about one week and then used to innoculate the experimental cell cultures.

Details of the various media are given on the following page.

The experimental cultures were grown on four percent carbon dioxide in air in 300 mls of liquid medium contained in 500 ml Drechsel flasks fitted with sintered bubblers. The flasks were suspended in a glass tank, immersed to neck level in water maintained at 22 $\pm$ 0.1 °C by means of a refrigeration unit and a Sunvic relay. Illumination was provided by a single row of 100 w tungsten bulbs placed about three inches from the tank surface (about nine inches from the mid-line of the culture flasks) and six inches apart.

Three to four day cultures were used.

The cells were harvested in an M.S.E. centrifuge , fitted with 200 ml swing head buckets , at 3000 r.p.m. , re-suspended in distilled water and re-centrifuged. The cells were usually suspended in potassium dihydrogen phosphate solution (10^{-4} M. pH 4.5 - 5.0) .

A cell density of 5 µl of packed wet cell volume of cells per ml. of suspension was used.

Chlorella Medium

Agar Slopes

1 M	KNO_3	5.0 mls
1 M	KH_2PO_4	0.5
1 M	K_2HPO_4	0.5
1 M	$MgSO_4.7H_2O$	2.0
0.1 M	$Ca(NO_3)_2$	0.25
"Trace"		1.0
Fe^{++}EDTA		1.0 ($\equiv$ 5 mg Fe^{++})
Agar		15 gms
Water		To 1 litre

("Trace" $MnCl_2.4H_2O$ 1.81 gm , H_3BO_3 2.81 gm, $ZnSO_4.7H_2O$ 0.22gm, $CuSO_4.5H_2O$ 0.04 gm, $(NH_4)_6Mo_7O_{22}$ 0.2 gm. Made up to 1 litre.)

(Fe^{++}EDTA EDTA 26.1 gms , $FeSO_4.7H_2O$ 24.9 gms , 1 N KOH 268 mls, Made up to 1 litre.)

Liquid Cultures

KNO_3	1.25	gm
KH_2PO_4	1.25	
$MgSO_4.7H_2O$	2.5	
"Trace"	1	ml
Fe^{++}EDTA	1	ml
Water	To 1 litre	

("Trace" and Fe^{++} as above)

2) Chemicals and gas mixtures

Analar grade reagents were used without further purification.

The following compressed gases were obtained from British Oxygen,Ltd:-

Air.

Oxygen.

Nitrogen (White spot)

Air (Free of carbon dioxide)

Air (Plus 4 % carbon dioxide)

Carbon dioxide.

These gases were used to produce other gas mixtures,as desired. These were made by trial and error. A small volume of carbon dioxide was introduced into an empty cylinder by means of a copper spiral.The cylinder was filled with air or oxygen etc. The mixture was analysed with a carbon dioxide gas analyser (Infra-red Development Co.Ltd.), at intervals until the required concentration of carbon dioxide was obtained.

The following inhibitors were used; Isonicotinyl hydrazide (Isoniazide. I.N.H.) Obtained from Lightand Co. This was added to a final concentration of 10^{-2} M. (Pritchard 1962) Iodoacetamide (I.O.D.) Obtained from British Drug Houses Ltd. This was recrystallised and added to a final concentration of 10^{-5}M.

The following radioactive compounds were fed.

Compound	Specific activity		Source
Sodium carbonate-C^{14}	3.6	mg/mC	Radio Centre Amersham .
Glucose-U-C^{14}	0.07	mg/.05 mC	"
Glucose-1-C^{14}	0.29	mg/.05 mC	"
Glucose-2-C^{14}	0.29	mg/.05 mC	"
Glucose-6-C^{14}	0.30	mg/.05mC	"
l-Serine-3-C^{14}	0.54	mg/.01mC	"
*dl-Serine-1-C^{14}	0.28	mg/.01mC	California Biochem.

Most experiments were performed in 10^{-4} molar potassium dihydrogen phosphate solution.

The following carbonate/bicarbonate buffers were used in the manometric experiments :- (Manometric Techniques - Umbreit 1959)

Buffer No.	1	2	* 3	4	5
0.1 M $KHCO_3$ mls.	15	25	50	75	95
0.1 M Na_2CO_3mls.	85	75	50	25	5
pH Measured at end of experiment	10.5	9.9	9.6	9.3	8.6
pH.Theoretical	10.42	10.19	9.79	9.32	8.69
CO_2 concentration x 10^6 M/l.	0.53	1.7	9.8	43.0	330.0

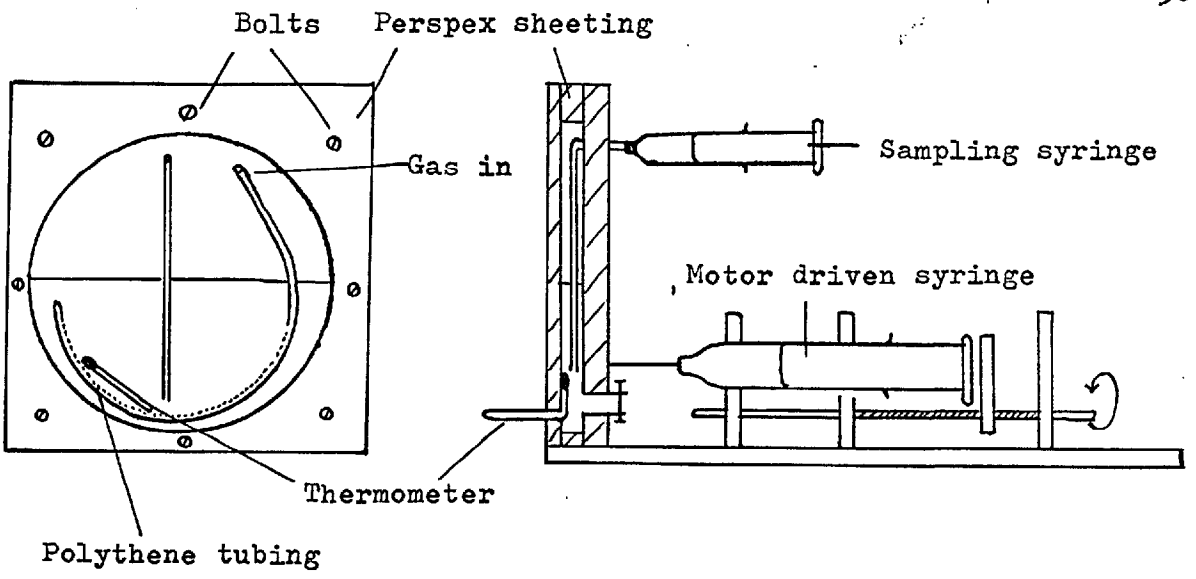
* This buffer has a carbon dioxide concentration close to that of distilled water in equilibrium with air at standard temperature and pressure.

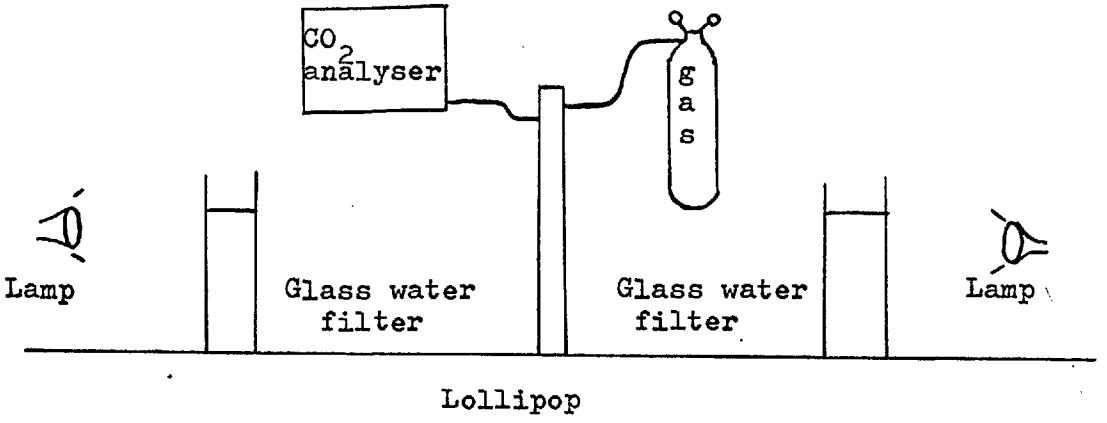
In order to overcome difficulties experienced in chromatography, diluted carbonate/bicarbonate buffers (Graham 1959) were used in carbon-14-dioxide feeding experiments .

Buffer	A	B
0.02 M $KHCO_3$	15	85
0.004 M Na_2CO_3	85	15
pH	10.2	8.97
$CO_2 \times 10^6$ M/L	0.36	66.6

3) Experimental

Most experiments were carried out with the cell suspension contained in a large perspex lollipop. This consisted of two plates of transparent perspex clamped together over a rubber vacuum ring 18.5 cms in diameter (Fig M.1). A ring of perspex 5 mm thick was used to space the two plates. The whole apparatus was supported vertically by a clamp and stand. An outlet tube 1 cm in diameter was placed at the bottom of the circle formed by the vacuum ring. This was fitted with a short length of polythene tubing which was closed near to the surface of the lollipop by a burette clip. The suspensions of cells were introduced into the apparatus through this tube. The cells were aerated by means of a length of perforated narrow bore polythene tubing. The movement induced by the escaping gas bubbles was sufficient to keep the algae stirred and in uniform suspension. Radioactivity was introduced into the vessel by means of a hypodermic syringe. The temperature of the suspension was measured by a right angled thermometer, the bulb of which was introduced into the suspension near the outlet tube, through a small hole sealed with a length of rubber tubing. The temperature of the vessel was not controlled, and varied from experiment to





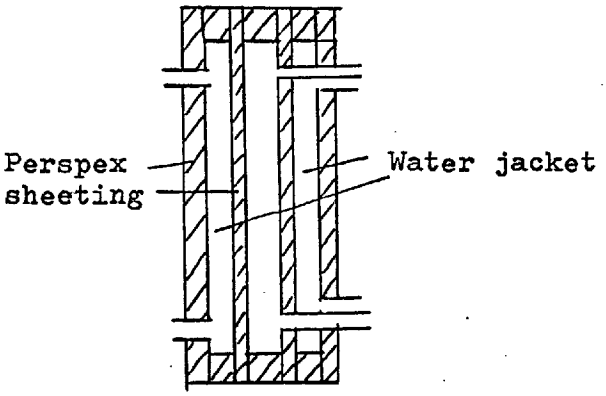


Fig M.1. Details of the lollipops used, and the arrangement of lights and water filters.

experiment between 18°C and 24°C , the variation in temperature during an experiment was always less than 2°C. In early experiments samples were taken by opening the spring clip on the outlet tube and running the required volume of suspension into a measuring cylinder. In later experiments the accuracy and speed of sampling was greatly increased by running a glass capillary into the vessel. A hypodermic syringe was used to withdraw the required sample through this capillary.

In long term experiments , the radioactivity was introduced into the vessel by means of a motor-driven hypodermic syringe (10 ml) The radioactivity was injected directly into the cell suspension at the rate of 1 ml/seven minutes.

The experimental vessel was illuminated by two Mazda 250 w floodlights , one placed each side of the vessel. A rectangular glass jar , containing water , was placed between the lamps and the experimental vessel to act as a heat filter , at a distance of 10 cms from the lamp surface. (In some experiments , at high light intensity , a perspex lollipop fitted with a water jacket was used.) The light intensity used in a given experiment was varied by altering the distance of the lamps from the experimental vessel. Three distances were chosen: 25 cms, 50 cms, and 100 cms.

The light intensity produced by these bulbs was measured by two methods:-

a. Using a Cambridge Instrument Co. thermopile and a Pye galvanometer.

b. Using a light meter.(Evans Electroselenium Ltd.)

In both cases the light intensity was measured against distance from the surface of the bulb , through a sheet of perspex 0.25 inches thick , the rectangular glass filter and an infra red filter. The results obtained were as follows:-

Distance. cms	Intensity. Method a. ergs/sq cm/sec.	Intensity. Method b. f.c.
100	2.5×10^4	370
50	5×10^4	860
25	19×10^4	2340

Unless otherwise specified all experiments were performed at the second light intensity i.e 860 f.c. This intensity is comparable with that used in the manometric experiments.

The rate of photosynthetic oxygen production was measured by the direct method of Warburg (Manometric Techniques - Umbreit 1959). The cells were suspended in carbonate/bicarbonate buffer solutions (page 28) of 0.1 molar concentration , at a density of 5 µl packed wet cell volume / ml of suspension. Conical glass flasks were used(15 mls in volume) fitted with side arm/arms and gas inlet. These were suspended in a 'Braun' circular type Warbugh bath and illuminated by means of 100w tungsten filament lamps supported beneath the bath at a distance of 4.5 cms below the bottom surface of the manometer vessel.

The vessels were shaken at a rate of 180 times per minute. The temperature of the bath was maintained at 25.0 ^{o}C. Each pair of vessels was illuminated by one lamp placed between them. Interchanging the position of the vessels did not significantly alter the rates of photosynthesis,i.e. the vessels were uniformly

illuminated.

The light intensity produced by the 100w tungsten filament bulbs was measured using the light meter (Evans Electroselenium Ltd), against distance from the surface of the bulb , through a sheet of perspex 0.25 inches thick and an infra red filter. The light intensity at a distance of 4.5 cms from the bulb had a value of 800 f.c.

In the serine feeding experiments , and in some light-stimulated dark fixation experiments , a similar lollipop to that described above was used . However this lollipop had a diameter of 5.5 cms and a capacity of about 10 mls.

In the radioactive carbonate/bicarbonate experiments the cells were illuminated in a small glass lollipop about 4 cms in diameter and 0.5 cms deep , which held about 5 ml of suspension. This was fitted with a glass tap , and could be sealed with a ' Suba Seal '.

Further details of the experimental vessels and methods used are given in the results section.

4) Killing

The algae were killed in boiling alcohol at a final concentration of 80 % v : v .

5) Extraction

The suspension of algae in 80 % alcohol was centrifuged and the insoluble residues further extracted with 20 % ethanol (twice). The extracts were combined and evaporated to dryness in a stream of air under reduced pressure. The evaporation flasks were immersed in a water bath maintained at 35° C . The distillate

did not contain a significant amount of radioactivity.

The residue was taken up in 0.5 ml of 80% ethanol followed by 0.5 ml of 20% ethanol. 0.5 ml of distilled water was then added to the evaporation vessel, which was gently heated. The three extracts were added together, centrifuged in a small conical centrifuge tube, and made up to 2 ml.

In some experiments the lipid fraction was removed by fractionation in ether/chloroform, prior to chromatography.

The ethanol insoluble fraction was hydrolysed in sulphuric acid (page 38)

6) Counting of samples, estimation of total fixation.

0.1 ml samples of the original alcohol suspension were pipetted onto 2 cm filter paper discs, on aluminium planchettes. These were dried under a Mazda infra red lamp, and counted using a G.C.E. 2-inch thin end window Geiger Muller tube and an I.D.L. series 1800 scaler, decatron counter and automatic timer. All counting was continued for sufficient time to give a maximum error of five percent. (S.Aronoff, Techniques of Radiobiochemistry)

Either three or five duplicates were usually taken ; if these varied by more than five percent further samples were taken.

The ethanol insoluble fraction was resuspended in 1 ml of distilled water, 0.1 ml duplicate samples were taken and dried at 80°C in an electric oven on paper discs and aluminium planchettes , and then counted as above.

The 'origins' of the chromatograms were also counted. The total ethanol soluble counts calculated from this figure were

usually in agreement with the difference between the two previous counts obtained , for total fixation and ethanol insoluble fraction.

1 μC of sodium carbonate-C^{14} counted as outlined above gave 2.5×10^5 c.p.m.

7) 2-D Chromatography and Autoradiography

The composition of the ethanol soluble fraction was determined by two dimensional chromatography (Benson 1950).

One tenth of the redissolved experimental solution was spotted out on each side of Whatman No.4 chromatography paper and run two dimensionally , first in phenol water (100 gm phenol . 40 ml water) and then in n-butanol:acetic acid:water (75:20:50)

Descending chromatographic techniques were used.

The chromatograms were dried at room temperature, and mounted on cardboard 'backs'. The location of radioactivity was determined by autoradiography, using Ilford Ilflex films. Radioactivity was counted directly on the paper, using the counting system detailed above.

8) Identification of Products

The following sprays were used to identify the given chemical compounds.

Ninhydrin	Amino Acids	Block et al 1955 p.88
O-Ophthaldehyde	Glycine	Patton 1949
P-anisidine	Sugars	Mukherjee 1952.from Block et al 1955 p.133
Analine phthalate	Sugars	Block et al 1955 p.133
Molybdate	Sugarphosphate	Haines 1949

The following compounds were identified by co-chromatography with authentic specimens, the location of the spots being determined by treatment with the appropriate spray:-

Alanine Glycine Serine Glutamate Aspartate Glucose Fructose Sucrose.

The following compounds were identified by co-chromatography with authentic specimens, the location of the spots was determined by autoradiography:-

Malate Fumarate Succinate Glycollate Glycine Serine Glutamate Aspartate Glucose Sucrose.

The following compounds were identifed by making the dinitrobenzine derivatives (Block 1955 p. 113, 115) :-

Serine Ethanolamine.

The above identifications were carried out in the solvents as indicated.in the table on the next page.

Sugar phosphates were also identified by their position on the 2-D chromatograms, compared with figures published by Calvin and co-workers. Phosphoglyceric acid was dephosphorylated, the resulting free acid did not move in solvent 4. which would distinguish it from glycollate with an r.f. of 0.52.

Glycollic acid was identified and estimated by the method of Calkins (1943). The glycollic acid was degraded by the method of Aronoff , Techniques of Radiobiochemistry 1956,and found to contain two carbon atoms.

Sucrose on hydrolysis was found to give two products which separated when run in solvent 3 to give two spots with equal

	Solvent	Author
1	n-Butanol:Acetic acid:Water. 75:20:50	Benson 1950
2	Phenol:Water 100:40	Benson 1950
3	Pyridine:Ethyl acetate:Water 1:2:2	Block 1955 p 128
4	Ether: Acetic acid:Water 156:36:12	Smith 1958 p 207
5	Water saturated n-butanol	Block 1955 p 112
6	Benzene:1% acetic acid	Block 1955 p 112

Compound	Solvent No.
Alanine.	1, 2.
Aspartate.	1, 2.
Glycine.	1, 2, 3.
Serine.	1, 2, 3.
Glutamate.	1, 2.
Serine-DNP	5, 6.
Ethanolamine-DNP	5, 6.
Glucose.	1, 2, 3.
Fructose.	1, 2, 3.
Sucrose.	1, 2, 3.
Glycollate.	1, 2, 4.
Glycerate.	1, 2, 4.
Malate.	1, 2, 4.
Fumarate.	1, 2.
Succinate.	1, 2.
Sugar-phosphate.	1, 2.

activity. These products had r.f. values corresponding to fructose and glucose.

The ethanol insoluble fraction was investigated as follows:

1. It was hydrolysed, under reflux, for 8 hours in N.H_2SO_4. The supernatant was taken as hydrolysed carbohydrate.

2. The residue was then hydrolysed for 8 hours in 6 N. H_2SO_4 . The supernatant was taken to be hydrolysed amino acids.

The acid was neutralised with barium chloride, and the precipitate spun off. The supernatant was evaporated to dryness and chromatographed as previously described. The location of radioactivity was derived by autoradiography and by use of the appropriate sprays. The composition of the ethanol insoluble fraction is given in a subsequent section. The main component of the ethanol insoluble fraction was glucose.

The glucose from sucrose and from the ethanol insoluble fraction was degraded by Dr A Marker and shown to contain six carbon atoms.

The final identification, of the products of photofixation of carbon-14-dioxide by Chlorella, after 2-D chromatography is shown on the following page.

Autoradiographs of 2-D paper chromatograms obtained from the ethanol extract of Chlorella after photosynthesis in carbon-14-dioxide for 15 minutes in the following conditions:-

1. Light intensity 860 f.c. Continuous injection of sodium carbonate-C^{14}. Cells bubbled with air containing 0.04% Carbon dioxide. (For further details of these experiments see pages 64 to 79)

2. As above plus 10^{-2} M I.N.H.

3. As 1. bubbled with oxygen containing 0.04 % carbon dioxide.

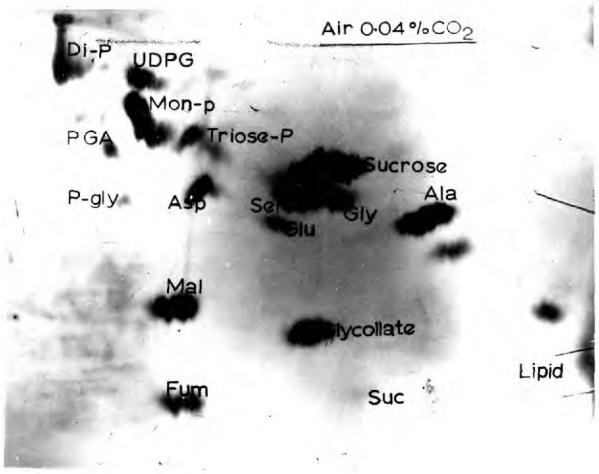
Chromatograms run in phenol left to right and in butanol top to bottom. Abbreviations used:-

Di-P	Sugar di phosphates.	Asp	Aspartate
Mon-P	Sugar mono phosphates.	Mal	Malate
Triose-P	Triose phosphate.	Fum	Fumarate
PGA	Phosphoglyceric acid.	Suc	Succinate
P-gly	Phosphoglycollate.	Ser	Serine
PEP	Phosphoenolpyruvate.	Gly	glycine
Glu	Glutamate	Ala	Alanine

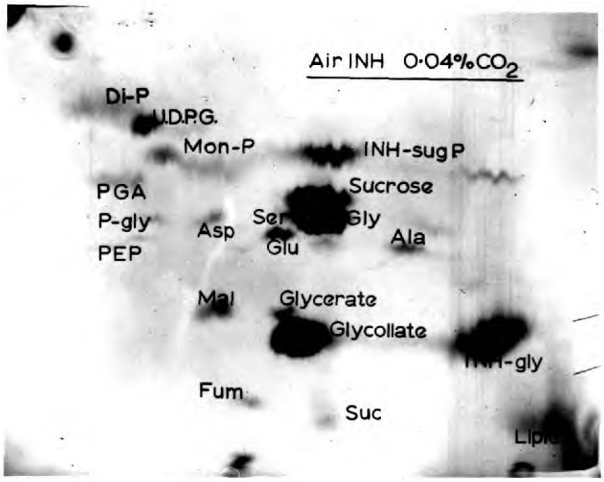
INH-sugP , INH-gly , addition products of INH with sugar phosphate and glycollate.

The various effects of the additions,which are further discussed in the following sections, can be seen.Addition of I.N.H. decreases serine formation,and increases glycine and glycollate.Alanine Aspartate and Glutamate are reduced, Succinate formation is increased. Oxygen increased the formation of serine, glycine and glycollate.

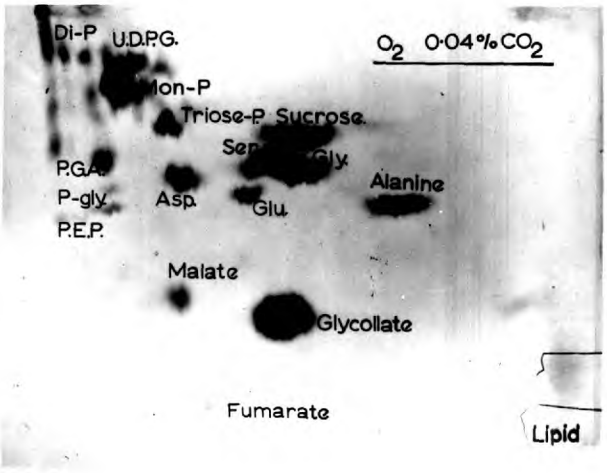
1


Air 0·04 %CO2
Di-P
UDPG
Mon-p
PGA
Triose-P
Sucrose
P-gly
Asp
Ala
Gly
Glu
Mal
Glycollate
Lipid
Fum
Suc

2


Air INH 0·04%CO2
Di-P
U.D.P.G.
Mon-P
INH-sug P
Sucrose
PGA
P-gly
PEP
Asp
Gly
Ala
Glu
Mal
Glycerate
Glycollate
Fum
Suc

3


Di-P
U.D.P.G.
O2 0·04%CO2
Triose-P
Sucrose
P.G.A.
P-gly
P.E.P.
Asp.
Glu.
Alanine
Malate
Glycollate
Fumarate
Lipid

EXPERIMENTAL RESULTS

The results of manometric experiments designed to investigate the effects of I.N.H. on the rate of photosynthetic oxygen production and to compare the degree of inhibition brought about by high oxygen p.p. on oxygen production and carbon-14-dioxide fixation are shown in Figs. E 1 and E 2.

The rates of oxygen gas exchange have been calculated as µl oxygen/µl packed wet cell volume/hour. The actual manometer readings were first corrected for the thermobarometer changes and then multiplied by a constant calculated from the appropriate flask constant , cell volume and time interval.

Khurdak (1962) found that I.N.H. inhibits the rate of photosynthesis in Chlorella. However Whittingham et al (Pritchard 1961. 1962.1963. Whittingham 1962.1963b.) reported little effect of I.N.H. on the rate of photofixation of carbon dioxide in Chlorella.

Under the experimental conditions reported here no inhibition of oxygen evolution , byI.N.H. , was found if the gas phase consisted of either nitrogen or air. However in high p.p of oxygen the addition of I.N.H. increased the inhibition of oxygen production brought about by the high oxygen tension.

Brittain (1957) found a considerable decrease in the assimilatory quotient (carbon dioxide/oxygen) in Chlorella transferred to high oxygen p.p. , whereas Gaffron (1940) reported no change or a slight increase in this quotient for Scenedesmus after similar treatment.

The results reported here confirm the observations of Brittain.

Fig. E 1 A. The effect of I.N.H. on the rate of photosynthetic oxygen production at various carbon dioxide concentrations in air.

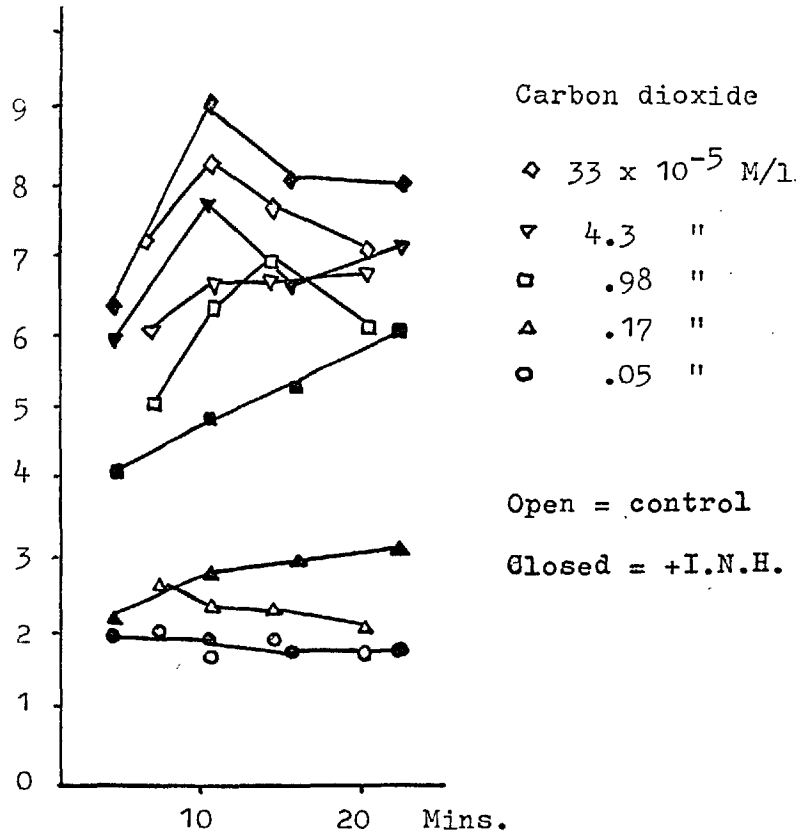
1.9 mls. of the required buffer was placed in the main vessel , and 10 µl of cells suspended in 0.1 mls of distilled water in the side arm , of a conical manometer flask. I.N.H. was added to the buffer before the cells were introduced to the side arm. After the vessels had equilibrated the taps were closed and the two solutions mixed by tipping the contents of the main vessel into the side arm and out once. The cells were allowed to photosynthesise for 30 mins. The manometers were then reset and readings taken as indicated.

Fig. E 1 B. The effect of I.N.H. on the rate of photosynthetic oxygen production at various oxygen p.p.

Conical manometer vessels fitted with two side arms were used. The cells were placed in the first arm and the I.N.H. in the second. The vessels were gassed for 30 minutes with the required carbon dioxide free gas prior to equilibration.The vessels were then equilibrated for ten minutes with the taps open , during this time some gas exchange could take place. The taps were closed the cells tipped into the buffer , and readings taken. After 20 minutes the I.N.H. was tipped into the cells and further readings taken.

µl Oxygen/µl Cells/Hour.

Fig.E 1 A



µl Oxygen/µl Cells/Hour.

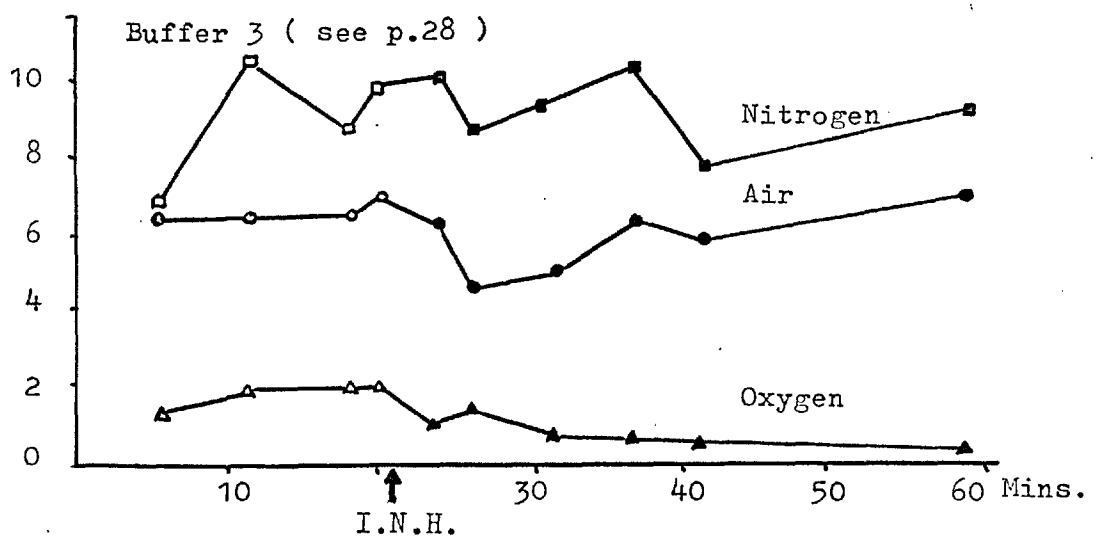


Fig.E 1 B

Fig E 2 The effect of oxygen p.p. and I.N.H. on the rate of photosynthetic oxygen evolution and carbon-14-dioxide fixation at various carbon dioxide concentrations.

The cells were made up to a concentration of 5µl/ml in the buffer and placed in the main vessel. 1 µC of sodium carbonate-C^{14} buffered with potassium bi-carbonate was placed in the side arm. The vessels were gassed as above. I.N.H, was added to the buffer. After equilibration the cells were allowed to photosynthesise for 30 minutes. The manometers were then reset and readings taken. After the given time interval the radioactivity was tipped into the cells. At the end of the experiments the cells were killed and the total fixation estimated as previously described.(page 34)

Total fixation c.p.m. x 10^{-3}

Fig A. Buffer 4.

Air	Air + I.N.H.	O_2	O_2+I.N.H.
45.0	39.0	18.4	11.7

Fig B. Buffer.

4		3		2	
air	O_2	air	O_2	air	O_2
55.8	28.0	43.4	14.4	20.0	6.2

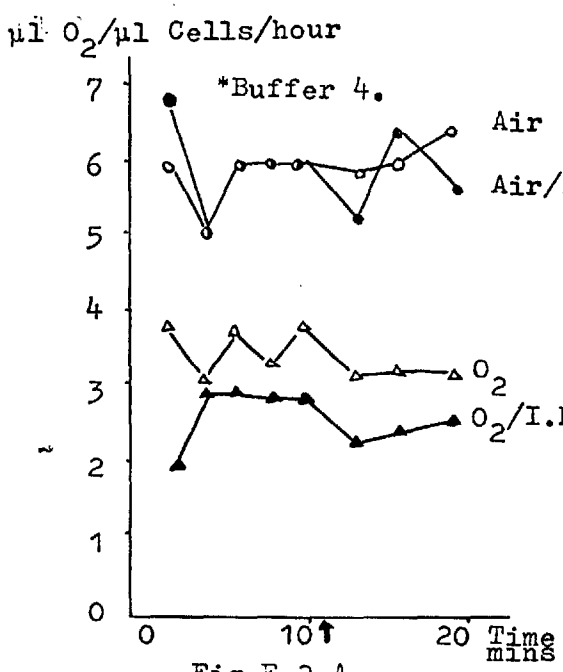


Fig.E 2 A

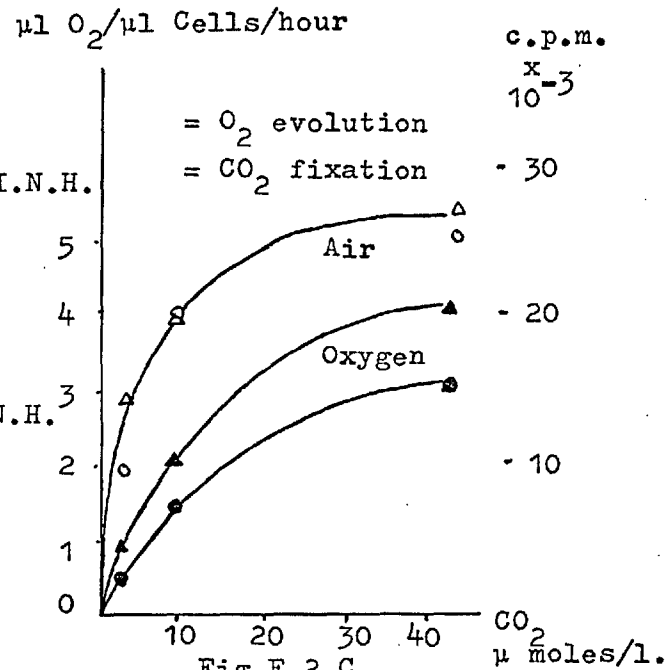


Fig E 2 C

Rate of photosynthesis.

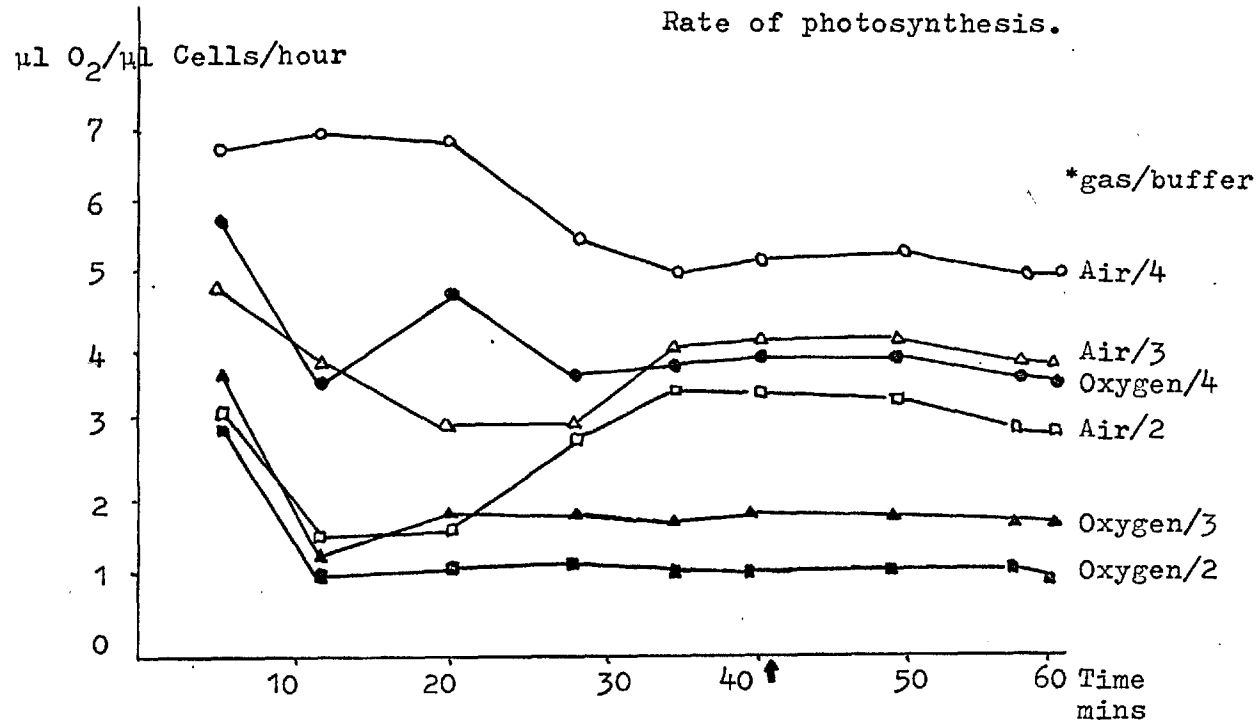


Fig E 2 B

* For details of buffers see p. 28 .

⬆ Indicates addition of radioactivity

That is carbon dioxide fixation was inhibited ,by high oxygen p.p. , to a greater extent than oxygen evolution. In Fig. E 2 C the rates of oxygen evolution , and the amounts of radioactive carbon dioxide fixed in experiment E 2 B are plotted against the carbon dioxide concentration of the buffers. The figures for the rates of photosynthesis as determined by both methods are plotted on a scale such that the air curves coincide. It can be seen that both the evolution of oxygen and the fixation of carbon dioxide were inhibited to a greater extent as the carbon dioxide concentration of the buffers was lowered. Furthermore the inhibition of carbon-14-dioxide fixation , by high p.p. of oxygen , was greater than that of oxygen evolution. This effect also increased as the carbon dioxide concentration was lowered.

The distribution of radioactivity into products of photo-fixation of carbon-14-dioxide in carbonate/bicarbonate buffers was investigated. It has been shown by Graham (1959) that in experiments involving the fixation of radioactive carbon dioxide , over periods of from six minutes to one hour , a large percentage of the total carbon fixed could be recovered from the ethanol soluble fraction in the form of an organic acid which increased in activity when the carbon dioxide concentration was decreased or the oxygen p.p. was increased. He suggested that this compound was free glyceric acid.

Graham found that the high concentration of ions present in these buffers led to streaking of the chromatograms. Therefore he used diluted buffers (see page 29) and passed the extracts through ion exchange columns.

However it was thought that the treatment on resin columns could lead to both losses and decomposition of the products. The streaking was therefore overcome by using very small samples. Tables 1 and 2.

Over periods of one to six minutes photosynthesis in these buffers considerable activity was incorporated into an organic acid which corresponded in position on the 2-D chromatograms to that found by Graham. A greater proportion of the carbon fixed was recovered in this compound in the buffer with the lower carbon dioxide concentration. The formation of this acid was enhanced by the addition of I.N.H. , and by high oxygen p.p. This acid was identified as glycollate by its r.f. values in solvents 1, 2, and 4 (page 37) , and by co-chromatography with authentic glycollate-C^{14}. The 2:7 dihydroxynaphthalein derivative was made and its absorption spectrum compared with that of authentic glycollate.

Table 1 shows that an increasing percentage of the carbon fixed was metabolised to glycollate in the buffer with the lower carbon dioxide concentration, which at the higher carbon dioxide concentration was recovered initially from the sugar phosphate fraction and after longer periods of time from the sucrose and ethanol insoluble fraction.

Table 2 shows that the total incorporation of carbon-14 dioxide under these conditions was inhibited by about 40% when the oxygen p.p. was raised to 100%. The addition of I.N.H. increased this inhibition. Incorporation of radioactivity into the sugar phosphates, sucrose, and the ethanol insoluble

Table 1 Time course of carbon dioxide fixation , and percentage distribution into metabolic products after photosynthesis in radioactive buffers A and B.

Table 2 Effect of I.N.H. on total fixation and the percentage distribution of carbon-14 into metabolic products after three minutes photosynthesis in buffer A in air and 100 percent oxygen.

Diluted buffers were used(page 29).These were made radio active by the addition of 100µC of sodium carbonate $-C^{14}$ to 100 mls of buffer. The calculated amount of potassium bicarbonate was added to maintain the $HCO_3^-/CO_3^=$ ratio. 25 µl of Chlorella were suspended in 5 ml of this buffer in a small glass lollipop,sealed with a 'Suba Seal'. The killing alcohol was acidified with acetic acid to remove the residual carbon-14. The cells were bubbled with the required gas by means of a hypodermic needle. A second needle served as a gas outlet.

Table 3 Percentage carbon-14 fixed into various products after five minutes photosynthesis in buffer A or in phosphate buffer, with and without I.N.H.

Experiments in carbonate buffer A as above. 25 µl of Chlorella were suspended in 5 ml of phosphate buffer in the small glass lollipop. The cells were aerated with 0.001 % carbon dioxide. This approximated to the CO_2 concentration of the carbonate buffer. 0.5 µC of sodium carbonate-C^{14} were injected into the lollipop.

Abbreviations used:- Sugar-P., sugar mono and di phosphates plus U.D.P.G. and P.G.A.

Amino & TCA acids, Glycine,Serine , alanine, glutamate, aspartate ,malate, fumarate,succinate.

EtOH insol, Ethanol insoluble fraction.

Table 1

Buffer	A			B		
Time mins.	1	3	6	1	3	6
Sugar-P	28 %	17	12	68	32	24
Amino & TCA acids	45	40	26	11	23	18
Sucrose	5	14	11	7	18	24
Glycollate	12	20	40	2	18	19
EtOH Insol	10	10	12	10	10	16
Total c.p.m. x 10^{-3}	202	326	466	110	208	305

Table 2

Treatment	Air	Air+I.N.H.	Oxygen	Oxygen+I.N.H.
Sugar-P	28 %	25	9	9
Amino & TCA acids	8	17	29	30
Sucrose	32	21	4	tr
Glycollate	17	33	53	55
EtOH Insol	16	14	5	5
Total c.p.m. x 10^{-3}	184	235	110	100

Table 3

Treatment	P	P.INH	PI/P	C	C.INH	CI/C
Glycollate	17 %	44	2.6	28	42	1.5
Glycine	9	36	4.0	7	22	3.0
Serine	13	3	0.2	7	1	0.15
Sucrose	33	5	0.15	26	3	0.11

fraction was decreased by both I.N.H. and high oxygen p.p. There was a corresponding increase in glycollate formation and in incorporation into the Krebs cycle and amino acid fraction.

Tolbert 1956.1963 has suggested that glycollate excretion consists of active exchange with bicarbonate ions from the external medium. If such a mechanism existed there could be competition between this exchange and the further metabolism of the C-2 precursor of glycollate via the I.N.H. sensitive pathway. Therefore I.N.H. would have less effect on the production of glycollate and glycine in carbonate buffers than in phosphate buffers with the same carbon dioxide concentration.

The results of such an experiment are shown in table 3. It was found that the relative increase in both glycine and glycollate caused by the addition of I.N.H. was greater in the phosphate buffer than in the carbonate buffer. These results would suggest that either an exchange mechanism exists , or as Tolbert 1963 has suggested the presence of phosphate ions inhibit the production of free glycollate.

Further experiments to investigate the effects of high p.p. of oxygen on the photometabolism of carbon were carried out in 10^{-4} M phosphate buffer, so that any secondary effects of glycollate/ bicarbonate exchange would be eliminated. In these experiments Chlorella was suspended in the large perspex lollipop at a density of 5 µl/ml. The cells were always preincubated in air for 30 minutes prior to any other treatment. I.N.H. was added at the

Table 4 The effect of I.N.H. on carbon-14-dioxide fixation in air and oxygen after 30 and 60 seconds photosynthesis in air or oxygen. Phosphate buffer bubbled with 0.04 % carbon dioxide.

Treatment	AIR		OXYGEN		AIR I.N.H.		OXYGEN I.N.H.	
Time.Secs	30	60	30	60	30	60	30	60
Di-P	25	14	5	7	9	9	8	3
Mono-P	38	29	23	22	46	30	24	23
P.G.A.	9	8	19	7	8	5	15	8
U.D.P.G.	7	7	5	4	9	16	6	6
Aspartate	3	4	3	3	2	2	2	3
Malate	4	5	8	3	1	2	3	4
Glycollate	4	11	15	23	3	19	21	26
Glycine/ Serine	8	11	7	10	9	8	4	10
Alanine	3	6	5	5	4	4	4	13
Sucrose	2	4	6	13	5	6	1	3
Total Fixation c.p.m. x 10^3	99.0	112.4	5[illegible]9	57.0	94.5	110.4	40.8	49.5

The cells were preincubated in the light in the required gas for 30 minutes at a density of 5µl/ml. Ten ml samples were run into a 1.5 x 12 cm test tube , sealed with a 'Suba Seal' and 3 µC of sodium carbonate-C^{14} injected. The cells were not aerated during this period.

commencement of illumination.

The effect of I.N.H. on the total fixation of carbon-14 dioxide, and the distribution of radio activity into ethanol soluble compounds, after 30 and 60 seconds photosynthesis in either air or oxygen is shown in table 4. These experiments were performed in phosphate buffer bubbled with 0.04 % carbon dioxide. After thirty seconds photosynthesis the main effect of I.N.H. was to change the ratio between the mono and di phosphate fractions. The formation of sucrose was stimulated. After a further 30 seconds photosynthesis U.D.P.G. and glycollate rose. I.N.H. had no marked effect on the total fixation of carbon-14-dioxide.

By contrast the high oxygen p.p. markedly lowered the total fixation. Although after 30 seconds the percentage of carbon-14 recovered in the total sugar phosphate fraction was less than in the control the formation of P.G.A. was stimulated. Glycollate and sucrose formation were also stimulated , the former was increased and the latter decreased by the addition of I.N.H. After 60 seconds the stimulation of P.G.A. formation could no longer be seen. The effects of oxygen and of oxygen/I.N.H. on glycollate and sucrose production became more marked. Again it was found that the increase in glycollate , produced by the addition of I.N.H. was greater in air than in oxygen.

Since the rate of photosynthesis in oxygen was lower than that in air these results represent the distribution after various amounts of carbon dioxide had been fixed. Therefore the effect

Fig E 3 The effect of oxygen p.p. on the distribution of activity into photosynthetic products , at air levels of carbon dioxide. The time of fixation was varied so that the same amount of carbon dioxide was fixed at all oxygen p.p.

Ten ml. samples were run into a test tube and sealed witha 'Suba Seal' . Three μC of sodium carbonate-C^{14} were injected into the tube. The oxygen sample was allowed to photosynthesise for three minutes . The other samples were allowed to fix the same amount of radioactivity. This was achieved by ' trial and error'. The cells were bubbled with the required gas prior to but not during the experimental period.

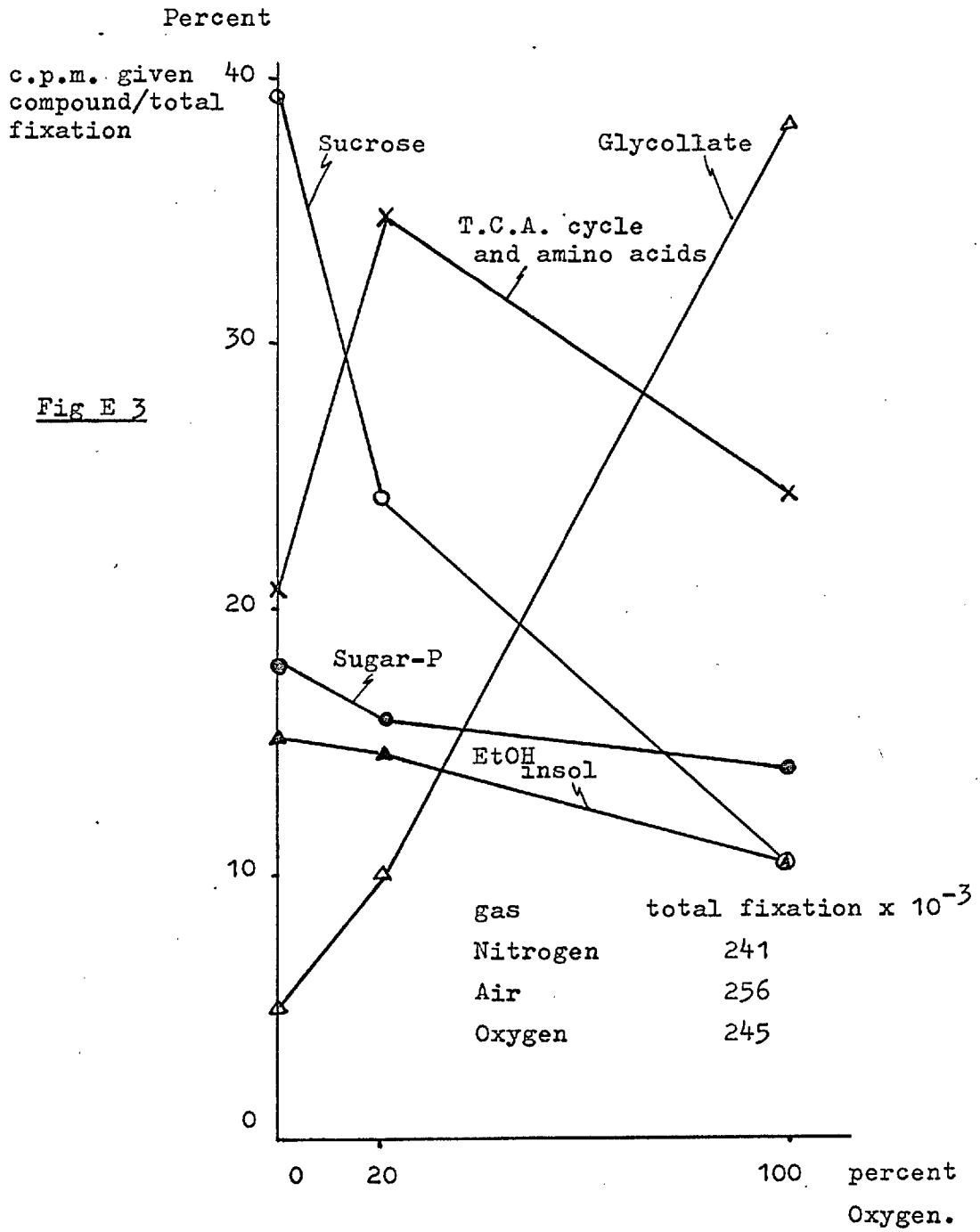

Percent
c.p.m. given compound/total fixation
40
30
20
10
0
Sucrose
Glycollate
T.C.A. cycle and amino acids
Sugar-P
EtOH insol
gas
total fixation x 10^{-3}
Nitrogen 241
Air 256
Oxygen 245
0
20
100
percent Oxygen.

Fig E 3

of oxygen p.p. on the percentage distribution of carbon-14-dioxide fixed into various products of photosynthesis, after the same amount of carbon had been incorporated , was investigated. Fig E 3.

The results of this experiment show that for a given amonnt of carbon fixed more carbon-14 was incorporated into glycollate, and less into sucrose and ethanol insoluble compounds, as the oxygen p.p. was raised . The incorporation into the amino and T.C.A. fraction showed a peak at air levels of oxygen.

Fig E 4 shows the effect of varying the carbon dioxide concentration,in an atmosphere of oxygen , on the percentage distribution of carbon-14 into various products of photosynthesis after three minutes photosynthesis.

The products of photosynthesis under these conditions could be divided into three groups:-

1.Sugar phosphates, sucrose, and ethanol insolubles. The percentage of the carbon-14 recovered in these compounds increased as the carbon dioxide concentration was raised.

2. The acid and lipid fractions. The incorporation of radioactivity into these compounds showed a peak, the former at 0.09% carbon dioxide and the latter at 0.2% carbon dioxide.

3. Glycollate. The percentage carbon-14 recovered in glycollate fell rapidly as the carbon dioxide concentration was raised from 0.03% to 0.09%, and then at a slower rate as the carbon dioxide concentration was raised further.

Fig E 4 The effect of carbon dioxide concentration on the total fixation of carbon dioxide, and the percentage incorporation into products, after three minute photosynthesis. The cells were bubbled with oxygen containing the given carbon dioxide concentration prior to but not during the experimental period.

The cells were preincubated for thirty minutes. 10 ml samples were taken, and incubated with the following amounts of sodium carbonate-C^{14} for three minutes in a closed tube.

CO_2 %	0.03	0.09	0.20	2.00
C^{14} μC.	3.0	9.0	20.0	200.0

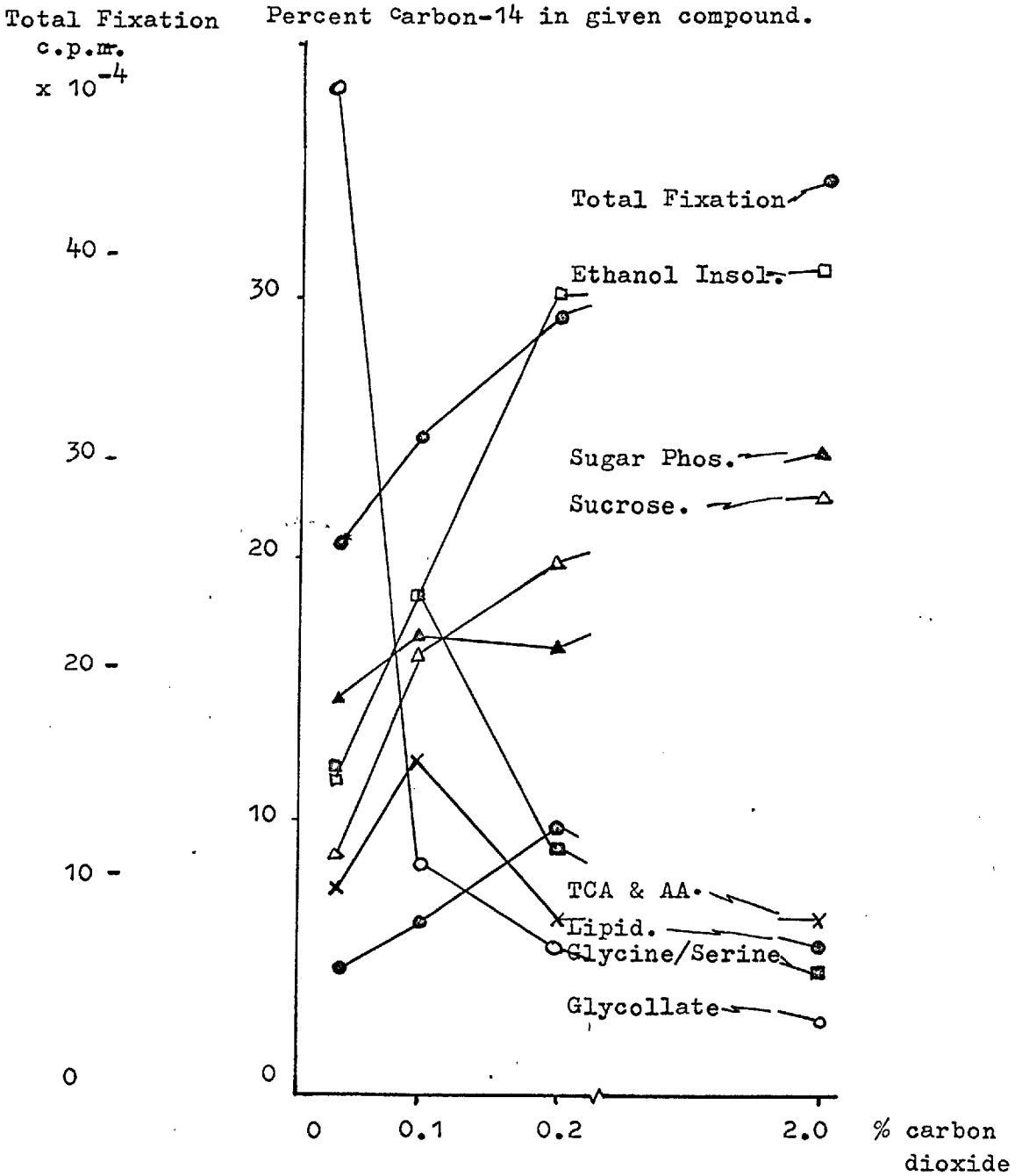

Total Fixation
c.p.m.
x 10^-4
Percent Carbon-14 in given compound.
40
30
20
10
0
30
20
10
0
Total Fixation
Ethanol Insol.
Sugar Phos.
Sucrose.
TCA & AA.
Lipid.
Glycine/Serine
Glycollate
0
0.1
0.2
2.0
% carbon dioxide

Fig E 4

In the experiments reported so far several assumptions had been made. First it was assumed that photosynthesis had reached a steady rate during the preliminary half hour preincubation period and that the rate did not change during the subsequent experimental period, and second it was assumed that the rate of photosynthesis and distribution of radioactivity into products was not affected by the transient changes in carbon dioxide concentration brought about by the injection of the radioactive carbonate into the sealed test tube. Experiments were performed to confirm these assumptions. Fig E 5 and E 6.

The results shown in Fig E 5 indicate that the rate of photosynthesis in both air and oxygen remained constant during a period of thirty minutes, following a period of thirty minutes preincubation in the given gas. The distribution of carbon-14 into photosynthetic products was not greatly affected by the length of the preincubation period, the results were in agreement with those previously reported. The incorporation of carbon-14 into all sugar phosphates except P.G.A. was decreased by high p.p. of oxygen, incorporation into the di-phosphates showed the highest degree of inhibition. The incorporation into sucrose, the ethanol insoluble fraction, malate, fumarate, serine, glycine, and alanine was also inhibited. The incorporation into glycollate was increased.

The injection technique of introducing the radioactive carbon dioxide to the cells caused a transient increase in the

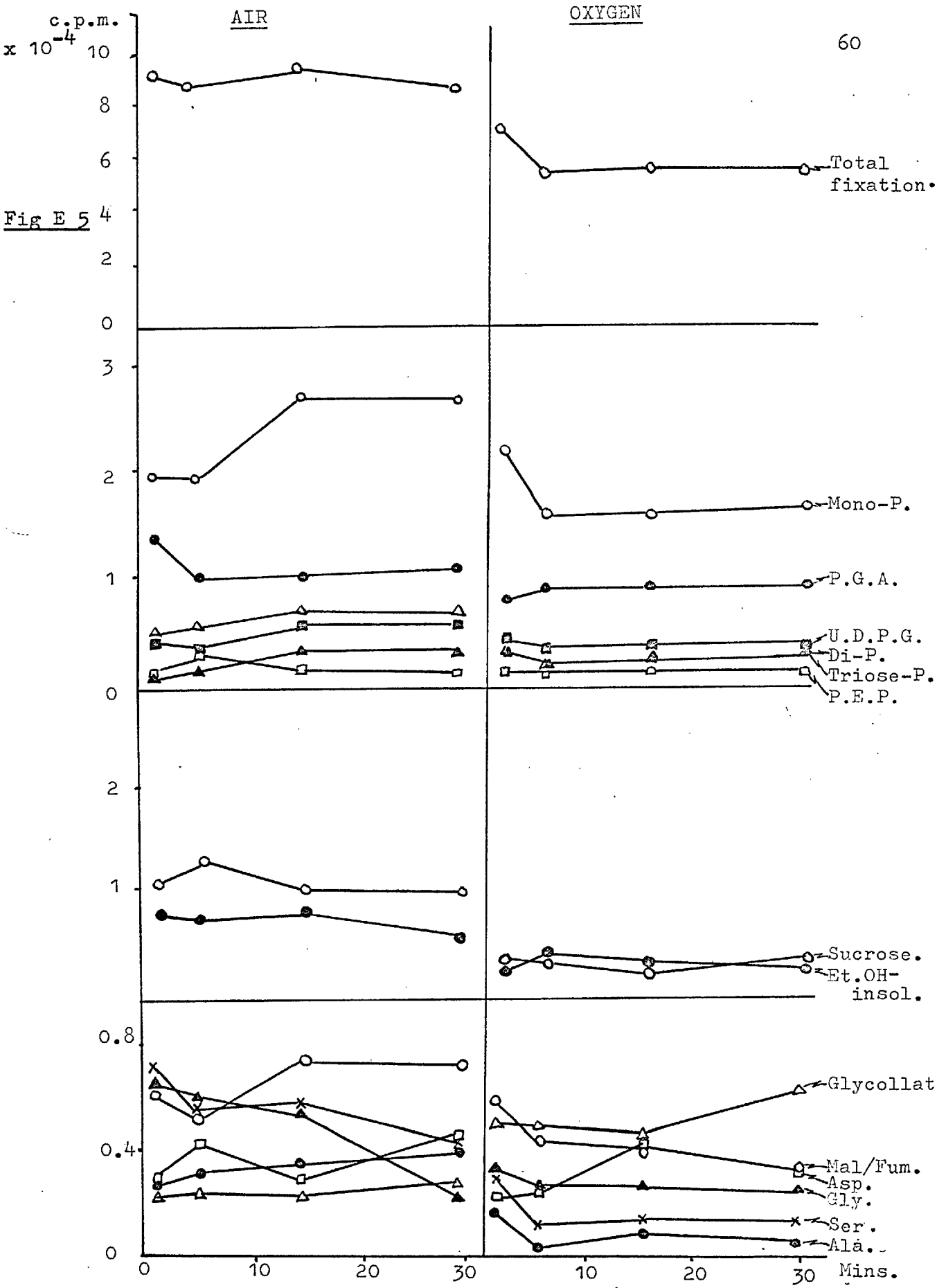
Fig E 5 The total fixation of carbon-14-dioxide , and distribution of activity into products of photosynthesis at various times after the half hour preincubation period. The cell suspension was bubbled with either air or oxygen containing 0.036% carbon dioxide.

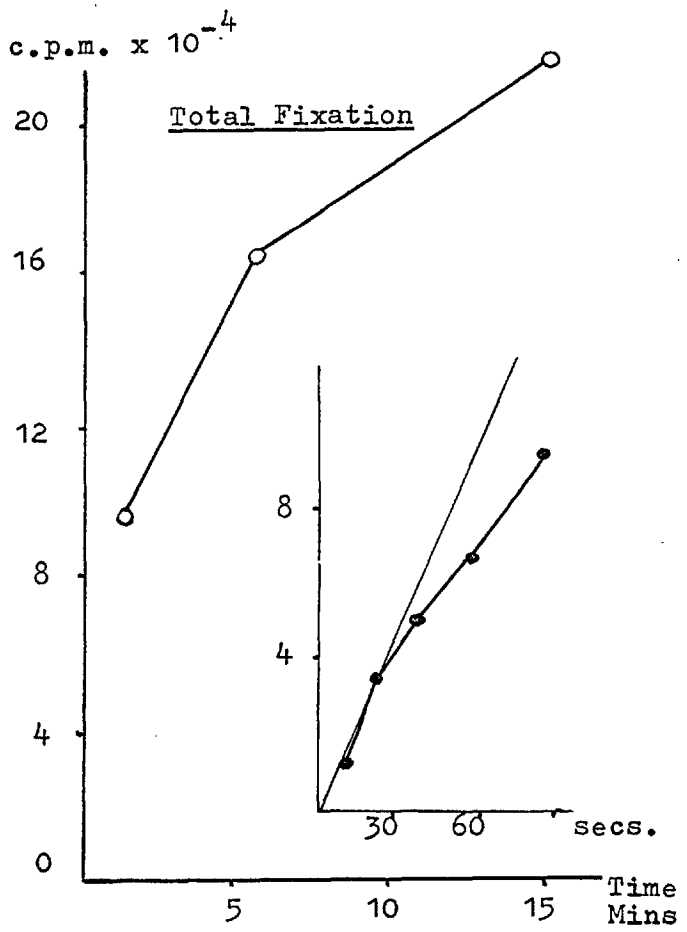
The cells were preincubated in air or oxygen for 30 mins. 10 ml samples were run into a test tube, sealed with a 'Suba Seal' and injected with 3 μC of sodium carbonate-C^{14}. The cells were killed after one minute photosynthesis in the closed tube.

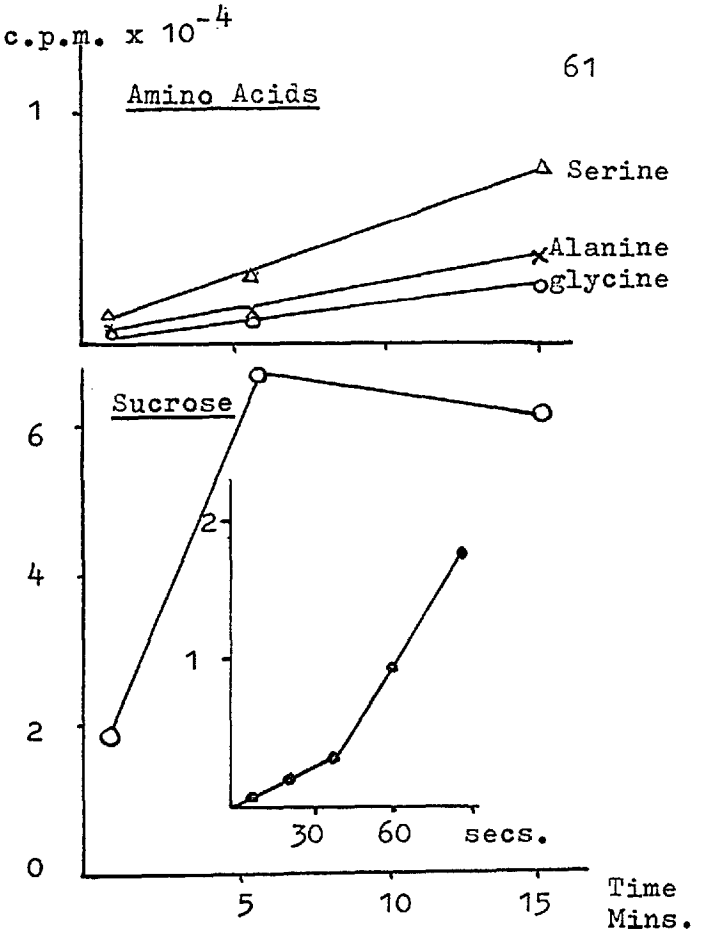
Fig E 6 Time course of fixation of carbon-14-dioxide in the closed tube. The cells were preincubated in air.

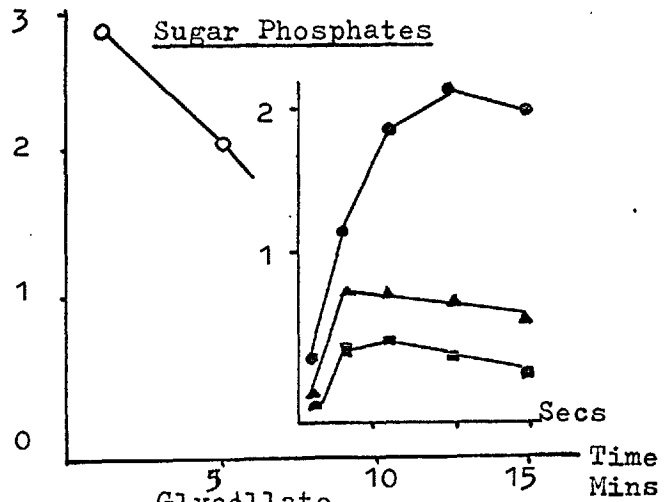
10 ml samples were taken and incubated with carbon-14 in a closed test tube as above. The cells were killed after periods of from ten seconds to fifteen minutes photosynthesis in the closed tube.

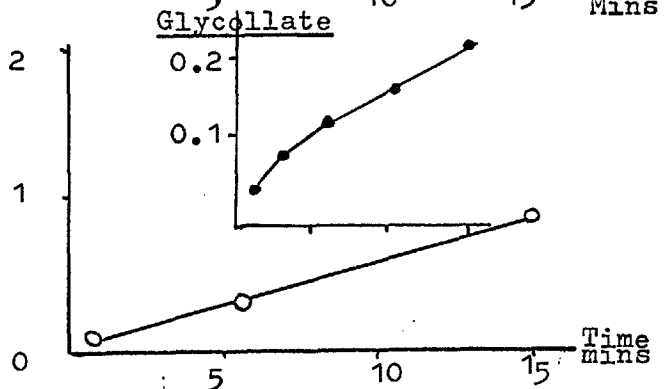
Fig E 5


AIR
OXYGEN
c.p.m.
x 10⁻⁴
60
Total fixation.
Mono-P.
P.G.A.
U.D.P.G.
Di-P.
Triose-P.
P.E.P.
Sucrose.
Et.OH-insol.
Glycollat
Mal/Fum.
Asp.
Gly.
Ser.
Ala.
Mins.









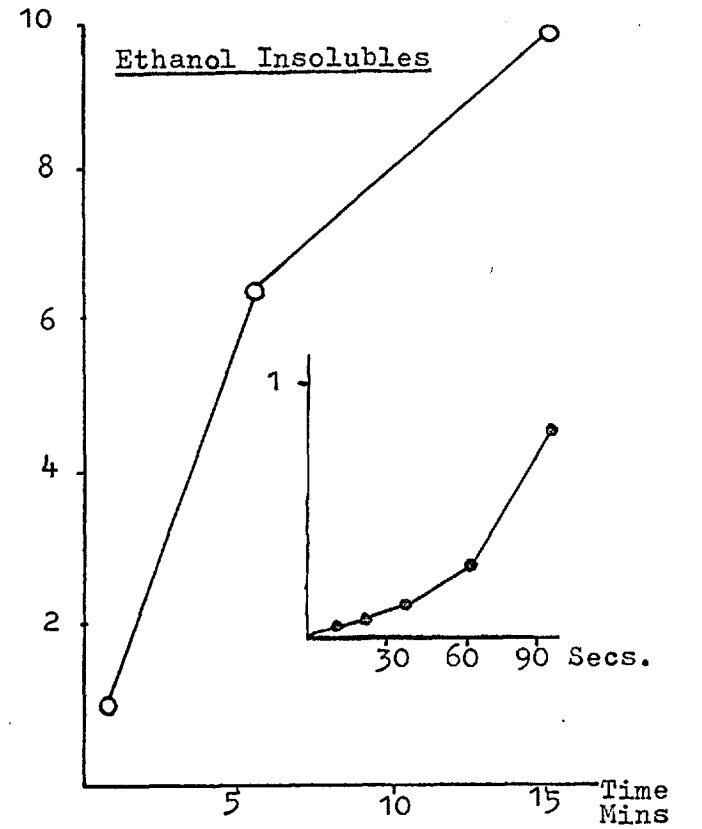


Fig E 6 Time course of photosynthesis in the closed tube.

carbon dioxide concentration within the test tube. (Three μC of sodium carbonate would produce sufficient carbon dioxide to raise the concentration within the tube from 0.036 percent to about 0.046 percent) This increase in the carbon dioxide concentration was followed by a decrease as the carbon dioxide was fixed by photosynthesis.

It was found that the rate of fixation of carbon-14-dioxide in the closed tube was linear for the first thirty seconds, and had not altered greatly after sixty seconds photosynthesis (at which point about 15 percent of the carbon supplied had been utilised) After fifteen minutes about 30 percent of the carbon supplied had been fixed. In short periods of photosynthesis most of the radioactivity fixed was recovered from the sugar phosphate fraction,and after longer periods in end products, mainly sucrose and ethanol insoluble compounds. The incorporation of radioactivity into the sugar phosphate fraction and into sucrose decreased as the carbon dioxide concentration fell. However the incorporation of carbon-14 into other products did not appear to be affected by the decreasing concentration of carbon dioxide.

As a result of these experiments it could be concluded that a steady rate of photosynthesis was reached during the preincubation period and that under these conditions the rate of fixation and distribution of radioactivity into end products of photosynthesis was not affected by the changing carbon dioxide concentration,in the closed tube,for periods of up to one minute.

The results so far reported are in agreement with those from the laboratories of Calvin, Tolbert, Warburg, Whittingham, and others reported in the introduction. Additional information has been derived from experiments in which I.N.H. was added at high p.p. of oxygen and at high concentrations of bicarbonate ions. These experiments show that under conditions where more free glycollate was produced less carbon was metabolised via an I.N.H. sensitive route. The inhibition of photosynthesis under high p.p. of oxygen was accompanied by a decrease in the incorporation of radioactivity into the sugar phosphate fraction and an increase in incorporation into glycollate. This could indicate that oxygen increased the production of glycollate from the intermediates of the P.C.R. cycle, leading to a decrease in the rate of photosynthesis.

This hypothesis was further investigated in two series of experiments:-

1. The steady state metabolism of carbon-14-dioxide in air and oxygen, at various carbon dioxide concentrations, was invetigated.

2. The changes in carbon metabolism, which occured during the transition from one gas phase to another, were investigated.

Long term steady state photosynthesis in phosphate buffer

The effects of I.N.H., I.O.D., and high p.p. of oxygen on the rate of steady state photosynthesis, and the distribution of carbon-14 into the end products of photosynthesis were investigated for periods of up to seventy minutes, using the automatic injection technique.(p.30)

Fig E 7 shows the rates of fixation obtained, at the three different carbon dioxide concentrations used, in the air control experiments. It was found that this method of introducing the carbon dioxide-C-14 gave a linear rate of fixation throughout the experimental period. It could also be seen that the cells were carbon dioxide limited at the lower concentrations.

In Fig E 8 the rates of photosynthesis obtained with other treatments are expressed as percentages of the fixation in air at that carbon dioxide concentration. The addition of I.N.H. caused a slight stimulation of fixation at air levels of carbon dioxide, this stimulation increased to about 20% as the carbon dioxide concentration was raised to 4.0%. At the lowest concentration of carbon dioxide I.O.D. had only a slight inhibitory effect after 15 minutes photofixation of carbon-14 (45 minutes incubation in I.O.D.) c.f. Kohn 1935. The degree of inhibition increased with time and as the carbon dioxide concentration was raised to reach a final value of about 70% inhibition. In contrast the inhibition of photosynthesis by high p.p. of oxygen was less dependent on the length of treatment , and was most marked at low

Fig E 7 to E 13 Long Term Photosynthesis in phosphate buffer.

In these experiments the radioactivity was continuosly injected into the base of the large perspex lollipop by means of a motor driven syringe, which delivered the solution at the rate of one ml. every seven mins. The cells were bubbled with the required gas containing the required level of carbon dioxide. The amount of carbonate-C-14 injected was not sufficient to significantly alter this carbon dioxide concentration. 10^{-2}M I.N.H. was used. 10^{-5}M I.O.D. was used. The following amounts of radioactive carbonate were injected over periods of 70 minutes at the various carbon dioxide concentrations.

CO_2 % .	0.04	0.40	4.0
µC	10	100	100

The c.p.m. fixed in 4% carbon dioxide were multiplied by a factor of ten to correct for the difference in specific activity of this carbon dioxide.

Fig E 7 a. Time course of fixation in air at the various carbon dioxide concentrations.

Fig E 7 b. Rate of fixation in air as a function of carbon dioxide concentration.

Fig E 8 Rate of photosynthetic carbon-14-dioxide fixation as a percentage of the rate in air at that carbon dioxide concentration.

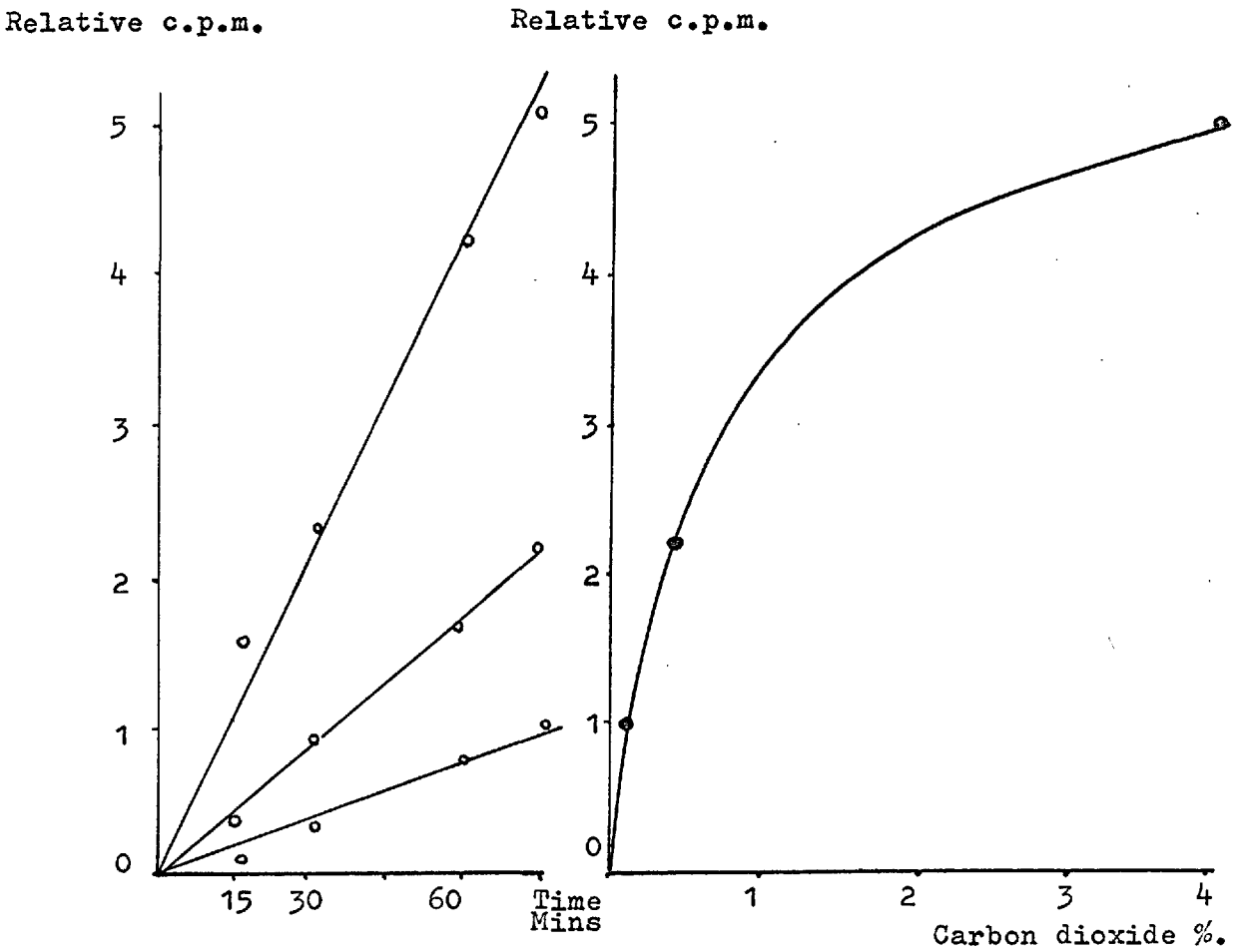


Fig E 7 a

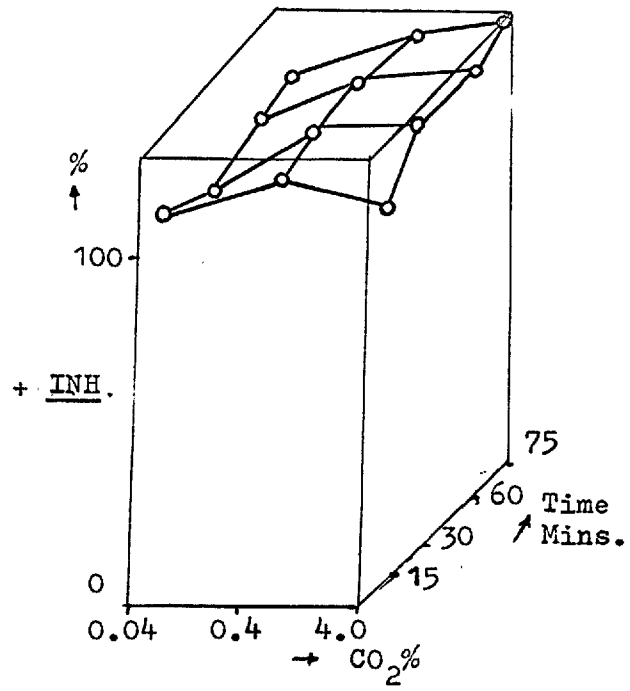
Time course of fixation in air.

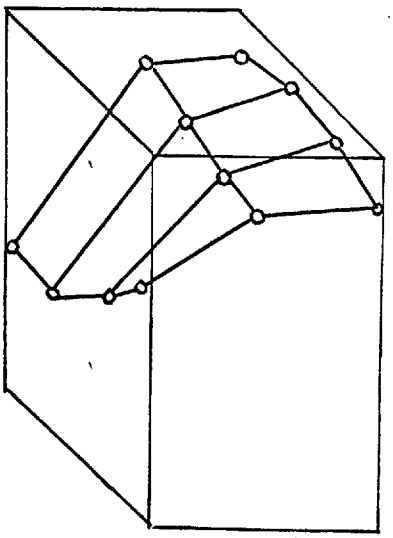
Fig E 7 b

Fixation in air as a function of carbon dioxide concentration.

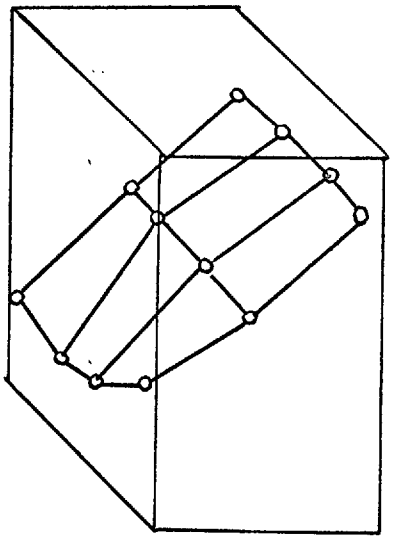
AIR

OXYGEN

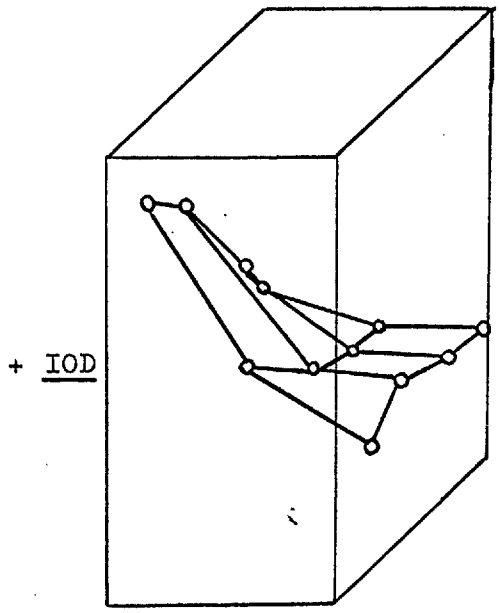




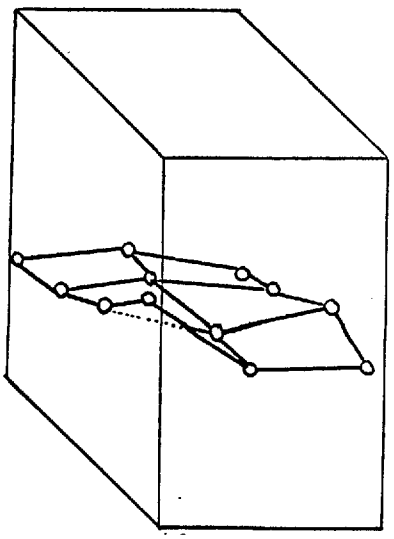
Control



+ INH



+ IOD



+ IOD

Fig E 8 Rate of fixation as a percentage of that in air.

concentrations of carbon dioxide. I.O.D. and high oxygen p.p. had a complimentary effect on the inhibition of photosynthesis, i.e. at low carbon dioxide concentrations the oxygen effect was seen and at the high concentrations of carbon dioxide, the I.O.D. effect was seen. This result would indicate that high p.p. of oxygen do not inhibit photosynthsis mearly as inhibitors of enzymes or reactions in which -SH groups are involved.

In Figs. E 9 to E 12 the percentage incorporation of radio activity into the given end product of photosynthesis is expressed as a function of carbon dioxide concentration and time, for the various treatments.

Over 95% of the total radioactivity fixed could be recovered from the following fractions:-

1. Ethanol Insoluble fraction.

The ethanol insoluble fractions from the various experiments were hydrolysed. (p. 38) The composition of this fraction was not found to be significantly altered by any of the recorded treatments. Where the incorporation of radioactivity into this fraction was decreased this could be accounted for mainly by a decrease in incorporation into glucose. Results, showing the distribution of radioactivity in the products of the degradation of the insoluble fraction from experiments with and without I.N.H. at air levels of carbon dioxide, are given on the following page. It can be seen that the main component of this fraction must have been a glucose polymer.

2. Glycollate

3. Sucrose The sucrose was hydrolysed by invertase to give two derivatives, with r.f's corresponding to glucose and fructose, in the pyridine solvent (p.37). These compounds gave the expected colour reactions when the chromatograms were sprayed with sugar reagents (p.35). The two spots were equally radioactive, i.e. the fructose and glucose were of the same specific activity.

4. Lipid Fraction The compounds which ran at the solvent front in both the phenol solvent and in the butanol/acetic solvent are termed the lipid fraction and included the photosynthetic pigments. This fraction could be separated from the ethanol soluble fraction in chloroform/ether.

In the air control the percentage incorporation of carbon-14 into the ethanol insoluble fraction was not affected by the carbon dioxide concentration, (Fig E 9), but was decreased by all other treatments when the cells were incubated with air levels of carbon dioxide. The degree of inhibition was fairly constant and independent of the duration of the treatment. As the carbon dioxide concentration was raised the inhibition of incorporation into the ethanol insoluble fraction decreased, and at 0.4% carbon dioxide in air/I.O.D. and in oxygen, and at 4.0% carbon dioxide in air/I.O.D., oxygen, and oxygen/I.N.H., was replaced by a stimulation of the percentage incorporation.

The percentage incorporation into sucrose (Fig E 10) was

Composition of the hydrolysed ethanol insoluble fraction from Chlorella after seventy minutes fixation of carbon dioxide-C^{14} in air (0.04 % carbon dioxide), with and without I.N.H. Results expressed as c.p.m. recovered from the 2-D chromatogram.

First hydrolysis	Spot no.	Compound	Control	+I.N.H.
Carbohydrates.	1	U.K	107	92
	2	"	49	tr
	3	"	214	72
	4	"	314	222
	5	"	276	106
	6	Glucose	26356	15071
	7	U.K.	1211	1766
	8	Fructose	689	420
	9	U.K.	139	tr
	10	"	986	727
	11	"	563	272
	12	"	364	tr
Second hydrolysis	1	"	47	tr
	2	Aspartate	tr	tr
	3	Glutamate	100	87
	4	Serine	260	260
	5	Glycine	1360	1271
	6	U.K	190	266
	7	Alanine	180	100
	8	U.K	70	tr
	9	"	180	115

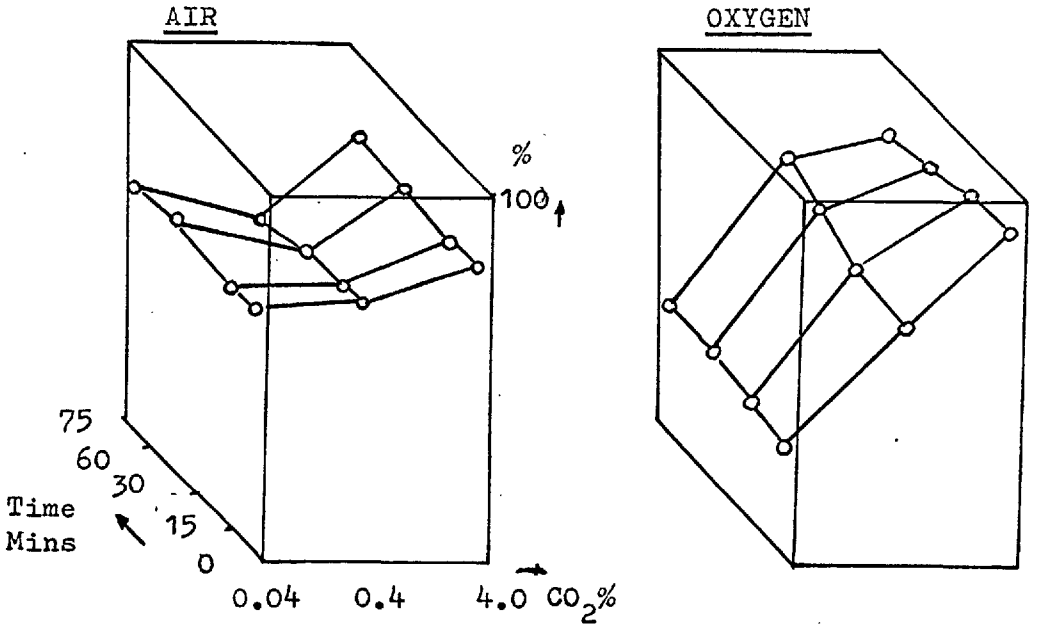
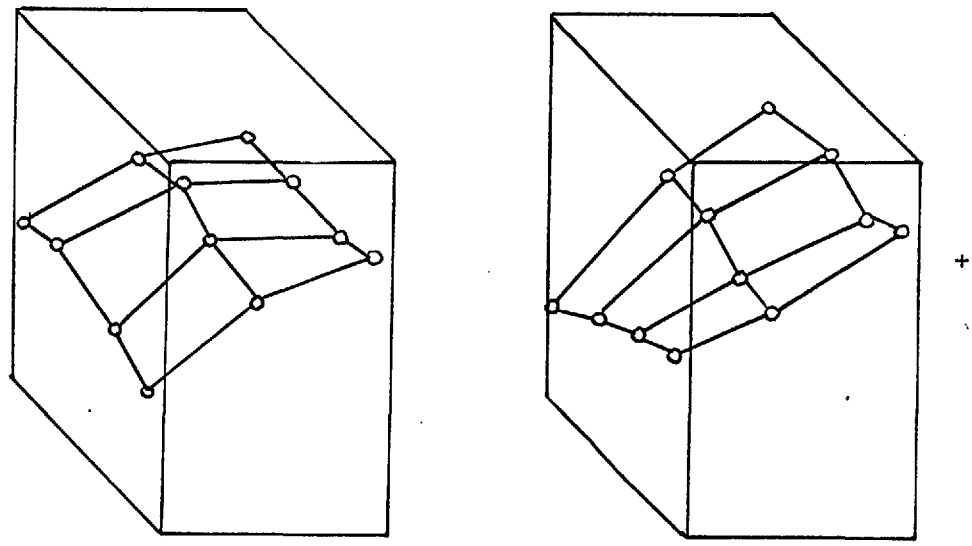
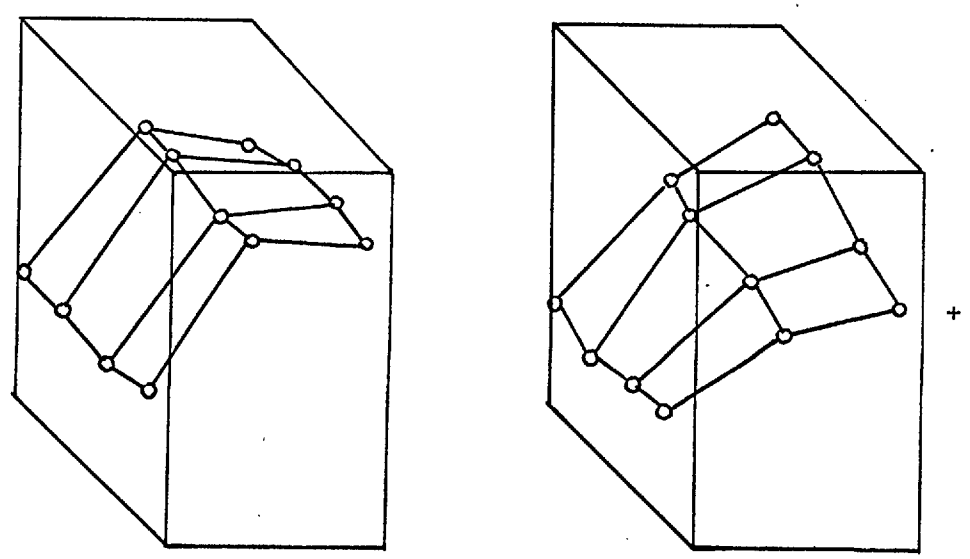


Fig E 9 Percentage incorporation into ethanol insolubles





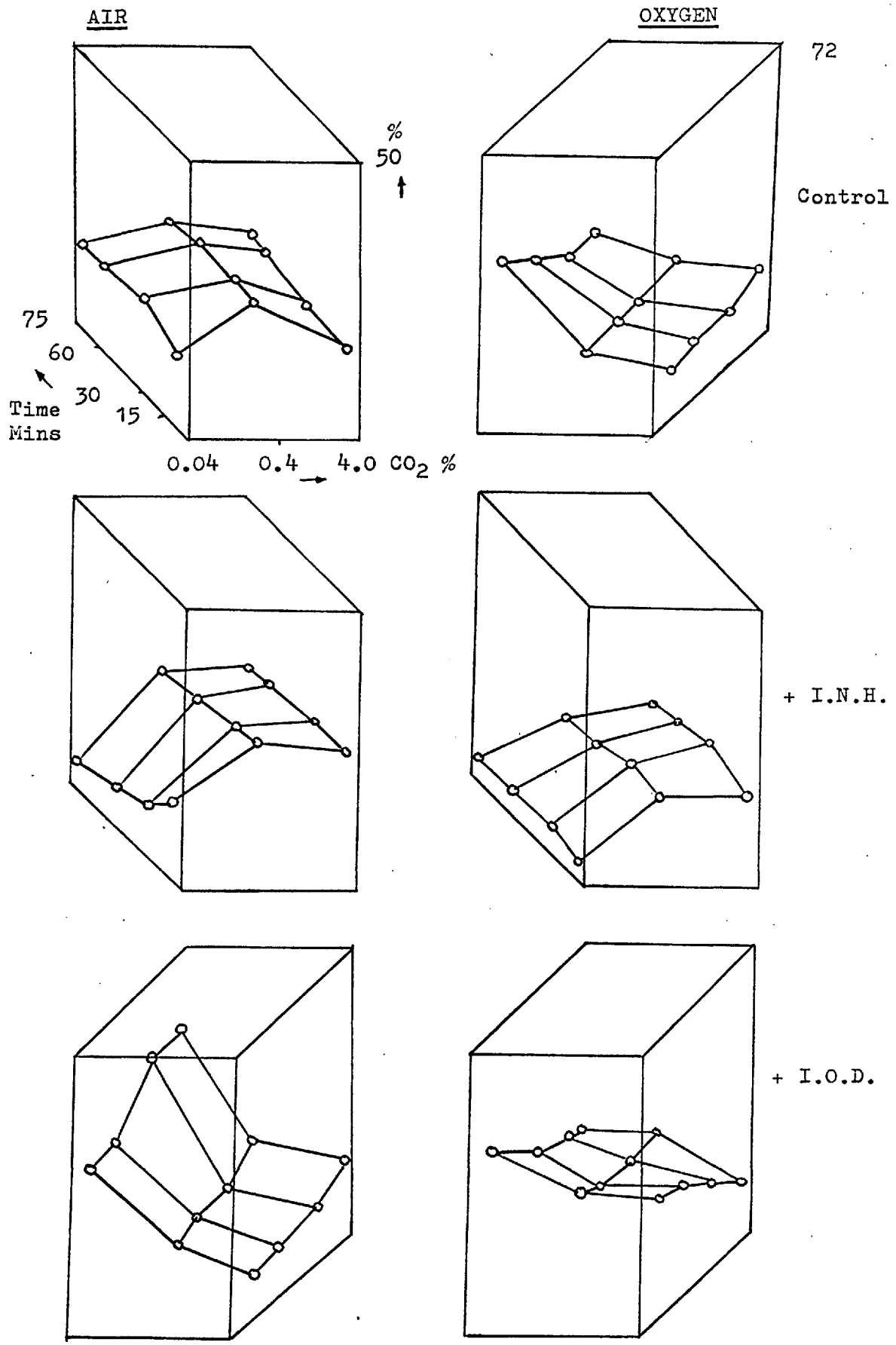


Fig E 10, Percentage incorporation into sucrose.

stimulated at 0.04 percent carbon dioxide by I.O.D. in air and by high oxygen p.p., and inhibited by I.N.H. in both air and oxygen. As the carbon dioxide concentration was raised these effects became less marked. At 4.0 percent carbon dioxide the percentage of the radioactivity incorporated into sucrose was decreased by both high p.p. of oxygen and by I.O.D., and stimulated by I.N.H.

In the air control the production of glycollate was negligible, under these conditions, even at low concentrations of carbon dioxide. (Fig E 11). However the addition of I.N.H. or raising the oxygen p.p., caused a marked increase in the incorporation of radioactivity into glycollate. I.O.D. caused an initial stimulation of the percentage incorporation into glycollate, at the lowest carbon dioxide concentration, this decreased with length of time of incubation with this inhibitor. In high p.p. of oxygen I.N.H. also enhanced the production of glycollate at 0.4 percent carbon dioxide. At the highest concentration of carbon dioxide the incorporation of radioactive carbon dioxide into glycollate was not enhanced by any of the given treatments.

At low concentrations of carbon dioxide the percentage incorporation of radioactivity into lipids was inhibited by both I.N.H. and high oxygen p.p. (Fig E 12) At other carbon dioxide concentrations and with other treatments the incorporation into lipids was not affected.

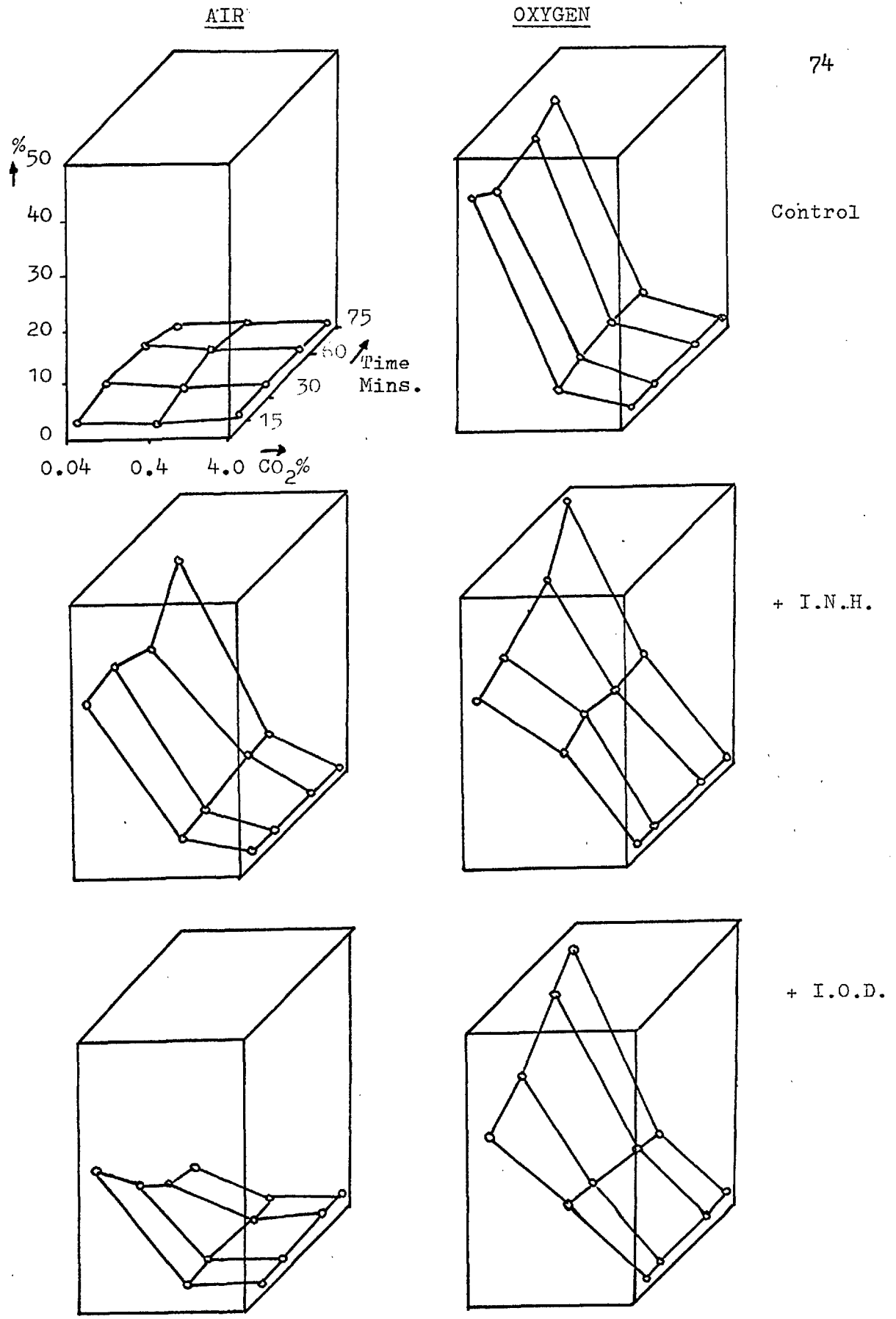


Fig E 11. Percentage incorporation into glycollate.

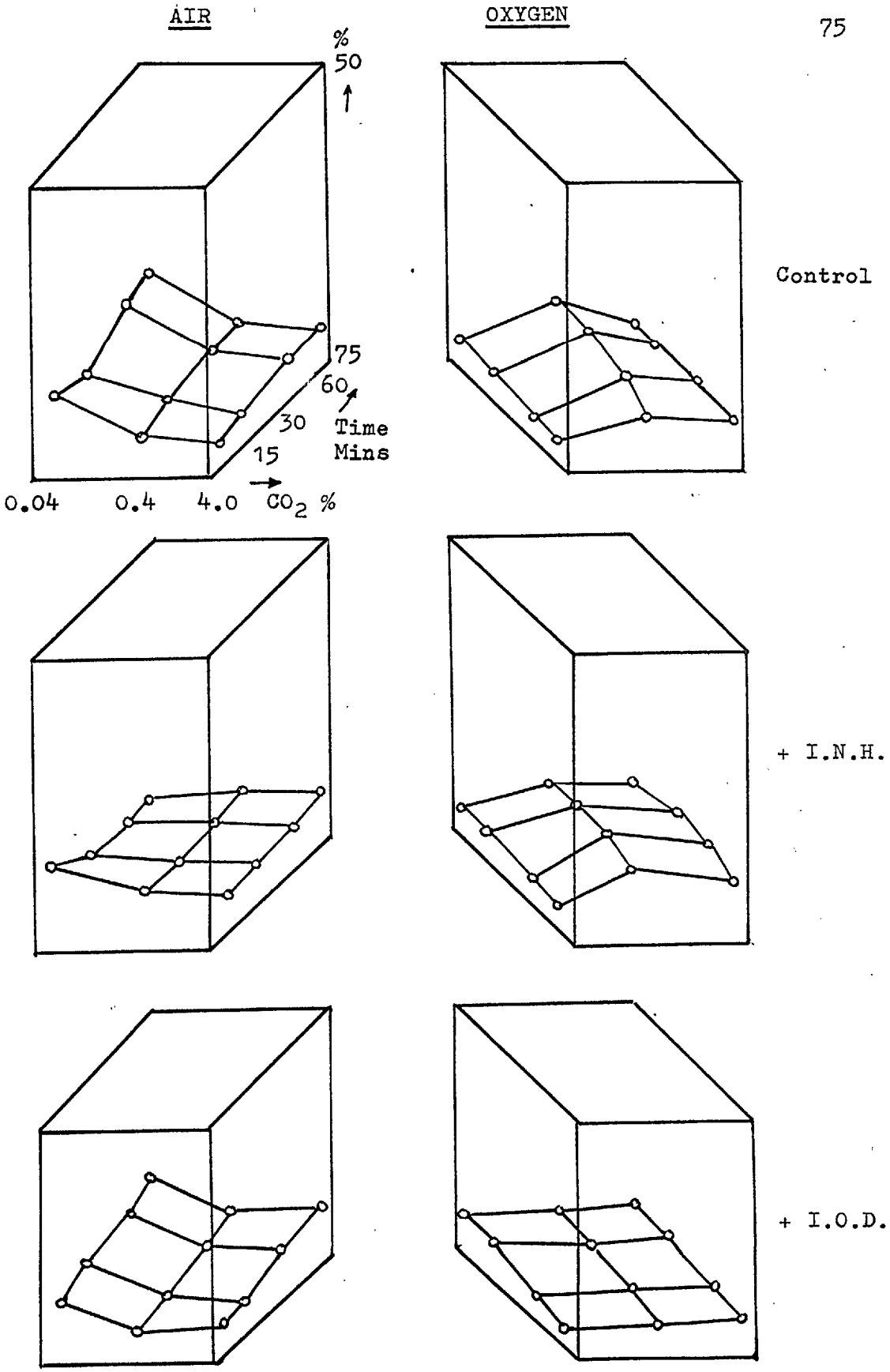


Fig E 12 Percentage incorporation into lipids.

From these results it can be seen that the major differences in the percentage distribution of radioactivity fixed into end products of photosynthesis occured only at the lowest concentration of carbon dioxide. Further discussion will therefore be restricted to results obtained under these conditions.

The following table (p. 77) shows the effects of the various treatments on the amount of radioactivity incorporated into the sugar phosphates, carboxylic acids and amino acids after seventy minutes photosynthesis at air levels of carbon dioxide. With the exception of glycine the various compounds had reached a steady level of activity in this time, and hence a comparison of the radioactivity fixed into a given compound with a given treatment gives an indication of the relative pool sizes.

The only sugar phosphate significantly reduced by the addition of I.N.H. was phospho enol pyruvate. Other treatments decreased the incorporation of carbon dioxide-C^{14} into all the sugarphosphates with the exception of monophosphate and U.D.P.G. in air/I.O.D.

The incorporation into malate was reduced by high p.p. of oxygen, whereas that into fumarate was increased. Fumarate was also increased by I.N.H. as was the incorporation into succinate.

The steady state level of activity in aspartate, glutamate, and alanine was decreased by both inhibitors and by high p.p of oxygen.

Glycine and/or serine did not reach a steady level of activity in this time. The amount of glycine formed was greatly increased by the addition of I.N.H. Those treatments, i.e. oxygen and I.O.D. which increase the formation of glycollate without apparently

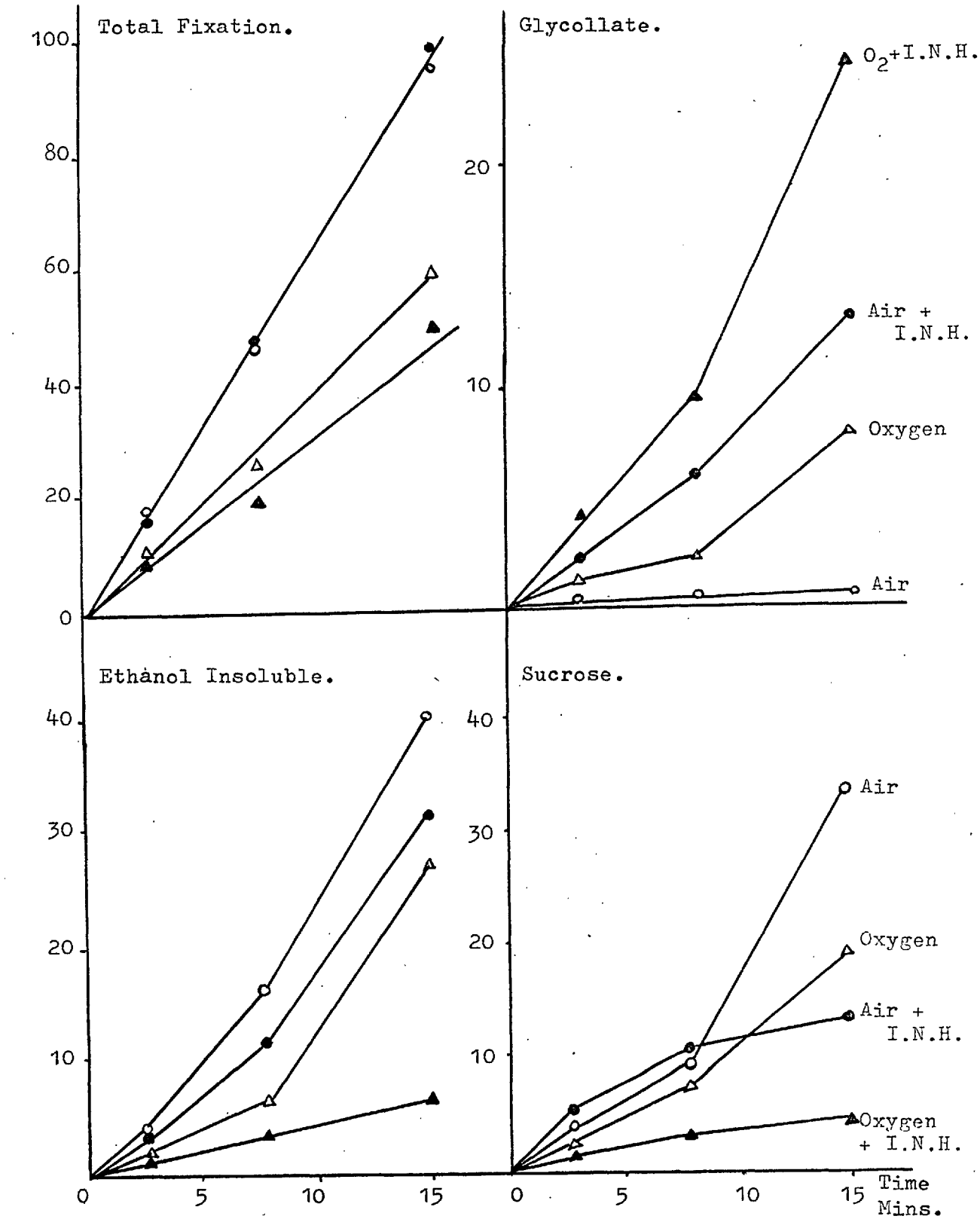
The incorporation of carbon-14 into various photosynthetic intermediates after seventy minutes photosynthesis in air and oxygen at air levels of carbon dioxide, with and without I.N.H. and I.O.D. (Arbitrary units based on the c.p.m. recovered from the paper chromatograms)

	AIR			OXYGEN		
	CON	INH	IOD	CON	INH	IOD
Di- P	3.39	3.36	2.99	1.23	0.50	tr
Mono-P	9.21	8.31	8.23	2.60	3.54	1.63
UDPG	1.70	1.85	2.60	.30	.28	.33
PEP	.98	.26	.19	.39	.25	.49
PGA	5.70	4.80	1.43	1.21	1.42	.62
Triose	2.00	1.70	.46	.60	.39	.72
Malate	1.30	1.39	1.67	.64	.59	.10
Fumarate	tr	.91	.68	.37	.12	.17
Succinate	tr	3.23	1.37	tr	1.08	tr
Glycine	1.30	49.97	3.50	9.8	16.58	4.36
Serine	2.3	.31	4.24			1.10
Aspartate	3.51	1.05	2.63	3.03	.26	.50
Glutamate	10.38	4.42	6.91	2.37	1.52	2.03
Alanine	4.03	2.17	2.37	2.10	1.20	1.25

affecting its further metabolism, caused an increase in both glycine and serine.

The incorporation of radioactive carbon dioxide was further investigated by the automatic injection technique over periods of up to fifteen minutes at air levels of carbon dioxide. (Fig E 13) The results of these experiments again indicated that carbon which in the air control was incorporated into sucrose and into poly-glucan, was incorporated into glycollate in the presence of I.N.H. or high oxygen p.p. The stimulated formation of sucrose production induced by the presence of I.N.H., which was noted in the short term experiments previously reported, was again seen. The stimulated sucrose formation occurred during the first seven minutes of fixation, after this time the sucrose became inhibited by I.N.H.

Fig E 13 Total fixation and c.p.m. incorporated into end products over fifteen minutes photosynthesis in air and oxygen, with and without I.N.H., at air levels of carbon dioxide.



Changes in the photometabolism of carbon-14-dioxide by Chlorella during transitions in the gas phase.

1. Changes in total fixation of carbon-14-dioxide and the distribution of radioactivity into products of photosynthesis during transitions from air to oxygen and from oxygen to air, studied using the test tube injection technique.

Where possible, in all these transition experiments the gas stream from the lollipop was monitored, using an infra-red carbon dioxide gas analyser. However the response time of this instrument was too long to enable any useful information to be obtained from the shape of the transient curve recorded for the onset of inhibition of photosynthesis by high p.p. of oxygen. The final steady state level of inhibition of photosynthesis measured in this way was comparable with that measured in terms of carbon dioxide fixation.

a. The effect of light intensity on the degree of inhibition of photofixation of carbon-14-dioxide by high p.p. of oxygen.

Due to the number of samples that had to be taken it was not possible to use cells from the same batch of cultures for the complete investigation into the effect of light intensity on the transitions. Hence the results from an experiment, with cells from the same culture, in which the change in total fixation of radioactive carbon dioxide after the transition from air to oxygen was investigated at various light intensities, are first presented. Fig E 14. The second Fig (Fig E 15) shows the percentage inhibition of photosynthesis by high oxygen p.p. at various times after the transition from air to oxygen and from

Figs 14 to 19. The effect of light intensity on the total fixation of carbon-14-dioxide ,and the distribution of radioactivity into products of photosynthesis, during transitions of gas phase, from air to oxygen and from oxygen to air.

50 mls of Chlorella were suspended at a density of 5 µl per ml. in phosphate buffer and preincubated for 30 minutes in the required gas mixture, at the required light intensity. A ten ml sample was run into a test tube ,sealed with a 'Suba Seal' and injected with 3 µC of sodium carbonate-C-14. The cells were killed after one minute photosynthesis in the closed tube. The gas flowing through the lollipop was changed and similar samples taken at various time intervals up to 30 minutes after the gas stream had been changed. Since different batches of cells were used for the experiments reported in Figs 16 to 19 the results have been expressed as a percentage ,as follows:-

$$\frac{\text{c.p.m. fixed into a given compound for a given sample} \times 100}{\text{Total c.p.m. fixed in the air sample at that light intensity}}$$

OPEN SYMBOLS = Without I.N.H. CLOSED SYMBOLS = With I.N.H.

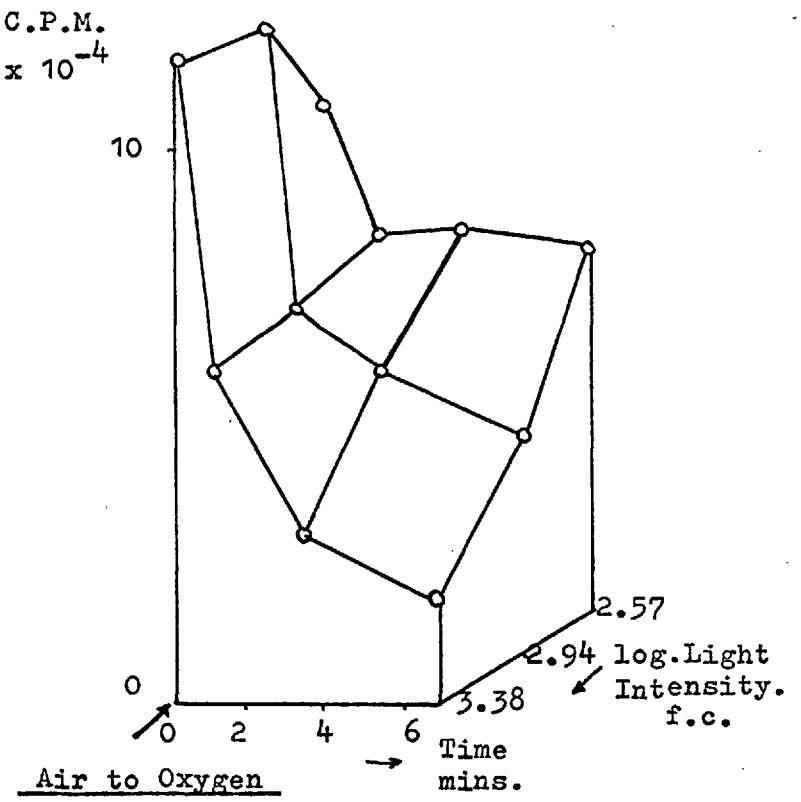


Fig E 14. The effect of light intensity on the rate of photofixation of carbon-14-dioxide by Chlorella, after the transition from air to oxygen.

oxygen to air, with and without I.N.H. at the various light intensities. These results were obtained from experiments in which cells from a number of different cultures were used.

Myers 1940 has shown that photosynthesis in very dilute suspensions of Chlorella may be markedly inhibited by high light intensities; this effect increased with the duration of the illumination. The following control experiment was therefore performed. The total fixation of carbon-14-dioxide was measured by the test tube injection technique, after 30 and 60 minutes exposure at the given light intensity in air, results as follows were obtained. (c.f. p.60)

Light intensity f.c.	Time of illumination 30 mins	60 mins	
370	85.7	87.1	c.p.m. x 10^{-3}
860	118.6	110.9	
2340	109.0	103.2	

It can be seen that under these conditions light induced inhibition of photosynthetic carbon dioxide fixation did not occur.

The results shown in Figs E 14 and E 15 indicated that as the light intensity was increased the inhibition of photosynthesis by high oxygen p.p. also increased. At low light intensities I.N.H. tended to lower the degree of inhibition, and to increase the rate of recovery after the cells had been returned to air. At the highest light intensity the inhibition appeared to become irreversible; little increase in photosynthesis was apparent after the cells were returned to air.

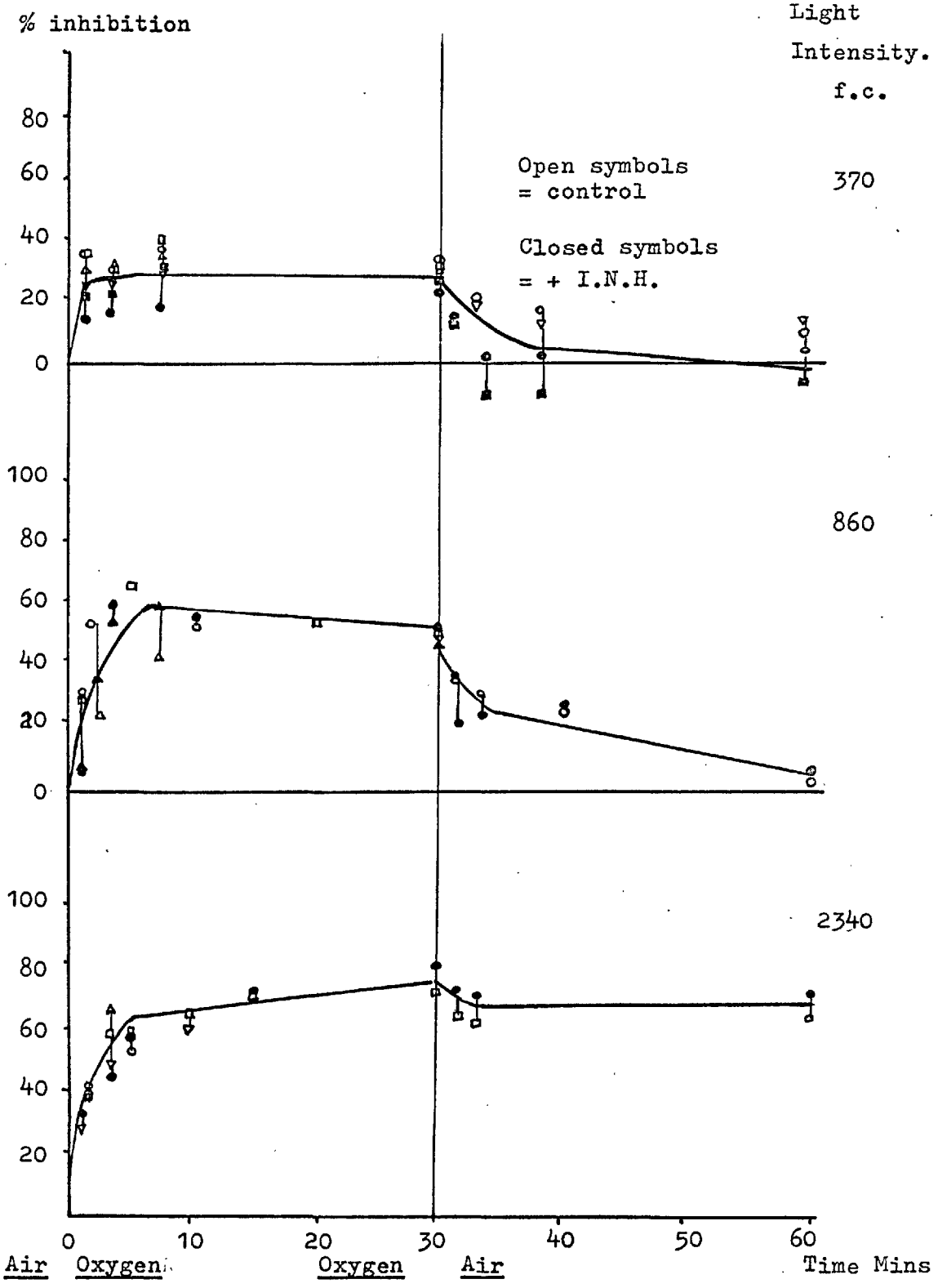


Fig E 15. The effect of light intensity on the percentage inhibition of carbon-14-dioxide fixation by Chlorella during transitions from air to oxygen to air.

b. Changes in the distribution of radioactivity into photosynthetic products during the transitions.

The sugar mono and di-phosphates.(Fig E 16.)

After the air to oxygen transition, at the lower light intensities the incorporation of carbon -14- dioxide into the sugar phosphate fraction dropped to reach a new steady level after about ten minutes. At the highest light intensity the incorporation into the sugar phosphates continued to drop throughout the experimental period. At low light intensities , on return to air the incorporation into the sugar phosphates recovered to reach the pre-transient level, the rate of recovery was stimulated by the addition of I.N.H. At the highest light intensity there was only a slight recovery on return to air.

These results reflect those shown in Fig E 15, for the percentage inhibition of photosynthesis by oxygen at the various light intensities, and therefore suggest that the amount of fixation at any time after the transition is dependent on the amount of sugar phosphate present at that time. The drop in rate of photosynthesis after the transition to oxygen could be caused by the oxidation of some intermediate resulting in a lowering of the size of the sugar phosphate pools. On return to air this oxidation would cease, and the pools would increase in size leading to a recovery of photosynthesis. Presumably at the highest light intensity a secondary oxidation led to irreversible damage of the photosynthetic apparatus.

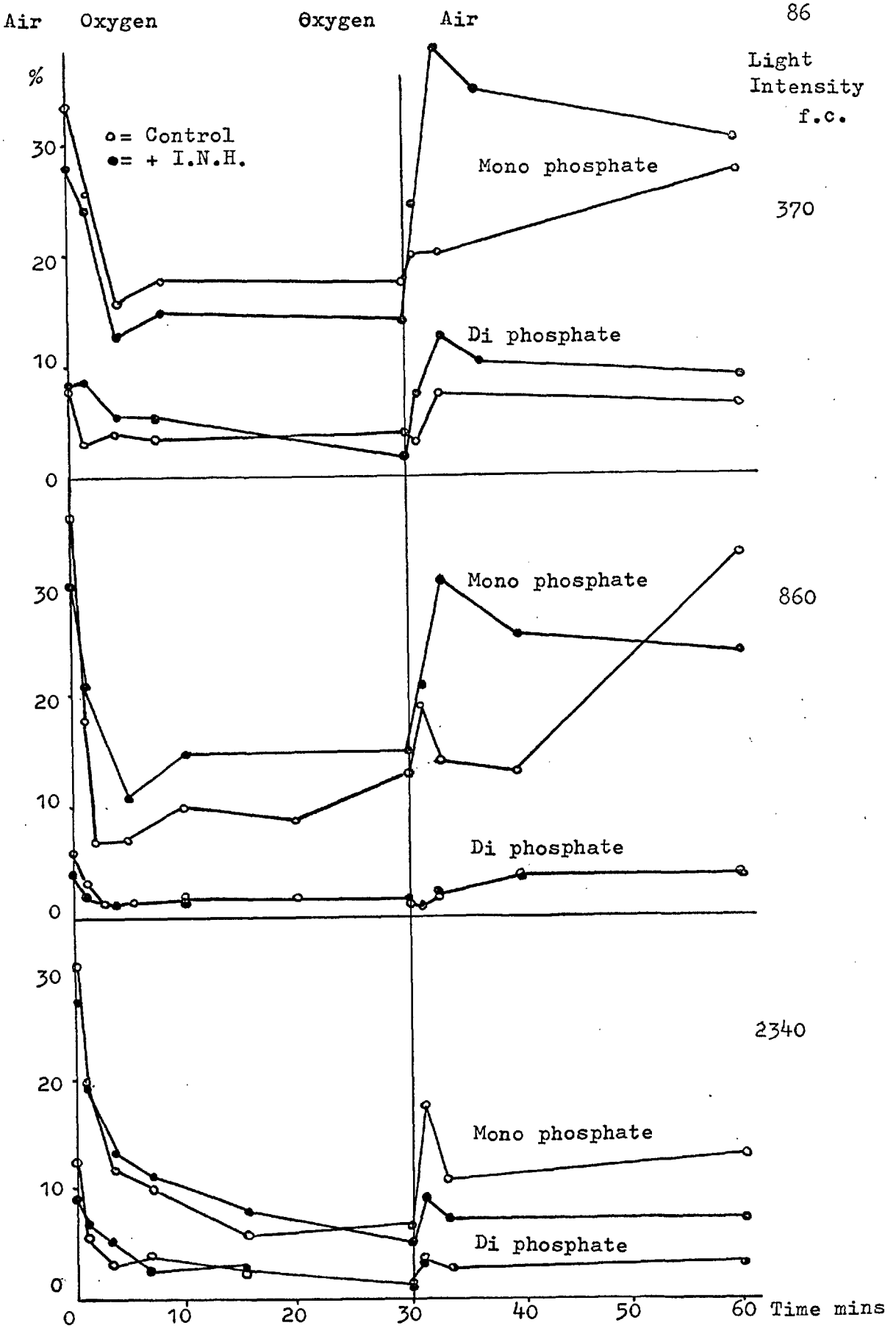


Fig E 16. Incorporation into sugar phosphates during the transitions.

Phosphoglyceric acid and alanine (Fig E 17)

The changes in incorporation into these compounds, after the transitions, are similar to those reported for the incorporation into the sugar phosphates i.e. the rate at which incorporation drops after the transition from air to oxygen was increased by increased light intensity, and the rate of recovery, on return to air decreased as the light intensity was raised.

Aspartate/Malate/Fumarate. (Fig E 18 a)

The changes in incorporation of radioactive carbon dioxide into these compounds again reflected the changes in total fixation.

Glycine and Glycollate (Fig E 18 b)

The formation of glycine/glycollate showed a peak at the second (saturating) light intensity. At all light intensities the incorporation of radioactivity into this fraction was increased by the addition of I.N.H. After the transition to oxygen incorporation into this fraction was increased at all light intensities, this was reversed on return to air. In oxygen at the highest light intensity there was a decrease in glycollate formation, as compared with that at a lower light intensity, even when I.N.H. was added.

Sucrose (Fig E 19 a)

In these experiments the short term stimulated formation of sucrose on the addition of I.N.H. was again seen in the air controls. After the transition to oxygen, at the lower light intensities, there was a drop in incorporation into this fraction, followed by an increase, which was I.N.H. sensitive. The decrease

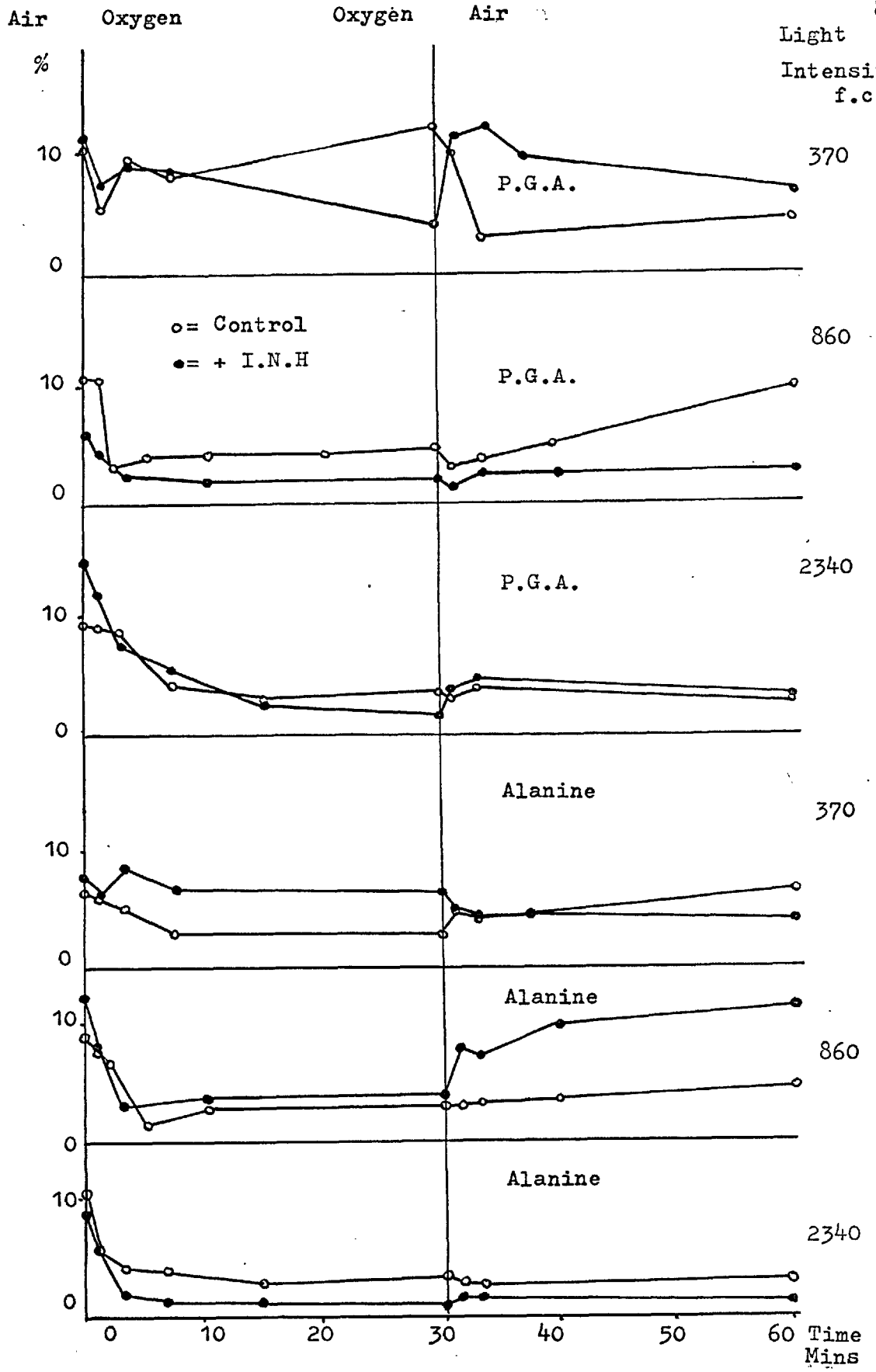


Fig E 17. Incorporation into P.G.A. and alanine .

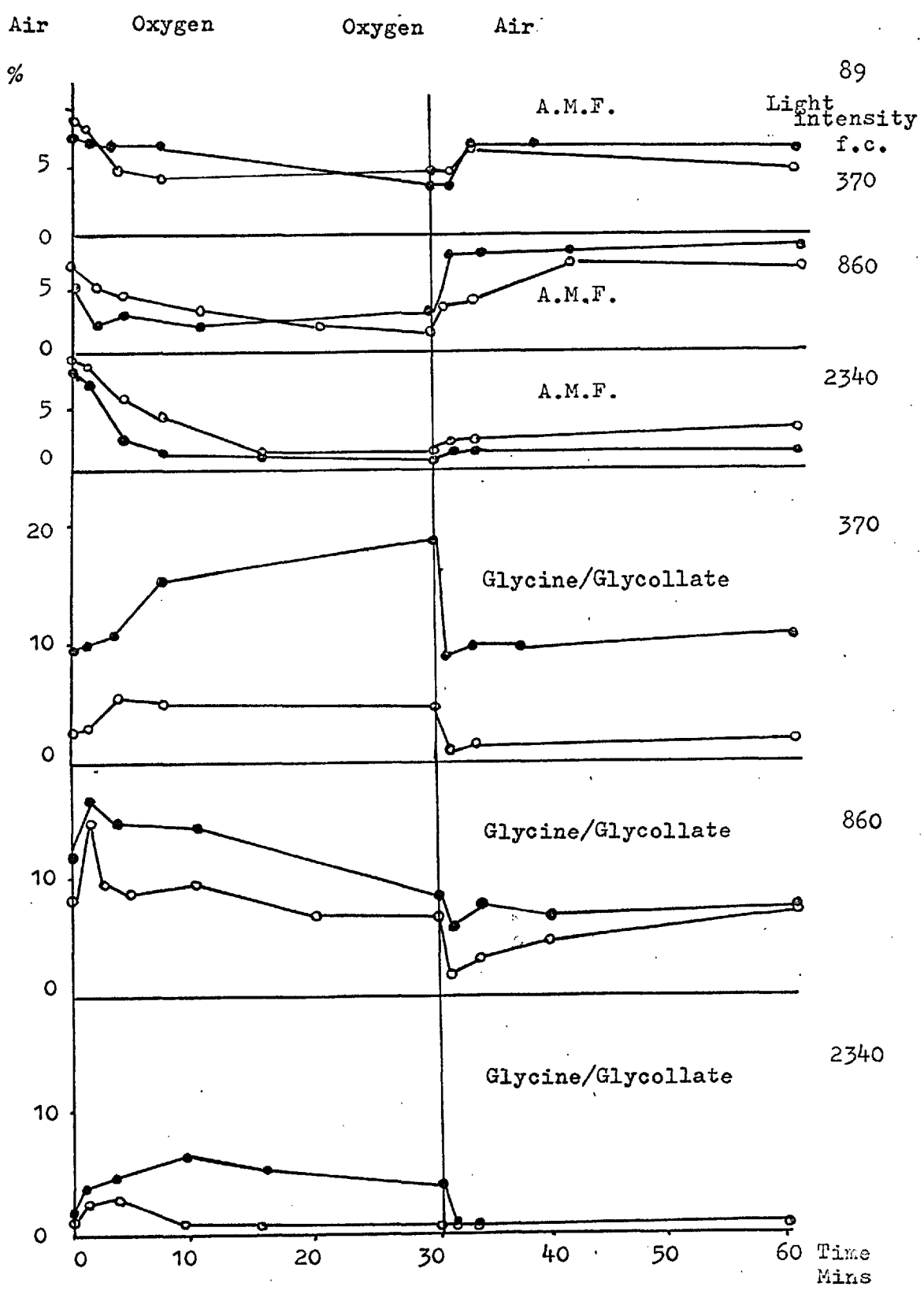


Fig E 18. Incorporation into amino and other acids.

o = Control ● = + I.N.H

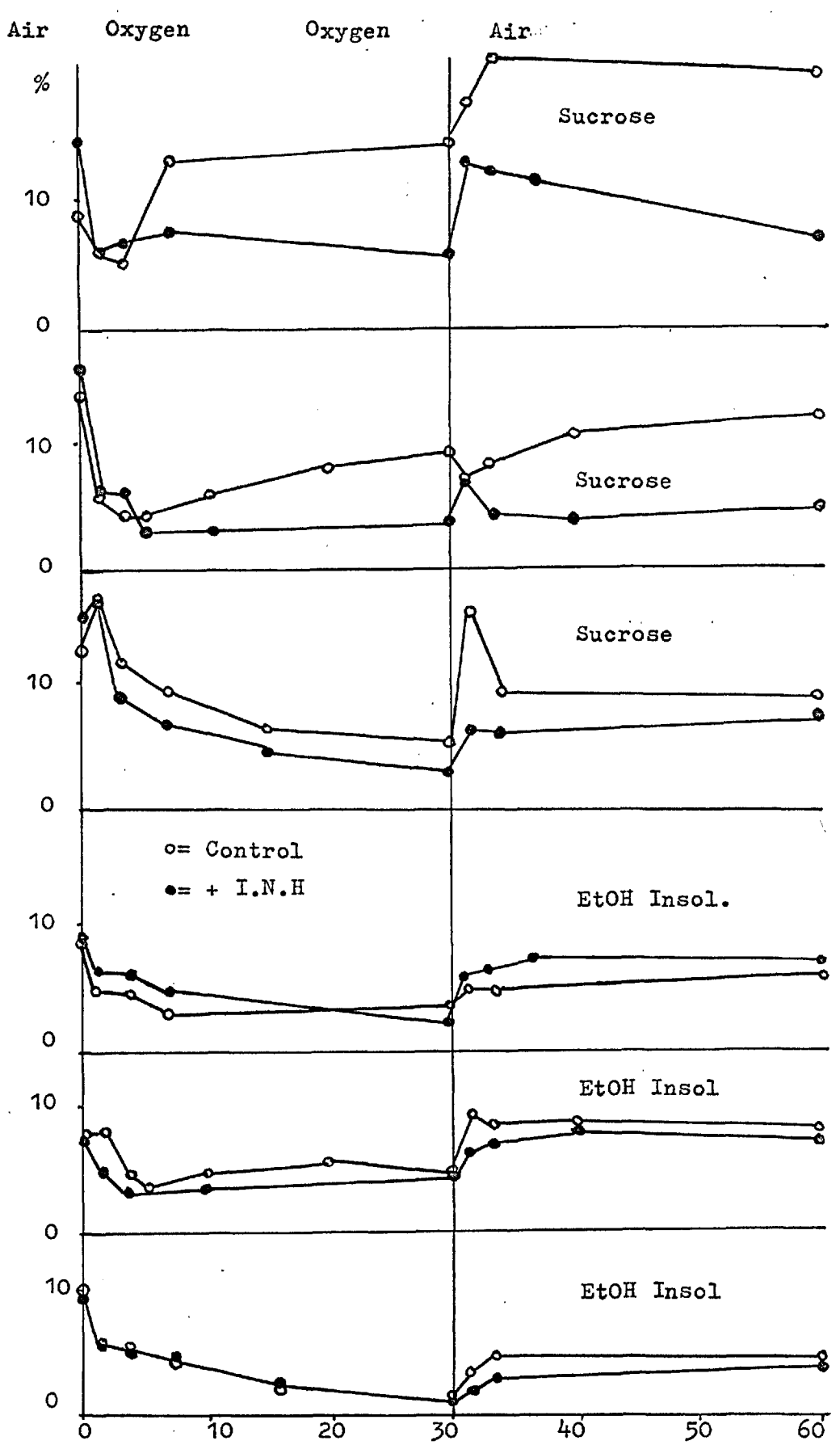


Fig E 19. Incorporation into carbohydrates.

in radioactivity incorporated into sucrose in oxygen/I.N.H. could be accounted for in terms of the increased incorporation into the glycine/glycollate fraction. On return to air the incorporation of radioactivity into sucrose increased. This sucrose was still I.N.H. sensitive, but in this case the difference was accountable for in the I.N.H. stimulated sugar phosphates. At the highest light intensity the incorporation into sucrose reflected the results obtained for the total fixation of carbon-14-dioxide.

Ethanol Insoluble compounds.

The incorporation of activity into the ethanol insoluble fraction reflected the changes in total fixation.

These results suggest that there is a close relationship between the inhibition of photosynthesis by high p.p. of oxygen and the oxidation of an intermediate of the P.C.R. cycle leading to a decrease in the size of the sugar phosphate pools and an increase in glycollate production. This was further investigated in experiments in which the effect of oxygen on the steady state pool sizes was studied.

2, Changes in the rate of photosynthesis and of glycollate production during transitions from air to oxygen,using the steady state injection technique.

It was found that in the long term steady state experiments previously presented (p.64 to 79) that after about fifteen minutes photosynthesis in carbon -14- dioxide the sugar phosphate

pools reached a steady level of radioactivity. It was assumed that this was reached when the sugar phosphates were uniformly labelled, and hence the radioactivity recovered from these compounds was a measure of the pool sizes of the sugar phosphates.

Hence the cells were allowed to fix carbon-14-dioxide in air until this level had been reached, the gas phase was then changed to oxygen and the changes in level of activity in a given compound taken as an indication of the change in pool size.

Fig E 20 shows the change in rate of photofixation of carbon dioxide ,and of excretion of radioactive glycollate, after the transition from air to oxygen. For convenience the time scale is expanded after the transition.

From this figure it can be seen that after the transition to oxygen the rate of fixation of carbon-14-dioxide dropped rapidly, this drop in fixation was accompanied by an increase in the rate of excretion of glycollate-C^{14}. The rate of fixation reached a new steady rate three to ten minutes after the transition to oxygen. Neither the new rate of fixation, or the equilibration time was affected by the addition of I.N.H. The rate of glycollate production increased, as the rate of fixation dropped, to reach a peak value about one minute after the transition to oxygen, and then dropped to reach a new level slightly higher than that in air. The peak rate of glycollate-C^{14} production was increased by the addition of I.N.H., and was in excess of the rate of fixation of carbon-14-dioxide at this time. This would indicate that some metabolic intermediate was being oxidised to glycollate-C^{14}.

Fig E 20 to E 22. Changes in the steady state rate of photosynthesis, and metabolism of carbon, during transitions from air to oxygen.

Fifty mls of Chlorella suspended at a density of 5µl per ml in phosphate buffer were injected with 10 µC of sodium carbonate-C-14 using the automatic syringe. (p. 30) One ml samples were taken at intervals of about ten seconds. In some experiments the samples were centrifuged prior to killing, in others the supernatant was removed by filtration. The radioactivity in the supernatant was measured. The supernatant was estimated for glycollate using Calkins method. The rates of fixation and of glycollate-C-14 production were determined from the average of five duplicate planchette counts. The rates were expressed in arbitrary units obtained by dividing the difference in c.p.m. between two succesive samples by the time in seconds between sampling.

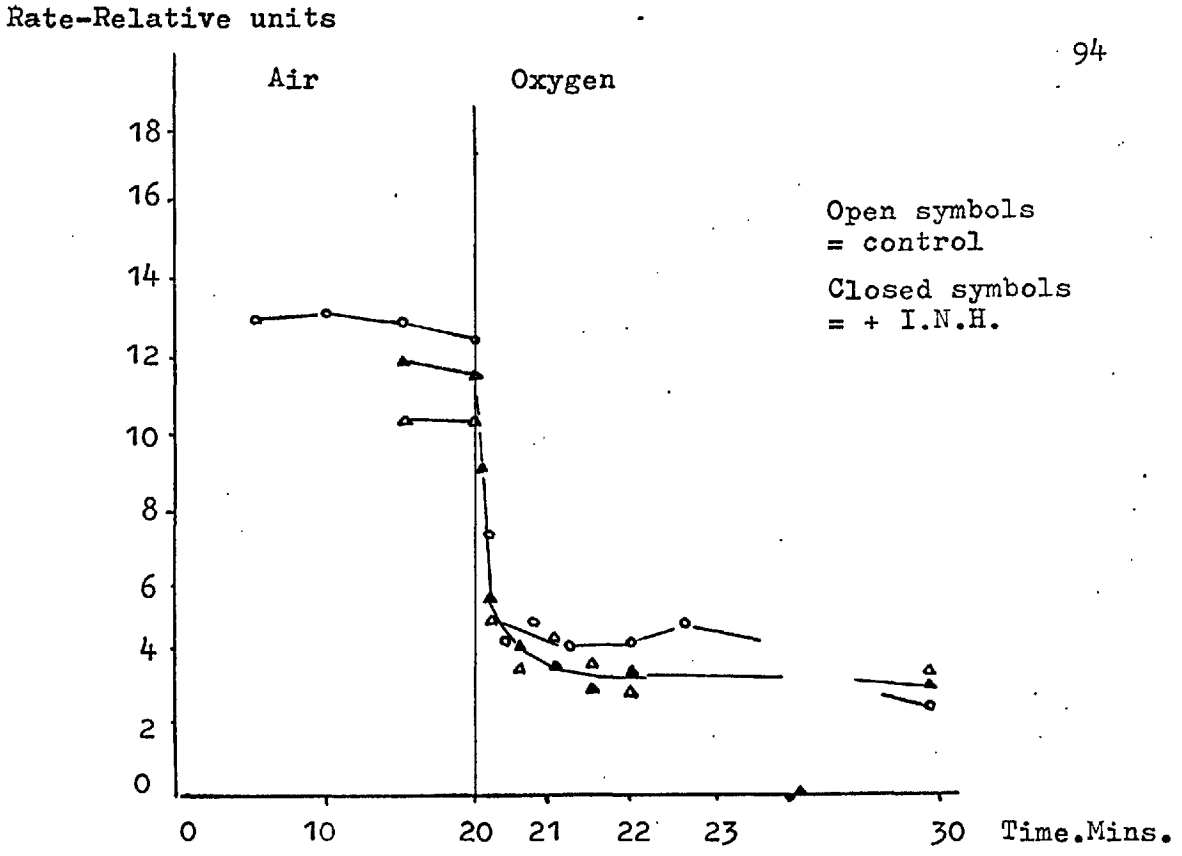


Fig E 20 a. Rate of fixation of carbon-14-dioxide.

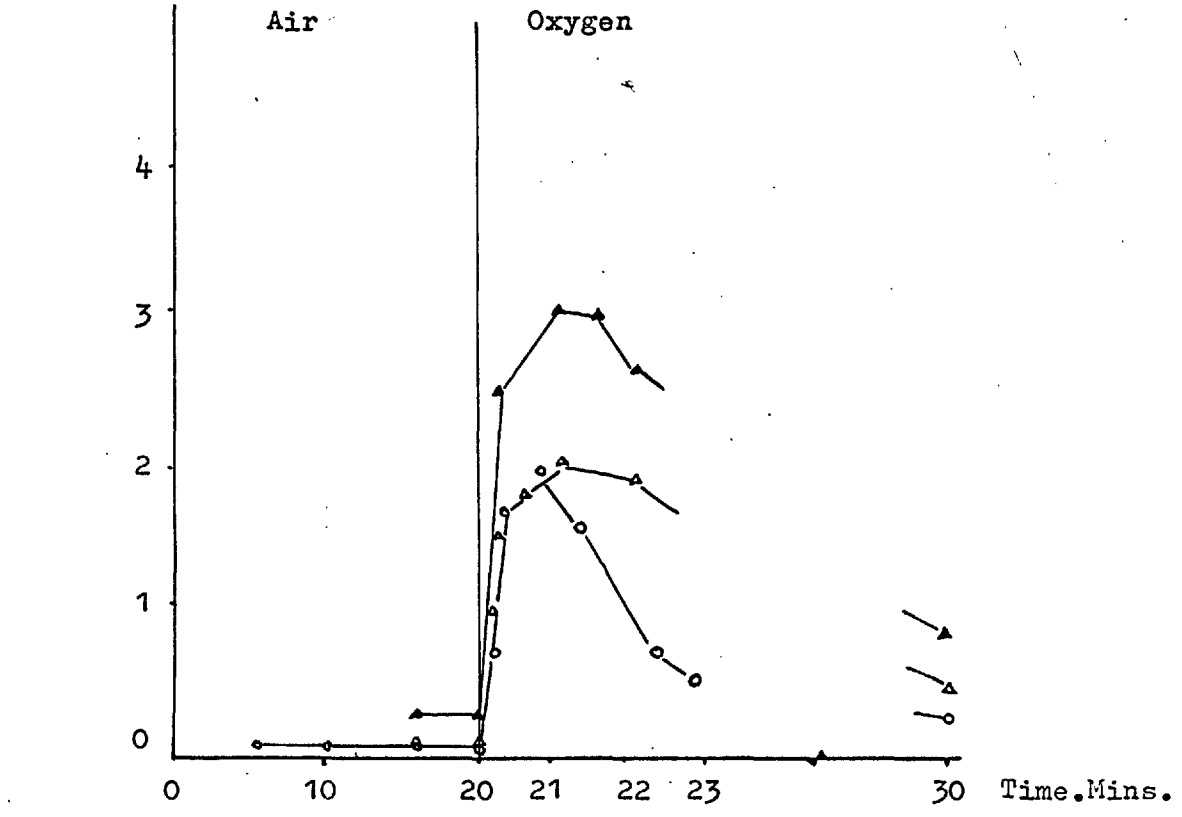


Fig E 20 b. Rate of glycollate-C-14 excretion.

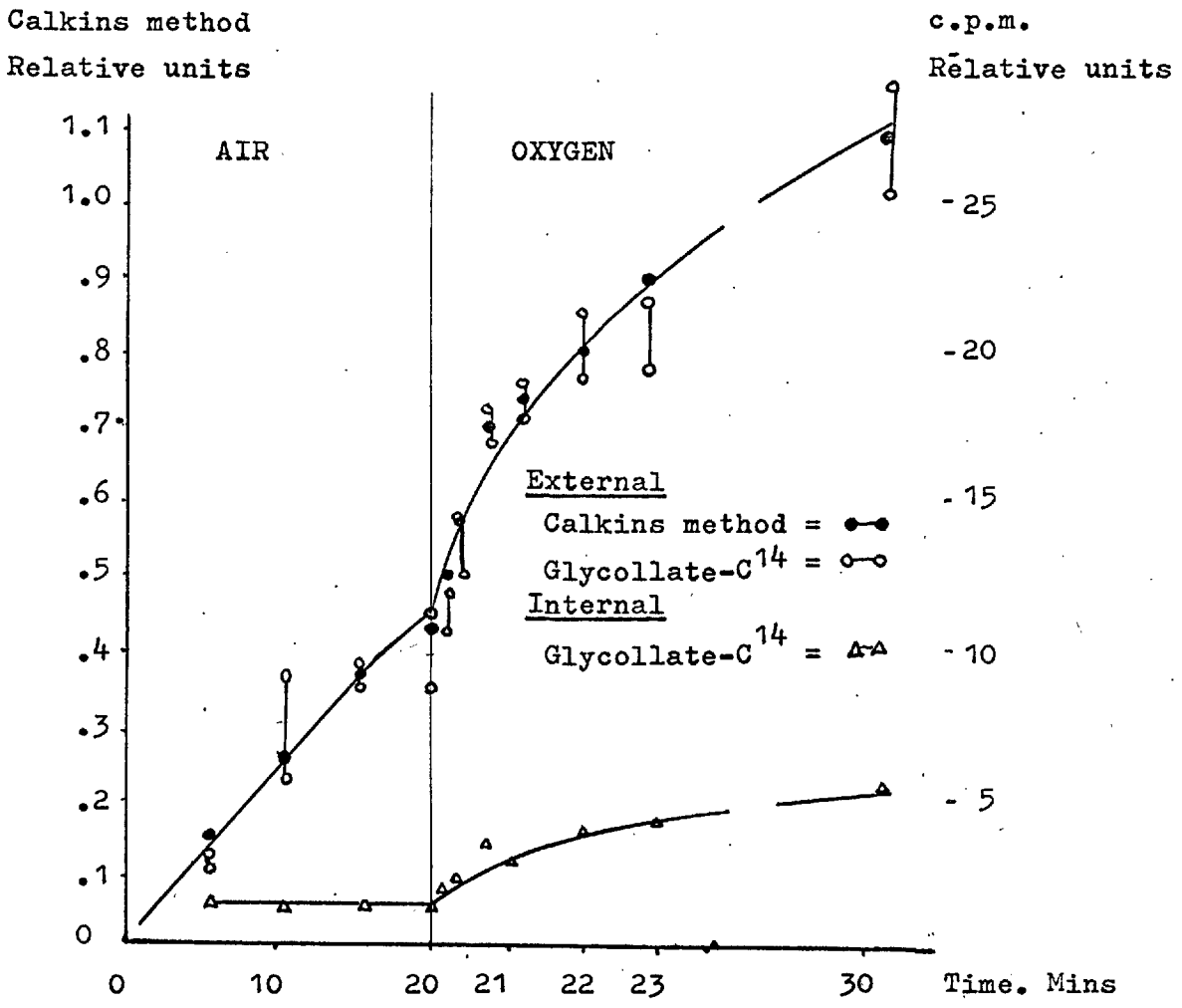


Fig E 21. Glycollate production during the transition from air to oxygen determined as glycollate-C^{14} and by Calkins method.

In the figure just discussed the data for glycollate production is presented on the assumption that glycollate is the only compound excreted under these conditions, and that most of the glycollate formed is excreted. Fig.E 21 shows the results of an experiment performed to test this hypothesis. In this figure the external c.p.m. and the readings from the spectrometer used in the estimation of glycollate by Calkins method, obtained from the supernatant of samples from a transition experiment of this type are plotted on scales such that the values at the point of transition coincided. The radioactive glycollate obtained from the cells after 2-D paper chromatography (internal glycollate) is also shown. There is close agreement between the two graphs for external glycollate, indicating that glycollate-C^{14} is the only radioactive product excreted. It was found that in air there was a constant internal pool of glycollate, whilst the external glycollate increased regularly throughout the experimental period. The internal glycollate also increased after the transition to oxygen,although for a shorter time. The final level of internal glycollate was about three times that in air.

3. Changes in the steady state pool sizes, during transitions from air to oxygen , using the steady state injection technique.

Fig E 22 shows the effects of changing the gas stream from air to oxygen on the steady state pool size of various photosynthetic intermediates. This method was only suitable for investigations into the changes in incorporation into intermediates which showed

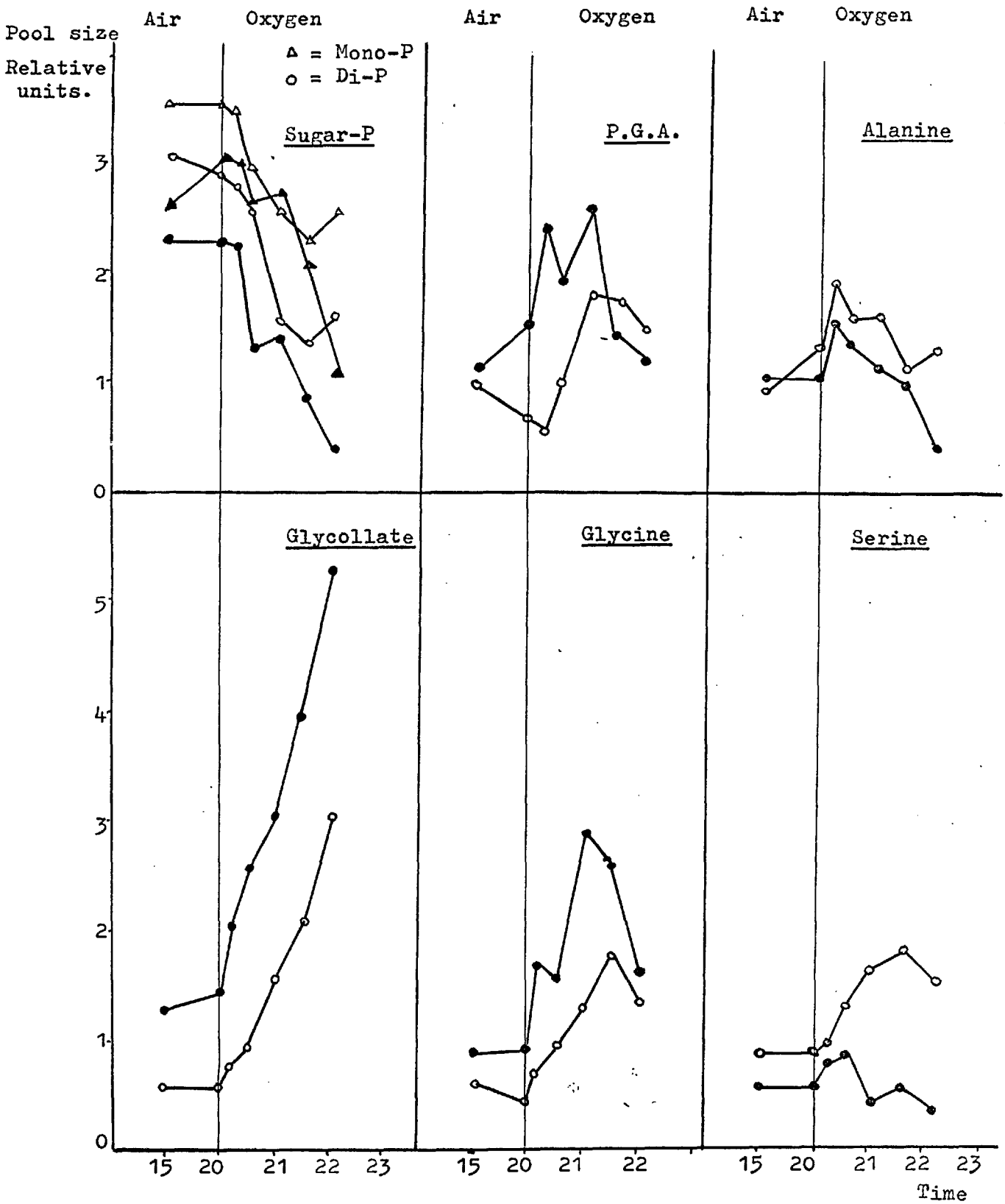


Fig. E 22. The effect of high oxygen p.p. on the pool size of various photosynthetic intermediates.

Open symbols=control Closed symbols=+I.N.H

a large percentage change after the transition. Changes could not be seen in end products such as sucrose, ethanol insolubles and lipids.

The size of the sugar phosphate pools decreased rapidly after the transition to oxygen. The drop in di-phosphate was greater than that in mono-phosphate. There was a transient rise in the activity of the following compounds after the change in the gas stream:- P.G.A., Alanine, Glycine, and serine. The incorporation of carbon-14 into glycollate increased rapidly. The addition of I.N.H. decreased the rise in alanine and serine and increased the rise in P.G.A., glycine, and glycollate. These results again suggest that the sugar phosphates are being oxidised to a C-2 fragment, by high oxygen p.p., which can be metabolised via an I.N.H. sensitive route to serine.

4. The effect of high oxygen p.p. on the further metabolism of radioactivity incorporated into the sugar phosphates.

a. Transitions to carbon dioxide free conditions.

In order to determine whether high oxygen p.p. had any effect on the rate of flow of carbon from the P.C.R.cycle to end products of photosynthesis Chlorella was allowed to fix carbon-14-dioxide for one minute in the air. After this time about fifty percent of the carbon-14 was incorporated into the sugar phosphate fraction. The further metabolism of the labelled sugar phosphate in carbon dioxide free conditions in either air or oxygen,with or without I.N.H. was studied. The results are shown in Fig E 23 and 24.

Carbon dioxide free conditions were used so that the rate of carbon-12-dioxide fixation, which is affected by the high oxygen p.p., would not affect the rate of dilution of carbon-14.

The rate of loss of carbon-14 from the sugar mono phosphates in air was slightly faster than that with other treatments,i.e. I.N.H./air, Oxygen, I.N.H./oxygen. The addition of I.N.H. increased the incorporation of radioactivity into phospho glyceric acid during the initial fixation period, and increased slightly the rate of loss of radioactivity from this compound. The loss of carbon-14 from these compounds was followed by a transient rise in the di-phosphate fraction. Triose-phosphate was decreased and U.D.P.G. increased, during the preliminary fixation period, by the addition of I.N.H. In oxygen incorporation into alanine showed a transient peak, after the transition to carbon dioxide free conditions, in air the radioactive alanine decreased without showing this peak.

The incorporation of carbon-14 into all other compounds rose with time after the transition. The main end products were sucrose, ethanol insolubles,lipids, glycine and glycollate. The various treatments had no effect on the total radioactivity recovered from these compounds. However the percentage distribution of the carbon-14 amongst the end products was markedly affected. Incorporation into sucrose and into the ethanol insoluble fraction showed marked inhibition by high oxygen p.p. and by I.N.H. Glycine and glycollate formation was stimulated by these treatments. The incorporation of carbon-14 into glycollate

Fig E 23 to E 25. The effect of I.N.H., oxygen p.p. and carbon dioxide concentration on the further metabolism of sugar phosphates labelled with carbon-14 dioxide in the air.

Fifty mls of Chlorella suspended at a density of 5µl per ml in phosphate buffer were preincubated in air for thirty minutes. The gas stream was stopped and fifteen µC of sodium carbonate-C^{14} were injected into the lollipop. The cells were allowed to fix the carbon-14 for one minute, and the excess carbon-14 dioxide blown off by a rapid stream of gas, either air or oxygen. A sample was taken as the gas stream was changed. In this sample about fifty percent of the carbon fixed could be recovered from the sugar-phosphate fraction. The further metabolism of these labelled sugar phosphates was studied by taking 10 ml samples at intervals after the transition. I.N.H. was added at the commencement of illumination.

Fig E 23. The further metabolism of labelled sugar phosphates to end products of photosynthesis, in carbon dioxide free air or oxygen.

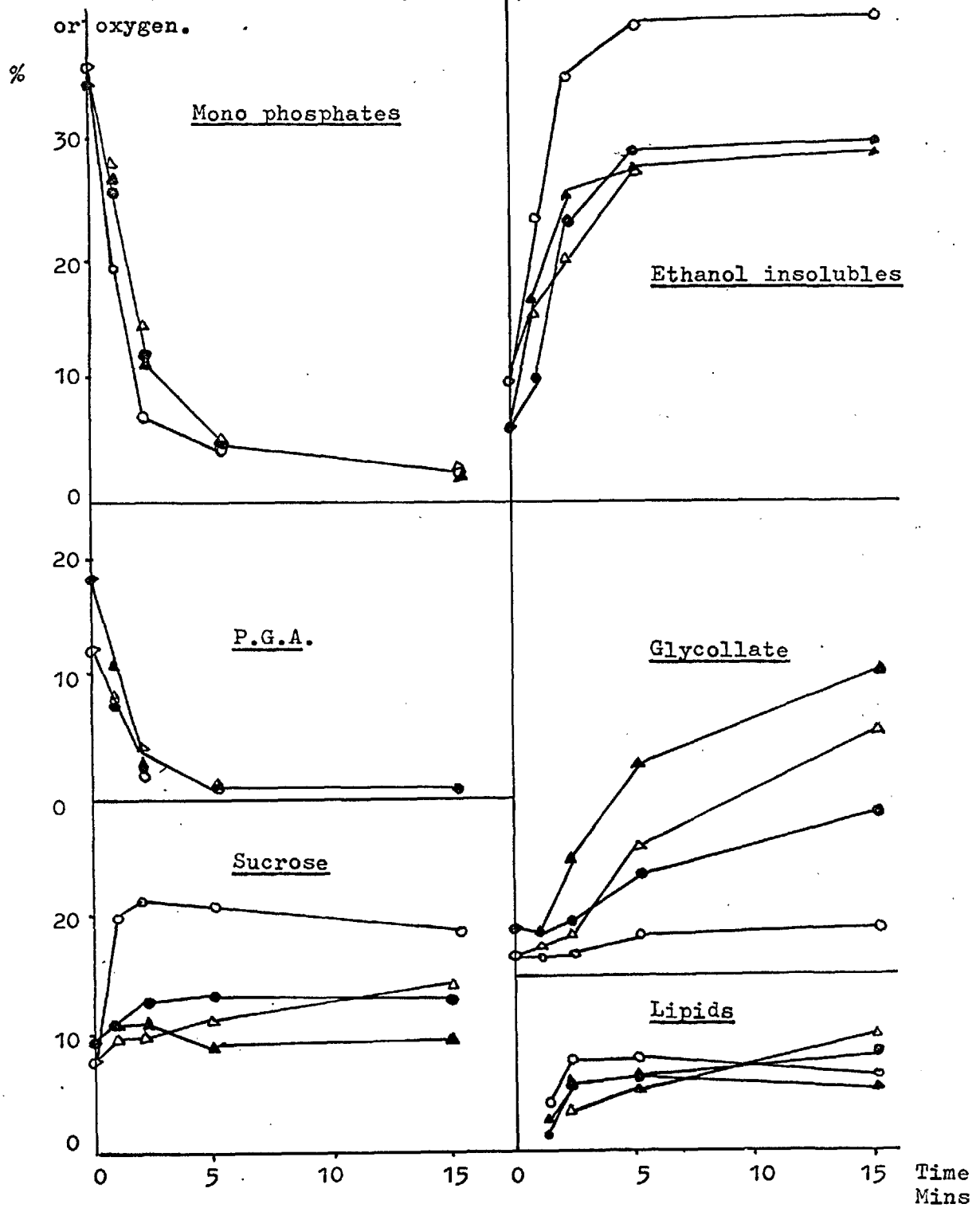
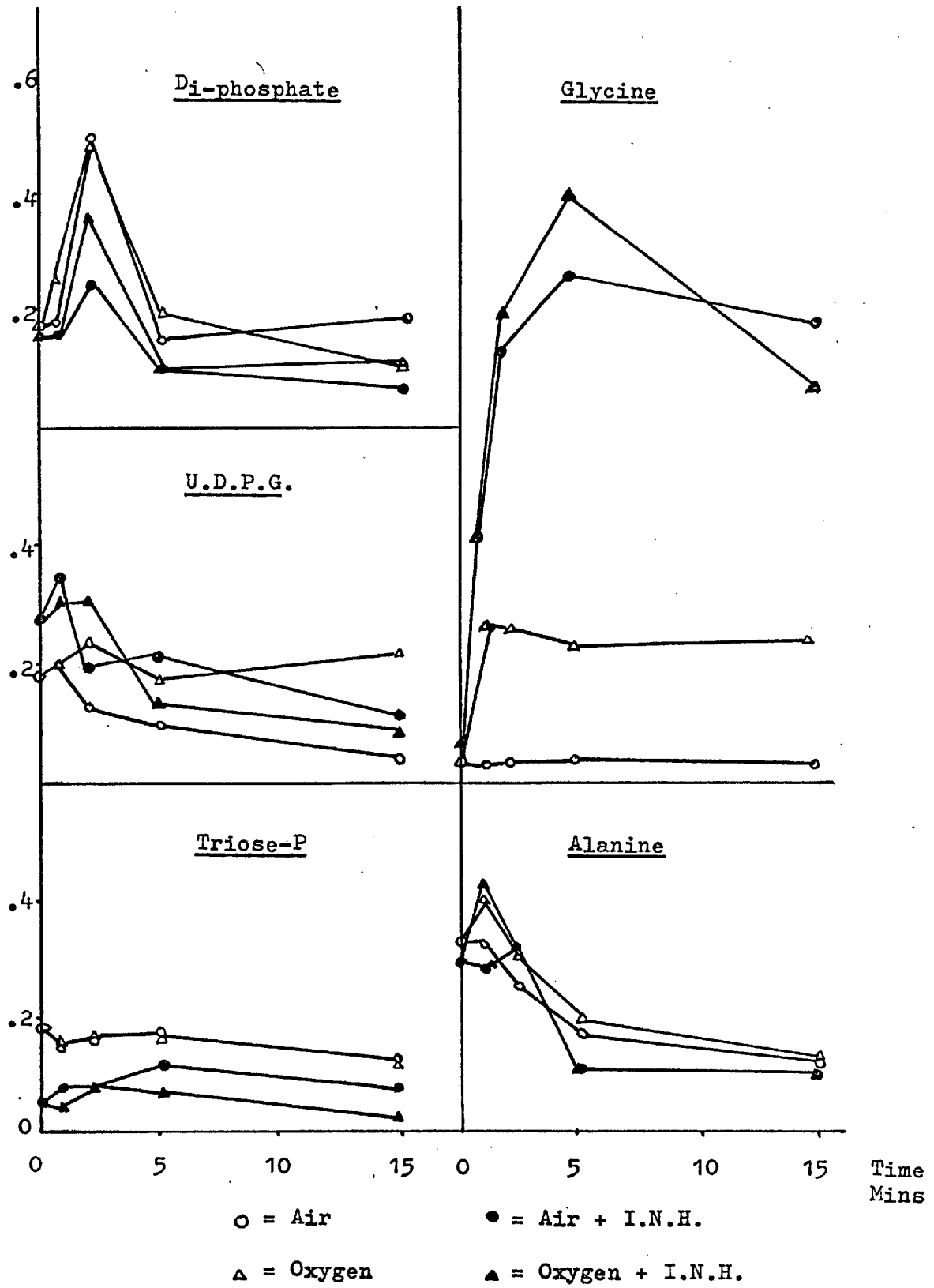


Fig E 24. Intermediates of the further metabolism of labelled sugar phosphates to end products of photosynthesis.



showed a lag of about three minutes, not rising until the sugar di-phosphates had reached a peak. This might indicate that the glycollate was derived from a sugar di-phosphate.

In this experiment no fixation took place after the cells had been transferred to oxygen, hence these results show without doubt that carbon which is normally incorporated into carbohydrates, from the intermediates of the P.C.R.cycle, is diverted to glycollate when the oxygen p.p. is raised or I.N.H. is added.

5. Transitions to oxygen containing 0.04% carbon dioxide

Further investigations into the effect of I.N.H. on the metabolism of sugar phosphates to end products in high oxygen p.p. were carried out as above except that after the period of fixation in air containing carbon-14-dioxide the cells were transferred to oxygen containing 0.04% carbon-12-dioxide. The results are shown in Fig E 25.

After the transition the rate of loss of activity from the sugar phosphate fraction was considerably faster than it was in the previous experiment. In this experiment I.N.H. had the effect of increasing the incorporation of radioactivity into P.G.A. at the expense of alanine. The main end products were glycollate and ethanol insolubles. The incorporation of carbon-14 into these compounds was reversed by the addition of I.N.H., ethanol insolubles were the main product in oxygen, and glycollate with I.N.H. The incorporation into glycine was increased and into serine and sucrose decreased by the addition of I.N.H.

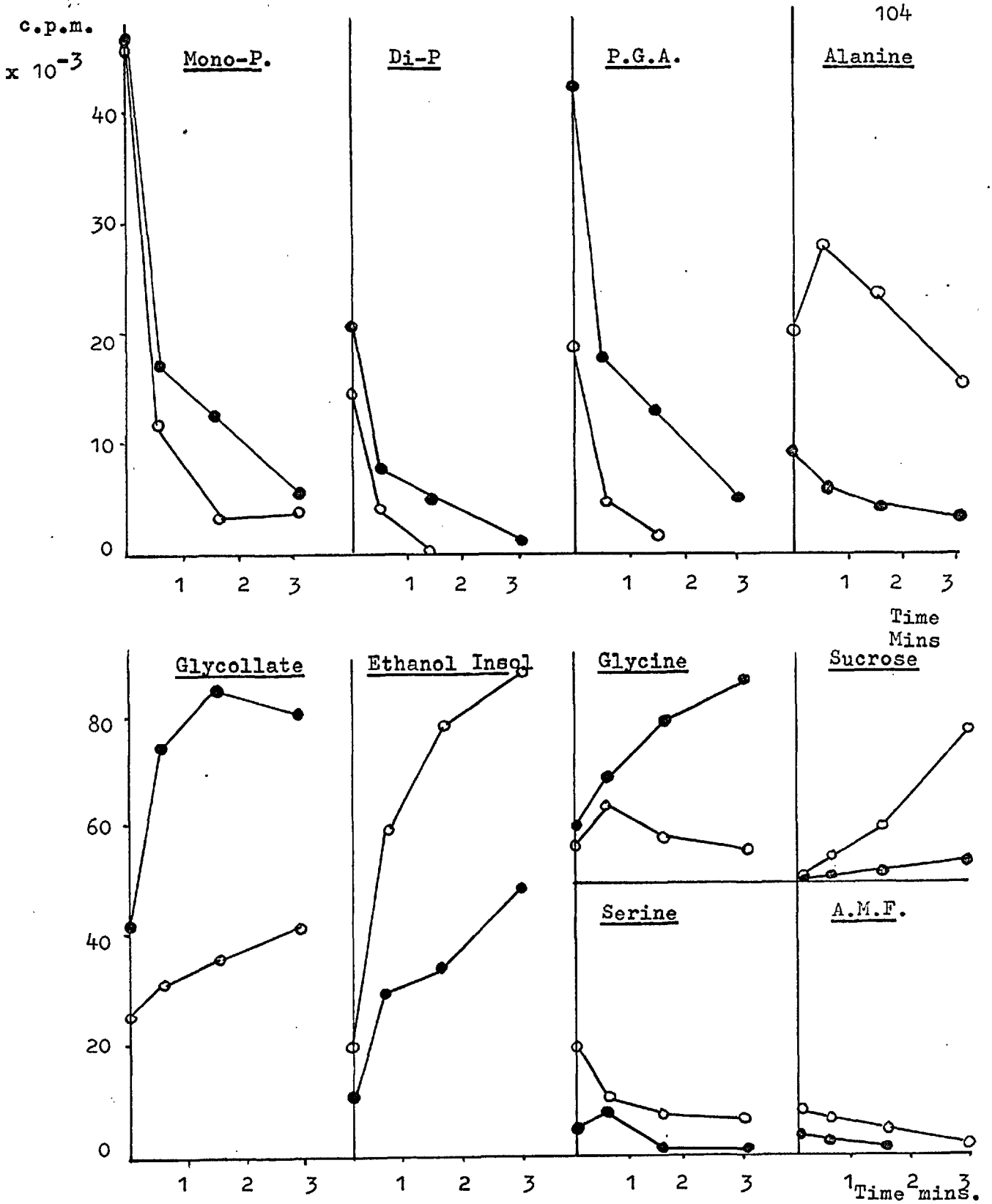


Fig E 25. Dilution of carbon-14 fixed in the air by carbon-12-dioxide fixation in oxygen (0.04% carbon dioxide)

Open symbols = Control Closed symbols = + I.N.H.

6. The effect of oxygen p.p. and I.N.H. on the end products of the further metabolism of sugar phosphates labelled with carbon-14 in the air.

Table 5 shows the results of another similar experiment in which carbon-14 was fixed into the sugar phosphates and then diluted out either in air or oxygen, with and without I.N.H. and/or carbon dioxide. A single sample was taken at the end of the treatment, after 20 minutes, so that the effects of these factors on the incorporation of activity into the end products could be seen. The samples were divided into two. One part was processed as usual, the other was filtered and the supernatant counted for excreted glycollate-C^{14}.

The addition of I.N.H. or raising the oxygen p.p. caused a reduction in the incorporation of carbon-14-dioxide into the ethanol insoluble fraction, with a corresponding increase in incorporation into glycollate. Lowering the carbon dioxide concentration also increased the incorporation of carbon-14 into glycollate at the expense of the ethanol insoluble fraction.

In the first set of experiments, when carbon-12-dioxide was being fixed, the incorporation of carbon-14 into sucrose was inhibited by high oxygen p.p. but not by I.N.H. In contrast the second set of experiments,in the absence of carbon dioxide, show sucrose inhibited by I.N.H. but not by high oxygen p.p.

The formation of lipid was stimulated by oxygen in the presence of carbon dioxide, and in air in the absence of carbon dioxide, conditions which increase the formation of C-2 compounds,

Table 5. The effect of oxygen p.p. and I.N.H. on the end products of the further metabolism of sugar phosphates labelled with carbon-14 in the air.

Experimental method as for Fig E 23 to E 25 with the exception that only one sample was taken. This sample was divided in two; one part was processed as usual, the other filtered and counted for excreted glycollate -C-14. The results are expressed as c.p.m. x 10^{-3}.

Compound	Air	Air/I.N.H.	Oxygen	Oxygen/I.N.H.
0.04 percent carbon dioxide				
Sucrose	3.9	4.2	2.5	3.1
Lipids	9.6	9.4	18.1	10.3
EtOH insol	70.0	36.0	37.0	30.0
External glycollate	4.0	38.0	30.0	50.0
Internal glycollate	3.0	4.3	7.6	8.0
Carbon dioxide free				
Sucrose	5.1	4.6	5.4	1.7
Lipids	13.1	9.3	7.1	6.0
EtOH insol	64.5	36.0	33.0	26.0
Ext. glycollate	15.0	41.0	31.0	50.0
Int. glycollate	3.5	2.3	6.5	8.7

and decreased by both I.N.H. and high oxygen p.p. in the absence of carbon dioxide. Most of the radioactive glycollate produced in these experiments could be recovered from the supernatant. However a small amount of internal glycollate-C^{14} was found under all conditions. The amount of internal glycollate was increased by raising the oxygen p.p. but not affected by the addition of I.N.H. It is possible that this internal glycollate is derived from phosphoglycollate which could be dephosphorylated during extraction.

7. The effect of carbon dioxide concentration and light on the end products of the further metabolism of sugar phosphates labelled in the air.

Table 6 shows the results of an experiment similar to that detailed above, in which carbon-14 was fixed into the sugar phosphates and then diluted out either in air or oxygen at air levels of carbon dioxide or four percent carbon dioxide, in the light or dark.

In the dark the incorporation of carbon-14 into alanine was increased, irrespective of the oxygen p.p. or carbon dioxide concentration. The dark incorporation of activity from the sugar phosphates into alanine at air levels of carbon dioxide has been discussed by Hiller 1963. At four percent carbon dioxide high oxygen p.p. had no affect on the incorporation of activity into end products. However at air levels of carbon dioxide high oxygen p.p. increased the incorporation of carbon-14 into glycollate at the expense of sucrose and

Table 6. The effect of carbon dioxide concentration and light on the end products of the further metabolism of sugar phosphates labelled in the air.

Experimental method as for Fig E 23 to E 25 with the exception that only one sample was taken. After the fixation period the cells were gassed with either air or oxygen containing 4.0% or 0.04% carbon dioxide in the light or in the dark for twenty minutes and then sampled. The results are expressed as c.p.m.x 10^{-3}.

0.04 percent carbon dioxide.	Air/light	Air/dark	Oxygen	Oxygen/dark
Alanine	6	19	5	18
Glycine/Serine	6	3	12	1
Glycollate	3	2	16	6
Sucrose	15	6	11	5
EtOH insol	53	44	30	45
Lipid	13	11	17	19
4.0 percent carbon dioxide.				
Alanine	5	17	5	22
Glycine/Serine	1	1	4	2
Glycollate	1	1	1	1
Sucrose	20	18	12	13
EtOH insol	51	49	50	44
Lipid	10	13	11	15

the ethanol insoluble fraction , as had previously been found. This oxygen stimulated glycollate production was not observed in the dark. At low concentrations of carbon dioxide the incorporation of carbon-14 into glycine and serine was also dependent on the light, whereas at high carbon dioxide concentration incorporation into these compounds increased in the dark.

The results reported in this section indicate that at low concentrations of carbon dioxide high p.p. of oxygen cause the production of C-2 compounds at an enhanced rate, in a light dependent reaction. This increase in production of C-2 compounds is associated with a decrease in both the total fixation of carbon dioxide and in the size of the sugar phosphate pools. The addition of I.N.H. increased the production of glycollate and glycine, and decreased the incorporation into carbohydrates.

Light Stimulated Dark Fixation

If plants are preilluminated and then placed in the dark they will continue to fix carbon dioxide for several seconds at a rate which is in excess of the rate of dark fixation. This phenomenon has been termed the 'R' effect (see Introduction) Considerable work has been done, on the effect of oxygen p.p. and sulfhydral reagents on the level of 'R' by the Japanese school under Tamiya. The main products of light stimulated dark fixation have been shown to be P.G.A. and alanine. e.g. Gaffron 1951.

The experiments reported here were performed to determine whether I.N.H. had any effect on the level of 'R' or on the distribution of the carbon fixed. The 'R' effect was also used as a means of introducing carbon-14 into the carboxyl group of phospho glyceric acid.

Preliminary experiments confirmed that the major products of this fixation were P.G.A. and alanine. Table 7. The ratio of these products to one another changed with the length of time the cells were left in the dark during fixation, although the total fixation was not affected.

Both the addition of I.N.H. and raising the oxygen p.p. increased the incorporation of carbon-14 into alanine at the expense of that into P.G.A. Table 8. The increase in oxygen p.p. decreased the total fixation of carbon-14 but did not affect the incorporation into the Malate/Aspartate/Fumarate fraction. The addition of I.N.H. had no effect on the total fixation.

Table 7 and 8 , Fig E 27. Light stimulated dark fixation

Three vessels were placed in series (Fig E 26), a light tight, blackened 100ml separating funnel, a large perspex lollipop, and a light tight blackened 100 ml conical flask. The cells were placed in the top flask. Ten ml samples were run into the lollipop and incubated for the required time in the light bubbled with the required carbon dioxide free gas. 3μC of sodium carbonate-C-14 were injected into the bottom flask which had previously been evacuated using a vacuum pump. After the required incubation period the tap between the two bottom vessels was opened, and the cells sucked into the darkened vessel. The cells were killed by the injection of hot ethanol. In those experiments in which the effect of I.N.H. was studied the cells were preincubated with I.N.H. for at least 20 minutes before being added to the top resevoir. In the experiment in which the further metabolism of P.G.A. was studied (Fig E 27) the dark fixation vessel was connected to a second smaller perspex lollipop. (Fig E 26) The cells were preincubated in the light for ten minutes in carbon dioxide free nitrogen. 15μC of sodium carbonate-C-14 were placed in the fixation vessel. The cells were allowed to fix radioactivity for five seconds. They were then run into the second lollipop and illuminated in air or oxygen containing 0.04 percent carbon dioxide. The further metabolism of the P.G.A. was studied by periodic sampling.

Apparatus for light stimulated dark fixation.

Apparatus for further metabolism of P.G,A. from dark fixation.

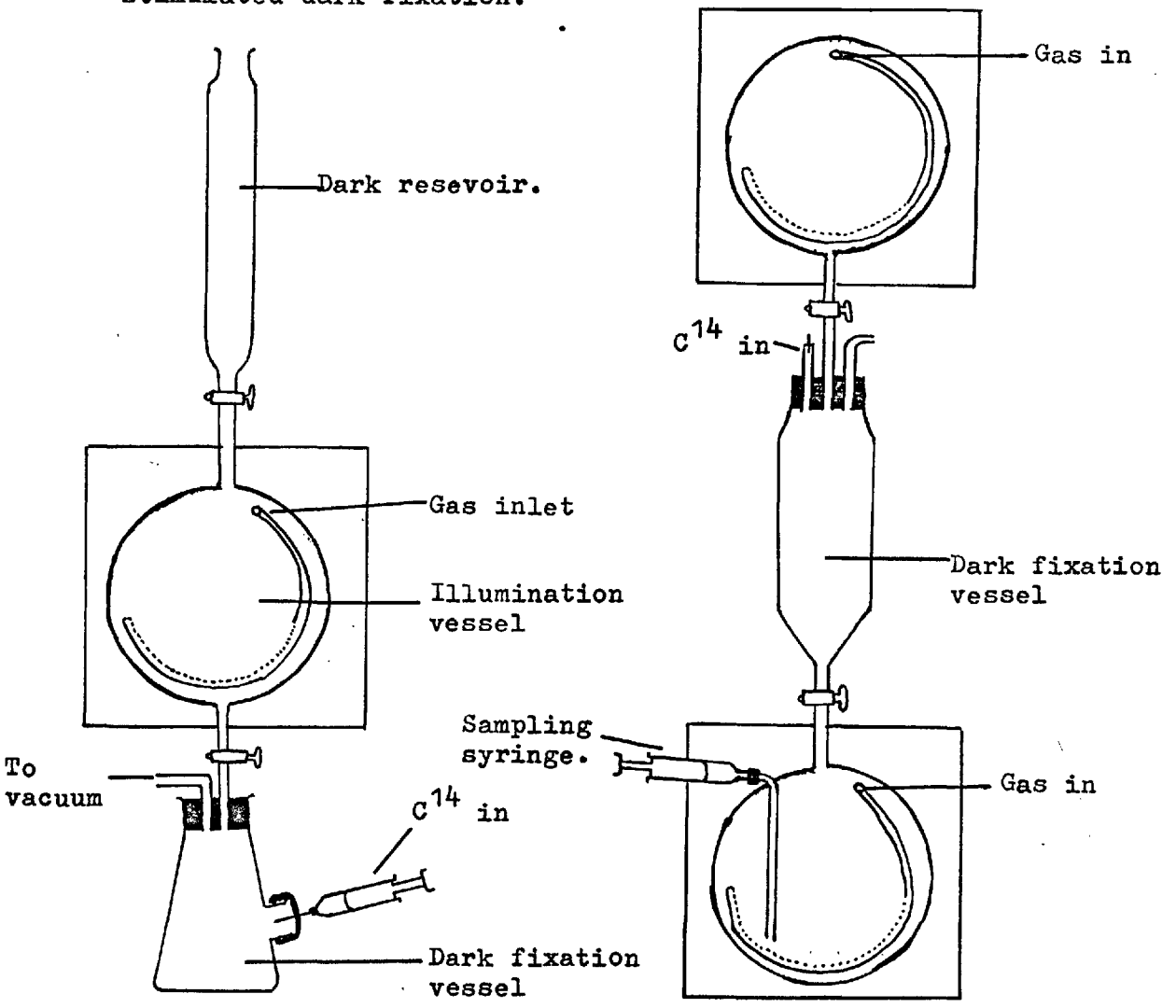


Fig E 26. Arrangement of glass vessels and perspex lollipops used in the dark fixation experiments.(Diagramatic not to scale)

Table 7. Incorporation of carbon-14 into various products after five seconds and thirty seconds light stimulated dark fixation, after ten minutes preillumination in carbon dioxide free nitrogen.

Compound %	5 seconds	30 seconds.
P.G.A.	76	34
P.E.P./P-Glycollate	6	6
U.D.P.G.	2	2
Mono phosphate	-	7
Aspartate	3	9
Malate	3	4
Fumarate	-	1
Glycine/Serine	3	5
Alanine	9	27
Total fixation c.p.m. $\times 10^{-2}$	87.9	88.6

Table 8. The effect of oxygen p.p. and I.N.H., on the total fixation of carbon-14 in thirty seconds light stimulated dark fixation and on the percentage distribution of radio activity into products.

	Nitrogen		Air		Oxygen	
I.N.H.	-	+	-	+	-	+
P.G.A. %	34	17	48	37	24	23
P.E.P.	1	1	1	2	2	3
P.Glycollate	15	2	10	5	4	4
U.D.P.G.	2	2	2	4	1	1
Mono-P	7	9	15	11	9	6
Aspartate	9	8	6	7	16	12
Malate	4	4	7	7	13	12
Fumarate	1	1	1	1	1	1
Glutamate	1	1	1	1	2	2
Glycine/Serine	5	3	3	tr	2	2
Alanine	27	48	5	18	23	28
Total fixation c.p.m. x 10^{-2}	78.4	77.8	63.9	54.5	36.8	39.1

Although phospho glycollate was produced as a result of light stimulated dark fixation no formation of free glycollate was detected.

The 'R' effect was used as a means of introducing carbon-14 into the -COOH group of phospho glyceric acid so that the effect of oxygen on its further metabolism could be studied.

After the five second period of dark fixation about sixty percent of the carbon-14 incorporated could be recovered as P.G.A. The alanine formed was found to contain a constant amount of the radioactivity after return to the light. The radioactivity in alanine was therefore subtracted from the total radioactivity. The amount of radioactivity in other compounds was expressed as a percentage of the resultant figure. Fig E 27.

High p.p. of oxygen had no effect on the rate of loss of activity from P.G.A., but affected its further metabolism. From P.G.A. the radioactivity flowed into the sugar phosphates; incorporation into both the mono and the di phosphates was decreased by the high oxygen p.p.; and from these into C-2 compounds, serine and end products. High oxygen p.p. increased the incorporation into glycollate and glycine and decreased that into serine, sucrose and ethanol insolubles. The incorporation of carbon-14 into lipid was not affected.

In this experiment the only carbon-14 available for further metabolism in the light was that in P.G.A. Hence the oxygen stimulated glycollate must have been derived from intermediates of the P.C.R. cycle.

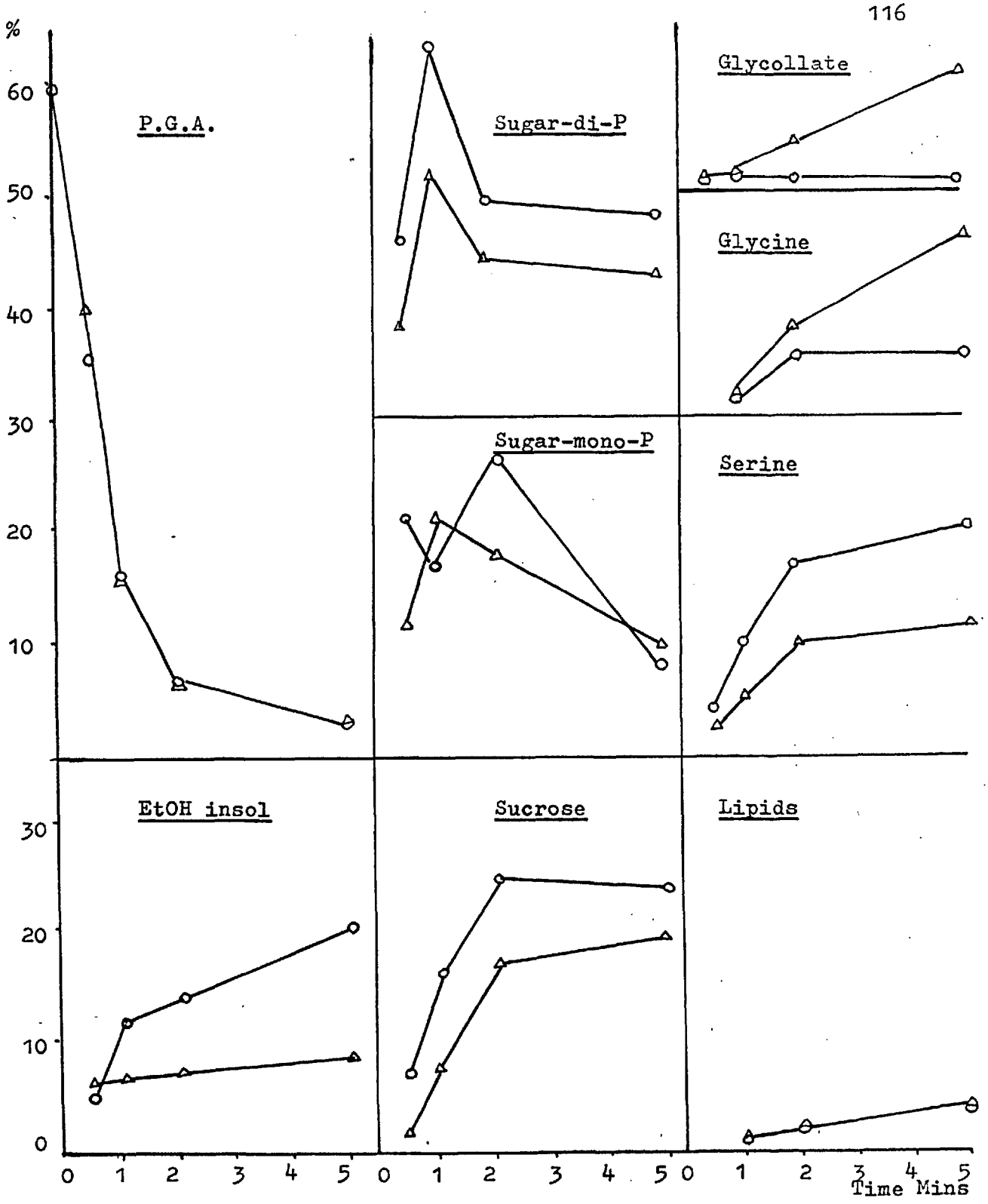


Fig E 27. The further metabolism, in the light, of carbon-14 fixed into P.G.A. in the dark. O =Air. Δ = Oxygen.

The photometabolism of glucose in carbon dioxide free conditions

Under certain conditions (high oxygen p.p.,high light intensity, carbon dioxide free air) normal photosynthesis may be partly reversed so that oxygen is consumed and carbon dioxide evolved in a light stimulated reaction. This process which will be termed photoxidation eventually leads to damage of the photochemical apparatus. Frank(1941) showed that this light injury could be prevented if the plant was supplied with an external supply of oxidisable substrate e.g. glucose.

Whittingham (1963) showed that Chlorella could photometabolise glucose to produce glycollate and that the relative production of glycollate increased as the carbon dioxide concentration was decreased.

The fact that both glycollate production and photoxidation occur under the same conditions suggests that the two processes may be related. It has already been shown (p. 89) that at high light intensities in oxygen, glycollate production was decreased suggesting that it was being oxidised completely to carbon dioxide. This possibility was further investigated in experiments in which radioactive glucose was fed in carbon dioxide free conditions.

Fig E 28 shows the rate of metabolism of glucose-U-C^{14} in carbon dioxide free air and oxygen, and the effect of oxygen p.p. and I.N.H. on the percentage incorporation of radioactivity into various products. The rate of metabolism of glucose was not

found to be affected by either the addition of I.N.H. or by raising the oxygen p.p. The glucose was metabolised as soon as it was taken up by the cells. No internal glucose could be detected. The major products of the photometabolism of glucose under these conditions were:- Sucrose, Glycine/Serine, Glycollate, Ethanol insolubles, and lipids.

High oxygen p.p. had the same effects on these compounds when they were derived from radioactive glucose as when they were derived from radioactive carbon dioxide. The incorporation of radioactivity into both sucrose and the ethanol insolubles was decreased by the addition of I.N.H. or by high oxygen p.p. The incorporation of radioactivity into glycollate was increased by these factors. Most of the glycollate produced was recovered from the supernatant. However there was a small constant amount of internal glycollate present.

If the radioactive glucose is the substrate for photoxidation then radioactive carbon dioxide should be produced under these conditions when radioactive glucose is fed. This was found to be the case. Table 9 shows the distribution of radioactivity into various products after the cells had been allowed to metabolise glucose-U-C^{14}, in the dark or in the light in 100% oxygen with or without I.N.H., for twenty minutes.

Fig E 28. The effect of oxygen and I.N.H. on the rate of photometabolism of glucose-U-C^{14}, and the percentage distribution of radioactivity into products, in carbon dioxide free conditions.

The cells were suspended in fifty mls of phosphate buffer at a density of five µl per ml., in the large perspex lollipop and bubbled with the required gas. The cells were preincubated for thirty minutes. Two µC of glucose-U-C-14 were injected into the lollipop and samples taken at the indicated intervals. The samples were divided, one part was processed as usual the other was filtered and the supernatant chromatogramed in butanol acetic acid. This resulted in the separation of the unmetabolised glucose and the excreted glycollate-C-14.

Table 9. The distribution of radioactivity into various products after the metabolism of glucose-U-C^{14} in the dark or in the light in 100% oxygen.

As above. The gas stream from the experimental vessel was passed through a normal solution of sodium hydroxide contained in a pyrex boiling tube fitted with a sintered glass bubbler. The carbonate was precipitated as barium carbonate, and heated to constant weight in a Gouch crucible. Equal weights of the dried precipitate were then counted on aluminium planchettes.

In this experiment and all other investigations into the photometabolism of glucose (except Fig E 28) the cells were allowed to metabolise the glucose for twenty minutes.

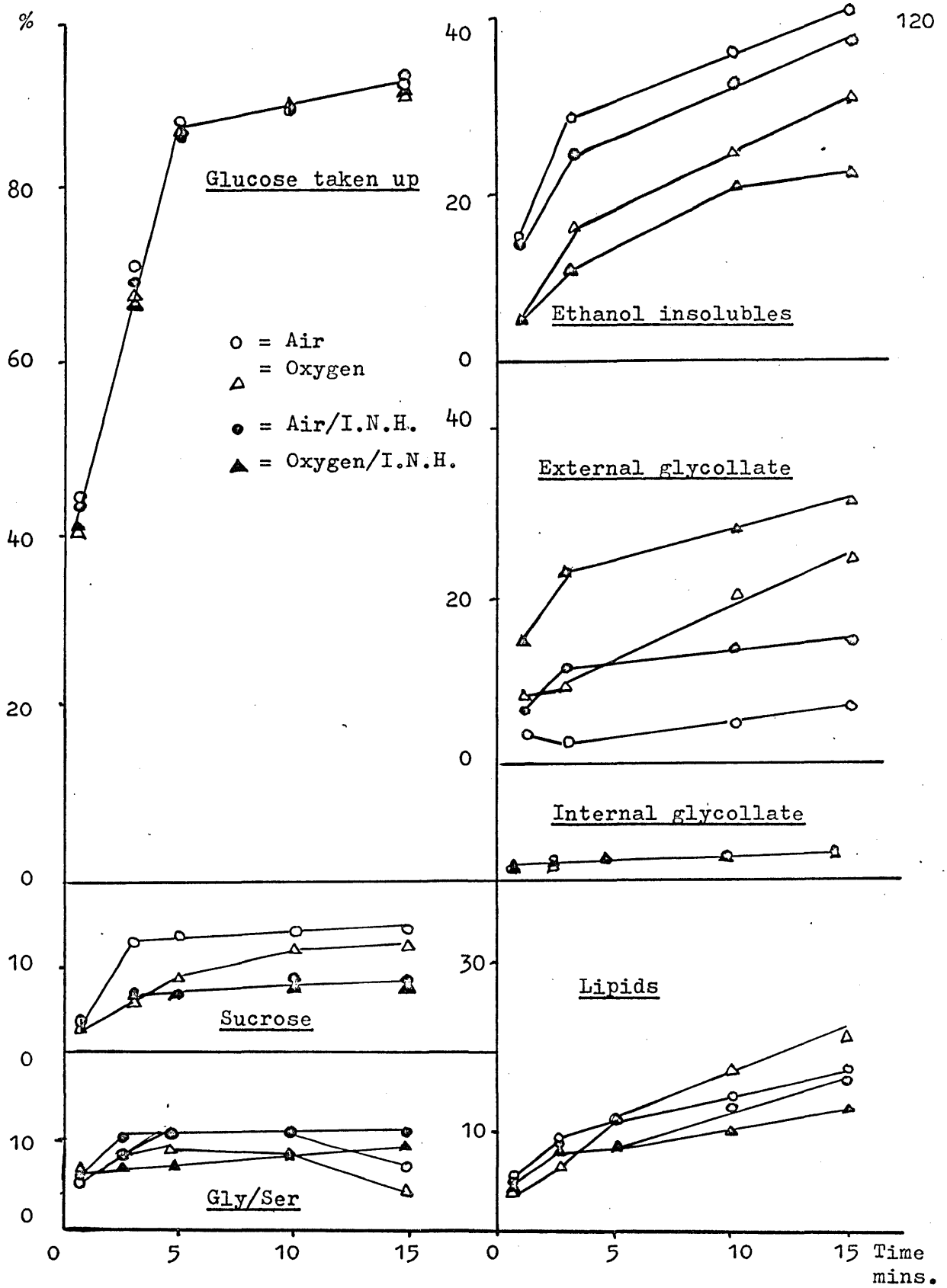


Fig E 28. The photometabolism of glucose-U-C^{14} in carbon dioxide free air or oxygen, with and without I.N.H.

Table 9. The distribution of radioactivity into various products after the metabolism of glucose-U-C^{14} in the dark or in the light in 100% oxygen.

%	Dark	Light/Oxygen	Light/O_2/I.N.H.
Sugar-P	6	6	3
Asp/Mal/Fum	2	1	1
Glycine/Serine/ Sucrose.	10	16	19
Glycollate	1	12	21
Alanine	4	2	1
Glutamate	4	1	-
Lipids	1	2	2
Ethanol Insol	60	47	42
Carbon-14-dioxide	10	16	12
Total c.p.m.x 10^{-2} Metabolised	31.2	34.6	31.7

In the dark the main product was ethanol insolubles (probably a poly-glucan) In the light the incorporation into this product was decreased, and the formation of glycollate, carbon-14 dioxide and the sucrose/glycine/serine fraction was enhanced. When I.N.H. was added in the light the amount of glucose incorporated into the ethanol insoluble fraction was further decreased, and the incorporation into glycollate increased. The production of radioactive carbon dioxide was also decreased by the addition of I.N.H.

The differences in the distribution of radioactivity incorporated from glucose-U-C^{14} into the ethanol insolubles, sucrose , glycollate and carbon-14-dioxide are quite marked. Hence the effect of light intensity on the distribution into these products was studied by the same method. Fig E 29.

As the light intensity was raised the incorporation into ethanol insolubles dropped rapidly, whilst the incorporation into sucrose, glycollate and carbon-14-dioxide increased.

These results indicate that as the light intensity is raised carbon is diverted from a direct metabolism to poly-glucan to a more complex metabolism which can result in the formation of glycollate and the photoproduction of carbon dioxide.

If glucose is respired via the glycolytic (E.M.P.) pathway and the Krebs cycle carbon dioxide will be produced preferentially from the C-3 and C-4 of glucose. Similarly if the glucose is

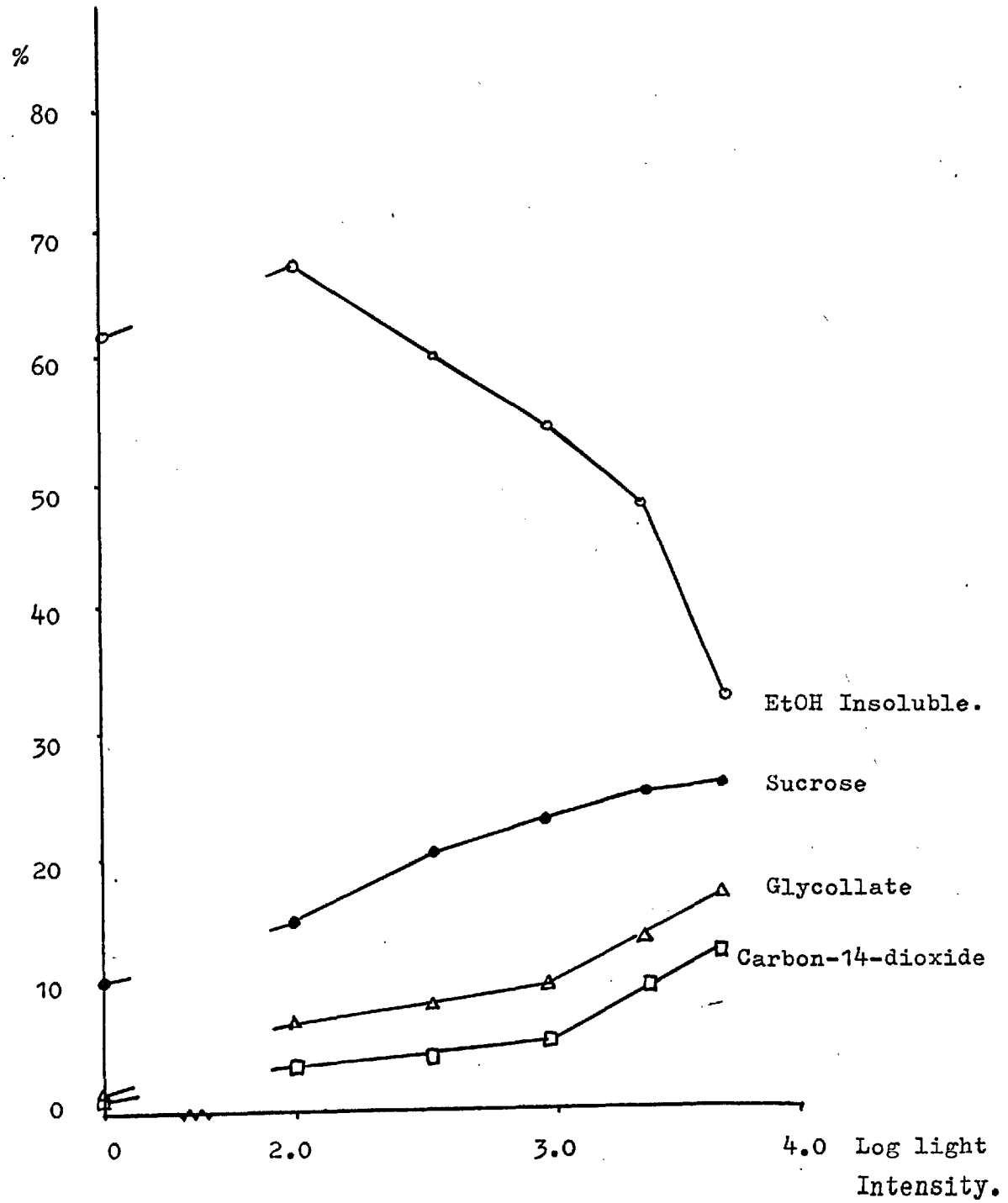


Fig E 29 The effect of light intensity on the metabolism of glucose-U-C^{14} by Chlorella.