# *Development of a Stable Isotope GC/MS Methodology for the Reinvestigation of the Path of Carbon in Photosynthesis*

by

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*of*

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## *Declaration*

The work described in this thesis is original and has not previously been submitted for a degree or diploma in any other University or College, and to the best of my knowledge, does not contain material previously published or presented by another person, except where due reference is made in the text.

Ian L Flanigan.

*Ian L. Flanigan*

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# *Abbreviations*

| | |
|---|---|
| Ald | Aldolase |
| All 6-P | Allose 6-phosphate |
| Anthrone | 9,10-dihydro-9-oxoanthracene |
| Ara 5-P | Arabinose 5-phosphate |
| ATP | Adenosine triphosphate |
| BSA | bis(trimethylsilyl)acetamide |
| Chl | Chlorophyll |
| CI | Chemical ionization |
| Dg Da octulose | D-*glycero*-D-*altro*-octulose |
| Dg Di octulose | D-*glycero*-D-*ido*-octulose |
| Dg Di oct 1,8-$P_2$ | D-*glycero*-D-*altro*-octulose 1,8-bisphosphate |
| Dg Di oct 8-P | D-*glycero*-D-*ido*-octulose 8-phosphate |
| DHAP | Dihydoxyacetone phosphate |
| DPM | Disintegrations per minute |
| EC | Enzyme Commission |
| EDTA | Ethylenediaminetetraacetic acid |
| EI | Electron impact ionization |
| EIMS | Electron impact mass spectrometry |
| Eox | Ethoxime |
| Ery 4-P | Erythrose 4-phosphate |
| ESI | Electrospray ionization |
| FAB | Fast atom bombardment |
| FBPase | Fructose bisphosphatase |
| Fru 1,6-$P_2$ | Fructose 1,6 bisphosphate |
| Fru 6-P | Fructose 6-phosphate |
| GAP-DH | Glyceraldehyde 3-phosphate dehydrogenase |
| GC | Gas chromatography |

| | |
|---|---|
| GC/MS | Gas chromatography / mass spectrometry |
| Glc 6-P | Glucose 6-phosphate |
| Gra 3-P | Glyceraldehyde 3-phosphate |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid |
| HMDS | Hexamethyldisilazane |
| HPLC | High performance liquid chromatography |
| LDH | Lactate dehydrogenase |
| *m/z* | mass to charge ratio |
| Mox | Methoxime |
| $NAD^+$ | Nicotinamide adenine dinucleotide |
| NADH | Nicotinamide adenine dinucleotide (reduced) |
| $NADP^+$ | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced) |
| NMR | Nuclear magnetic resonance |
| Orcinol | 3,5-dihydroxytoluene |
| PAD | Pulsed amperometric detection |
| PC | Paper chromatography |
| PCR | Photosynthetic carbon reduction |
| PGA | Phosphoglyceric acid |
| Pi | Inorganic orthophosphate |
| PPE | Pentose phosphate epimerase |
| PPI | Pentose phosphate isomerase |
| PPP | Pentose phosphate pathway |
| PRK | Phosphoribulokinase |
| Rib 5-P | Ribose 5-phosphate |
| RLEP | Rat liver enzyme preparation |
| Rt | Retention time |
| Ru 1,5-$P_2$ | Ribulose 1,5 bisphosphate |
| Ru 5-P | Ribulose 5-phosphate |
| Rubisco | Ribulose bisphosphate carboxylase/oxygenase |
| SAX | Strong anion exchange |
| Seh 1,7-$P_2$ | Sedoheptulose 1,7-bisphosphate |
| Seh 7-P | Sedoheptulose 7-phosphate |

| | |
|---|---|
| TA | Transaldolse |
| TFA | Trifluoroacetate |
| TK | Transketolase |
| TLC | Thin layer chromatography |
| TMCS | Trimethylchlorosilane |
| TMS | Trimethylsilyl |
| TPI | Triose phosphate isomerase |
| U | Units |
| UV | Ultraviolet |
| Xlu 5-P | Xylulose 5-phosphate |

# Contents

**CHAPTER 3: Photosynthesis Experiments**

# Abstract

The inception of this project was in the recognition of the growing body of evidence that the photosynthetic carbon reduction pathway may be more complex than that originally proposed (Calvin, 1956, Figure1.1). The evidence upon which the acceptance of this pathway rests is examined in Chapter 1 together with subsequent evidence which does not conform with the established theory, and which has led to the proposal of a modified photosynthetic carbon reduction pathway (Clark, 1974, Figure 1.3). Methods available for an examination of the pathway are discussed and an analysis by gas chromatography / mass spectrometry (GC/MS) of the products of a $^{13}CO_2$ incorporation study using isolated chloroplasts is postulated as being the most suitable. The sugars, and the carbon atoms within them, which potentially reveal the labelling data that are the most reliable for such a study are identified (Figures 1.5 and 1.6).

Chapter 2 is concerned with the gathering of information which is necessary to enable a $^{13}CO_2$ incorporation study to be made. The syntheses of specifically $^{13}C$-labelled sugars and sugar phosphates are described and the available mass spectrometric techniques are considered. Decisions are made concerning the most suitable derivatives for the analysis of the sugars. The mass spectral fragmentation characteristics of the derivatized sugars are described and the ions suitable for a $^{13}CO_2$ incorporation study are identified (Table 2.4). The procedures for sample handling and purification prior to GC/MS analysis are established and described.

Chapter 3 addresses the conceptual and practical matters relating to the plant physiology involved in the investigation. The design of a suitable apparatus and the experimental procedures necessary for the conduct of the $^{13}CO_2$ incorporation study using isolated intact spinach chloroplasts are presented. The sugar phosphates of the Calvin pathway together with other sugar phosphates not included in that pathway were found in extracts from isolated photosynthesizing chloroplasts. The procedures for the calibration of the metabolic sugars against an internal standard are given, and the

chloroplast sugar concentrations which were measured using these procedures are reported.

The results of a GC/MS analysis of a series of $^{13}CO_2$ incorporation experiments using isolated intact spinach chloroplasts are reported in Chapter 4. The patterns of label distribution between sugars show some most surprising results. Some of the Calvin pathway intermediates were amongst the sugars which incorporated the least $^{13}C$ label, whilst other sugars, some of which are more closely related to sugars of the modified carbon reduction pathway were amongst those which incorporated the most $^{13}C$ label. The results of this analysis show that the methodologies developed here are capable of providing the information needed for a more definitive study and have pointed out some areas in which further development of the methods may be required. They also add further evidence to that which already exists suggesting that the path of carbon in photosynthesis may be more complex than it was previously believed to be.

# *Publications*

The following publications are relevant to the work carried out in this investigation:

(1) Flanigan, I., Collins, J.G., Arora, K.K., MacLeod, J.K., and Williams, J.F. (1993). "Exchange reactions catalyzed by group-transferring enzymes oppose the quantitation and unravelling of the identity of the pentose pathway." *Eur. J. Biochem.*, **213**, 477-485.

(2) Flanigan, I.L., Graham, K., Collins, J.G., MacLeod, J.K., and Williams, J.F. (1995). "Assignment of the $^{1}H$ NMR resonances of D-*glycero*-D-*ido*- and D-*glycero*-D-*altro*-octulose mono- and bisphosphates." *Magnetic Resonance in Chemistry*, **33**, 231-232.

(3) Irvine, R.W., Flanigan, I.L., MacLeod, J.K., Collins, J.G., and Williams, J.F. (1992). "Mass Spectrometric studies of the path of carbon in photosynthesis: Positional isotopic analysis of $^{13}C$-labelled 2-octulose phosphates." *Org. Mass Spectrom.*, **27**, 1052-1060.

# CHAPTER 1

## GENERAL INTRODUCTION

## 1.1 Introduction

Photosynthesis is the principal means by which organic molecules are synthesized on earth. It involves the reduction of carbon dioxide to sugars using energy from sunlight that has been converted into a chemical form. Light is not the only source of energy used in nature to reduce inorganic carbon to sugars. There are a few micro-organisms, classed as chemolithotrophes, such as the hydrogen, sulphur, iron and denitrifying bacteria (e.g. *Thiobacillus* and *Nitrosomonas*), which are able to use various forms of chemical energy (Anderson et al., 1991) for this conversion. Whether the energy needed to drive this reduction comes from chemical reactions or from light, or whether the electron donor is water or some other compound, the only known reaction sequence providing a net flow of carbon into the biosphere is the Calvin or photosynthetic carbon reduction (PCR) cycle (Curmi et al., 1991) shown in Figure 1.1*. Hence the reactions of this pathway account for the origin of almost all of the biomass on the planet, making it overwhelmingly its most important biochemical pathway.

The importance of photosynthesis was recognized by Julius Robert Mayer (1814-1878) as long ago as 1845. In his review of this period of the history of photosynthesis, Florkin (Florkin, 1979) reported that Mayer realized that plants captured energy from sunlight, converted it into chemical energy and stored it in a form which gave mankind a permanent supply of potential chemical energy (Mayer, 1845). With this understanding of the energy relations in photosynthesis, the essential elements of the process were known. Oxygen had been shown to be evolved during photosynthesis by Joseph Priestley in 1772; Ingen-Housz in 1779 recognized the need for light for the green parts of plants to evolve oxygen; Senebier in 1783 showed that carbon dioxide was a necessary participant; de Saursure in 1804 demonstrated that

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* Throughout this thesis, all sugars are shown in the Fischer projection for simplicity and all sugars have the D-configuration.

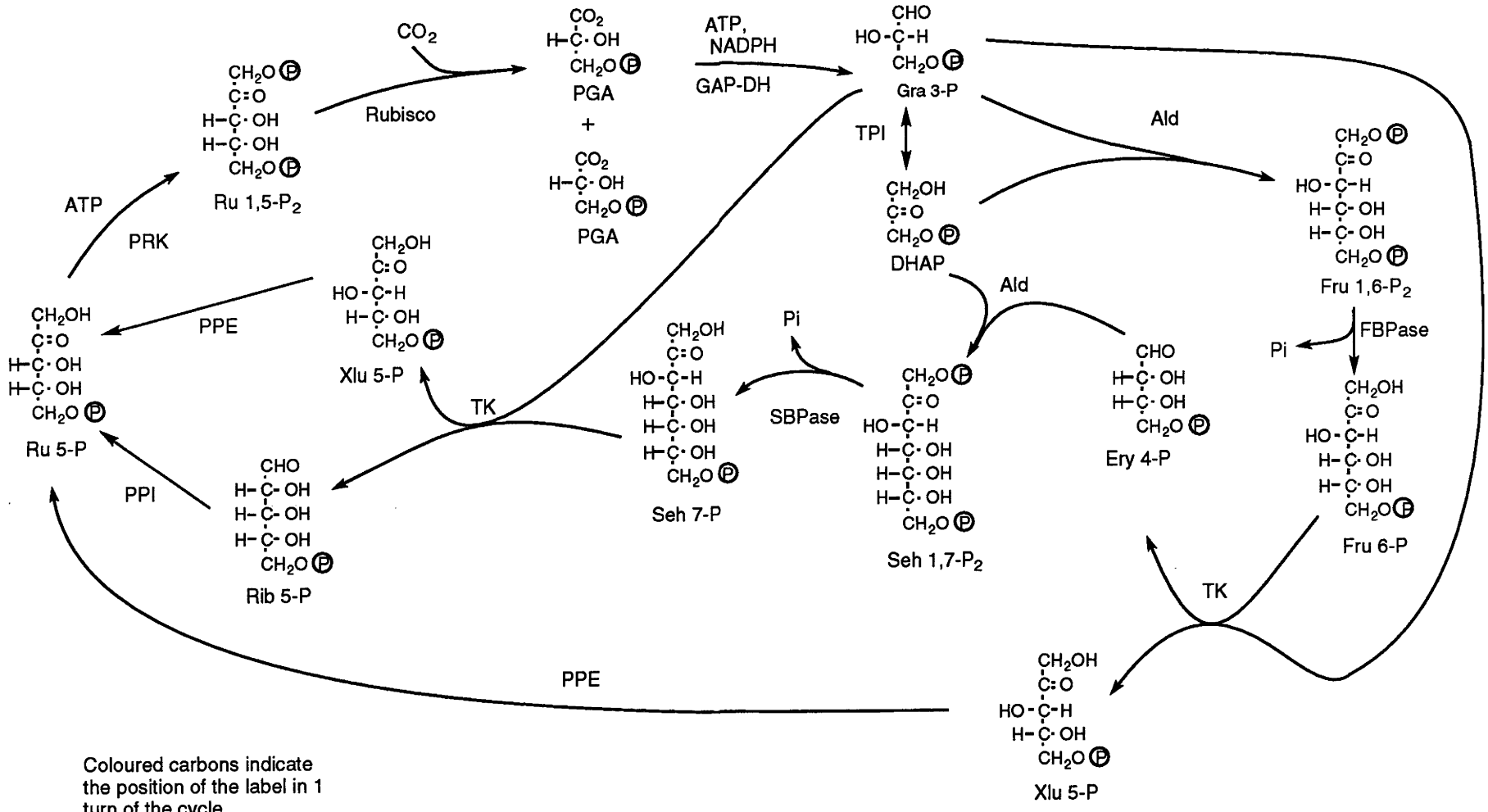


Figure 1.1. The PCR pathway (Calvin, 1956).

water is involved in the process. When Boussingault determined the overall quotient $O_2/CO_2$ to be unity in 1864, it became possible to write an equation for plant photosynthesis as

$$CO_2 + H_2O + \text{light} \longrightarrow O_2 + \text{organic matter}$$

but the precise nature of the end product was unknown.

Blackman (Blackman et al., 1905) reported that assimilation was controlled by light intensity, leaf temperature, and $CO_2$ concentration and also that there were non-photochemical processes (or dark reactions) involved. In 1936, Van Niel postulated that the process of light absorption was concerned only with the splitting of the water molecule and the removal of the hydrogen from the oxygen, and that this hydrogen was in such a form that it could later reduce the carbon dioxide molecule. Thus it was recognized that photosynthesis consisted of two distinct parts: 1) the capture and storage of sunlight energy in a chemical form capable of carrying out the reduction of carbon dioxide; and 2) the reduction reactions themselves which resulted in the formation of carbon reduced to the level of carbohydrate and which did not depend directly on sunlight. The above events are more fully described in the review by Florkin (Florkin, 1979) from which these exerpts have been taken.

This thesis is concerned only with the second part of the process of photosynthesis, that is, the reactions by which carbon dioxide is reduced to sugars and known here as the photosynthetic carbon reduction (PCR) pathway. Although the PCR pathway as presented by Calvin has been widely accepted by the international community of chemical and plant scientists, its re-examination is warranted for three main reasons.

1. The evidence upon which Calvin based the pathway was incomplete.

2. More recent evidence suggests that other intermediates may be involved.

3. There have been substantial advances in technologies since Calvin's data were collected using $^{14}C$-isotopes and paper chromatography (PC) in the 1950's and these have the potential to provide more definitive results than Calvin was able to obtain.

## 1.2 Calvin's evidence for the PCR pathway

Figure 1.1 shows that photosynthetic carbon reduction takes place when a molecule of $CO_2$ combines with the enediol form of ribulose 1,5-bisphosphate to form a six-carbon ß-ketoacid intermediate which ultimately cleaves into two molecules of 3-phosphoglyceric acid which, in turn, become further reduced to triose phosphate. Calvin's discovery of these initial fixation and reduction steps was a remarkable piece of chemistry and solved a problem that had intrigued chemists for many years. It was Calvin's greatest achievement and this thesis intends to cast no doubt on the accuracy of these steps. The remainder of the pathway is concerned with the regeneration of the $CO_2$ acceptor, ribulose 1,5-bisphosphate via the various $C_4$ to $C_7$ intermediates shown in Figure 1.1. These are the steps that make it a sustainable cycle and although they were consistent with the available data, there was little direct evidence for the sequence of reactions proposed.

The evidence on which the proposed regenerative steps were based was of three types: identification of most of the intermediates involved, erythrose 4-phosphate being a notable exception; identification of the labelling patterns in some of these intermediates; and knowledge of the possible enzymic reactions leading to the interconversion of sugar phosphates that had been recently discovered in other laboratories, particularly those of Horecker and Racker.

Armed with this information Calvin drew up a sequence of reactions which allowed ribulose 1,5-bisphosphate to be reformed from the triose phosphate products of the fixation and reduction steps. It was not the only scheme possible and an alternative version involving transaldolase, that was

consistent with all of the data then available was published (Horecker et al., 1955). This version fell out of favour, however, when insufficient transaldolase was found to sustain the observed rates of carbon fixation. The Calvin version is a highly plausible and relatively simple scheme which found immediate, largely uncritical acceptance from most of the scientific community at the time with remarkably few dissenters, an exception being Stiller (Stiller, 1962). In recognition of his contribution to photosynthesis research Calvin was awarded the Nobel Prize for chemistry in 1961. His pathway has become one of the canons of metabolic biochemistry. In view of the importance of the PCR pathway, it is surprising that little further evidence has been added to that which Calvin used in the formulation of his pathway.

In order to obtain evidence, Calvin made good use of the PC and radioautography techniques of Fink (Fink et al., 1948) which had been developed from those introduced by Martin and Synge (Martin et al., 1941). These methods permitted the rapid separation of a wide range of compound classes and led to the identification of those compounds into which the $^{14}C$ isotopic label had become incorporated after short periods of exposure to $^{14}CO_2$, and which could thus be intermediates in the carbon pathway. The PC methods were unable, however, to provide adequate resolution between groups of similar compounds such as the sugar monophosphates and sugar bisphosphates. Small amounts of other compounds may have been unidentified. The intermediates of the regenerative phase of the cycle that were positively identified are fructose monophosphate (Calvin et al., 1949), sedoheptulose monophosphate (Benson et al., 1951), ribulose bisphosphate (Benson, 1951), ribose monophosphate, ribulose monophosphate, (Buchanan et al., 1952) and sedoheptulose bisphosphate (no data reported) (Calvin et al., 1956). "Hexose DP" was mentioned in many papers, but fructose 1,6-bisphosphate was not specifically mentioned, presumably because the presence of other hexose bisphosphates could not be ruled out.

Further evidence came from the degradation of the chromatographically resolved compounds by the microbial and chemical methods then available (Bassham et al., 1954). Using these methods Calvin and collegues obtained qualitative information about the $^{14}C$ isotopic label distribution within ribulose bisphosphate, sedoheptulose monophosphate, phosphoglyceric acid and "hexose monophosphate".

Calvin also attempted to determine the sequential relationships between the labelled compounds, by plotting curves representing the appearance of radioisotope within selected compounds. By calculating the percentage of the total isotope that each compound contained at each time point and projecting the slope of the curve back towards zero time, Calvin hoped to show that, at the earliest time, most of the isotope would be contained in a single substance. If this compound was then eliminated from the calculations and the procedure repeated he hoped to identify the compound that was next in the sequence and so on. Whilst this procedure was successful in confirming the first labelled compound as phosphoglyceric acid, (Bassham et al., 1954) it failed to allow them to assign any order of precedence amongst the remainder of the intermediates. Thus he was forced to rely on the labelling and degradation studies referred to above.

Combined with these two types of evidence from his own laboratory, Calvin was able to draw on the pentose pathway enzyme discoveries being made at much the same time in the laboratories of Horecker and Racker. These were applied to the solution of his own problem of regenerating ribulose bisphosphate from the radiolabelled compounds he had identified. Horecker had shown that rat liver enzymes were able to form sedoheptulose phosphate from pentose phosphate (Horecker et al., 1953) while the enzyme involved was identified as transketolase by Racker (Racker et al., 1953). The reaction was reversible and in the reverse direction it was the very reaction Calvin needed to form ribulose phosphate from sedoheptulose phosphate and produce the labelling distributions he had observed. Horecker reported that

aldolase catalyzed the condensation of triose phosphate and erythrose 4-phosphate to form sedoheptulose 1,7-bisphosphate (Horecker et al., 1952) and Calvin saw that such a reaction would provide the required labelling pattern in sedoheptulose 7-phosphate after dephosphorylation from the bisphosphate. Finally, Calvin also recognized that the appropriately labelled erythrose 4-phosphate could be formed from fructose 6-phosphate in a transketolase reaction of the type reported by Racker (Racker et al., 1953). The labelling patterns Calvin used to make these deductions are illustrated in Figure 1.2. The resulting scheme that Calvin proposed appeared to be the most likely means for the regeneration of ribulose phosphate from the triose phosphate products of carbon fixation and reduction. However there was some uncertainty associated with this because the $^{14}C$ isotope distribution observed in sedoheptulose, after a very short exposure of soybean leaves to $^{14}CO_2$, was that shown in Table 1.1. This labelling pattern was inconsistent with an aldolase route of formation but Calvin nevertheless concluded that it was the most likely route even though other routes were possible. His reasoning for this was based on the finding, in the soybean leaf extract, of a small amount of the sedoheptulose bisphosphate that was assumed to be the product of the aldolase condensation. He proposed that incomplete equilibration between the triose phosphates might have accounted for the non-compliance of the sedoheptulose labelling reported in Table 1.1 with that predicted by an aldolase route of formation.

Based on these inconclusive labelling patterns and a series of deductions regarding the above transformations Calvin published the outline of an entire photosynthetic carbon reduction cycle involving the crucial transketolase-aldolase-transketolase sequence of reactions in April 1954 (Bassham et al., 1954). This scheme remained unaltered until workers in Racker's group found that the true substrate of the transketolase reaction was xylulose 5-phosphate and not ribulose 5-phosphate (Srere et al., 1955).

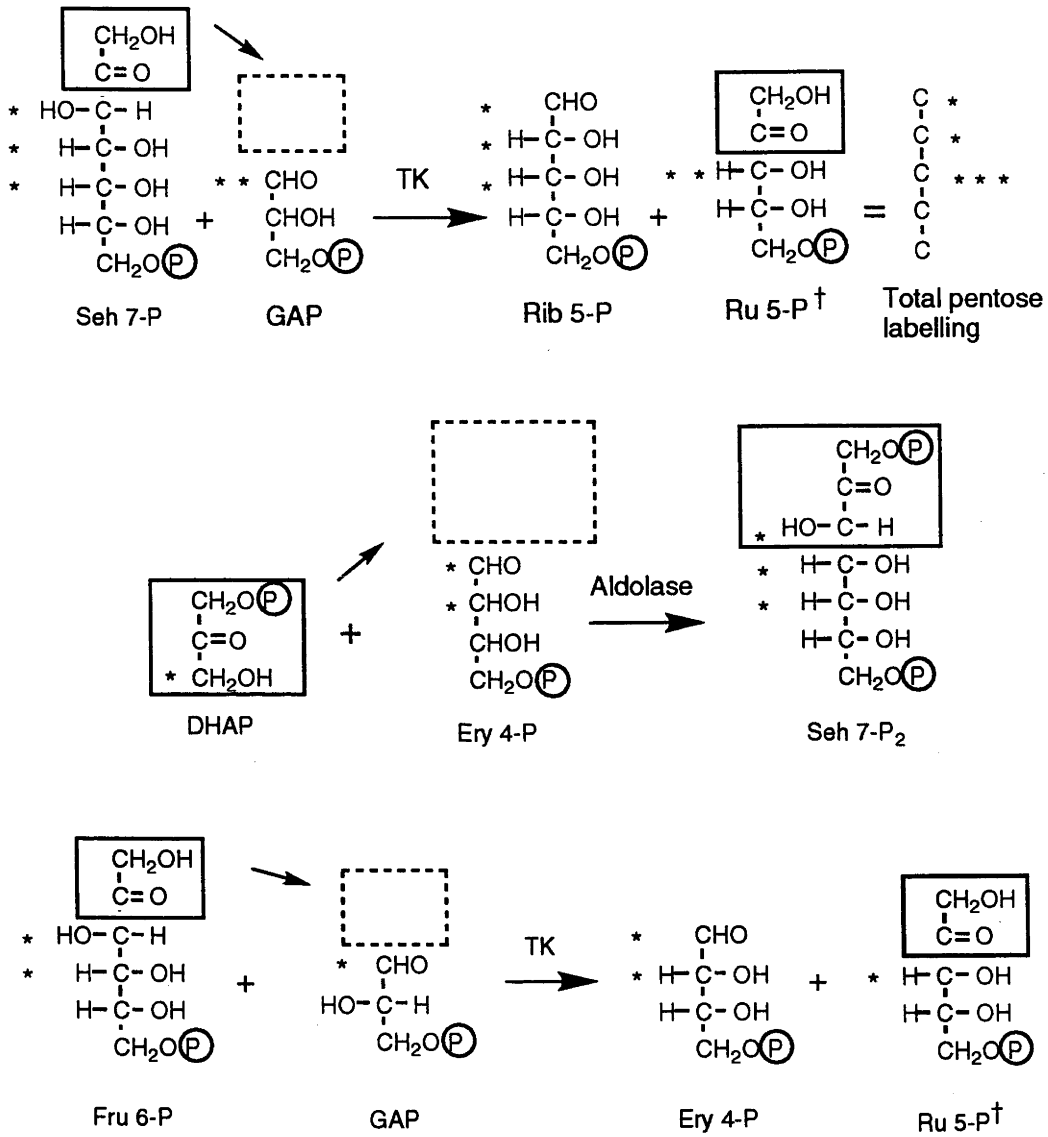


**Figure 1.2.** The $^{14}C$ labelling patterns Calvin used to help deduce the sequence of reactions in the PCR pathway (Bassham, 1954). † This compound was later shown to be Xlu 5-P and not Ru 5-P.

**Table 1.1.** The labelling distribution in sedoheptulose after short exposures of Soybean to $^{14}CO_2$ (Calvin et al., 1956).

| Sedoheptulose carbon number | Time of exposure to $^{14}CO_2$ | |
|---|---|---|
| | 0.4 sec | 0.8 sec |
| 1 | 0 | 2 |
| 2 | 0 | 2 |
| 3 | 33 | 39 |
| 4 | 8 | 18 |
| 5 | 49 | 38 |
| 6 | 0 | 2 |
| 7 | 0 | 2 |

Calvin incorporated this modification into the photosynthetic PCR scheme and presented the final version in October, 1955 in a Centenary Lecture to the Chemical Society in London (Calvin, 1956). This reaction scheme is plausible, generally consistent with the data then available, and involved many of the same intermediates and reactions that were included in the pentose phosphate pathway in liver (Horecker et al., 1954) and pea tissue (Gibbs et al., 1954). Each pathway thus added to the credibility of the other.

## 1.3 Evidence not accommodated by the Calvin pathway.

### 1.3.1 Presence of octulose phosphates

In the early 1950's the Horecker and Racker groups had been conducting experiments which resulted in the discovery of the enzymes and reactions of the pentose phosphate pathway (PPP). This pathway contains reactions which enable transformations to take place between pentose and hexose phosphates. Calvin was confronted with the reverse problem of devising transformations between hexose and pentose phosphates. The reversibility of the reactions discovered by Horecker and Racker suggested to Calvin the means by which he could complete his cycle and regenerate ribulose bisphosphate from the hexose phosphate products of the fixation and

reduction steps he had already discovered. When Calvin used these reactions to formulate a pathway which could account for most of the labelling patterns he had observed, it was clear that the PPP and the PCR pathway had many reactions in common.

Whilst Calvin's labelling results had reasonably closely matched those predicted by his pathway, the evidence gathered in investigations into the PPP in a variety of tissues consistently failed to support PPP theory. A search therefore began for an alternative PPP reaction scheme which better fitted the available evidence (Clark et al., 1974).

An alternative scheme for the PPP was proposed (Clark et al., 1974; Williams et al., 1978) in which reactions involving the new intermediates, D-*glycero*-D-*ido*-octulose 8-phosphate and D-*glycero*-D-*ido*-octulose 1,8-bisphosphate and arabinose 5-phosphate were distinguishing features. The detailed history of the development of the original "F-type" and the alternative, "L-type" pentose pathways has been comprehensively reviewed (Williams et al., 1987) and it now seems clear that the original formulation of the PPP by Horecker may be an inadequate description of non-oxidative pentose metabolism in most tissues. Labelling studies using chlorella produced labelling patterns that were incompatible with the classical "F-type" pentose pathway but did furnish evidence for the operation of the "L-type" pentose pathway (Heath, 1984).

Octulose phosphates do not feature in any of the conventional metabolic pathways but their formation from an aldolase-catalyzed condensation of dihydroxyacetone phosphate and pentose 5-phosphate has been known for many years (Dische, 1958). They have also been observed as products when erythrocytes were incubated with ribonucleosides (Bartlett et al., 1968; Vanderheiden, 1964) and when rat liver enzyme preparation (RLEP) was incubated with ribose 5-phosphate (Williams et al., 1978; Williams et al., 1985; Williams et al., 1984). Their involvement in normal rat liver metabolism was demonstrated when Paoletti (Paoletti et al., 1979a)

isolated and measured the concentrations of the mono-and bisphosphates of both D-*glycero*-D-*altro*- and D-*glycero*-D-*ido*-octulose in fresh rat liver.

As was the case for the original PPP, it is also possible to draw a modification to the Calvin PCR pathway which involves octulose phosphates (Clark et al., 1974). A version of this scheme is shown in Figure 1.3. In view of the close relationship, both mechanistically and historically, between the PPP and Calvin's PCR pathway, it seemed pertinent to look for octulose phosphates in photosynthesizing plants.

Both D-*glycero*-D-*ido*- and D-*glycero*-D-*altro*-octuloses and arabinose have been identified and measured by gas-liquid chromatography (GC) in the extracts of whole spinach leaves. The concentrations measured for the octuloses were 11.8 and 8.2 nmol per mg chlorophyll respectively, although this represented both free sugars and phosphate esters (Kapuscinski et al., 1985). When intact spinach leaves were allowed to photosynthesize in an atmosphere of 0.2% $^{14}CO_2$ in air, [$^{14}$C]D-*glycero*-D-*ido*-octulose 1,8-bisphosphate was amongst the early labelled intermediates that were isolated (Kapuscinsci et al., 1984), (Kapuscinski, 1985). The kinetics of $^{14}$C incorporation into D-*glycero*-D-*ido*-octulose 1,8-bisphosphate were similar to those which occurred in sedoheptulose 1,7-bisphosphate and glucose 6-phosphate. Furthermore, an experiment using the same "chase" technique as that used in Calvin's group, in which the period of $^{14}CO_2$ incorporation was followed by a period of exposure to unlabelled $CO_2$, showed that the $^{14}$C label in octulose bisphosphate was rapidly "washed out" (Kapuscinski, 1985). This is not a characteristic of the end products of metabolism such as sucrose and starch, and serves as evidence that the octulose bisphosphate is in dynamic equilibrium with other intermediates of the PCR pathway. Intact isolated chloroplasts subjected to short term exposure to either $^{14}$C-bicarbonate or $^{32}$P-orthophosphate substrates also rapidly incorporated these labels into D-*glycero*-D-*ido*-octulose 1,8-bisphosphate (Kapuscinski, 1985). The concentration of D-*glycero*-D-*ido*-octulose 1,8-bisphosphate was estimated to

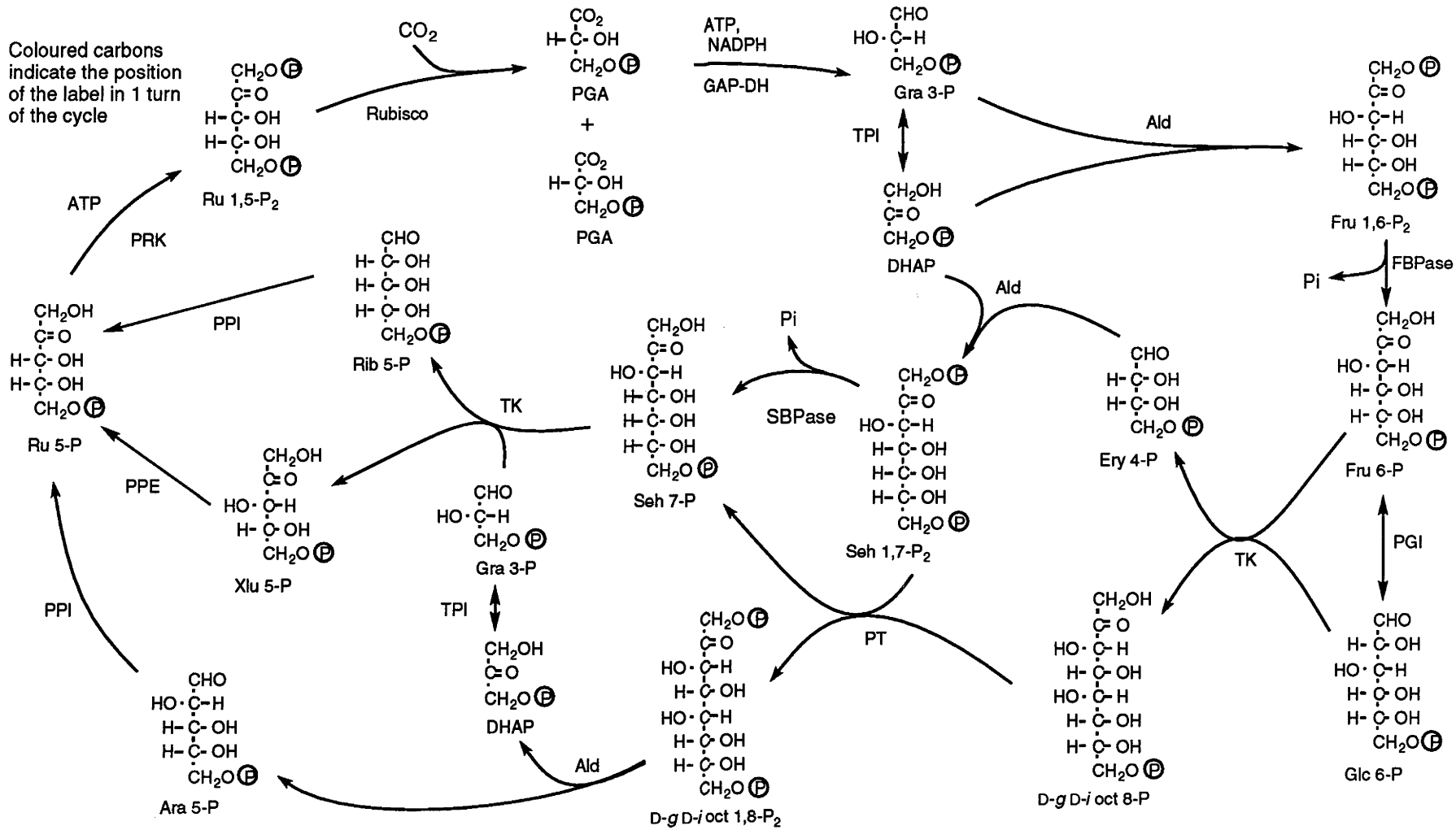


Figure 1.3. The modified PCR pathway (Clark, 1974).

be 50 μM in the stroma of isolated chloroplasts. Both intact leaves and isolated chloroplasts exposed to $^{14}CO_2$ produced a labelling distribution in D-*glycero* D-*ido* octulose 1,8-bisphosphate, as determined by degradation methods, which implied that the route of synthesis was most likely via a transketolase mechanism rather than an aldolase or transaldolase mechanism as illustrated in Figure 1.4.

$^{13}C$ NMR experiments using intact spinach leaves and $^{13}CO_2$ showed the presence of D-*glycero*-D-*altro*-octulose 1,8-bisphosphate which was $^{13}C$ enriched in the 3,4,5 and 6 positions (Bartlett et al., 1989) consistent with an aldolase route of formation. The presence of D-*glycero*-D-*ido*-octulose 1,8-bisphosphate was ambiguous because of the inherent insensitivity and poor resolution of NMR spectroscopy in the relevant region of the spectrum.

### 1.3.2 Gibbs effect

Labelling predictions based on the Calvin cycle (Figure 1.1) assume that there is chemical and isotopic equilibration of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate prior to an aldolase catalyzed condensation of the two equivalent triose phosphate moieties to form fructose bisphosphate. This would give rise to symmetrically labelled fructose bisphosphate, fructose 6-phosphate and glucose 6-phosphate with the labelling pattern of carbons 1, 2 and 3 being the same as that of carbons 6, 5 and 4. This may be a false assumption. In most early work asymmetrical distributions of isotope labelling have been observed amongst the hexoses (Kandler, 1956, Gibbs, 1957, Havir, 1963) but symmetrical distributions were also reported (Bassham, 1954). When asymmetrical distributions were found, carbon 4 of hexoses was more heavily labelled than carbon 3, whilst carbons 1 and 2 were usually more heavily labelled than carbons 6 and 5 respectively. This phenomenon has been termed the "Gibbs effect" and there are many results attesting to the asymmetrical relationships described above. Attempts to account for the "Gibbs effect" have usually invoked various combinations of dilution effects

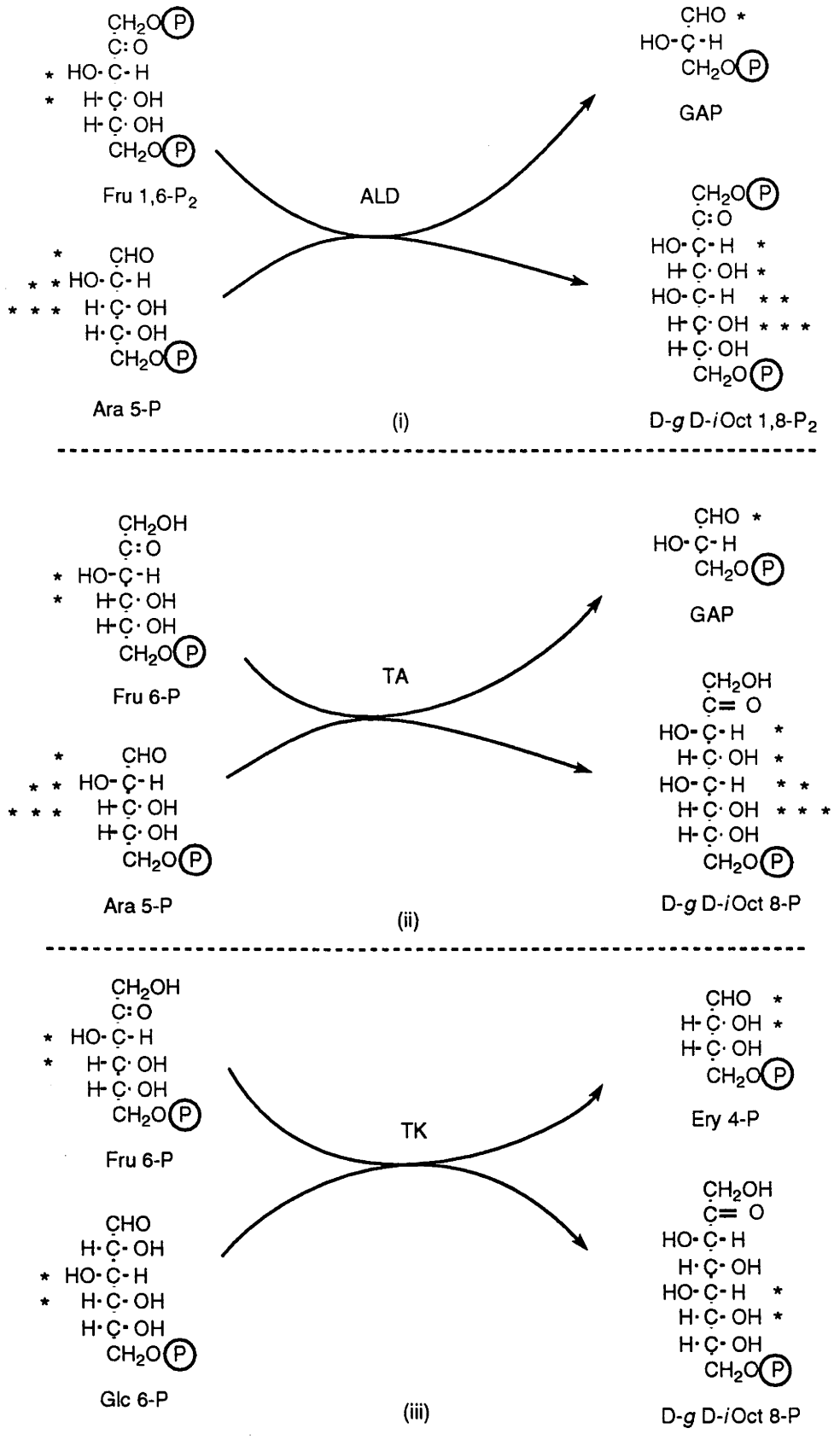


**Figure 1.4.** Possible reactions for the synthesis of D-g D-i octulose phosphates. (i) ALD= Aldolase ; (ii) TA = Transaldolase; TK = transketolase; * denotes $^{13}C$ label. The label distribution in arabinose assumes isotopic equilibration in the pentose pool.

and exchange reactions involving transketolase, aldolase and transaldolase, although this effect has also been shown to be possible through the operation of the modified version of the PCR pathway (Clark et al., 1974).

For the purpose of this discussion, the significance of the "Gibbs effect" is important only to the extent that it is derived from reactions related to the operation of the PCR cycle. Thus distortions to the PCR pathway-derived labelling patterns arising from the presence of exogenous metabolites and enzymes are excluded from consideration. The cytosol contains all of the enzymes of the oxidative pentose phosphate pathway (Emes et al., 1979; Simcox et al., 1977). Cytosolic aldolase may cause unlabelled aldo acceptors, glyceraldehyde 3-phosphate, erythrose 4-phosphate and ribose 5-phosphate from the PPP, to combine with labelled triose phosphate exported from the chloroplast to form fructose bisphosphate, sedoheptulose 7-phosphate and D-*glycero*-D-*altro*-octulose 1,8-bisphosphate respectively, with labelling patterns different to those formed in the chloroplast by photosynthetic PCR. Subsequent actions of phosphatases, transketolase, transaldolase, phosphoglucose isomerase, ribose phosphate isomerase and pentose phosphate epimerase have the potential to produce exogenous metabolites whose labelling patterns have little relationship to those produced by the PCR cycle within the chloroplast. When these exogenous metabolites are combined with the PCR metabolites in whole cell extracts misleading labelling patterns may emerge. *Thus for the purpose of understanding observed labelling patterns in PCR pathway intermediates, only data derived from isolated intact chloroplasts can be regarded as reliable.* Data derived from whole cell, or whole leaf studies should be disregarded until the labelling patterns derived from isolated intact chloroplast data are understood. Whilst it must be acknowledged that the operation of the PCR pathway in chloroplasts isolated from their normal regulatory processes in the intact organism is an artificial system, there are at present no indications that the PCR reaction sequence is any different under these conditions.

In addition, the assumption that all hexoses within the chloroplast will contain the same labelling pattern should not be made as there have been some suggestions that the labelling of fructose bisphosphate may be symmetrical, but that in glucose 6-phosphate is not (Havir et al., 1963). Frequently the source of the hexose examined was not reported and for the purpose of this discussion, only data from specified hexoses extracted from isolated, intact chloroplasts are considered to be useful. Data meeting these criteria are few. In isolated spinach chloroplasts Havir (Havir et al., 1963) found only a slight asymmetry between carbons 3 and 4 of glucose 6-phosphate and chloroplast polysaccharide glucose after fermentation and degradation with *Leuconostoc mesenteroides* (Table 1.2).

**Table 1.2.** Ratio of radioisotope in $C_4$ to $C_3$ in glucose from two sources in isolated spinach chloroplasts after exposure to $^{14}CO_2$ for varying times.*

| Source of glucose | $C_4$:$C_3$ ratio | | |
|---|---|---|---|
| | 4 min | 10 min | 20 min |
| Glucose 6-phosphate | 1.03 | 1.16 | 1.09 |
| Polysaccharide | 1.16 | 1.06 | - |

*(Havir et al., 1963)

Earlier work by the same group produced data showing that spinach chloroplasts containing a substantial amount of cytoplasmic material produced $C_4$:$C_3$ ratios of 2.42 (3 min) and 1.57 (4 min) (Gibbs et al., 1958), but when the chloroplasts were washed free of the cytoplasmic material the ratio was found to be only 1.09 (30 min). No trend over time was revealed in the later work from isolated chloroplasts.

Returning to the data of Havir (Havir et al., 1963), the asymmetry between carbons 1,6 and 2,5 was more pronounced with ratios of $C_1$:$C_6$ and $C_2$:$C_5$ ranging from 1.00 to 1.45 for glucose 6-phosphate and ranging from 1.01 to 1.60 for polysaccharide glucose. That study was not able to measure the label distribution in fructose bisphosphate, fructose 6-phosphate or the triose phosphates, so it is impossible to explain the asymmetry. However, if the only significant asymmetry proved to be confined to the $C_{1,2}$ and $C_{5,6}$ pairs of

carbons then this is consistent with a transketolase exchange of $C_1$ and $C_2$ of fructose 6-phosphate with a more highly labelled ketol group. It is not clear, however, what this highly labelled ketol group may be.

Asymmetry between carbons 3 and 4 of glucose 6-phosphate from isolated spinach chloroplasts exposed to $^{14}CO_2$ for various periods was more pronounced in the results of Kapuscinsci, shown in Table 1.3.

**Table 1.3.** Ratio of $^{14}C$ in related carbons of the glucose 6-phosphate from isolated spinach chloroplasts showing asymmetrical labelling of the glucose 6-phosphate.*

| Ratio | 40 sec | 60 sec |
|---|---|---|
| C4:C3 | 1.70 | 1.84 |
| C2:C5 | 0.86 | 1.57 |
| C1:C6 | 1.30 | 1.25 |

*(Kapuscinski, 1985).

In summary, this brief treatment of the "Gibbs effect" intends to make the following points:

1. There have been few degradative studies conducted to determine the extent of the asymmetry between the upper and lower three carbons of the hexose phosphates.

2. Those that have been made have mostly used extracts of whole leaves or whole organisms (algae) and the labelling distributions observed are necessarily influenced by metabolites and processes occurring in extrachloroplast cellular compartments.

3. The only evidence of asymmetry in hexoses produced by the operation of the PCR cycle comes from isolated intact chloroplast data. They show a range of degrees of asymmetry between $C_3$ and $C_4$ and significant asymmetry between $C_{1,2}$ and $C_{5,6}$ of glucose 6-phosphate and polysaccharide glucose.

4. The results are insufficient to be certain of the extent of the asymmetry in carbons 3 and 4. The source of the asymmetry remains unclear, although the

actions of unspecified exchange reactions by the broad specificity group transferring enzymes, transketolase, transaldolase and aldolase offer an attractive and feasible mechanism.

The "Gibbs effect" therefore remains an as yet unexplained departure from Calvin cycle theory.

## 1.4 Methods available for the analysis of PCR pathway intermediates using tracer isotopes

### 1.4.1 Chromatographic methods

A study of the intermediates of the photosynthetic carbon reduction pathway in isolated chloroplasts involves the analysis of a complex mixture of the phosphates of many monosaccharides. These have not been easy compounds to separate or detect and a variety of approaches have been used.

Since its first application to the analysis of carbohydrates (Partridge, 1946) paper chromatography (PC) has been an invaluable technique for the separation of mixtures of sugars. The identification of these resolved sugars was made by their co-chromatography with authentic standards and by a variety of colorimetric and chemical tests. The unravelling of the PCR pathway in the years following World War II became possible only with the development of paper chromatographic methods, coupled with the extraordinary sensitivity provided by radioautography ($10^{-12}$ mole). By subjecting photosynthesizing tissues to short periods of exposure to $^{14}CO_2$ and then quickly denaturing the plant material and analyzing the extracts by PC and radioautography, the identity of most of the photosynthetically active intermediates was deduced. Together, PC and radioautography permitted the separation of those compounds which had become labelled, and thus were deemed to be closely involved with photosynthesis, from the highly complex mixture of all classes of compounds found in the crude extracts of living plant

tissue. The distribution of isotope within the photosynthetically active intermediates could then be partially determined using the laborious microbial and chemical degradation methods then available. Subsequently, thin layer chromatography (TLC) provided increased speed and sensitivity over PC, but both PC and TLC are now generally regarded as inferior methods for the analysis of carbohydrates (Robards et al., 1986).

During the 1960's, low pressure column chromatography techniques were perfected and were used for the analysis of sugar phosphates extracted from tissues exposed to $^{14}C$- or $^{32}P$-labelled substrates. In these cases, detection was by liquid scintillation counting and quantification was by measurement of the peak areas from a liquid scintillation counting chromatogram. For the separation of small amounts of material when radioactive tracers were used, it was necessary to include unlabelled carriers to reduce adsorption losses (Heldt et al., 1980; Kapuscinski et al., 1985; Williams et al., 1971). Resolution between the hexose and pentose monophosphate fractions was poor, and only in one case (Williams et al., 1971) were sedoheptulose phosphates detected. Further purification by PC was necessary prior to degradation of the sugars to determine the position of the isotopic incorporation (Kapuscinski, 1985; Kapuscinski et al., 1985; Williams et al., 1971). A microscale anion exchange chromatography technique was used to obtain resolution between sugar phosphates extracted from isolated spinach chloroplasts which had been exposed to $^{14}CO_2$ (Lilley et al., 1977) but degradation of the sugar phosphates was not attempted. Enzymic assay of sugar phosphates isolated from chloroplast extracts by anion exchange chromatography has also been carried out but this was possible for only a few sugar phosphates (Heldt et al., 1980).

HPLC has been a favoured method in carbohydrate analysis because of its speed and minimal pre-treatment of samples. The short run times, low solvent volumes, good reproducibility and high sample throughput give HPLC distinct advantages when compared with traditional low pressure

chromatography. The interpretation of the chromatograms is simple because the anomeric forms of the sugars normally remain unresolved (Schaffler et al., 1984). This preference for HPLC is particularly true in the food industry for non-phosphorylated sugars where the high concentrations of sugars permit the use of low sensitivity detectors. For low concentrations of sugars, HPLC detection has depended on pre- or post-column derivatization to incorporate chromophoric or fluorophoric groups necessary for UV or fluorescence detection. More recently, pulsed amperometric detection (PAD) has permitted the detection down to 10 pmol of a range of monosaccharides resolved on a Dionex anion exchange column under alkaline conditions (Lee, 1990). For the separation of sugar phosphates extracted from photosynthetic tissue exposed to radioactive substrates, strong anion exchange columns (LiChrosorb AN (Giersch, 1979), Partisil 10 SAX (Giersch et al., 1987), (Giersch et al., 1980; Marques et al., 1986)) have been used. In these cases, detection was by liquid scintillation counting. When radioactive substrates were not used, detection has been by chemically suppressed conductivity (Smith et al., 1988). In one case, post-column treatment with phosphatase was carried out so that a molybdate reagent could react with the released inorganic phosphate (Pi) enabling spectrophotometric detection to be used (Meek et al., 1986). In no case was satisfactory resolution obtained between the hexose and pentose monophosphates and only in one case was sedoheptulose reported (as the bisphosphate rather than the monophosphate) (Marques et al., 1986).

The development since the 1950's of suitable techniques for the preparation of volatile derivatives of carbohydrates permitted an improvement in resolution to be obtained by the use of packed column gas chromatography (GC). Sugar phosphate standards prepared as the TMS derivatives were resolved on a packed column filled with 5% methylsilicon fluid (DC 430) on a pretreated support of Diasolid H (Hashizume et al., 1966). GC has been used for the analysis of the sugar phosphate products of the incubation of ribose 5-P with a rat liver enzyme preparation (Williams et al., 1978; Williams et al.,

1984), and for sugars extracted from whole spinach leaves (Kapuscinski, 1985). After dephosphorylation and derivatization as the TMS ethers, separation between at least 10 compounds was very good, and far superior to the results obtained with HPLC methods. For both HPLC and GC methods, however, interest has focussed on the identification and quantification of the analytes, since the question of the PCR pathway mechanism has been generally regarded as settled. If further analysis of labelling information within compounds was required, it was necessary to return to traditional methods of separating the sugars by PC followed by microbial and chemical degradation procedures (Kapuscinski, 1985), as analytical HPLC and GC methods do not lend themselves to the recovery of sufficient material for degradative purposes.

The most significant advance in separation technology came with the development of capillary GC using fused silica columns with bonded stationary phases. With a suitable choice of derivatives, this method provided short run times and outstanding resolution. The retention times are highly reproducible, the equipment is reliable, simple to use and suitable for routine analysis. It can be interfaced to sensitive universal detection systems such as the flame ionization detector and the mass spectrometer.

### 1.4.2 The choice of tracer isotope: $^{14}C$ vs $^{13}C$

Isotopic tracer studies of metabolic pathways have most frequently been conducted using radioactive isotopes. These methods have many advantages over non-radioactive techniques and upon their introduction in the 1940's, they revolutionised the study of metabolic pathways. The radioisotope provides a convenient and readily identifiable marker which allows the detection and tracing of the labelled compound with high sensitivity, as well as providing a built-in means of measuring sample recoveries.

As Calvin's techniques using $^{14}CO_2$ developed, he soon adopted what appeared to be a simple and straightforward approach for determining

the sequence of the intermediary steps in the pathway. The experiments would "consist of feeding a photosynthesizing organism radioactive carbon dioxide for various lengths of time and stopping the reaction by killing the plant. By determining those compounds into which the radioactive carbon has been incorporated for each period of illumination and, further, by determining the distribution of radioactivity within each compound, these data could then be used to construct a family of curves depicting the increase in radioactivity in each compound (and in each carbon atom of each compound) as a function of time. From a complete set of such curves it should be possible to draw a map of the path of carbon as it flows into the plant in the form of carbon dioxide and distributes itself among all the plant constituents." (Calvin et al., 1949). Using this technique he was successful in identifying the class of compounds which appeared to be most heavily involved in photosynthesis as the organic phosphates (Badin et al., 1950), and that amongst the organic phosphates, phosphoglyceric acid (PGA) was the first labelled intermediate (Calvin, 1949). However, he soon realized that this approach was not as simple as it first appeared. He began to recognize the complexity of the system he was dealing with and the need to be most careful about the maintenance of the photosynthesizing organism in the metabolic steady state. He also recognized the inapplicability of "precursor-product relationships" in the highly organized system of the intact cell, where the presence of separate metabolite pools may render specific activity measurements unreliable (Benson et al., 1952). Calvin then proceeded to propose the use of a mathematical model in which differential equations are written for the rate of change in specific activity of individual carbons in each compound. The solution to these equations would provide calculated appearance curves which could be compared with the observed appearance curves to test the validity of any proposed sequence of reactions (Calvin et al., 1952). This was a somewhat surprising approach since he had already recognized that the use of the whole organism precluded obtaining valid measurements of specific activity (Calvin, 1951). It is

therefore not surprising that it was later noted that the method of plotting such experimental appearance curves failed him completely in endeavouring to assign an order of precedence amongst the labelled compounds that were observed (Calvin, 1956) and he the therefore had to abandon this approach.

These observations concerning Calvin's experience with $^{14}CO_2$ illustrate that while the method led to many discoveries, there were many limitations in the use of $^{14}C$ as a tracer in unravelling metabolism *in vivo*. Apart from their hazardous nature, a principal disadvantage of radioisotopes in metabolism is that the detection methods tell nothing about the nature of the compound containing the radioisotopic label. Other analytical methods must be used to obtain information about compound identity, structure, purity, specific radioactivity and the location and degree of incorporation of the label within the molecule. Specific radioactivity measurements of the whole molecule are an inadequate value for assessing the order of precedence of intermediates in a pathway in which there are not necessarily simple, ordered transformations from one compound to the next. Reactions frequently involve the splitting of molecules and the rejoining of fragments that come from different parts of the pathway and contain different degrees of isotopic labelling. A further disadvantage is that degradation methods must be used if the degree of isotopic incorporation into specific atoms of labelled compounds is to be determined. It is in part because of the above problems that recent attention has been given to stable isotope methods using NMR spectroscopy and mass spectrometry.

### 1.4.3 $^{13}C$ NMR Spectroscopy

It is theoretically possible to study the PCR pathway in intact chloroplasts using $^{13}CO_2$ by nuclear magnetic resonance (NMR) spectrometry. This method has the very great advantage that it does not need the extensive extraction and purification steps that other methods require before an analysis can be made. As the natural abundance of $^{13}C$ is approximately 1.1% of $^{12}C$, small enrichments in individual carbons can be readily detected. Disadvantages are that the technique has low sensitivity and produces a $^{13}C$ NMR spectrum which can be very complex in the case of sugar phosphates most of which have a number of contributing forms at equilibrium. Thus the interpretation of the spectrum is very difficult or impossible, particularly when the investigation involves large differences in the concentrations of the various intermediates. As part of this investigation, consideration was given to the feasibility of conducting photosynthesis experiments on actively photosynthesizing chloroplasts in an NMR spectrometer (Graham, Flanigan, Collins, 1993 unpublished). Using published data for chloroplast metabolite concentrations (Gerhardt et al., 1987; Lilley et al., 1977; Stitt et al., 1980) it was estimated that a single chloroplast preparation equivalent to 8 mg chlorophyll would be necessary for the detection of all metabolites of interest using NMR. Natural abundance $^{13}C$ NMR was not a feasible option because of the low sensitivity of the method to this nucleus. Indirect detection methods, which offer high sensitivity, were investigated. Other practical difficulties included the illumination of such high densities of chloroplasts; the interference of protons and carbons from compounds of the suspension medium, distinguishing between chloroplastic and exogenous metabolites; the relatively short time during which isolated chloroplasts remain active; the long acquisition times needed for compounds present in low concentration; and the rate at which compounds of the PCR cycle are labelled. All of these combined to render the NMR approach totally impractical.

### 1.4.4 $^{13}C$ Mass spectrometry

Mass spectrometry is an an extremely sensitive analytical technique which has great potential in the investigation of reaction sequences of metabolic pathways. This technique overcomes many of those problems which hampered Calvin in the use of the $^{14}C$ isotope with PC and radioautography and the many practical difficulties associated with $^{13}C$-NMR metabolic studies. It has the potential to provide more definitive answers to questions relating to pathway reaction sequences and the identity of intermediates than Calvin was able to obtain.

When a mass spectrometer is used as the detector for a capillary GC separation system, the analytical power of the combination is unsurpassed. Extremely small samples, down to the picogram level, can be fully characterized using the combination of retention time ($R_t$) and electron impact (EI) mass spectrum. This not only gives an unequivocal identification of the components of a complex mixture of compounds, but it also enables detailed isotopic compositional analyses to be carried out on each component in the mixture from the mass spectral fragmentation pattern, without the need for laborious manual degradational methods. It is thus well suited to the conduct of isotopic incorporation studies on the components of a metabolic pathway such as the PCR pathway. Few studies of this type have been conducted. Desage used GC/MS to investigate the sites and extent of $^{13}C$ labelling in glucose produced by isolated hepatocytes from the gluconeogenic precursors, $^{13}C$-labelled glycerol or $NaH^{13}CO_3$ plus pyruvate or lactate (Desage et al., 1989). By studying glucose alone, deductions were made about the route of metabolism based on known biochemical pathway reactions. To date, other than the investigation reported here, the method has not been used for the study of all of the detectable components of a metabolic pathway in order to elucidate the reaction sequences involved.

## 1.5 Specific evidence required to clarify further the operation of the PCR pathway

### 1.5.1 Path of carbon reactions

Transketolase and aldolase are enzymes which not only play key roles in the normal operation of the PCR pathway, but also have a powerful capacity to catalyze exchange reactions. In fact the exchange capacity of these enzymes far exceeds the flux of carbon through the pathways in which these enzymes are active. Exchange reactions play no part in the net flow of carbon through the pathway but they do serve to equalize the isotope enrichments in the appropriate carbons of participating keto sugars. The capacity of these enzymes to obscure predicted labelling patterns in pathway tracer studies has been well documented (Clark et al., 1971; Clark et al., 1974; Williams et al., 1987), and was recognized by Racker (Racker, 1952), the Calvin group (Benson, 1954; Katz, 1992) and others. It is of note that these investigations recognized the theoretical consequences of exchange catalyzed by the group transferring enzymes. However only one study has been made of the quantitative measures of exchange rates for these enzymes versus the maximum pathway flux rates of tissue and chloroplast preparations (Flanigan et al., 1993). Williams and colleagues used controls to correct for transketolase exchange in all of their quantitative measurements of the L-type pentose pathway in liver and tumor tissues.

It therefore seems that there is little point in monitoring carbons subject to exchange reactions in an attempt to determine reaction sequences during tracer studies of metabolic pathways that include transketolase, aldolase or transaldolase. Thus C1 and C2 of the keto sugar monophosphates and C1,2,3 of the keto sugar bisphosphates need not be monitored during such studies. Instead, attention in PCR pathway investigations should be focussed on those carbons which are free from the influence of these exchange reactions and which may therefore more truly reflect reactions in the

path of carbon. If a distinction can be made between the two versions of the PCR pathway, then it is in these carbons a to d and e to i, identified by dashed boxes in Figures 1.5 and 1.6 respectively, that the necessary labelling information will be found. These carbons should show a consistency of labelling pattern and a progressive decrease in enrichment down the sequence of sugars from fructose 6-phosphate to ribose 5-phosphate and from glucose 6-phosphate to arabinose 5-phosphate. Inspection of Figure 1.3 shows that the sequence of sugars indicated in Figure 1.5 is the same as that appearing in the Calvin pathway of Figure 1.1 and may be termed the Calvin branch of the PCR pathway. Figure 1.3 also shows that the sequence of sugars indicated in Figure 1.6 appears only in the modified pathway and may be termed the alternative branch of the PCR pathway. The relative labelling intensities in these two branches should reveal the comparative importance of the two pathways.

### 1.5.2 Gibbs effect

As mentioned in section 1.3.2., past interpretations of the asymmetrical labelling in hexoses referred to as the "Gibbs effect" may have been based on incomplete and misleading results. To shed light on the extent and source of this effect, it is necessary to obtain the degree of label incorporation into each carbon of phosphoglyceric acid, glyceraldehyde 3 phosphate, dihydroxyacetone phosphate, fructose 1,6 bisphosphate, fructose 6 phosphate and glucose 6 phosphate. An understanding of the true labelling relationships between each of these compounds extracted from isolated intact chloroplasts after short term exposures to tracer isotope is a first step on the path that may lead to an understanding of the significance of the asymmetrical labelling in the top three and bottom three carbons of hexose and their relevance to PCR cycle theory.

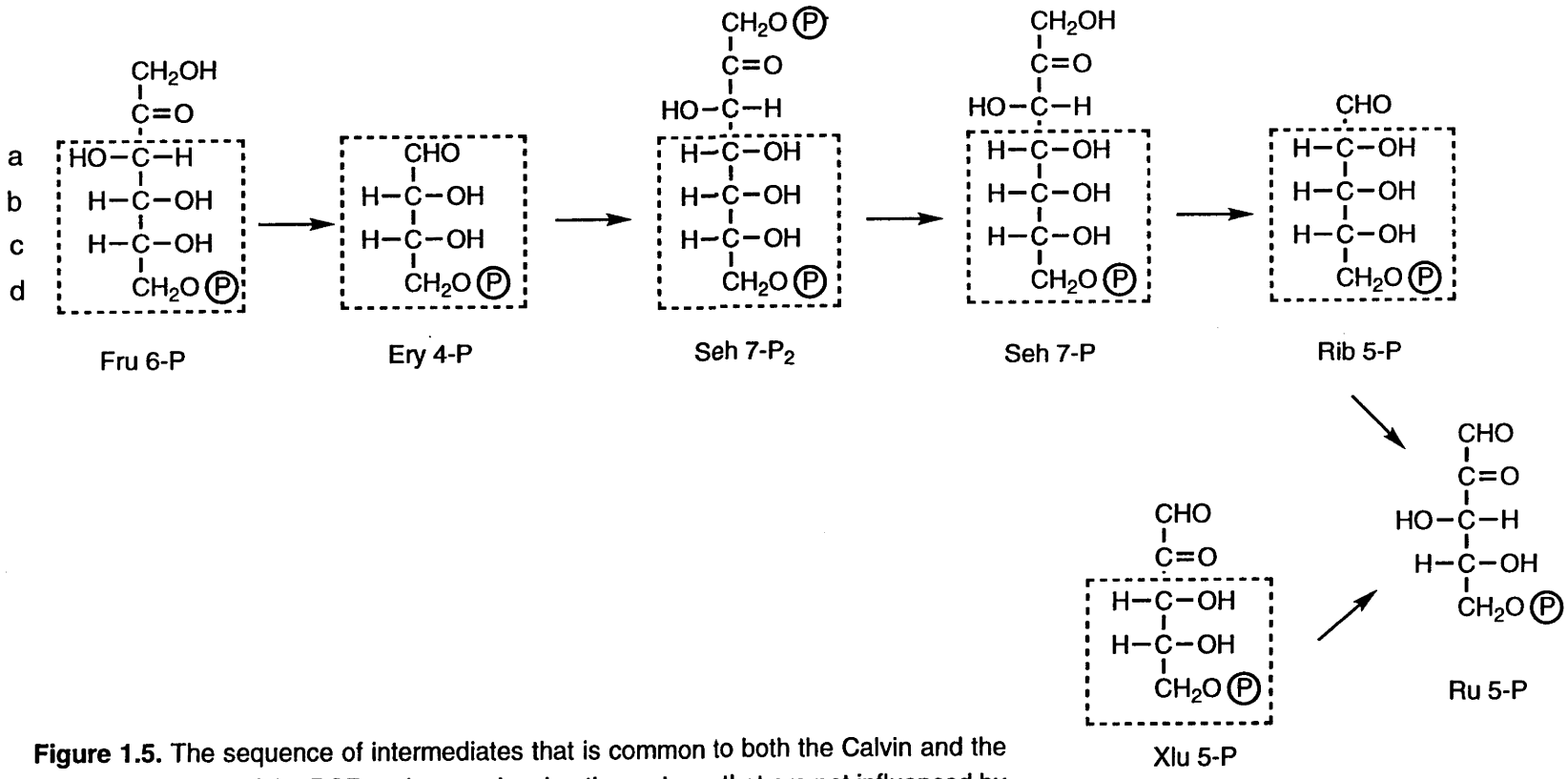


**Figure 1.5.** The sequence of intermediates that is common to both the Calvin and the modified versions of the PCR pathways showing the carbons that are not influenced by exchange reactions. Carbons 1 and 2 of Ru 5-P are subject to exchange, but C3,4,5 of Ru 5-P should be the resultant of the labelling in C3,4,5 of both Rib 5-P and Xlu 5-P.

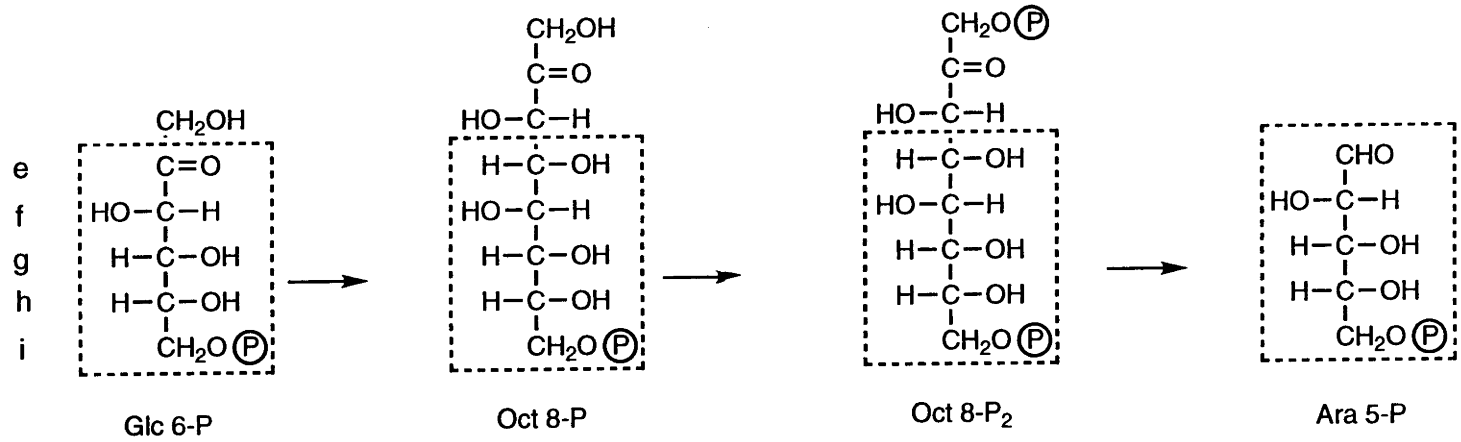


**Figure 1.6.** The sequence of intermediates that isexclusive to the modified version of the Calvin PCR pathway showing the carbons that are not influenced by exchange reactions.

## 1.6 Aims of this thesis

### 1.6.1 General approach

The aim of the research described in this thesis is to devise experimental procedures which would provide the specific labelling information needed to distinguish between the two alternative versions of the PCR pathway shown in Figures 1.1 and 1.3. These methods would be tested by conducting experiments on isolated, intact chloroplasts from spinach (*Spinacea oleracea*) which were subjected to short term exposure to $^{13}CO_2$. The sugar phosphates extracted from the chloroplasts would be dephosphorylated, derivatized and analyzed by GC/MS in order to determine the extent and pattern of $^{13}C$ incorporation into specific carbon atoms of each of the sugars identified in the extract. The effectiveness of the methodology would then be assessed.

### 1.6.2 Specific aims

In order to carry out the above experiments, it was first necessary to devise procedures and define operating parameters which would ensure the rigour and reproducibility of acquired data. In particular, the following were required.

1. Synthesis of specifically labelled $^{13}C$ sugars to carry out a GC/MS analysis of the fragmentation patterns of selected derivatives (Chapter 2).

2. GC/MS characterization (GC conditions, derivatization, acquisition procedures) of all sugars of interest to the study (Chapter 2).

3. Quantification of each of the sugar phosphates identified as an aid in the interpretation of the labelling enrichments observed. This aspect is discussed in Chapter 3.

4. Design of apparatus and procedures for the conduct of the photosynthesis experiments. This aspect is discussed fully in Chapter 3.

5. Development of procedures for the preparation and purification of chloroplast extracts prior to their analysis by GC/MS. This aspect is also discussed in Chapter 3.

6. Analysis of the $^{13}C$-labelling information accessible from a set of photosynthesis experiments. This aspect is also discussed fully in Chapter 4.

# CHAPTER 2

## SYNTHESIS AND

## GC/MS ANALYSIS OF

## SPECIFICALLY LABELLED SUGARS

## 2.1 Introduction

The analysis of the sugar phosphates extracted from isolated chloroplasts depends upon the correct identification of the individual components of a complex mixture of compounds. Often the components have similar structures and their separation can be difficult. In this study, capillary GC/MS of derivatized, dephosphorylated sugar phosphates was chosen as the analytical tool because of its superior ability to resolve and identify compounds at low concentrations, on the basis of both their retention time and mass spectrum.

A mass spectrometric positional isotopic analysis of photosynthetically $^{13}C$-labelled chloroplast metabolites generated by assimilation of $^{13}CO_2$ can only be carried out if the origins of the fragment ions appearing in the mass spectrum of each compound are known. Only ions that have a single origin are useful if accurate enrichment measurements to the 1% level are to be achieved. In order to determine the origins of the fragment ions, it is necessary to obtain, for each sugar, specifically labelled $^{13}C$-isotopomers for as many positions of the molecule as possible. For a few sugars, such as glucose, specifically labelled $^{13}C$-isotopomers are commercially available, but for most, especially the octuloses, which are of particular interest to this study, these isotopomers are not available and must be synthesized.

To follow the PCR pathway, it was not only necessary to identify and analyze the $^{13}C$-labelling patterns of each of the sugars, but also desirable to be able to quantify the individual sugars as an aid in the interpretation of the results of the observed labelling patterns. This involves the preparation of standard curves for each of the sugars, plotting the response of the measurement system to the sugar relative to an internal standard. For routine identification and quantification purposes it was therefore also necessary to

have available reasonable quantities unlabelled sugar or sugar phosphate standards. Details of the quantification procedure are described in Chapter 3.

A necessary and significant part of this research, therefore, was the synthesis of specifically labelled $^{13}C$-isotopomers of the $C_4$ to $C_8$ sugars implicated in either the Calvin or modified version of the PCR pathway.

### 2.1.1 Enzymic synthesis of $^{13}C$ sugar phosphate isotopomers.

As the sugar phosphates of interest to this study are intermediates of normal carbohydrate intermediary metabolism in a variety of tissues (Chapter 1), the most convenient means of synthesizing them was by use of the purified enzymes that are responsible for their formation *in vivo*. Several enzymes of carbohydrate metabolism catalyze the transfer of groups of carbon atoms from a donor to an acceptor molecule. The first preparations of isotopically labelled octuloses and heptuloses using these "group-transferring" enzymes were described by Clark (Clark et al., 1972). Transketolase and aldolase are group transferring enzymes that have been used for the synthesis of sugar-phosphates labelled with $^{14}C$ or $^{13}C$ in specific positions of the molecule (Kapuscinski et al., 1985), (Arora et al., 1988).

Transketolase-catalyzed reactions available for the synthesis of labelled or unlabelled sugar-phosphates are listed in Table 2.1. Reactions selected from the range in Table 2.1 were used to synthesize specifically labelled $^{13}C$-isotopomers from some commercially available specifically $^{13}C$-labelled substrates with high $^{13}C$ enrichment using the methods described in section 2.2. The reactions were not always straightforward, however, and it was not possible to prepare all of the specifically labelled $^{13}C$-compounds required for a complete analysis of all the sugars under investigation. It was desirable, however, that the synthetic procedures used resulted in a high level (>99%) of incorporation of the $^{13}C$-label in a given position on the sugar with no labelling elsewhere in the molecule.

**Table 2.1.** Reactions catalyzed by the "group transferring" enzymes, transketolase and aldolase.

| No. | Reaction[a] |
|---|---|
| 1 | ßOH-pyruvate + GAP ⇌ $CO_2$ + Xlu 5-P |
| 2 | ßOH-pyruvate + Ery 4-P ⇌ $CO_2$ + Fru 6-P |
| 3 | ßOH-pyruvate + Rib 5-P ⇌ $CO_2$ + Seh 7-P |
| 4 | ßOH-pyruvate + Glc 6-P ⇌ $CO_2$ + Dg Di Oct 8-P |
| 5 | ßOH-pyruvate + All 6-P ⇌ $CO_2$ + Dg Da Oct 8-P |
| 6 | Xlu 5-P + GAP ⇌ GAP + Xlu 5-P |
| 7 | Xlu 5-P + Ery 4-P ⇌ Fru 6-P + GAP |
| 8 | Xlu 5-P + Rib 5-P ⇌ GAP + Seh 7-P |
| 9 | Xlu 5-P + Glc 6-P ⇌ GAP + Dg Di Oct 8-P |
| 10 | Xlu 5-P + All 6-P ⇌ GAP + Dg Da Oct 8-P |
| 11 | Fru 6-P + Ery 4-P ⇌ Ery 4-P + Fru 6-P |
| 12 | Fru 6-P + Rib 5-P ⇌ Ery 4-P + Seh 7-P |
| 13 | Fru 6-P + Glc 6-P ⇌ Ery 4-P + Dg Di Oct 8-P |
| 14 | Fru 6-P + All 6-P ⇌ Ery 4-P + Dg Da Oct 8-P |
| 15 | Seh 7-P + Rib 5-P ⇌ Rib 5-P + Seh 7-P |
| 16 | Seh 7-P + Glc 6-P ⇌ Rib 5-P + Dg Di Oct 8-P |
| 17 | Seh 7-P + All 6-P ⇌ Rib 5-P + Dg Da Oct 8-P |
| 18 | DHAP + Ery 4-P ⇌ Seh 1,7-$P_2$ |
| 19 | DHAP + Rib 5-P ⇌ Dg Da Oct 1,8-$P_2$ |
| 20 | DHAP + Ara 5-P ⇌ Dg Di Oct 1,8-$P_2$ |
| 21 | Fru 1,6-$P_2$ + Ery 4-P ⇌ GAP + Seh 1,7-$P_2$ |
| 22 | Fru 1,6-$P_2$ + Rib 5-P ⇌ GAP + Dg Da Oct 1,8-$P_2$ |
| 23 | Fru 1,6-$P_2$ + Ara 5-P ⇌ GAP + Dg Di Oct 1,8-$P_2$ |
| 24 | Seh 1,7-$P_2$ + Ery 4-P ⇌ Ery 4-P + Seh 1,7-$P_2$ |
| 25 | Seh 1,7-$P_2$ + Rib 5-P ⇌ Ery 4-P + Dg Da Oct 1,8-$P_2$ |
| 26 | Seh 1,7-$P_2$ + Ara 5-P ⇌ Ery 4-P + Dg Di Oct 1,8-$P_2$ |

[a] Reactions 1-17 are catalyzed by transketolase; reactions 18-26 are catalyzed by aldolase.

The variety of substrates and products of the reactions of Table 2.1 shows that although these enzymes have very broad specificities they are usually assigned specific reactions with phosphorylated sugars in defined metabolic networks.

Recently, chemists have used these enzymes because of their ability to catalyze the stereospecific synthesis of compounds that are not easily made by conventional chemical methods (Toone et al., 1989), (Demuynck et al., 1990), (David et al., 1991) although chemical means are still used for the synthesis of some sugars (Walker et al., 1988). In many of these applications, the reactions were carried out using non-phosphorylated sugars and this was accomplished by the use of a large excess of enzyme. When only a very small amount of a specifically labelled compound is required, for example for GC-MS analysis, these reactions may also be carried out on a much reduced scale using small quantities of the enzyme. In this investigation, reactions of the latter type were especially useful for the synthesis of $^{13}C$-labelled erythrose, which could not be produced in sufficient yield using the phosphorylated forms of the reaction substrates.

### 2.1.2 Choice of ionization method

Electron impact (EI) ionization is known as a "hard" ionization technique, that is, it produces molecular ions many of which possess sufficient excess energy to undergo fragmentation, producing a fingerprint mass spectrum that contains a significant number of ion fragments. This compares with "soft" ionization techniques such as chemical ionization (CI), electrospray ionization (ESI) and fast atom bombardment (FAB) which produce mainly molecular ion species and few fragment ions. The production of diagnostic fragment ions is an essential element in the analysis of the isotopic enrichment in each of the carbon atoms of the sugars under investigation in this study. Although this might also be achieved using other techniques such

as MS/MS with other "soft" ionization methods, it is most conveniently carried out using EI ionization.

There are several other advantages of EIMS that are applicable to this study. In particular, being a unimolecular process, EI mass spectra show a very high degree of reproducibility, not only between repeated measurements using the same instrument, but also between different instruments, provided that ionization is carried out at approximately the same energy level (~70 eV). Thus the resulting mass spectrum is able to be compared with those published in standard EIMS libraries for compound identification. EIMS also has high sensitivity and is considered to be a "universal detector" for GC, enabling the combination to be used for a wide variety of applications and for quantitation purposes. When gas chromatography is combined with EI mass spectrometry, the system constitutes a simple, inexpensive technique which lends itself to routine use as a computer-controlled benchtop instrument.

One of the few disadvantages of EIMS is that compounds must be sufficiently volatile for gas phase ionization to occur. When compounds to be analyzed are very polar (e.g. sugars) they require derivatization to increase their volatility, a requirement which is also common to GC and thus enhances the compatibility of the combination. In spite of this additional workup step, EI was considered to be much the most suitable ionization method for this investigation.

### 2.1.3 Choice of derivatives for GC/MS analysis

The experiments described in this thesis are concerned with the analysis of the phosphate esters of $C_4$ to $C_8$ sugars. Since the introduction of gas chromatography in 1952 by James (James et al., 1952) it has proven to be a most effective technique for the separation of complex mixtures of compounds. Because of the low volatility of sugar phosphates they are unable to be analysed by GC unless they are first converted into more volatile derivatives. Although it is possible to silylate the sugar phosphates and carry

out separations by GC, silylation does present some difficulties (Pierce, 1968). The derivatization of some sugar phosphates was first accomplished by esterification of the phosphoric acid groups with diazomethane in a mixture of methanol and diethyl ether, followed by TMS derivatization (Wells et al., 1964a). Sugar monophosphates were derivatized as the TMS ethers (Hashizume et al., 1966), although some dephosphorylation may occur during the reaction. In order to avoid such side reactions, the analysis of sugar phosphates by GC is therefore usually best accomplished by their prior dephosphorylation.

The first report of the use of GC in the analysis of carbohydrates was in 1958 when some monosaccharides were converted to the fully methylated methyl glycosides and separated on an Apiezon M/Celite 545 packed column (McInnes et al., 1958). The separations were satisfactory and the recovery of the unchanged compounds demonstrated the stability of the compounds under these conditions. The retention times for the methyl mono-*O*-methyl D-glucopyranosides were very long and in an effort to find more volatile derivatives with reduced retention times, the monosaccharides were reduced with sodium borohydride and acetylated to give alditol acetate derivatives (Bishop et al., 1960), (Gunner et al., 1961) which had satisfactory GC characteristics.

A further improvement to the volatility of carbohydrates was provided by the introduction of the trimethylsilyl derivative (Hedgley et al., 1960), although it was not until a simple derivatization procedure was described in a classical paper (Sweeley et al., 1963) that the use of the method became widespread. The addition of a 2:1 mixture of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in anhydrous pyridine to the dried sugars resulted in rapid derivatization at room temperature in virtually quantitative yields.

The TMS derivative is the most preferred of the volatile derivatives of sugars, and more powerful silyl donors such as N-trimethylsilylimidazole, N,*O*-

bis(trimethylsilyl)acetamide (BSA) and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) have also been developed, with TMCS included as a catalyst. Wells (Wells et al., 1964b) has written an early history of the application of gas chromatography to the analysis of carbohydrates while Robards (Robards et al., 1986) has reviewed more recent developments.

Aqueous solutions of carbohydrates consist of an equilibrium mixture which can include the linear form, together with the α and β anomers of both the pyranose and furanose ring forms. All of the direct derivatization procedures can therefore give rise to multiple chromatographic peaks for each isomeric compound which greatly complicates the analysis. Where a considerable number of sugars are present as in the PCR pathway, direct derivatization techniques can make the analysis impracticable.

The problem of the various anomeric forms of sugars giving rise to multiple chromatographic peaks can be circumvented by the use of acyclic derivatives. The simplest of these methods is the reduction of the sugars to alditols before derivatization of the hydroxyl groups as either the peracetates or the pertrimethylsilyl ethers. Although this procedure has been widely used, it involves a loss of structural information because the same alditol can be formed from different ketose and aldose precursors e.g. the reduction of both D-mannose and, D-fructose produces mannitol (Figure 2.1).

An alternative acyclic derivative introduced by Sweeley (Laine et al., 1971) for the analysis of sugars by GC/MS is the formation of the methoxime of the carbonyl group with the subsequent derivatization of the free hydroxyls as the TMS ethers. This avoids the loss of structural information and results in two straight chain forms only, the *syn-* and *anti-* methoximes (shown in Figure 2.2) whilst involving a minimum of chemical procedures.

Sugar *O*-methyloxime TMS (Mox-TMS) derivatives have been reported to give the best overall results by capillary GC where accurate quantification of mixtures that were not too complex was required (Andrews, 1989). However, where the mixtures were more complex, improved resolution

could be obtained with the use of *O*-benzyloxime TMS derivatives. Trifluoroacetylation (TFA) of the *O*-benzyloximes provided even better resolution (Andrews, 1989). For the routine quantification of sugars by GC using autosampler techniques, derivatization was best accomplished as either the TMS or oxime-TMS derivatives of aqueous sugar solutions (Schaffler et al., 1984). This avoided the time-consuming preliminary drying step, but required the use of a 100-fold excess volume of silylating reagent.

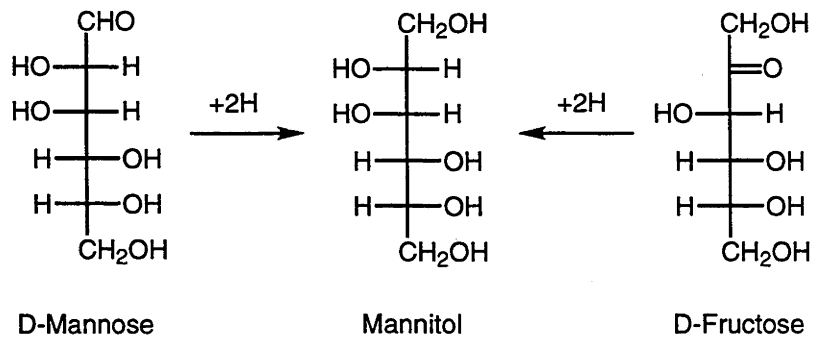


**Figure 2.1** Both D-mannose and D-fructose are reduced to mannitol.

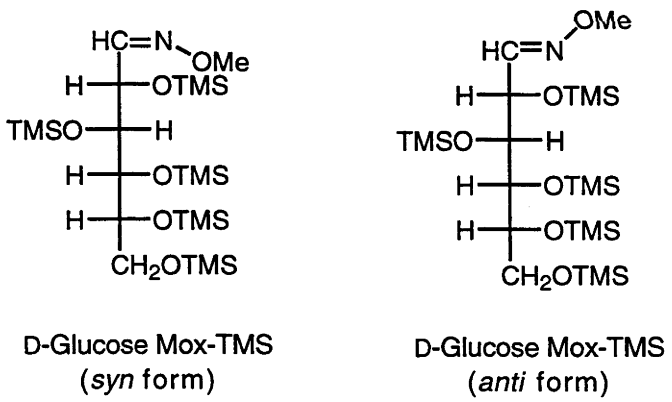


**Figure 2.2** The *syn* and *anti* forms of the methoxime - TMS derivatives of glucose.

For the chosen derivative to be suitable for the GC/MS analysis of the photosynthetic carbon reduction cycle intermediates encountered in this study, it was necessary that it conform to the following requirements: (i) in its EI mass spectrum, the derivatized sugar should show a range of discrete fragment ions each having a single origin; (ii) there should be a minimum number of isomers

produced for each sugar; (iii) when derivatized, the complex of sugars involved in the photosynthetic cycle should be readily separable by capillary GC; (iv) derivatization should be quantitative for all sugars and involve minimum manipulations; and (v) the molecular weight of the derivatized sugar should be kept as low as possible.

Using the two octulose isomers of interest to this study as model compounds, three derivatives, per-TMS, methoxime-TMS (Mox-TMS) and ethoxime-TMS (Eox-TMS) were assessed for their suitability for use in this investigation. The Mox-TMS was found to be the most suitable for routine analyses whilst the Eox-TMS could be used in a few instances where additional information was deemed necessary (Irvine et al., 1992). This was in agreement with Desage, who examined four derivatives of glucose and found the Mox-TMS to be the most suitable for a positional isotopic analysis of $^{13}C$-labelled glucose in a study of gluconeogenesis in liver cells (Desage et al., 1989).

## 2.2 Materials and Methods

### 2.2.1.Materials

All enzymes were obtained from either Sigma Chemical Co., St. Louis, MO63178, U.S.A. or from Boehringer-Mannheim Corp., D-6800 Mannheim, Germany (Table 2.2). [1,3-$^{13}C$] Glycerol was purchased from ISOTEC Inc., Miamisburg, Ohio, 45342 and all other $^{13}C$-labelled compounds were purchased from Cambridge Isotopes Limited, Andover, MA 01810-5413, U.S.A. All $^{13}C$-labelled compounds were 99 % enriched except for the [6-$^{13}C$]-glucose which was 90 % enriched. Enzymes used in this investigation are listed in Table 2.2, along with their designated E.C. numbers. All sugars referred to have the D-configuration.

All other chemicals and solvents were of analytical grade and were purchased from either Ajax chemicals, Sydney, Australia or from BDH, Poole BH12 4NN, UK, or from E. Merck, D-6100 Darmstadt, Germany. All solvents

**Table 2.2.** Enzymes referred to in this thesis, together with their designated EC numbers.

| Enzyme | EC Number |
|---|---|
| Acid phosphatase | EC 3.1.3.2 |
| Aldolase | EC 4.1.2.13 |
| Alkaline phosphatase | EC 3.1.3.1 |
| Arabinosephosphate isomerase | EC 5.3.1.13 |
| Catalase | EC 1.11.1.6 |
| Fructose bisphosphatase | EC 3.1.3.11 |
| Glucose 6-phosphate dehydrogenase | EC 1.1.1.49 |
| Glucosephosphate isomerase | EC 5.3.1.9 |
| Glyceraldehyde phosphate dehydrogenase | EC 1.2.1.12 |
| Glycerokinase | EC 2.7.1.30 |
| Glycerol 3-phosphate dehydrogenase | EC 1.1.1.8 |
| Hexokinase | EC 2.7.1.1 |
| L-Lactate dehydrogenase | EC 1.1.1.27 |
| Phosphoriboisomerase | EC 5.3.1.6 |
| Phosphoribulokinase | EC 2.7.1.19 |
| Phosphotransferase | Not assigned |
| Ribulosephosphate 3-epimerase | EC 5.1.3.1 |
| Ribulose bisphosphate carboxylase (Rubisco) | EC 4.1.1.39 |
| Sedoheptulose bisphosphatase | EC 3.1.3.37 |
| Transaldolase | EC 2.2.1.2 |
| Transketolase | EC 2.2.1.1 |
| Triosephosphate isomerase | EC 5.3.1.1 |

were distilled and pyridine was pre-dried over solid potassium hydroxide and distilled from calcium hydride under an argon atmosphere. Water was obtained from a Milli-Q water purification system (Millipore).

Anion exchange resins were from BioRad, Richmond, CA 94804, U.S.A. and phenylboronate agarose gel (Matrex PBA 60) was purchased from Amicon Corp., Danvers, MA 01923, U.S.A.

### 2.2.2 Chromatography

When larger quantities of specifically labelled purified compounds were required for 13C NMR studies or further syntheses sugar phosphates were separated or purified by the formate anion exchange chromatographic method of Bartlett and Bucolo, (1968) (Bartlett et al., 1968) except for the hexose monophosphates. In the case of the hexose monophosphates separation was unsatisfactory by this method but good separation was achieved the using borate anion exchange chromatographic method of Williams et al, (1971) (Williams et al., 1971).

#### 2.2.2.1 Formate anion exchange chromatography.

The sample containing the synthetic labelled or unlabelled sugar phosphates to be separated was passed through a column (Pharmacia K15/30) packed with anion exchange resin (Bio Rad AG 1 x 8, 200-400 mesh) in the formate form. After washing the sample through the column with 100 mL deionized water, sugar phosphates were eluted with a linear gradient formed with water (0.6 L) in a mixing beaker and a solution 1 M ammonium formate + 4 M formic acid (0.6 L) in a reservoir beaker. Liquid from the mixing beaker was pumped onto the column using a peristaltic pump (Pharmacia P-3, Pharmacia Biotech, Uppsala, Sweden) (0.8 mL $min^{-1}$) and liquid was drawn from the reservoir into the mixing beaker through an interconnecting overhead bridge.

Fractions of 6-8 mL were collected using a Golden Retriever Model 820 fraction collector (Instrument Specialties Co. Inc., Lincoln, Nebraska, U.S.A.). Appropriate colourimetric tests (section 2.2.3) were used to locate fractions containing sugar phosphates. Ammonium ions were removed from the pooled fractions testing positive to the colourimetric tests by cation exchange chromatography (Bio Rad AG50W 200-400 mesh resin in the acid form). The size of the cation exchange column depended on the volume and strength of the formate eluate containing the sugar phosphate and the sample was washed through with at least two bed volumes of deionized water (Milli-Q). The eluate was reduced to 50 mL by rotary film evaporation under reduced pressure (Rotovapor, Büchi), then continuously extracted (Soxhlett liquid/liquid extractor) with diethyl ether for up to 48 h to remove formic acid. The sugar phosphate solution was then neutralized (pH 6.8) with NaOH (0.02 M) and lyophilized.

#### 2.2.2.2 Borate anion exchange chromatography

Solvents for borate anion exchange chromatography were degassed by filtration through a Waters solvent clarification kit (Waters Corporation, Milford M 01757 U.S.A.) and subjected to reduced pressure for 15 min. prior to use. Anion exchange resin (BioRad AG1x8, 200-400 mesh) in the chloride form was converted to the borate form by elution with 0.8 M sodium tetraborate until the eluate was free of chloride ions. The resin was then washed with starting buffer (0.1 M ammonium tetraborate) until the conductivity of the eluate was equal to that of the starting buffer (3.8 mmho) as measured using a Radiometer Copenhagen Type CDM 2e conductivity meter (Radiometer Copenhagen, Copenhagen NV, Denmark).

The pH of samples containing the hexose phosphates to be separated was adjusted to 8.0 with a dilute solution of ammonium hydroxide and the samples were applied to the column as for the formate chromatography in section 2.2.2.1. Sugar phosphates were eluted using a

linear concentration gradient that increased from 0.1 to 0.4 M ammonium tetraborate. The volume of 0.1 M ammonium tetraborate in the mixing beaker was 0.8 L and an equal volume of 0.4 M ammonium tetraborate was used in the eluent beaker. Fractions containing hexose phosphates were identified using the anthrone reagent referred to in section 2.2.3. Ammonium tetraborate was removed as methyl borate and ammonia from fractions containing glucose 6-P or fructose 6-P by evaporation with repeated additions of methanol in a rotary film evaporator under reduced pressure. The sample was redissolved in deionized water and the pH of the solution was adjusted to 6.8 before lyophilization.

#### 2.2.2.3 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) using a Waters strong anion exchange (SAX 8 x 10) Radial Compression cartridge and Waters HPLC system permitted the rapid separation of $^{13}C$-labelled fructose 6-phosphate from ATP and ribose 5-phosphate when small amounts of $^{13}C$-labelled fructose 6-phosphate were produced in small-scale reaction mixtures.

All samples were filtered through a 4 mm filter unit (Activon, AGS1104N4P, 0.45 μm nylon) prior to HPLC purification. Solvents were filtered using a Waters solvent clarification kit followed by sonication for 30 min to degas the solvent. A Waters SAX 8 x 10 Radial Compression cartridge was prepared for use by carrying out a blank run using a programmed gradient routine in the Waters HPLC system. The gradient programme consisted of a 3 min wash with deionized water followed by a linear concentration gradient from 0 to 1 M sodium acetate pH 4.6 over 20 min. The final concentration of 1 M sodium acetate was maintained for a further 7 min. before employing another linear gradient which reduced the concentration to 100 % water over 10 min. The column was equilibrated for 15 min. with water before commencing a run in which 500 μL of sample was injected through a Rheodyne injector and the above gradient routine applied. The above

procedure permitted the elution of sugar monophosphates but sugar diphosphates were not eluted by this procedure as synthetic diphosphates were not required. However, if necessary, the sugar diphosphates could be eluted using a similar gradient system to that described above, but using 2 M ammonium formate + 4 M formic acid pH 3.2 as the elution buffer. Fractions of 1 mL were collected in a Golden Retriever Model 1200 "Pup" fraction collector (Instrument Specialties Co. Inc., Lincoln, Nebraska, U.S.A.). Fractions containing hexose phosphates were identified by the anthrone reagent method referred to in section 2.2.3 or by specific enzymic assay (Bergmeyer, 1974).

#### 2.2.2.4 Phenylboronate agarose gel chromatography

The epimeric mixture of aldopentose phosphates arising from the synthesis of [1-$^{13}$C]-ribose 5-phosphate was resolved into [1-$^{13}$C]-ribose 5-phosphate and [1-$^{13}$C]-arabinose 5-phosphate fractions on phenylboronate agarose gel and purified using previously described methods (Arora et al., 1988).

### 2.2.3 Colourimetric tests for synthetic sugar phosphates

Sugar phosphates which eluted from liquid chromatography columns were located in eluate fractions by standard colourimetric tests which distinguish different classes of sugars. Hexose phosphates were detected by the anthrone reagent method (Graham et al., 1965); pentose phosphates were detected using orcinol reagent (Blackmore et al., 1974) and octulose phosphates were detected using cysteine-$H_2SO_4$ (Paoletti et al., 1979c). Absorption spectra in the visible region were obtained on a Gilford 2600 single beam spectrophotometer coupled with a Hewlett Packard 7225 A plotter.

## 2.2.4 Synthesis of labelled and unlabelled sugars and sugar phosphates

### 2.2.4.1 $^{13}$C-Labelled erythrose isotopomers

[1-$^{13}$C]-, [2-$^{13}$C]-, and [4-$^{13}$C]-Erythrose were prepared using the reaction scheme shown in Figure 2.3. The reaction mixture contained triethanolamine (25μmol), fructose 1,6-bisphosphate (0.25 μmol), either [3-$^{13}$C]-, or [4-$^{13}$C]- or [6-$^{13}$C]-fructose (0.4 μmol) (prepared by dephosphorylation (section 2.2.5.2) of the phosphate esters prepared according to section 2.2.4.4), $MgCl_2$ (5 μmol), thiamine pyrophosphate (0.5 μmol), aldolase (0.9 units) and transketolase (0.5 units) and was made up to a volume of 0.375 mL with water. The reaction was conducted at 25 °C for 22 h and was terminated by heating at 100 °C for 2 min. After cooling, salts and phosphorylated sugars were removed from the reaction mixture according to the method described in section 2.2.5.3 and the resultant sugars were derivatized for GC/MS analysis (section 2.2.5.4). The mass spectra showed that there was no dilution of the $^{13}$C-label arising from the presence of the fructose 1,6-bisphosphate.

### 2.2.4.2 $^{13}$C-Labelled xyluloses

[1-$^{13}$C]-Xylulose was prepared from [1-$^{13}$C]-fructose 6-phosphate in a reaction catalyzed by transketolase with glyceraldehyde as the acceptor molecule for the ketol group (Figure 2.4). The reaction mixture (1 mL) contained triethanolamine HCl (100 μmol), $MgCl_2$ (15 μmol), glyceraldehyde (2 μmol), thiamine pyrophosphate (2 μmol) and [1-$^{13}$C]-fructose 6-phosphate (6 μmol). The pH was adjusted to 7.6 with 2 M NaOH and the reaction was commenced with the addition of transketolase (2 units) and left for 8 h at 37 °C. The reaction was terminated by heating for 2 min. at 100 °C. The cooled reaction mixture was then deionized according to section 2.2.5.3 and lyophilized for GC/MS analysis.

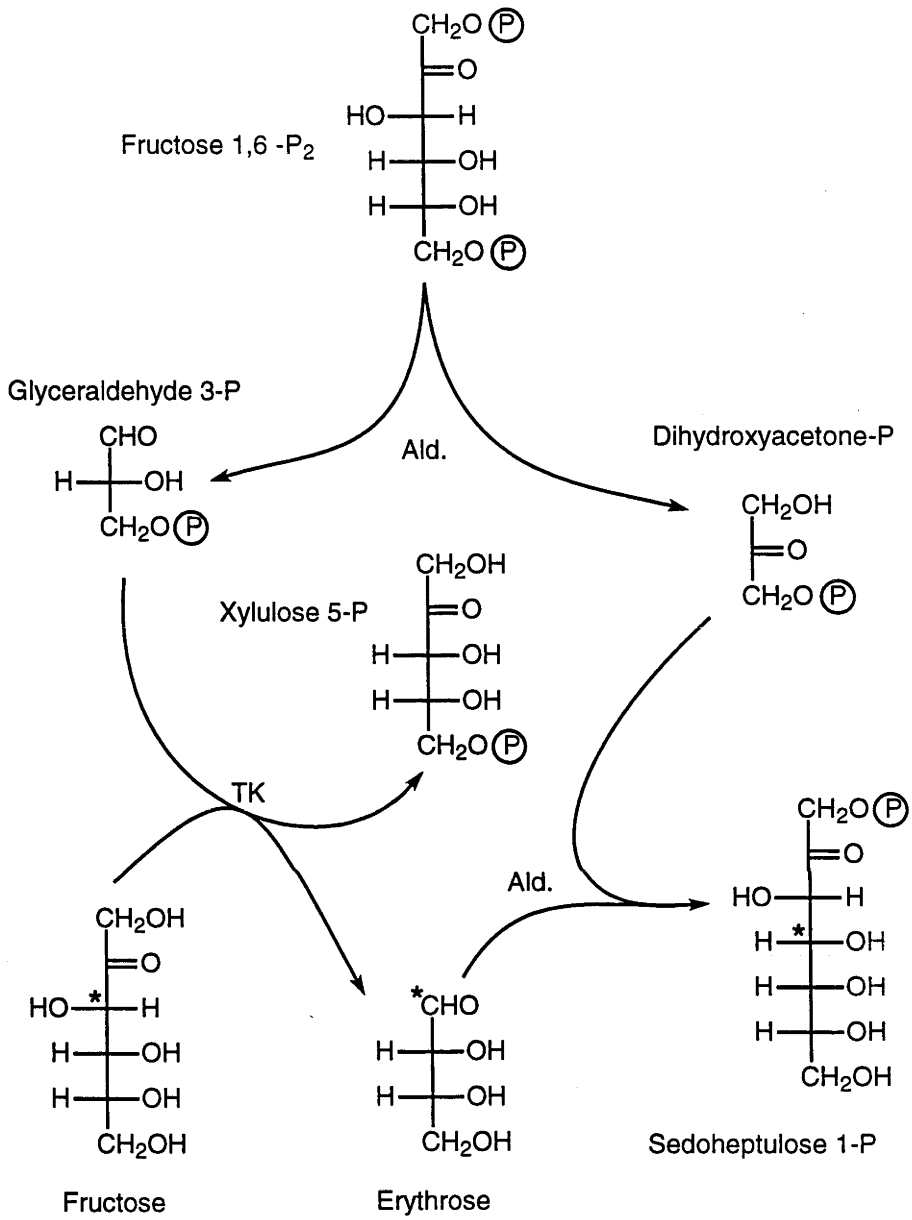


**Figure 2.3** Reaction scheme for the synthesis of [1-$^{13}$C]-erythrose from [3-$^{13}$C]-fructose.

* Denotes the position of the $^{13}$C-label. Ald. = aldolase; TK = transketolase.

[4-$^{13}$C]-Fructose produces [2-$^{13}$C]-erythrose and [5-$^{13}$C]-sedoheptulose 1-P.

[6-$^{13}$C]-Fructose produces [4-$^{13}$C]-erythrose and [7-$^{13}$C]-sedoheptulose 1-P.

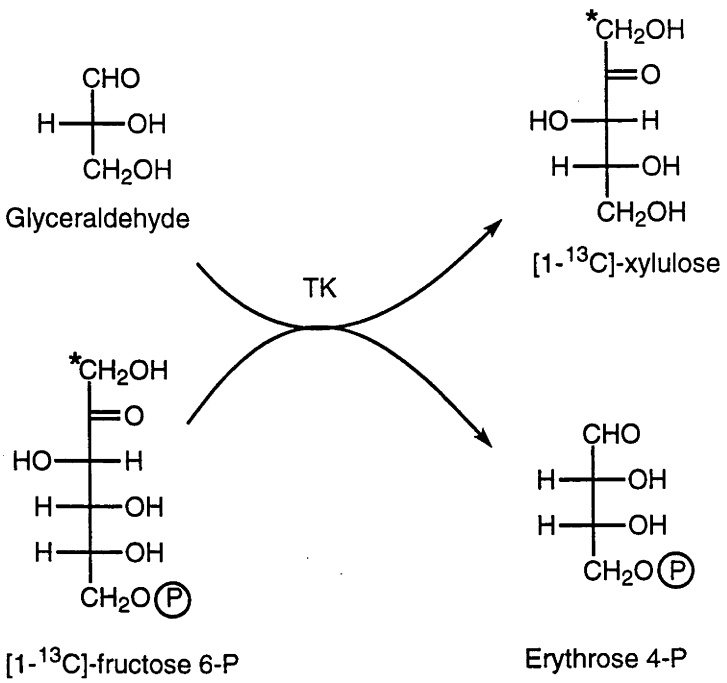


**Figure 2.4** Reaction scheme for the synthesis of [1-$^{13}$C]-xylulose from [1-$^{13}$C]-fructose 6-P.
* denotes $^{13}$C-label; TK = transketolase.

Attempts were made to prepare [4-$^{13}$C]- and [3,5-$^{13}$C]-xylulose from [2-$^{13}$C]-glycerol and [1,3-$^{13}$C]-glycerol respectively using the reaction scheme shown in Figure 2.5. The reaction mixture (0.25 mL) contained triethanolamine-HCl (25 μmol), [2-$^{13}$C] glycerol (0.25 μmol), fructose (1.25 μmol), pyruvate (2.5 μmol), adenosine triphosphate (1.25 μmol), nicotinamide adenine dinucleotide (oxidized form, 2.5 μmol), $MgCl_2$ (5 μmol), thiamine pyrophosphate (0.5 μmol), glycerokinase (1 unit), glycerol 3-phosphate dehydrogenase (0.5 units), lactate dehydrogenase (1 units) and transketolase (0.25 units). The pH of the reaction mixture was adjusted to 7.6 with 2 M NaOH prior to the addition of the enzymes. The reaction was terminated either by heating at 100 °C for 2 min. or by the addition of cold methanol (1mL), or by the addition of cold 0.6 M $HClO_4$ followed by neutralization with dilute KOH solution. The denatured protein was then sedimented by centrifugation at 12,000 rpm for 10 min. and the supernatant solution containing the sugars and sugar phosphates was transferred to a rotary film evaporator and reduced to approximately 0.2 mL. The mixture was then dephosphorylated (section 2.2.5.2), deionized (section 2.2.5.3) and derivatized for GC/MS analysis (section 2.2.5.4). The mass spectra of the derivatized [4-$^{13}$C]- and [3,5-$^{13}$C]-xyluloses showed that there was significant dilution of the label and these compounds could therefore not be used for studies aimed at identifying the origins of diagnostic ions in xylulose.

### 2.2.4.3 [1-$^{13}$C]-Ribose 5-phosphate

[1-$^{13}$C]-Ribose 5-phosphate and [1-$^{13}$C]-arabinose 5-phosphate were synthesized from erythrose 4-phosphate and K$^{13}$CN using the classical Kiliani synthesis method (Arora et al., 1988) and purified prior to use by the phenylboronate agarose gel chromatography method described therein.

### 2.2.4.4. Specifically labelled $^{13}$C-glucose 6-phosphates and fructose 6-phosphates

[1-$^{13}$C]-, [2-$^{13}$C]-, [3-$^{13}$C]-, [4-$^{13}$C]-, and [6-$^{13}$C]-Glucose 6-phosphates were synthesized from the corresponding commercially available specifically labelled $^{13}$C-glucose isotopomers in order to provide the substrates necessary for the synthesis of [3-$^{13}$C]-, [4-$^{13}$C]-, [5-$^{13}$C]-, [6-$^{13}$C]-, and [8-$^{13}$C]-D-*glycero* D-*ido* octulose 8-phosphates respectively, and other $^{13}$C-labelled sugar phosphates.

The appropriately labelled glucose (20 mg) was phosphorylated in a solution (10 mL) containing triethanolamine HCl (400 μmol), adenosine triphosphate (500 μmol), $MgCl_2$, (80 μmol), and set to a pH of 7.6 with 2 M NaOH. The reaction was commenced by the addition of hexokinase (2 units) and allowed to proceed for 90 min. at 37 °C after which it was terminated by heating for 5 min. at 100 °C.

Nucleotides were removed from the cooled solution by stirring with 1 g of activated charcoal for 10 min. three times followed by filtration of the suspension through Whatman (Whatman Asia Pacific Pte Ltd., 08-01 San Centre Singapore 169877) No 542 filter paper. The clear filtrate, which contained the specifically labelled glucose 6-phosphate, was then further purified by borate anion exchange chromatography (section 2.2.2.2).

Specifically $^{13}$C-labelled fructose 6-phosphates were prepared from appropriately $^{13}$C-labelled glucose using the above procedure for $^{13}$C-glucose 6-phosphates, except that phosphoglucose isomerase (5 units) was included in the reaction mixture. Anion exchange chromatography by the borate method described in section 2.2.2.2 resolved the two hexose phosphates.

### 2.2.4.5 Specifically $^{13}C$-labelled Sedoheptulose

(i) [3-$^{13}C$]-Sedoheptulose was prepared from [1-$^{13}C$]-ribose using the transketolase catalyzed reaction with β-hydroxypyruvate illustrated in Figure 2.6. The reaction mixture (0.25 mL) contained triethanolamine (50 μmol), β-hydroxypyruvate (1.5 μmol), [1-$^{13}C$]-ribose (0.75 μmol), $MgCl_2$ (5 μmol), thiamine pyrophosphate (0.5 μmol) and transketolase (1 units). The reaction was allowed to proceed at 25 °C for 27 h and was terminated by heating at 100 °C for 2 min. The cooled reaction mixture was then passed through a bed of mixed resin as described in section 2.2.5.3. The sample was redissolved in water (200 μL) and an aliquot (50 μL) was taken for derivatization (section 2.2.5.4) and GC/MS analysis (section 2.2.5.4).

The same reaction could have been used to prepare [1-$^{13}C$]- or [2-$^{13}C$]-sedoheptulose if the ribose was unlabelled and when either [1-$^{13}C$]- or [2-$^{13}C$]-fructose replaced β-hydroxypyruvate. This was not carried out as the mass spectrum of sedoheptulose does not produce any significant cleavage between carbons 1 to 3 and thus little information would have been added to that already provided by the [3-$^{13}C$] isotopomer of sedoheptulose.

(ii) [4-$^{13}C$]-, [5-$^{13}C$]- and [7-$^{13}C$]-Sedoheptulose 1,7-bisphosphates were prepared in a two-step process, the first of which was the synthesis and partial purification of the precursor [3-$^{13}C$]-, [4-$^{13}C$]- and [6-$^{13}C$]-fructose 6-phosphates from the correspondingly labelled glucoses. The second step involved the use *in vitro* of the aldolase-transketolase-aldolase sequence of reactions that Calvin described for the formation of sedoheptulose bisphosphate *in vivo* during photosynthesis (Figure 2.7).

Either [3-$^{13}C$]-, or [4-$^{13}C$]- or [6-$^{13}C$]-labelled glucose (3 mg) was phosphorylated in a solution (1 mL) containing triethanolamine HCl (80 μmol), adenosine triphosphate (30 μmol) and $MgCl_2$, (8 μmol), and adjusted to a pH of 7.6 with 0.2 M NaOH. The reaction was commenced with the addition of hexokinase (0.5 units) and phosphoglucose isomerase (0.3 units) and was

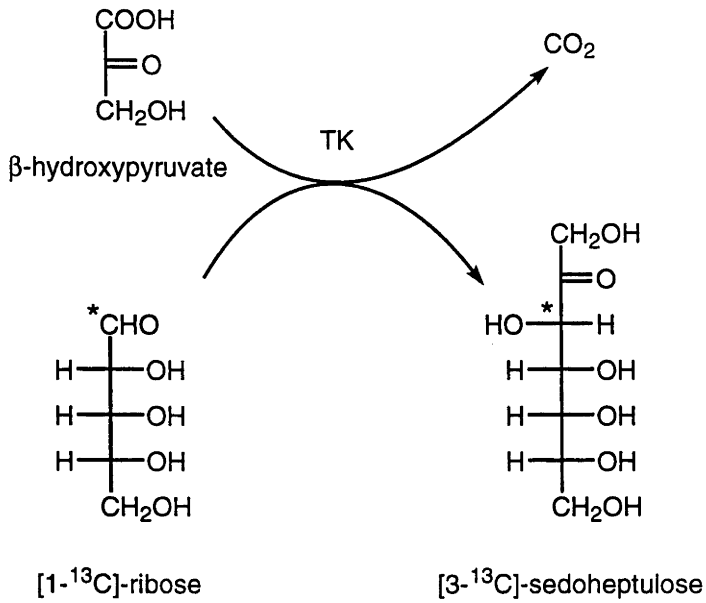


**Figure 2.6.** Reaction scheme for the synthesis of [3-$^{13}$C]-Sedoheptulose.
TK = transketolase; * denotes $^{13}$C-label.

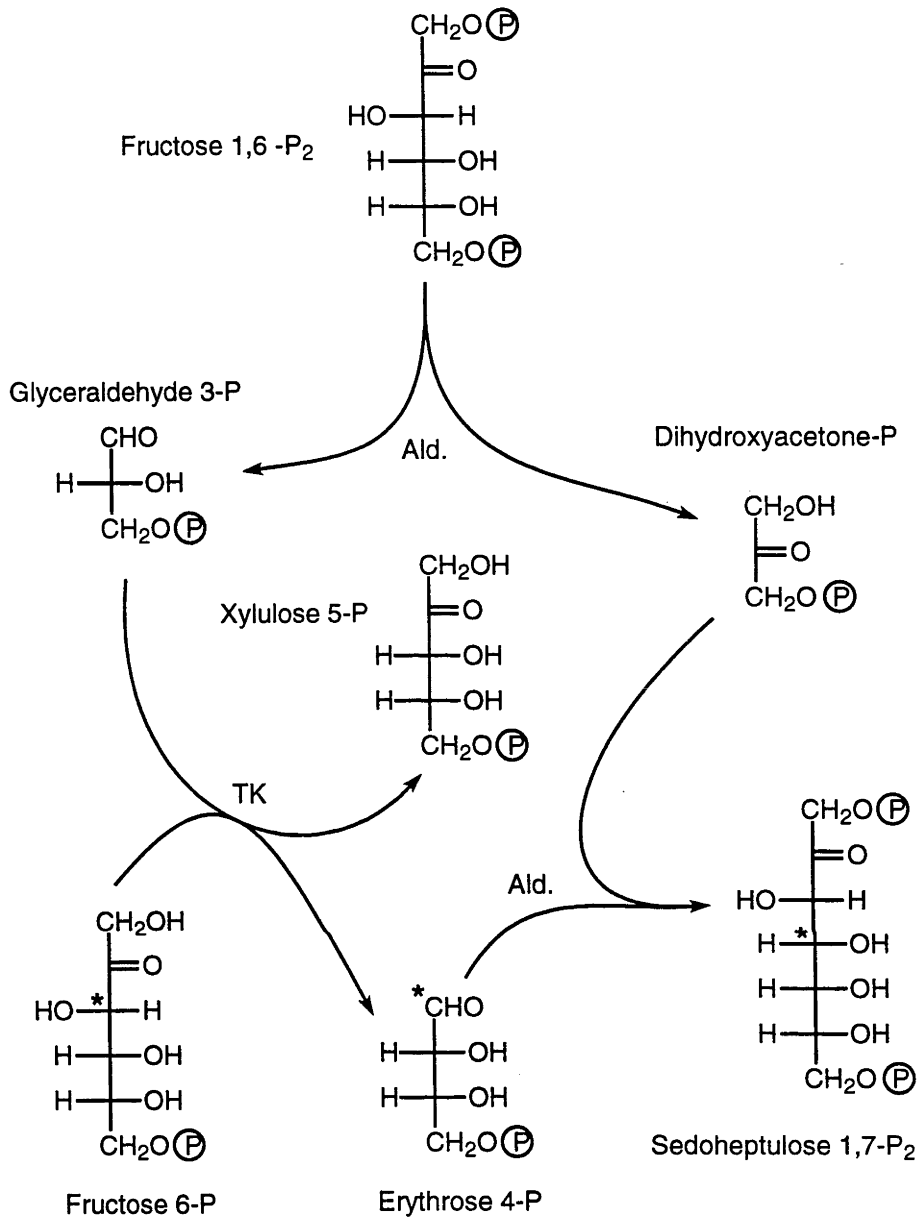


**Figure 2.7** Reaction scheme for the synthesis of [4-$^{13}$C]-sedoheptulose 1,7-$P_2$ from [3-$^{13}$C]-fructose 6-P. * Denotes the position of the $^{13}$C-label. [4-$^{13}$C]-Fructose 6-P produces [5-$^{13}$C]-sedoheptulose 1,7-$P_2$. [6-$^{13}$C]-Fructose 6-P produces [7-$^{13}$C]-sedoheptulose 1,7-$P_2$. Ald. = aldolase; TK = transketolase.

allowed to proceed for 180 min. at 30 °C before it was terminated by heating for 2 min. at 100 °C. The samples were then filtered through 4 mm filter units (Activon, AGS1104N4P, 0.45 μm nylon) and purified by HPLC (section 2.2.2.3). The maximum volume for each HPLC injection was 500 μL and therefore each of the above samples was purified by HPLC in two parts. ATP and ribose 5-phosphate were separated from the $^{13}C$-labelled fructose 6-phosphates by this HPLC procedure but $^{13}C$-labelled fructose- and glucose 6-phosphates were only partly resolved. Fractions containing $^{13}C$-labelled fructose- and glucose 6-phosphates were identified spectrophotometrically using specific enzymic assays (Bergmeyer, 1974).

Fractions (2 mL) containing $^{13}C$-labelled fructose 6-phosphate from two runs were combined and passed through a cation exchange column (Bio-Rad AG 50W x 8 200-400 mesh, $H^+$ form, 2 g dry weight) and washed through with water (25 mL). After reducing the volume to approximately 1 mL by rotary film evaporation, the pH was adjusted to 7.0 with 0.2 M NaOH and the samples were then assayed as above. The final mixtures containing $^{13}C$-labelled glucose 6-phosphates (approximately 6 μmol) and $^{13}C$-labelled fructose 6-phosphates (approximately 3 μmol) were lyophilized and used without further purification in the reactions for the following synthesis of [4-$^{13}C$]-, [5-$^{13}C$]- and [7-$^{13}C$]-sedoheptulose 1,7-bisphosphates.

The reaction mixture (0.5 mL) for the synthesis of [4-$^{13}C$]-, [5-$^{13}C$]- and [7-$^{13}C$]-sedoheptulose 1,7-bisphosphates contained triethanolamine (100 μmol), fructose 1,6-bisphosphate (3 μmol), either [3-$^{13}C$]-, or [4-$^{13}C$]- or [6-$^{13}C$]-fructose 6-phosphate (3 μmol) $MgCl_2$ (10 μmol), thiamine pyrophosphate (1 μmol), aldolase (0.9 units) and transketolase (0.5 units). The reaction was allowed to proceed at 25 °C for 18 h and it was terminated by heating at 100 °C for 2 min. The sample was then passed through a cation exchange column (Bio-Rad AG 50W x 8 200-400 mesh, 0.5 g dry weight) to remove the triethanolamine buffer and washed through with water (8 mL). The pH of the sample was then adjusted to 4.6 by the addition of dilute ammonium

hydroxide solution. The sample was then dephosphorylated by the addition of 0.1 M ammonium acetate-acetic acid buffer pH 4.6 (1 mL) and prostatic acid phosphatase (1 unit) and incubated for 24 h at 30 °C. The reaction was terminated by heating at 100 °C for 5 min. After cooling, the sample was deionized by stirring for 30 min. with 0.6 g dry weight of mixed bed resin prepared using equal weights of anion (Bio-Rad AG 1x8, 200-400 mesh, $HCO_3^-$ form) and cation (Bio-Rad AG 50Wx8, 200-400 mesh, $H^+$ form) exchange resins together with deionized water. The resin was removed by filtration through a column fashioned from a pipette tip (5 mL) plugged with cotton wool. The sample was washed through with water (15 mL) and collected in a polypropylene centrifuge tube (50 mL) and lyophilized. The residue was washed with deionized water (1 mL) into an Eppendorf tube (1.5 mL) and again lyophilized. The residue was redissolved in water (0.5 mL) and 20 µL was sampled for derivatization according to section 2.2.5.4. followed by GC/MS analysis (section 2.2.6).

### 2.2.4.6 Specifically $^{13}C$-labelled octulose phosphates

Methods for the synthesis of D-*glycero* D-*ido* and D-*glycero* D-*altro* octulose phosphates based on reactions catalyzed by aldolase or transketolase have been published (Franke et al., 1984), (Kapuscinski et al., 1985), (Arora et al., 1988). $^{13}C$-Isotopomers of these compounds were prepared from suitably $^{13}C$-labelled starting compounds using these methods. The transketolase-catalyzed transfer of the ketol group from β-hydroxypyruvate to either $^{13}C$-labelled glucose 6-phosphate or $^{13}C$-labelled allose 6-phosphate permitted the synthesis of the $^{13}C$-labelled monophosphates of D-*glycero* D-*ido* and D-*glycero* D-*altro* octulose, respectively (Figure 2.8). Specifically $^{13}C$-labelled octulose diphosphates were synthesized by the aldolase-catalyzed condensation of dihydroxyacetone phosphate with either ribose 5-phosphate or arabinose 5-phosphate labelled in the appropriate position (Figure 2.9). Purification was by

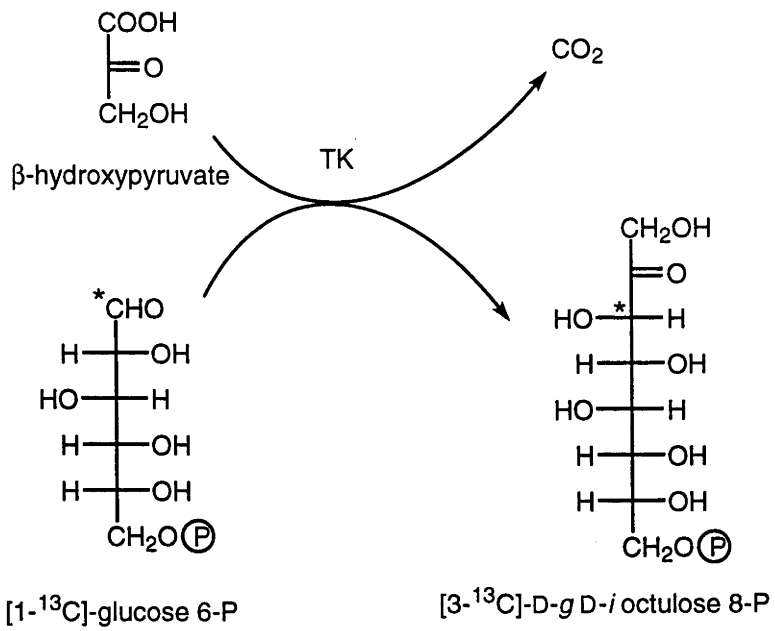


**Figure 2.8.** Reaction scheme for the synthesis of [3-$^{13}$C]-D-*g* D-*i* octulose 8-P from [1-$^{13}$C]-glucose 6-P. * Denotes $^{13}$C-label; TK = transketolase. Similarly, [2-$^{13}$C]-, [3-$^{13}$C]-, [4-$^{13}$C]- and [6-$^{13}$C]- glucose 6-P produced [4-$^{13}$C]-, [5-$^{13}$C]-, [6-$^{13}$C]-, and [8-$^{13}$C]- D-*g* D-*i* octulose 8-P respectively.

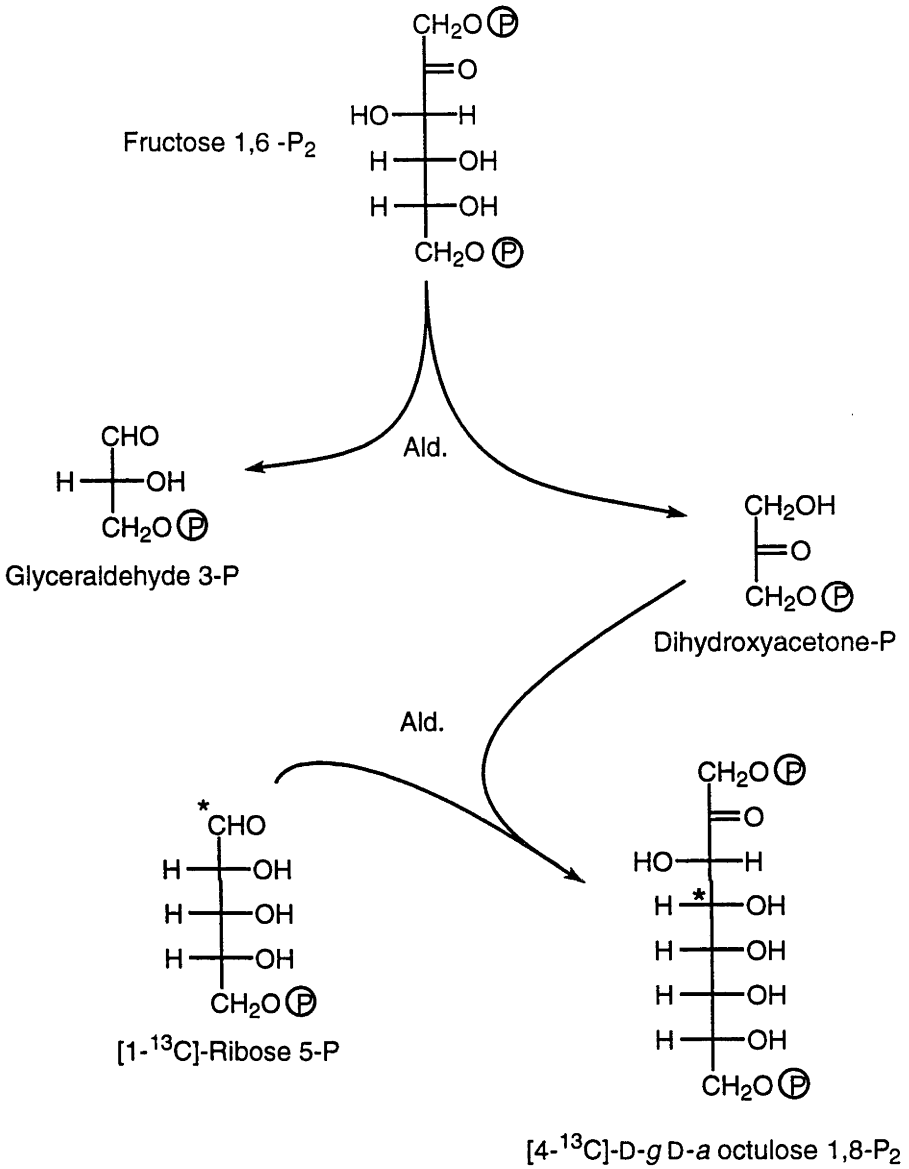


**Figure 2.9.** Reaction scheme for the synthesis of [4-$^{13}$C]-D-*g* D-*a*-octulose 1,8-$P_2$ from [1-$^{13}$C]-ribose 5-P. * Denotes $^{13}$C-label; TK = aldolase.

Similarly, [1-$^{13}$C]-arabinose produces [4-$^{13}$C]-D-*g* D-*a*-octulose 1,8-$P_2$.

the formate anion exchange chromatography method (section 2.2.2.1). When small quantities of these compounds were required exclusively for GC/MS analysis the reaction mixture used was approximately one twentieth the scale used previously (Kapuscinski et al., 1985). In these syntheses, the octulose phosphates were not separated from the unreacted starting compounds. Instead, on completion of the reaction, the mixture was heated at 100 °C for 2 min. to denature the protein, the cooled mixture was then dephosphorylated (section 2.2.5.2), the free sugars were separated (section 2.2.5.3) and derivatized for GC/MS analysis (section 2.2.5.4).

### *2.2.5 Preparation of samples for GC/MS analysis of sugar phosphates*

#### 2.2.5.1 SAX chromatography

Sugar phosphate samples to be analyzed by GC/MS was applied to an Elut Bond strong anion exchange (SAX) solid phase extraction column (500 mg, Varian Associates, Sunnyvale, CA 94089, U.S.A.) and washed through with deionized water (30 ml). The eluate was discarded and the combined sugar mono- and bisphosphate fractions were eluted with 0.5 M $NH_4HCO_3$ (5 mL) and collected in a polypropylene centrifuge tube (50 mL). Most of the buffer was removed by blowing the sample dry with a stream of dry nitrogen gas, whilst warming the sample in a water bath at 37 °C. The samples were then dephosphorylated as described below.

#### 2.2.5.2 Dephosphorylation of sugar phosphates

It was essential that the enzymic dephosphorylation reaction was completed and caused no transformation of the sugar phosphates due to impurity enzymes contained in the phosphatase. Several phosphatases had previously been investigated and human prostatic acid phosphatase was chosen for its ability to catalyze dephosphorylation without causing the transformations produced by other phosphatases as determined by GC/MS

analysis of the derivatized products (Irvine et al., 1992). Dephosphorylation of up to 0.5 μmoles of sugar phosphates was carried out by the addition of 0.5 units of prostatic acid phosphatase to the sugar phosphate solution in 0.1 M ammonium acetate-acetic acid buffer pH 4.6 (typically 200 μL) and incubated for 24 h at 30 °C. The reaction was terminated by heating at 100 °C for 2 min.

#### 2.2.5.3 Deionization of dephosphorylated sugars

After dephosphorylation, the sugar solutions were deionized by stirring for 30 min with 0.5 g of mixed bed resin prepared using equal weights of anion (Bio-Rad AG 1x8, 200-400 mesh, $HCO_3^-$ form) and cation (Bio-Rad AG 50Wx8, 200-400 mesh, $H^+$ form) exchange resins together with deionized water. The resin was removed by filtration through a small column fashioned from a pipette tip (5 mL) plugged with cotton wool and containing a further 0.25 g of the mixed bed resin. The sample was washed through with water (15 mL) and collected in a polypropylene centrifuge tube (50 mL) and lyophilized. The residue was washed with deionized water (1 mL) into an Eppendorf tube (1.5 mL) and again lyophilized. The residue was redissolved in water (120 μL) and the entire sample was transferred to a Reacti-Vial (200 μL) and dried over $P_2O_5$. The sugars were subsequently dried under high vacuum for 1 h and derivatized for GC/MS analysis.

#### 2.2.5.4 Derivatization of dephosphorylated sugars

Sugars were prepared for GC-MS analysis as the methyl or ethyl oxime-TMS derivatives using methods similar to those first described by Sweeley (Sweeley et al., 1963) for 1 mg scale derivatizations. In this investigation, smaller-scale derivatizations were required. Up to 350 nmol (approx. 50 μg) of the dried sugars in Reacti-Vials (0.2 mL) were derivatized by the addition, under dry nitrogen, of 1% methyl oxime (Mox) or ethyl oxime (Eox) in pyridine (40 μL) and refluxing at 80 °C for 2 h in a Reacti-therm heater. The mixture was cooled to room temperature then silylation was accomplished by the addition under dry nitrogen, of 10 μl of Regisil (Pierce) followed by

heating for 0.5 h at 80 °C. The mixture was cooled to room temperature then diluted as appropriate with dry pyridine for GC/MS analysis. Injections of 1 µl ideally contained no more than 125 pmol of the sugar under analysis in order to avoid saturation of the mass selective detector, as determined by sensitivity runs with derivatized glucose.

### 2.2.6 GC/MS analysis of sugars

Derivatized sugars were analyzed by capillary GC/MS on a Hewlett Packard 5890 gas chromatograph interfaced with a Hewlett Packard HP5970B mass-selective detector and controlled by a Hewlett Packard HP59970C ChemStation. Gas chromatography was performed on an HP-1 capillary column (12.5 m x 0.2 mm i.d., 0.33 µm cross-linked stationary phase) in the splitless mode. The carrier gas was helium and the flow rate was 1 mL $min.^{-1}$ The column temperature was held at 100 °C for 2 min for the passage of the solvent front through the column, then the temperature was increased to 250 °C at the rate of 10 °C $min^{-1}$, then held at 250 °C for 3 min. Spectra were obtained by electron impact (EI) ionization at 70 eV. Full scan spectra were usually obtained by scanning from *m/z* 100 to 660 at a rate of 0.77 scans per sec. and represent an average of scans taken across the top of each relevant chromatographic peak. Selected ion monitoring (SIM) experiments on clusters of ions of interest were carried out in groups of 8-12 ions with dwell times of 30 msec per ion. All results represent the mean of four separate injections unless otherwise stated. Corrections for natural abundance isotopes of H, C, N, O and Si and any contributions from adjacent ions were made in calculating $^{13}C$ enrichments in individual ions. For the purpose of this study, only ions which were found to contain a high incorporation of the $^{13}C$ isotope from specifically $^{13}C$-labelled sugar standards were considered to have a single origin.

High resolution accurate mass measurements, where it was necessary to confirm the composition of a fragment ion, were made on a VG

7070F instrument by peak matching using perfluorokerosene as a reference, with samples introduced via the direct insertion probe.

## 2.3 Results and Discussion

### 2.3.1 Synthesis of unlabelled and $^{13}C$ labelled sugars and sugar phosphates

An important feature of the enzymic methods of synthesis employed was that time-consuming procedures for separation and purification of the $^{13}C$-labelled sugars in the reaction mixtures were unnecessary prior to the GC/MS analysis. The sugars could be analyzed in an unpurified form by exploiting the resolving and analytical powers of the GC/MS measurement system.

#### $^{13}C$-Labelled erythroses.

The synthesis of $^{13}C$-labelled erythrose was based on the aldolase-transketolase-aldolase sequence of reactions shown in Figure 2.3. The natural substrates for these reactions are the corresponding phosphate esters of these sugars and consequently the reactions normally give rise to sedoheptulose 1,7-bisphosphate with little or none of the intermediate erythrose 4-phosphate being observed. However, when free fructose was used as a starting compound rather than fructose 6-phosphate, the aldolase catalyzed formation of sedoheptulose phosphate was much less favourable and a satisfactory yield of erythrose resulted. The reaction may also be carried out using glyceraldehyde 3-phosphate as the other starting compound thus avoiding the production of sedoheptulose 1-phosphate. However, fructose 1,6-bisphosphate together with aldolase provided a convenient source of glyceraldehyde 3-phosphate which avoided the need to prepare glyceraldehyde 3-phosphate from the diethylacetal monobarium salt form in which it was commercially available.

## $^{13}$C-Labelled xyluloses and riboses

Of the possible enzymic reaction procedures for the synthesis of $^{13}$C-labelled sugars or their phosphate esters, several proved to be unsatisfactory, including those for the synthesis of [4-$^{13}$C]- or [3,5-$^{13}$C]-xylulose. When an attempt was made to synthesize $^{13}$C-labelled xylulose 5-phosphate from either [2-$^{13}$C]- or [1,3-$^{13}$C]-glycerol and a suitable ketol group donor in a transketolase-catalyzed reaction, partially $^{13}$C-labelled sedoheptulose 7-phosphate was formed as determined by GC/MS of the derivatized, dephosphorylated reaction mixture. This is believed to be due to the release of ribose 5-phosphate from the ribonucleotides, ATP and/or $NAD^+$ used in the phosphorylation and oxidation of glycerol to glyceraldehyde 3-phosphate. Pentose phosphate epimerase and phosphoribose isomerase are present as slight but sufficiently active contaminants in the glycerol 3-phosphate dehydrogenase and triose phosphate isomerase. These contaminants allow an interconversion of the pentose phosphates and the transketolase-catalyzed formation of sedoheptulose 7-phosphate to take place. It also resulted in the mixing of the $^{13}$C-label in the pentose pool, thus diluting the enrichment of xylulose 5-phosphate. The release of ribose 5-phosphate occurred whether the reactions were terminated by heating or by the addition of the enzyme denaturing agents, methanol or cold $HClO_4$. Tests of all other reaction schemes involving these cofactors also produced evidence of unlabelled ribose 5-phosphate and rendered these methods unsuitable for the synthesis of fully enriched, specifically $^{13}$C-labelled compounds.

An alternative set of reactions for the synthesis of the $^{13}$C-labelled pentoses from $^{13}$C-labelled hexoses using the oxidative decarboxylation reactions of the pentose phosphate pathway also proved to be unsuitable because this approach also required the cofactor, $NADP^+$ which was expected to release interfering ribose 5-phosphate (although this was not verified). As a result of these difficulties, the information necessary to identify

unequivocally the sources of most of the pentose ions was not available. The alternative of preparing $^{13}C$-labelled xylulose 5-phosphate from previously prepared $^{13}C$-labelled fructose 1,6-bisphosphate followed by the reactions of Figure 2.3 was considered. However this would require the purification of aldolase as contaminating triose phosphate isomerase in the commercial enzyme would spread the isotope between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate thus diluting the enrichment of the resulting xylulose 5-phosphate product. The purification of such commercial enzymes was not attempted. As a result of these difficulties, only the [1-$^{13}C$]-xylulose was able to be prepared using the reaction scheme of Figure 2.4.

**$^{13}C$-Labelled glucoses and fructoses.**

Conversion of $^{13}C$-labelled glucose to the corresponding glucose 6-phosphate was rapid and greater than 99% as measured by the specific enzymic assay (Bergmeyer, 1974). The further reaction of $^{13}C$-labelled glucose 6-phosphate to $^{13}C$-labelled fructose 6-phosphate resulted in a ratio of glucose 6-phosphate to fructose 6-phosphate of 2.7 : 1. This mixture of $^{13}C$-hexose 6-phosphates could be used in the synthesis of appropriately $^{13}C$-labelled erythrose and sedoheptulose after partial purification to remove the ATP and ribose 5-phosphate.

**$^{13}C$-Labelled sedoheptuloses.**

In the synthesis of [4-$^{13}C$]-, [5-$^{13}C$]- or [7-$^{13}C$]-sedoheptulose 1,7-bisphosphates, the reaction mixture contained not only the substrate $^{13}C$-fructose 6-phosphates but also the corresponding $^{13}C$-glucose 6-phosphates. The $^{13}C$-glucose 6-phosphate was present because the reaction product resulting from the synthesis of $^{13}C$-fructose 6-phosphate from $^{13}C$-glucose was used and this was only partially purified. It is therefore of interest to note that the reaction described in section 2.2.4.5(ii) for the synthesis of $^{13}C$-sedoheptulose 1,7-bisphosphate also gave rise to D-*glycero* D-*ido* octulose 8-phosphate in 0.5 - 3.5% yield in addition to the 28% yield of sedoheptulose

1,7-bisphosphate. Thus the formation of octulose is an unavoidable consequence of the enzyme activities present, even in the simplified system used *in vitro*. The reaction for the formation of D-*glycero* D-*ido* octulose 8 phosphate from glucose 6-phosphate and fructose 6-phosphate was first described by Williams et al, (1978) and is shown in Reaction 13 (Table 2.1).

**Unlabelled octuloses and $^{13}$C-labelled octuloses.**

The mono- and bisphosphate esters of both D-*glycero* D-*ido* and D-*glycero* D-*altro* octulose together with the following seven $^{13}$C-labelled octulose phosphates were prepared by the methods described in section 2.2.4.6: [3-$^{13}$C]-D-*glycero* D-*altro* octulose 8-phosphate, [1,3-$^{13}$C]- and [4-$^{13}$C]-D-*glycero* D-*altro* octulose 1,8-bisphosphate and [3-$^{13}$C]-, [5-$^{13}$C]-, [6-$^{13}$C]- and [8-$^{13}$C]-D-*glycero* D-*ido* octulose 8 phosphate. All of the $^{13}$C-labelled octuloses contained 99% $^{13}$C enrichment with the exception of the [6-$^{13}$C]-D-*glycero* D-*ido* octulose, which was 90% $^{13}$C-labelled as determined by GC/MS and these enrichments reflected the enrichments of the precursor sugars.

**Yields of synthesized sugars**

The approximate yields of the synthesized compounds are indicated in Table 2.3. Only the values marked with an asterisk represent the isolated yield of purified product. The values given for the remaining compounds represent the percentage conversion to the nominated product as determined by the peak areas of the GC/MS chromatogram of the dephosphorylated Mox-TMS derivative.

### 2.3.2 GC/MS analysis of sugars

Figures 2.10 to 2.16 show the mass spectra and the origins of the major fragment ions for most of the sugars of interest to this investigation. In most of the spectra, the molecular ion is either very small or non-existent with the highest mass ion usually being the $[M-CH_3]^+$ fragment. Mass spectra of isomeric sugars were virtually identical e.g. ribulose/xylulose,

**Table 2.3** Yields obtained for the syntheses described in Section 2.2.4..

| Compound | Section reference | Yield (%) |
|---|---|---|
| [1-$^{13}$C]-, [2-$^{13}$C]-, [4-$^{13}$C]-erythrose | 2.2.4.5 | 10 |
| [1-$^{13}$C]-xylulose | 2.2.4.1 | 20 |
| [4-$^{13}$C]-, [3,5-$^{13}$C]-xylulose | 2.2.4.2 | N.A. |
| [1-$^{13}$C]-, [2-$^{13}$C]- glucose 6 phosphate | 2.2.4.3 (i) | 73 |
| [3-$^{13}$C]-, [4-$^{13}$C]-, [6-$^{13}$C]-glucose 6 phosphate | 2.2.4.3 (ii) | 73 |
| [3-$^{13}$C]-, [4-$^{13}$C]-, [6-$^{13}$C]-fructose 6 phosphate | 2.2.4.3 (ii) | 27 |
| [4-$^{13}$C]-, [5-$^{13}$C]- and [7-$^{13}$C]-sedoheptulose 1,7 bisphosphates | 2.2.4.4 (i) | 28 |
| [3-$^{13}$C]-sedoheptulose | 2.2.4.4 (ii) | 20 |
| D-*g* D-*i* octulose 1,8-bisphosphate | 2.2.4.6 | 60 * |
| D-*g* D-*a* octulose 1,8-bisphosphate | 2.2.4.6 | 70 * |
| [3-$^{13}$C]-D-*g* D-*a* octulose 8 phosphate | 2.2.4.6 | 38 |
| [4-$^{13}$C]-D-*g* D-*a* octulose 1,8-bisphosphate | 2.2.4.6 | 23 |
| [1,3-$^{13}$C]-D-*g* D-*a* octulose 1,8-bisphosphate | 2.2.4.6 | N.A. |
| [3-$^{13}$C]-, [5-$^{13}$C]-, [6-$^{13}$C]-,[8-$^{13}$C]-D-*g* D-*i* octulose 8 phosphate | 2.2.4.6 | 10 - 40 |

N.A. = Not available

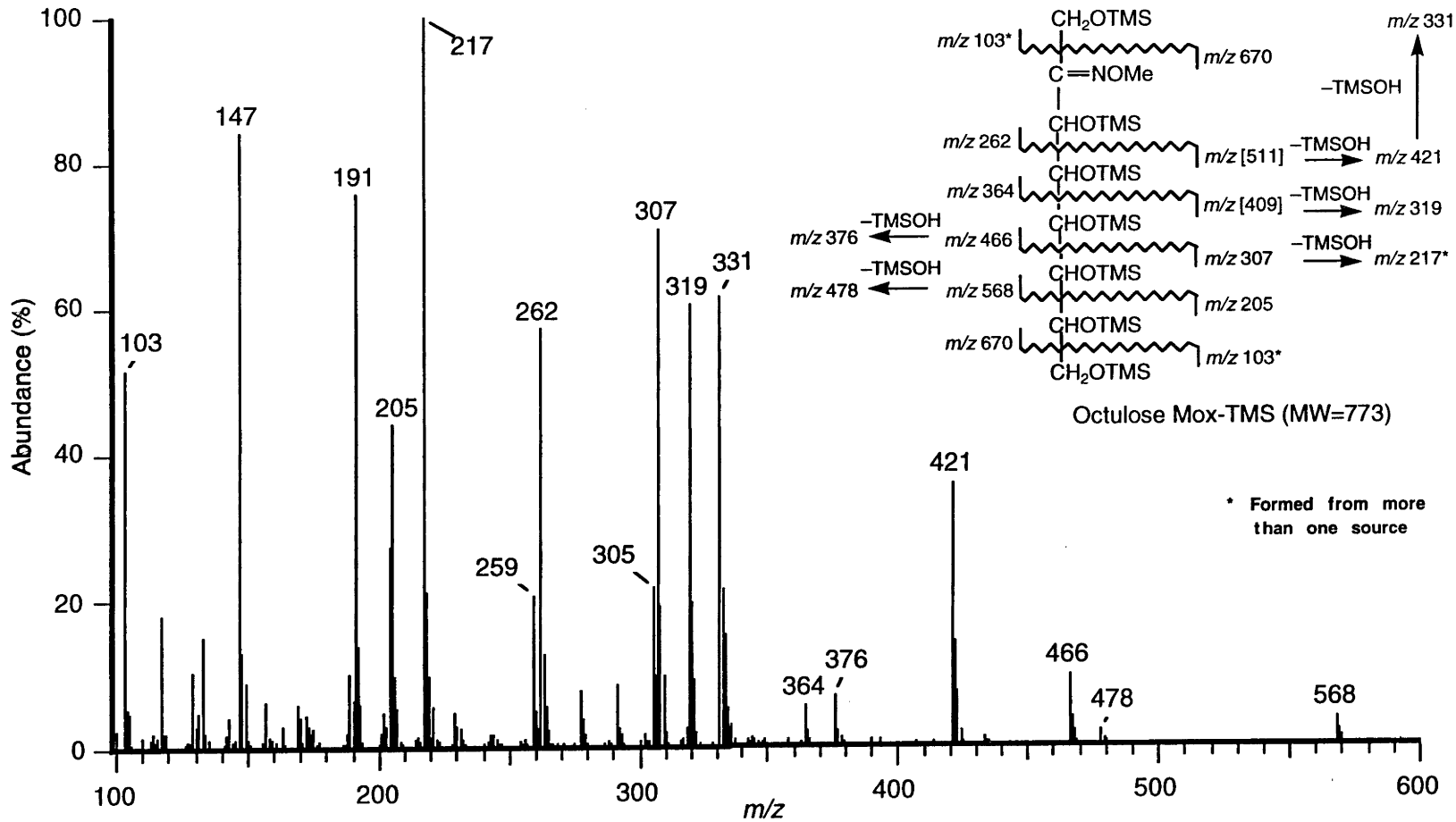


Figure 2.10. The mass spectrum of the Mox-TMS derivative of D-*glycero*-D-*altro*-octulose. The Fig. includes the cleavage pattern showing the origin of major ions.

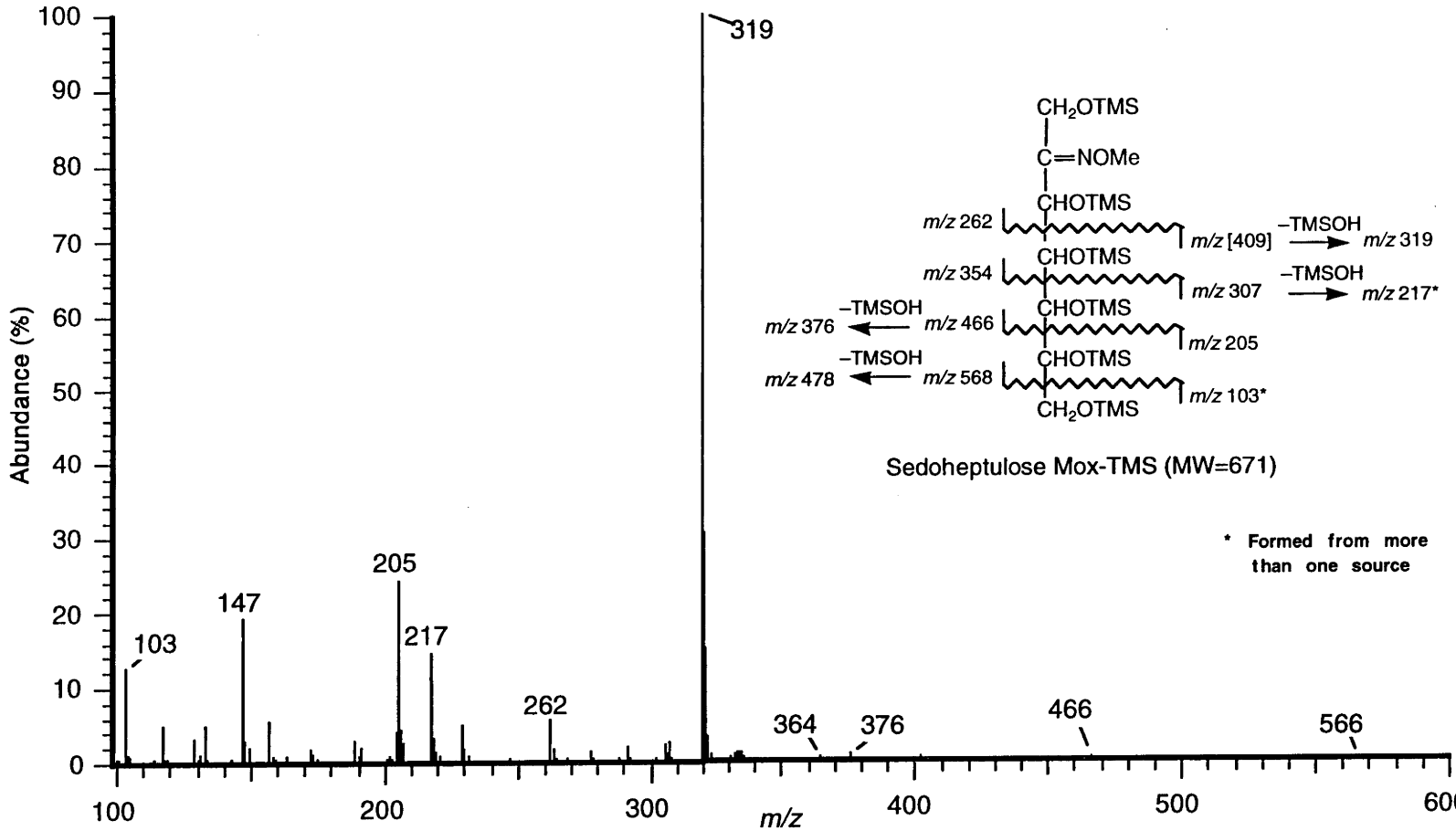


Figure 2.11. The mass spectrum of the Mox-TMS derivative of D-sedoheptulose. The Fig. includes the cleavage pattern showing the origin of major ions.

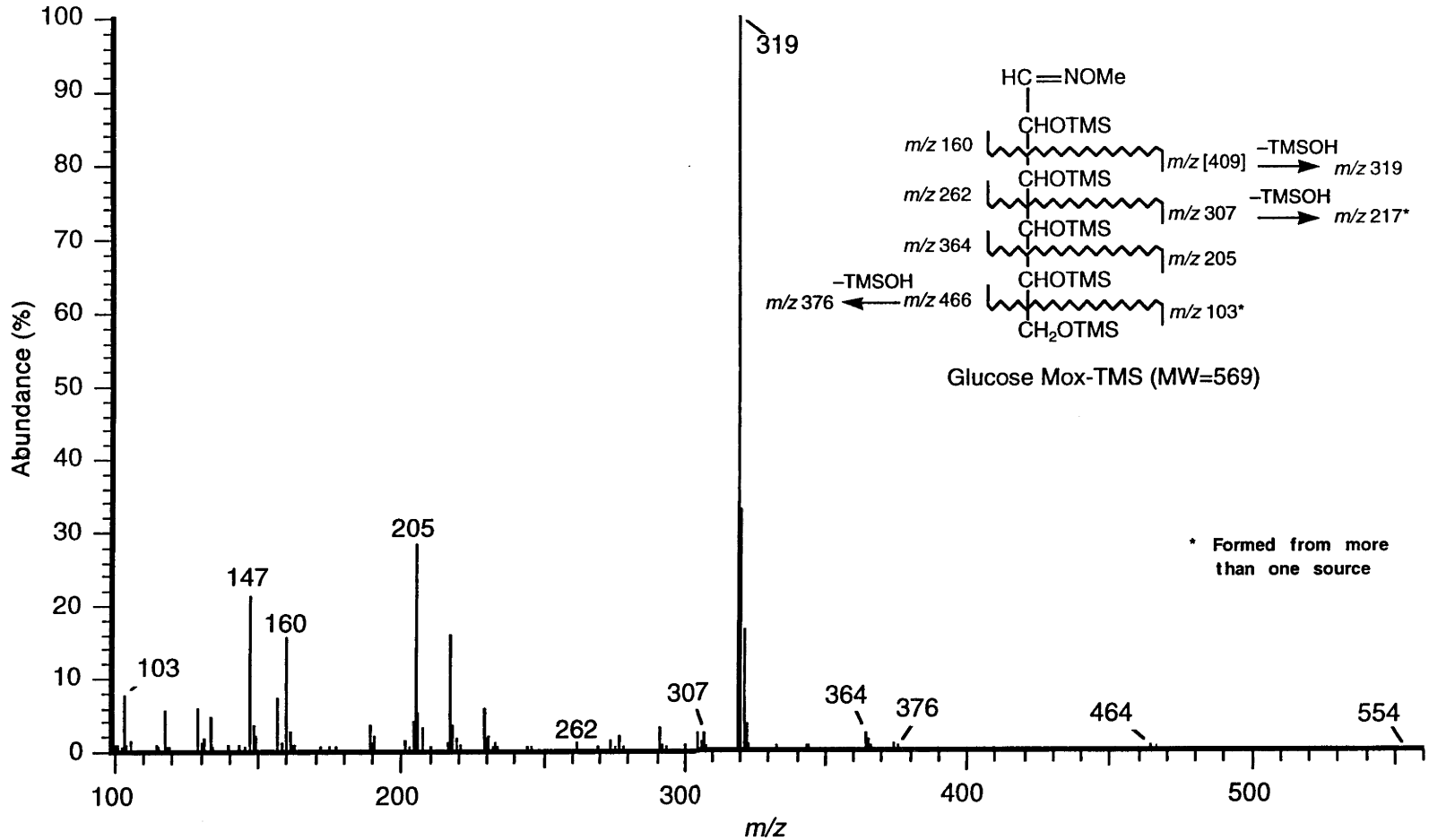


Figure 2.12. The mass spectrum of the Mox-TMS derivative of D-glucose. The Fig. includes the cleavage pattern showing the origin of major ions.

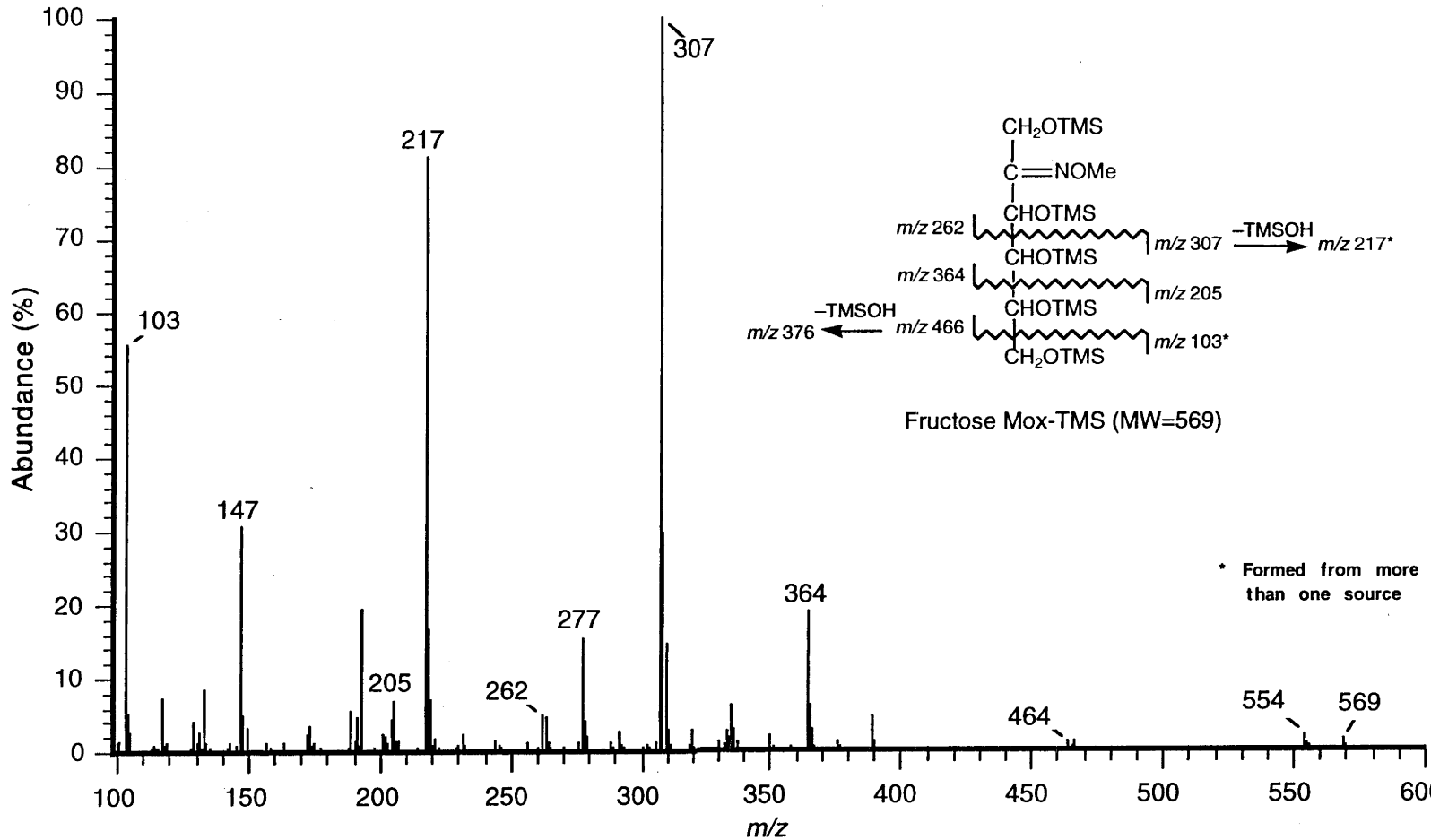


Figure 2.13. The mass spectrum of the Mox-TMS derivative of D-fructose.
The Fig. includes the cleavage pattern showing the origin of major ions.

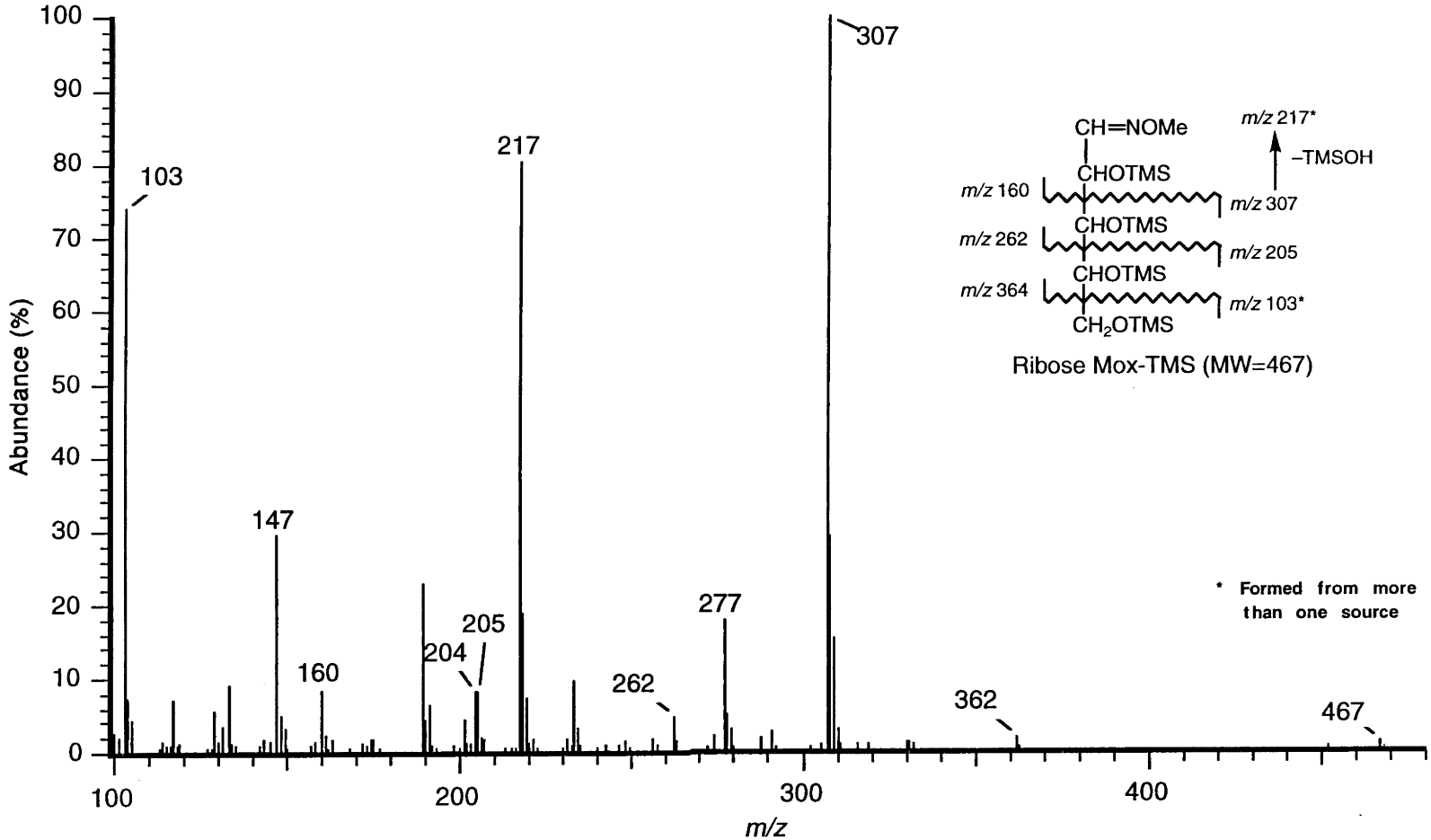


Figure 2.14. The mass spectrum of the Mox-TMS derivative of D-ribose.
The Fig. includes the cleavage pattern showing the origin of major ions.

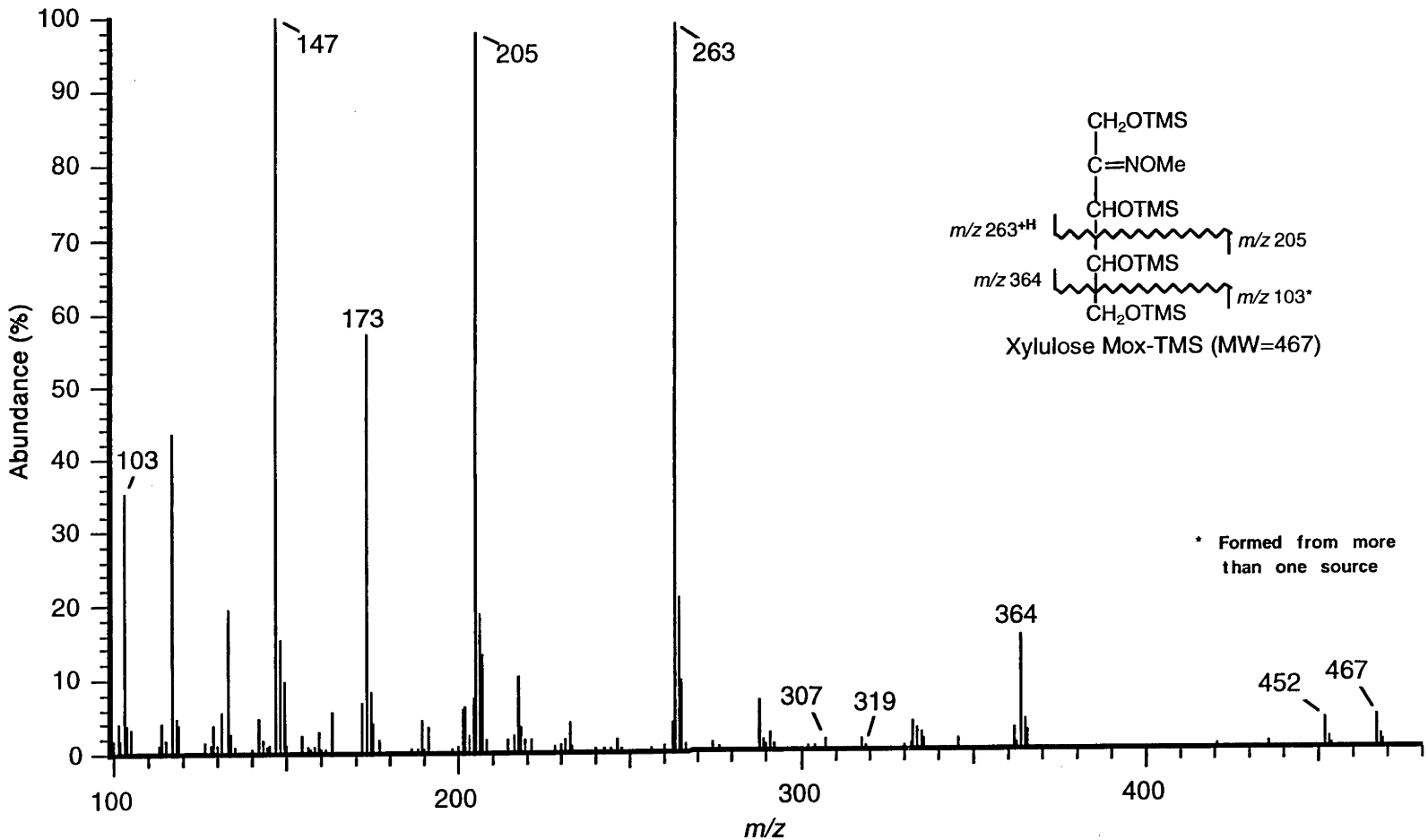


Figure 2.15. The mass spectrum of the Mox-TMS derivative of D-xylulose.
The Fig. includes the cleavage pattern showing the origin of major ions.

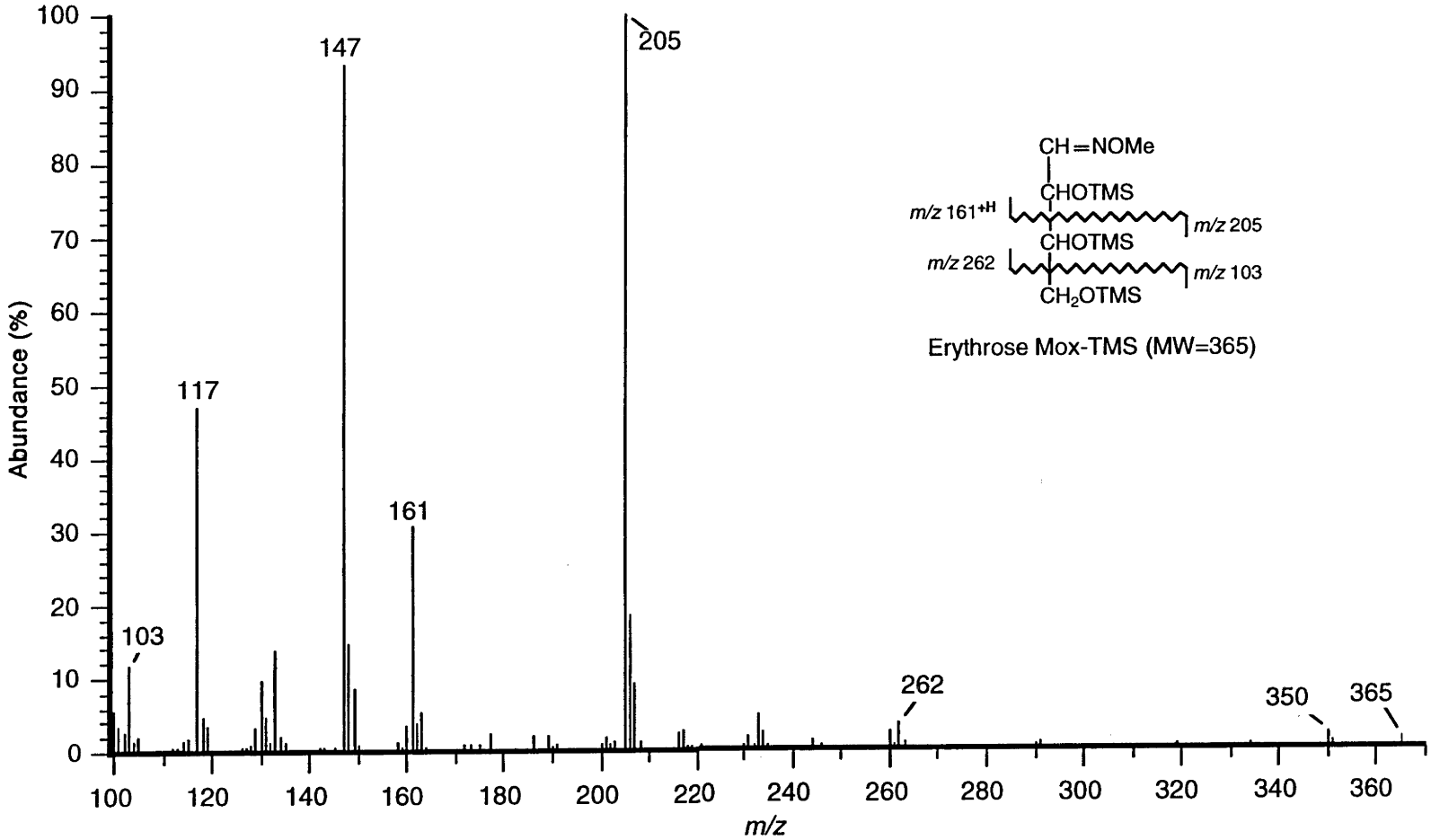


Figure 2.16. The mass spectrum of the Mox-TMS derivative of D-erythrose. The Fig. includes the cleavage pattern showing the origin of major ions.

ribose/arabinose and D-*glycero* D-*ido*-octulose/D-*glycero* D-*altro*-octulose, and the mass spectrum of only one of the respective isomers is shown. Differences were sometimes observed in the intensities of some diagnostic fragment ions between the *syn*- and *anti*-isomers of the Mox-TMS derivatives of a sugar, particularly the keto-sugars in which these isomers were formed in about equal amounts. The presence of the two isomers provided trwo sets of data that could be utilized in the calculation of fragment ion shifts in the 13C labelled compounds.

Not all sugars have EI mass spectra which show a sufficient range of fragment ions to allow a complete analysis to be made. A comparison of figure 2.10 (D-*glycero* D-*ido*-octulose) with Figure 2.11 (sedoheptulose) illustrates the substantial differences between the sugars in the usefulness of the mass spectrum of the Mox-TMS derivatives of these compounds. Although sedoheptulose has a carbon chain length just one carbon shorter than that of the octulose its mass spectrum is less amenable to analysis for the purpose of this study. In the mass spectrum for sedoheptulose Mox-TMS there is only one ion above *m/z* 300 that has a high intensity and that is the base ion at *m/z* 319. Other ions above *m/z* 300 have either a very low intensity or adjacent ions within one or two mass units which interfere with the analysis. The octulose mass spectrum by contrast shows at least six useable ions in this region of the spectrum.

The mass spectra of the octuloses show a useful range of high intensity ions which represent cleavages between most of the carbons of the sugar backbone of the Mox-TMS derivative (Figure 2.10). By contrast, the mass spectra of the other sugars of metabolic significance show fewer cleavages that produce ions of sufficient intensity and consequently the sources of the ions are less readily identified. The effect of this is seen in Table 2.4, which shows those ions that it has been possible to demonstrate have a single origin (as determined by the quantitative shift of the ions in the $^{13}C$-labelled compounds). There is a marked difference between the results for

**Table 2.4.** Shows, for each sugar examined, the percentage $^{13}C$ incorporation into the listed ion when synthesized with isotope in the specific carbon given.

| Sugar | Ion | Origin | Position of label and percent incorporation [a,b,c,] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Erythrose | 161 | C1-2 | 97.6 | 97.7 | | | | | | |
| | 205 | C4-5 | | | | 98.2 | | | | |
| | 262 | C1-3 | 99.3 | 99.9 | | | | | | |
| | 350 | C1-4 | 98.4 | 98.7 | | | | | | |
| Xylulose | 263 | C1-3 | 92.4* | | | | | | | |
| Ribose | 160 | C1-2 | 95.0 | | | | | | | |
| Fructose | 205 | C5-6 | | | | | | 100.0 | | |
| | 263 | C1-3 | | 91.1 | 98.1 | | | | | |
| | 307 | C4-6 | | | | 98.0 | | 97.0 | | |
| | 364 | C1-4 | | 97.0 | 98.0 | 98.0 | | | | |
| Glucose | 160 | C1-2 | | | | | | 92.0 | | |
| | 262 | C1-3 | | | | | | | | |
| | 319 | C3-6 | | | 98.0 | 99.0 | | 99.0 | | |
| | 364 | C1-4 | | 96.0 | 98.0 | 98.0 | | | | |
| | 205 | C5-6 | | | | | | 100.0 | | |
| Sedo- | 205 | C6-7 | | | | | | | 92.0 | |
| heptulose | 262 | C1-3 | | | 91.8 | | | | | |
| | 319 | C4-7 | | | 100.0 | 100.0 | 100.0 | | 100.0 | |
| Octulose | 205 | C7-8 | | | | | | | | 95.3 |
| | 259 | C4-8 | | | | 99.7 | 99.0 | 100.0 | | 99.4 |
| | 262 | C1-3 | 98.0 | | 99.6 | | | | | |
| | 307 | C6-8 | | | | | | 100.0 | | 99.7 |
| | 319 | C5-8 | | | | | | 99.6 | | 98.5 |
| | 344 | C2-7 | | | 96.8 | 96.9 | 97.7 | 99.5 | | |
| | 364 | C1-4 | | | 95.5 | | | | | |
| | 376 | C1-5 | 95.0 | | 98.9 | | 97.8 | | | |
| | 421 | C4-8 | | | | 100.0 | 99.3 | 100.0 | | 100.0 |
| | 466 | C1-5 | 94.0 | | 98.3 | 97.8 | 98.6 | | | |
| | 568 | C1-6 | 100.0 | | 100.0 | | 100.0 | 100.0 | | |

* Estimate only; values are based on corrections for interfering ions taken from full scan results

a . Means of three selected ion monitoring runs with a maximum standard deviation of 1.0.

b. Corrected for the contributions from natural abundance and interfering ions (where appropriate).

c. Corrected for the degree of enrichment of the commercially available precursor.

octulose compared with those for the other sugars. There are approximately as many results for octulose as there are for all of the other sugars combined. In general, the ions listed in Table 2.4 are those considered to be the most useful in a positional isotopic analysis, which aims to measure accurately the incorporation of isotope in a $^{13}CO_2$ photosynthesis study.

The less favourable mass spectral fragmentation patterns of the other sugars compared with the octuloses was not the only factor contributing to the less than complete set of results in Table 2.4. In addition, the synthesis of many of the other sugars from $^{13}C$ substrates is less easily accomplished by the enzymic methods used in this study than is the synthesis of the octuloses. Octulose phosphates can be synthesized from commercially available $^{13}C$-labelled hexoses and these are available with isotopic label in any carbon position of the molecule. As a result, the octulose phosphates may be synthesized with $^{13}C$-label in any of the eight carbon atoms and after dephosphorylation and conversion to the methyl or ethyl oxime trimethylsilyl (TMS) derivatives, the mass spectral fragmentation characteristics can be determined. This was carried out in an earlier study and the origins of all of the major ions were determined (Irvine et al., 1992).

In summary, a combination of the limitations of the mass spectra for some of the sugar Mox-TMS derivatives and of the synthesis procedures for some of the $^{13}C$-labelled sugars has meant that not all of the information necessary for a complete analysis of the proposed sugar phosphate intermediates in the PCR pathway was available for this study. Nevertheless it was considered that useful information could be acquired using the range of ions listed in Table 2.5.

**Table 2.5** Summary of the carbons that can be determined with the mass spectral data obtained in $^{13}C$ positional isotopic studies.

| Sugar | Ion | Carbons | Calculation | Carbon or group determined |
|---|---|---|---|---|
| Erythrose | 161 | 1,2 | - | 1,2 |
| | 262 | 1,2,3 | 262-161 | 3 |
| | 350 | 1,2,3,4 | 350-262 | 4 |
| | 205 | 3,4 | - | 3,4 |
| Keto pentose | 263 | 1,2,3 | - | 1,2,3 |
| Aldo pentose | 160 | 1,2 | - | 1,2 |
| Fructose | 263 | 1,2,3 | - | 1,2,3 |
| | 364 | 1,2,3,4 | 364-263 | 4 |
| | 307 | 4,5,6 | 307-205 | 3,4 |
| | 205 | 5,6 | - | 5,6 |
| Glucose | 160 | 1,2 | - | 1,2 |
| | 262 | 1,2,3 | 262-160 | 3 |
| | 364 | 1,2,3,4 | 364-262 | 4 |
| | 319 | 4,5,6 | 319-205 | 4 |
| | 205 | 5,6 | - | 5,6 |
| Sedoheptulose | 262 | 1,2 | - | 1,2 |
| | 319 | 5,6,7 | 319-205 | 4,5 |
| | 205 | 6,7 | - | 6,7 |
| Octulose | 262 | 1,2,3 | - | 1,2,3 |
| | 364 | 1,2,3,4 | 364-262 | 4 |
| | 376 | 1,2,3,4,5 | 376-364 | 5 |
| | 466 | 1,2,3,4,5 | 466-364 | 5 |
| | 568 | 1,2,3,4,5,6 | 568-466 or 376 | 6 |
| | 344 | 2,3,4,5,6,7 | - | - |
| | 421 | 4,5,6,7,8 | 421-319 | 4 |
| | 259 | 4,5,6,7,8 | 259-319 | 4 |
| | 319 | 5,6,7,8 | 319-307 | 5 |
| | 307 | 6,7,8 | 307-205 | 6 |
| | 205 | 7,8 | - | 7,8 |

# CHAPTER 3

## PHOTOSYNTHESIS

## EXPERIMENTS

## 3.1 Introduction

Early photosynthesis experiments were conducted on the whole leaf (soy bean) or on whole organisms such as the single-celled algae *Scenedesmus* or *Chlorella* (see e.g. (Fink et al., 1948), (Fager et al., 1950), (Bassham et al., 1954), (Kandler et al., 1956)). H.G. Wood warned of the hazards of interpreting the results of whole-organism experiments when the reactions under investigation were confined solely to the chloroplasts (Wood, 1950). As stated in Chapter 1, the photosynthetic carbon reduction cycle within the chloroplast is the principal source of all carbohydrate produced on earth. Hence the principal function of the chloroplast is to export the newly formed carbohydrate (mainly in the form of triose phosphates) to the cytoplasm for use by the rest of the growing plant. Although triose phosphates are the principal products of photosynthesis exported from the chloroplast, other small sugar phosphates are also released across the chloroplast envelope at lower rates (Heldt, 1980), (Lilley et al., 1977), (Stokes et al., 1972), (Heldt et al., 1971)?, (Heldt, 1980). In the intact cell, many metabolites occur in the cytoplasm at levels comparable with those measured in the chloroplasts (Gerhardt et al., 1987), (Stitt et al., 1980) and therefore any labelled PCR pathway metabolites released from the chloroplasts into the cytoplasm during a $^{13}CO_2$ experiment are expected to contribute to a pool of exogenous metabolites that have a substantially lower enrichment than that existing within the chloroplast. They may also undergo intermediary metabolic transformations due to cytoplasmic enzymes that have the potential to alter labelling patterns significantly. Thus the labelling levels and patterns obtained from extracts of the whole leaf or from an organism may provide a misleading picture of events taking place within the chloroplast. It is not surprising therefore, that it was found that the $^{14}C$ labelling levels in isolated chloroplasts were much higher than those found in the whole leaf (Bassham et al., 1968). Although it had become possible to isolate chloroplasts by the time Calvin was conducting his

photosynthetic studies (Hill, 1939), (Arnon et al., 1954), techniques at that time were still not sufficiently advanced to justify their use in path of carbon photosynthesis experiments. Advances in biochemistry have produced an increased appreciation of the roles of metabolic compartmentation within the plant cell. With the development since the late 1960's of effective techniques for the isolation of chloroplasts, it is now mandatory to take into account the compartmentation of metabolites during experiments involving chloroplastic metabolites. This has been done in a variety of ways, for example 1) following experiments with wheat and spinach protoplast suspensions, the outer membrane was rapidly ruptured and the chloroplasts were separated by a silicon oil centrifugation technique allowing the chloroplast metabolites to be analyzed relatively free of cytoplasmic interference as judged by the distribution of marker enzymes (Giersch et al., 1980); 2) following experiments with whole spinach leaves, the leaves were rapidly frozen, lyophilized and subjected to a non-aqueous fractionation technique (Gerhardt et al., 1984). The use of modern methods for the preparation of isolated intact chloroplasts (Walker, 1988) for path of carbon studies eliminates most of the difficulties associated with the existence of different metabolically active pools of the same metabolites, leaving only the problem of chloroplastic metabolites exported into the metabolically inactive medium. This matter is dealt with later.

In this investigation, while it was important to have low interference from other cellular compartments, other factors needed to be taken into consideration. The general requirement for this study was to establish steady-state photosynthesis using $^{12}CO_2$ and then to replace the $^{12}CO_2$ with $^{13}CO_2$ rapidly so that the course of the heavy isotope of carbon could be traced through the chloroplast intermediates using GC-MS. Carbon is taken up by the chloroplast in the form of dissolved carbon dioxide and in a typical aqueous chloroplast suspension this is provided by dissolved 10 mM $HCO_3^-$. A 3 mL chloroplast suspension therefore contains 30 µmoles of bicarbonate which equilibrates with carbon dioxide. Unlike the traditional approach of adding a small pulse of high

specific radioactivity $^{14}CO_2$ as a tracer isotope, the use of the stable isotope, $^{13}CO_2$, in GC/MS studies necessitates a complete and rapid replacement of the $^{12}CO_2$ with $^{13}CO_2$. It is not possible to replace this amount of dissolved $^{12}CO_2$ with the same concentration of $^{13}CO_2$ without seriously disrupting the steady state of the photosynthetic reactions. Since this cannot be accomplished with chloroplasts in aqueous suspension, an alternative approach needed to be found. In this context, it was of interest to note that Cerovic (Cerovic et al., 1987; Cerovic et al., 1991) had developed a method of mounting freshly prepared chloroplasts on a filter membrane which permitted the measurement of chloroplast activity in the sealed chamber of a leaf-disc oxygen electrode as illustrated in Figure 3.1 (Delieu et al., 1981). The volume of the chloroplast medium was therefore greatly reduced and consequently the amount of dissolved $CO_2$ was also reduced to a minimum. The chamber was then charged with an atmosphere containing $CO_2$. Upon illumination of the chloroplasts through a transparent window, photosynthesis could be measured by the evolution of oxygen in the same manner as in the conventional Clark-type oxygen electrode which measures the evolution of oxygen from illuminated chloroplasts in suspension.

Isolated chloroplasts have previously been immobilized on a filter paper support (Coombs et al., 1970), and in polyacrylamide gel (Karube et al., 1979). An interesting feature of the Cerovic (1987) method, however, was the use of a phosphatase in the medium. The phosphatase had the function of dephosphorylating the exported triose phosphates so that the free phosphate ions were returned to the chloroplasts in a one for one exchange with triose phosphate via the phosphate translocator (Heldt et al., 1971). It was still necessary to include inorganic phosphate in the medium and the concentration required for optimum chloroplast activity was much closer to that existing in the intact cell than is usually the case for chloroplast preparations in suspension (Cerovic et al., 1991). Although rates of oxygen evolution (and hence carbon dioxide fixation) by this method were slightly lower than those

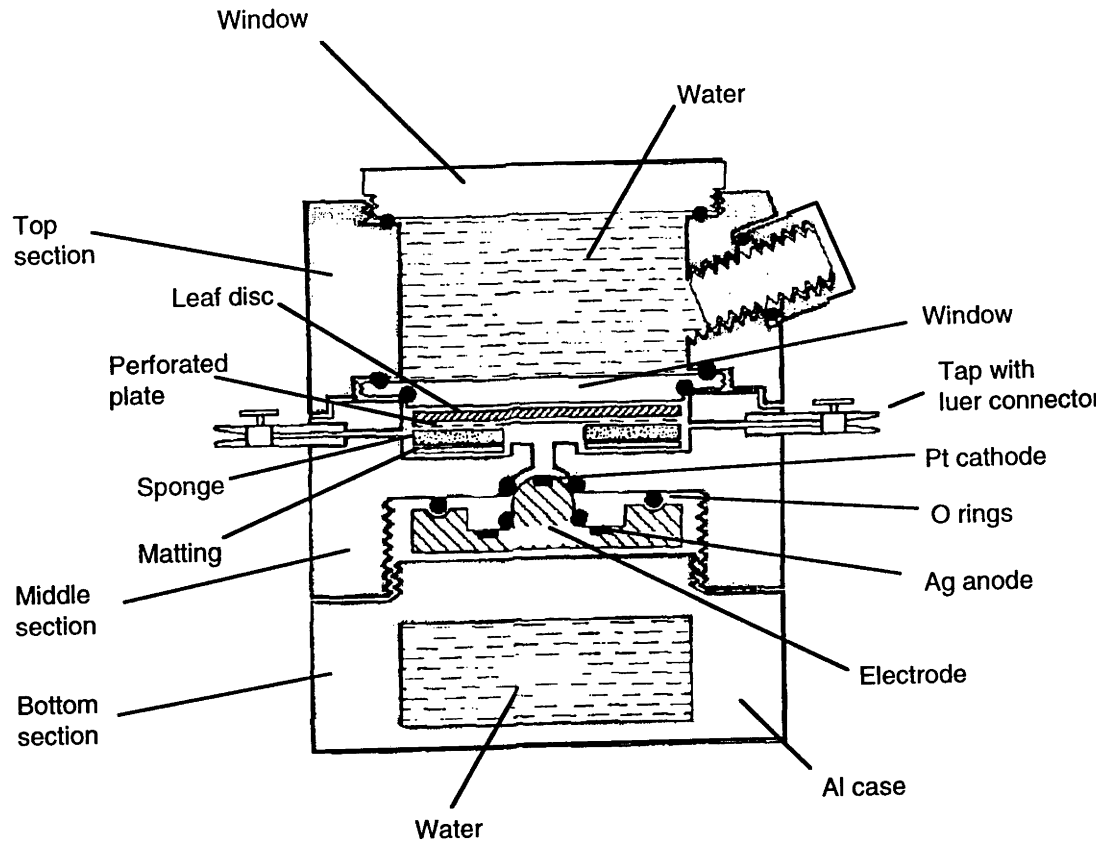


Figure 3.1. The leaf disc oxygen electrode (Delieu, 1972).

obtained by chloroplasts in suspension, the activity was sustained over a longer period. Since the exported metabolites are dephosphorylated, they are chemically distinguishable from the phosphorylated metabolites within the chloroplast and can by separated by ion exchange chromatography after extraction. Thus the major conceptual and practical difficulties of the proposed experiment are all addressed by this approach: metabolically active pools of exogenous metabolites are virtually eliminated; exported metabolites are distinguishable from chloroplastic metabolites; the $^{12}CO_2$ can be replaced rapidly with $^{13}CO_2$ with minimal disruption to the steady state of photosynthesis by switching to a different gas source; and the "dead volume" of the medium containing unlabelled dissolved $CO_2$ is minimized. The method conceptually appeared ideally suited to the purpose of tracing the path of carbon in photosynthesis using the $^{13}C$ isotope and is the approach that was adopted in this investigation. It was therefore necessary to design, build and test an appropriate apparatus in which the experiments could be conducted and this is dealt with in section 3.2.

Another important consideration in this investigation was the method by which the photosynthesis reactions would be quenched. Various methods for quenching photosynthesis reactions have been used. In algal suspensions, Bassham (Bassham et al., 1960) found no significant differences in the $^{14}C$ labelling patterns when using 80% methanol at room temperature, boiling ethanol or ethanol at -40C. Working with whole leaves, Hatch, M.D. (1966) used a sequence of boiling 80% ethanolic and aqueous extractions (after initially quenching the reactions using liquid nitrogen) as did Kapuscinski, M.K. (1984) and Bartlett M.R.E. (1986). For early work with isolated chloroplasts, (Bassham et al., 1968), (Jensen et al., 1968) and (Kaiser, 1979) chloroplast suspensions were injected into methanol.

More recent work with isolated chloroplasts favoured the use of cold 0.6 M perchloric acid, usually to a final concentration of about 0.3M, and neutralized with $K_2CO_3$, (Lilley et al., 1977), (Heldt et al., 1980), (Giersch et al.,

1980), (Giersch et al., 1987). This procedure is not without its problems as it has been found that aldolase and triose-P isomerase present in the same solution provide mutual protection against denaturation by cold perchloric acid (Salerno et al., 1982). In the study reported here, ribose 5-P was always released from sugar synthesis reactions (Chapter 2) in which adenine-, purine- or pyrimidine nucleotides were present regardless of whether the reactions were terminated using boiling ethanol, cold perchloric acid, or 1% sodium dodecyl sulfate (SDS) solutions.

Methods of quenching reactions that involve heating with alcoholic solutions always suffer from the flaw that the very reactions one is attempting to stop are in fact being speeded up during the initial heating phase. In a multi-enzyme pathway, different enzymes may be denatured at different rates by the hot alcohol and may thus allow transformations to take place which misrepresent the pathway under investigation. Although Bassham established that that question was not an issue for the algal system (Bassham et al., 1960), one cannot assume that it will be true for all photosynthetic tissue systems.

Ultimately, the method of choice for quenching the reactions can depend more on the practical limitations of the apparatus in use than any theoretical considerations. In this investigation the chloroplasts were mounted on a membrane and enclosed in an apparatus that could not be rapidly dismantled or immersed in liquid nitrogen. Thus the reactions had to be quenched by injecting an appropriate solution in a volume of approximately 6 mL, in order to ensure the complete coverage of the membrane.

Solutions tested for quenching photosynthesis reactions in the photosynthesis apparatus were hot 80% aqueous ethanol, 10% perchloric acid, and a 1% solution of SDS. Because of the relatively large volume of perchloric acid required, and the small amounts of metabolites involved, the subsequent repetitive neutralization and centrifugation steps involved meant that the sample recoveries were always poor and this method was

abandoned. Similarly, the removal of the SDS as the potassium salt was incomplete and the suffered from the same low sample recoveries. The methods finally chosen to terminate the photosynthetic reactions in this investigation are given in section 3.5.

**Pentose resolution**

It was stated in Chapter 1 that the carbons of particular interest are those indicated in Figures 1.5 and 1.6. In this investigation, sedoheptulose 7-phosphate and sedoheptulose 1,7-bisphosphate are indistinguishable as they become dephosphorylated before analysis to form a common pool of sedoheptulose. Erythrose 4-phosphate has never been convincingly demonstrated in any metabolic pathway and if present in the extracts from this investigation, it is likely to be present in very small concentrations. Therefore any investigation of the Calvin branch of the regenerative stage of the PCR pathway is likely to depend on the analysis of just three compounds: fructose, sedoheptulose, and ribose. Similarly, an investigation of the alternative branch of the regenerative stage of the PCR pathway depends on the analysis of glucose, D-*glycero*-D-*ido*-octulose and arabinose. The retention times of Table 3.1 show that all derivatized compounds are well resolved by GC on a non-polar column except for the pentoses. Although ribose and arabinose are well resolved, ribulose and xylulose interfere with the analysis of ribose. It was therefore desirable to obtain better resolution between the pentoses and the method used to achieve this is described in section 3.4.5(ii).

**Quantification of metabolites**

The use of an internal standard is usually the preferred method for quantitative analysis by GC, and it is commonly believed to produce results which are independent of the volume and concentration introduced into the column. However, it has been pointed out that this is not necessarily so (Shatkay et al., 1977), (Shatkay, 1978). For an injection volume of 2 μL, an error of 1 μL was shown to introduce an error of 20 % into the analysis, and a

tripling of the standard concentration introduced an error of 30 % into the analysis (Shatkay, 1978). It is relatively easy to control the injection volume so that errors of the magnitude reported above are avoided. Careful operation of a 5 µL syringe can achieve volume accuracies of +/- 10 % (as determined by the resulting peak areas) and as a result the errors introduced by variations in injection volume are likely to be minimal. Variations in the concentration of the standard introduced onto the column may be a more significant factor. One of the major advantages of the internal standard method is said to be that the losses during sample processing prior to analysis are of little consequence. However, when large losses are encountered during sample processing, the standard curve prepared for higher levels of internal standard is not applicable (Shatkay, 1978). Consequently, large errors can result and the major reason for using the internal standard method is lost. To remedy this, the sample should be concentrated so that the concentration of the standard approximately equals that at which the standard curve was prepared.

**Choice of internal standard**

The quantification of analytes is best achieved by the inclusion in the sample mixture of a known amount of an internal standard having similar chemical characteristics to those of the analytes. The internal standard would then undergo the same extraction, purification and derivatization procedures as the sample and if the compounds are sufficiently similar, then the losses for both the internal standard and the analyte may be assumed to be the same. In the GC chromatogram the area of the analyte peak may then be compared with the area of the peak for the internal standard and the amount of analyte present can then be read off a previously prepared standard curve.

Ideally, for GC/MS analysis the internal standard will be a stable isotope analogue of the analyte under investigation. When there are multiple analytes, a stable isotope of each analyte is preferred. In this way it can be certain that the processing losses, chromatographic behaviour and the

detector responses will be virtually identical for both the standard and the analytes and the greatest accuracy in quantification of the analytes will be achieved. In addition, the added stable isotope labelled analogue can act as a carrier for smaller quantities of the endogenous analyte. In this investigation however, the analytes are themselves expected to become multiply labelled with the $^{13}C$ isotope during photosynthesis in an atmosphere containing $^{13}CO_2$ and the stable isotope dilution method therefore cannot be used. The alternative approach of using a compound that is similar to a sugar but unlikely to be encountered in chloroplasts and would be well separated from the analytes had therefore to be used.

Although multiple internal standards for different parts of the chromatogram are desirable, it is also necessary to minimize the interference that multiple standards can provide to the analysis of a mixture of compounds.

The mass spectrum of the derivatized standard chosen for this GC/MS investigation needed to contain an ion for monitoring which was common to all of the sugars being measured. Additionally, this ion must not become labelled in a $^{13}CO_2$ experiment. The sugar alcohols seemed to be an obvious choice. The mass spectra of the TMS derivative of these compounds show a prominent *m/z* 147 ion representing the pentamethyldisiloxonium ion ($[(CH_3)_3SiOSi(CH_3)_2]^+$) as do all of the sugar methoxime TMS derivatives, and since this ion does not contain any carbons derived from the sugar, it cannot become labelled during an isotope incorporation experiment. Thus, selected ion monitoring of the m/z 147 ion was the method of choice for quantitation studies.

In the selection of the internal standard mannitol was shown to give a decreased response and consequently unreliable results when used with higher levels of sugars. Xylitol eluted too closely to the pentoses to be used. Erythritol was shown to give a linear and reproducible response with all levels of sugars up to 120 pmol and it eluted well separated from the sugars.

Consequently, erythritol was chosen as the single internal standard for this investigation.

## 3.2 Design of photosynthesis apparatus.

The basic requirements in the design and construction of the photosynthetic apparatus were 1) that the chloroplasts be mounted on a filter membrane inside a small chamber with a transparent window for the admission of light; 2) the filter membrane should be large enough to hold sufficient chloroplasts to provide enough extracted material for GC/MS analysis; 3) the atmosphere above the membrane must allow the establishment of steady state photosynthesis with $^{12}CO_2$ and the rapid replacement of the $^{12}CO_2$ with the same concentration of $^{13}CO_2$ whilst causing minimum disturbance to the steady state; 4) the volume of the chamber above and below the membrane should be small in order to minimize the time taken to flush out the $^{12}CO_2$ with $^{13}CO_2$ using a reasonable gas flow rate. Also the volume of the chamber below the membrane should be made small in order to minimize the "dead volume" of residual $^{12}CO_2$; and 5) there must be provision for an appliance to inject a liquid that will quench the photosynthetic reactions and for the convenient removal of the chloroplast extract after the quench.

Filter holders were available (Sartorius SM 165 08B) which, with minimal modification, provided a suitable basis on which to construct a prototype photosynthesis apparatus. The rapid replacement of $^{12}CO_2$ with $^{13}CO_2$ was provided for by the inclusion of two gas inlet ports each supplying an internal gas manifold around the outer perimeter of the chloroplast-supporting membrane. The manifolds directed $^{13}CO_2$ across the entire membrane and drove excess gas out through a series of 6 gas outlet holes drilled in the housing above the centre of the membrane which vented the expelled gases to the atmosphere. An electro-mechanical timer unit was constructed to permit the automatic and rapid switching of the gas supply from a gas cylinder containing 1 % $^{12}CO_2$ in nitrogen to another cylinder containing

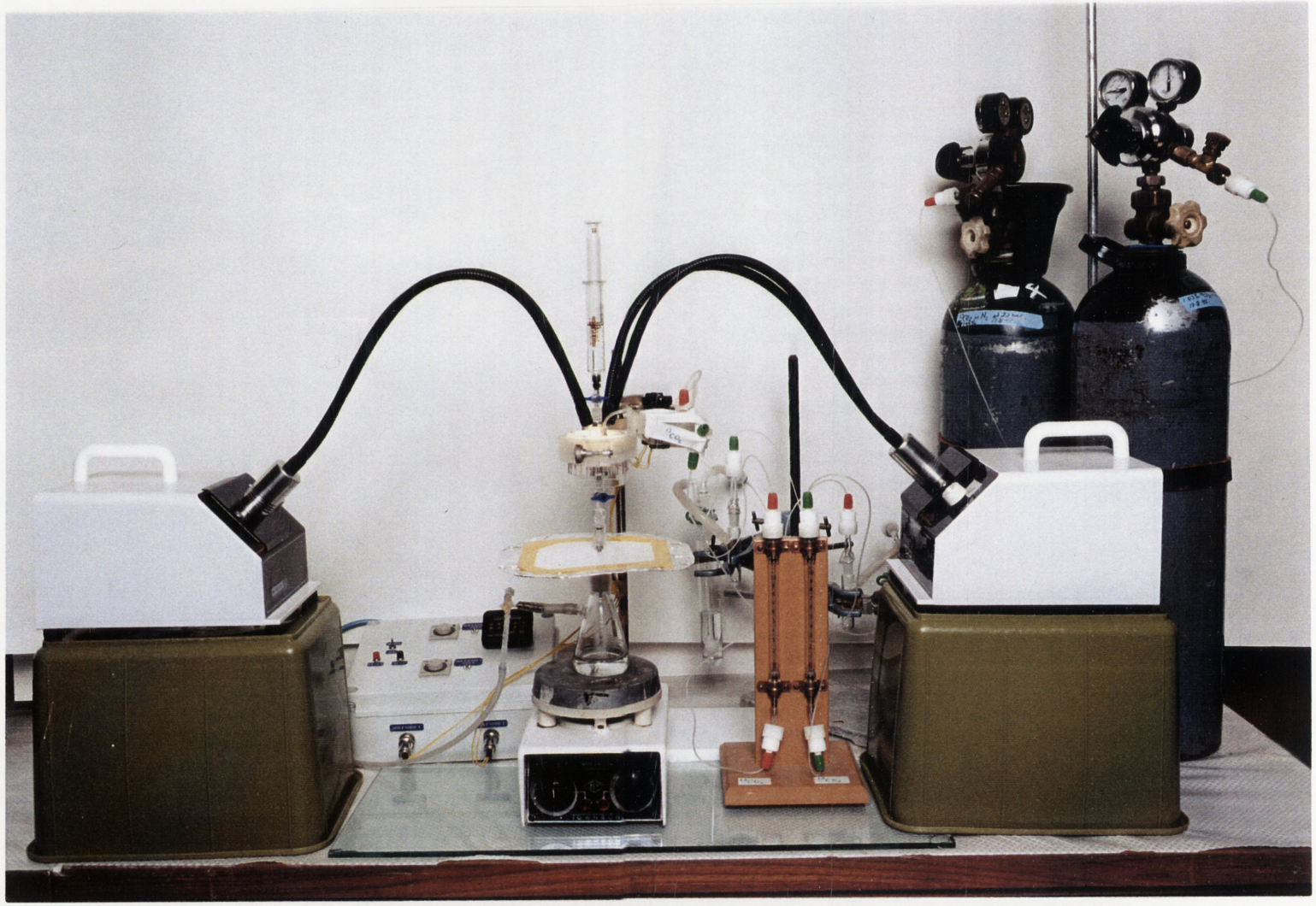

**Figure 3.2** Full view of photosynthesis set-up incorporating specially designed apparatus.

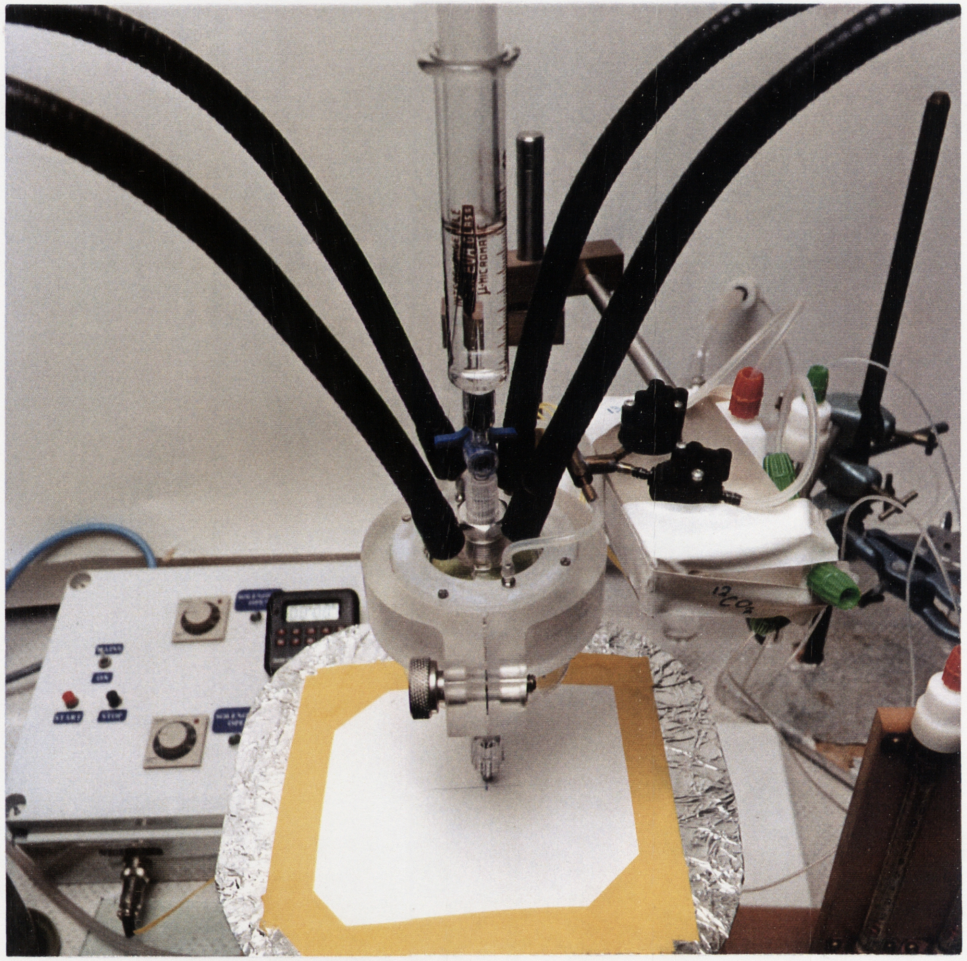

**Figure 3.3** Close-up of specially designed photosynthesis apparatus. Membrane containing chloroplast layer (green) can be seen at centre.

1 % $^{13}CO_2$ in nitrogen via a pair of electrically operated gas valves with minimal disruption to the steady state of photosynthesis.

The inlet and outlet ports for the filter holder provided suitable ports for the entry and withdrawal of the quenching solution. A 10 mL syringe containing the quenching solution was attached to the filter inlet port via a tap which was opened to allow the injection of the quenching solution at the completion of the photosynthesis experiment. By applying a gentle suction via a tap at the filter outlet port, the extract could be withdrawn into a flask containing boiling 80 % ethanol located below the apparatus. The chloroplasts and apparatus were protected from the heat produced by the hotplate by the provision of a reflective heat shield. Light was provided via fibre optic cables which greatly reduced the transmission of heat from the light source and reduced the need for cooling of the apparatus. The arrangement of the apparatus is illustrated in Figures 3.2. and 3.3.

The concentrations reported for chloroplast metabolites are variable but have usually been found to be in the range from 5 - 50 nmol per mg chlorophyll (Gerhardt et al., 1987), (Giersch et al., 1980), (Giersch, 1979). A filter membrane loaded with chloroplasts equivalent to 250 µg of chlorophyll should therefore contain 1.25 - 12.5 nmol of most metabolites of interest. Assuming minimal losses during processing for GC/MS analysis, this was considered to be adequate chloroplast material for each experiment.

## 3.3 Materials and equipment

Spinach (*Spinacia oleracea*, Yates Hybrid 102) and pea (*Pisum sativum* var. Greenfeast) seeds were obtained from Henderson Seed Co., Pty. Ltd., Lower Templestowe, Vic., Australia.

Sorbitol was purchased from Koch-Light Ltd.,Haverhill, Suffolk, CB9 8PB, UK and sodium pyrophosphate was purchased from Mallinckrodt Chemical Inc., Chesterfield, Missouri 63017 and Percoll was purchased from Pharmacia Biotech, Uppsala, Sweden. Suppliers of all other chemicals and enzymes were listed in section 2.2.1.

Leaf material was homogenized with a Polytron blender (Kinematica GmbH,CH-6010 Kriens, Luzern, Switzerland) (speed setting 7) fitted with a PT35K probe. Light measurements were made with a Hansatech Quantum Sensor 3/2897 (Hansatech Instruments, King's Lynn, Norfolk, PE32 1JL. UK). Oxygen evolution from isolated chloroplasts was measured in the gas phase using a leaf-disc oxygen electrode (LD1) and control unit (CB1D) (also from Hansatech Instruments. For measurements in the liquid phase the oxygen electrode was purchased from Rank. Oxygen evolution was recorded on a chart recorder (BBC Goerz Metrawatt SE120). The filter holder (SM 165 08B) and the cellulose nitrate filter membranes (50 mm, 8 μm nominal pore size) were purchased from Sartorius GmbH.

Isolated chloroplasts mounted on filter membranes were illuminated with a pair of 150 watt tungsten lamps each directed to the chloroplasts via bifurcated fibre optic light guides (Microlight 150) purchased from Fibre Optic Lightguides, Waitara, N.S.W. Australia.

The $^{13}CO_2$ gas was purchased from Cambridge Isotopes Limited and was prepared to 1 % in nitrogen along with 1 % $^{12}CO_2$ in nitrogen by Dr. Chin Wong of the Research School of Biological Sciences at the A.N.U. Gas analysis was carried out on a Varian 6000 gas chromatograph using a 1.83m x 0.32 cm glass column packed with Porapak N 100-200 mesh stationary

phase. A carrier gas of He was supplied at a flow rate of 20 mL $min^{-1}$ and the sample volume was 300 µL. The two gases eluted with baseline resolution within five minutes under isothermal conditions (30 °C). Detection was by thermal conductivity detection. Peak areas were automatically determined using a Hewlett Packard 3396A integrator.

Radioactivity was measured using a Beckman LS6000IC liquid scintillation counter and the scintillant was ReadySafe (Beckman Instruments, Inc., Fullerton, California).

## 3.4 Methods

### *3.4.1 Isolation of intact chloroplasts*

#### (i) Growth of plant material

Spinach was grown by water culture in a glasshouse under natural lighting according to established methods (Anderson et al., 1966). Seeds were sown in vermiculite moistened with nutrient solution and maintained at a temperature of 20-23 °C. After germination, the seedlings were exposed to incandescent light (800 µE $m^{-2}$ $sec^{-1}$) for a further three or four days during the initial expansion of the cotyledons. Before the emergence of the first pair of foliage leaves, the seedlings were removed from the vermiculite and transplanted into a water culture facility in a glasshouse. Basins (15 or 25 L) containing nutrient solution were covered with a lid containing 1 cm diameter holes through which the roots of the seedlings were passed. Small pieces of forked plastic prevented the above-ground parts of the seedlings from falling through the holes, whilst the roots were able to dangle freely in the nutrient solution, which was continuously aerated. Leaves were harvested five to six weeks after sowing the seeds, depending on the season.

Peas were grown in a glasshouse in trays containing a mixture of equal parts of vermiculite and pearlite to ensure good aeration. The trays were lightly watered initially and with a nutrient solution (Aquasol) typically every

three days after that until harvesting at 10-12 days after sowing. Less frequent watering was necessary in cooler weather. Supplementary lighting was provided during winter months.

**(ii) Chloroplast isolation**

The procedure for the isolation of intact chloroplasts from spinach or pea leaves was based on a method which involved a 2-step gradient of the silica-sol, Percoll (Pharmacia Biotech, Uppsala, Sweden) (Robinson, 1983). Five to six weeks old spinach leaves were harvested in the early morning in order to avoid the accumulation of starch granules, which can rupture the chloroplast envelope during centrifugation. For the isolation of chloroplasts from pea, the leaves were 10 - 12 days old. The amount of leaf material required to produce a satisfactory yield of chloroplasts was not critical but the yield depended on the quality of the leaves and increased with the amount of material used. Twenty-five to 65 grams of leaves produced between three and eight mg of chlorophyll. The harvested leaves were de-ribbed and floated in basins of water. Immediately before isolating the chloroplasts, the leaves were illuminated for 30 minutes with incandescent light at an intensity of 800 $\mu Em^{-2}sec^{-1}$. During this period, crushed ice was added to the water to maintain its temperature at 15 - 20 °C. Typically, 55 g of coarsely chopped leaves were homogenized in 300 mL of medium for 1.5 to 2 sec using a Polytron blender (speed setting 7) fitted with a PT35K probe. The homogenizing medium at pH 6.5 was chilled to a semi-frozen consistency before use and contained 330 mM sorbitol, 10 mM $Na_4P_2O_7.10H_2O$, 5 mM $MgCl_2$, 2 mM isoascorbic acid and 0.1% bovine serum albumin (BSA). The brie was filtered through either a double layer of Miracloth (Calbiochem) or a pad of cotton wool sandwiched between eight layers of muslin. The filtrate was then centrifuged at 1200 x g for 1 min in a Sorvall (DuPont Medical Products, Newtown, CT 0647-5509 U.S.A.) refrigerated centrifuge using either an SS34 or SA-600 rotor. After decanting and discarding the supernatant solution, the sedimented material was gently

resuspended, using a fine, soft paint brush, in 6 mL of a medium at pH 7.6 containing 330 mM sorbitol, 50 mM HEPES-KOH, 2 mM EDTA, 1 mM $MgCl_2$ and 1mM $MnCl_2$. An aliquot (3 mL) of the suspension was then placed into each of two 40 mL Corex tubes and carefully underlaid with 4 mL of Percoll medium made to 40% with resuspension medium. These tubes were centrifuged at 1200 x g for 1 min as before, but this time the centrifuge was allowed to come to rest without braking in order to avoid agitating the gradient formed. Using this procedure, intact chloroplasts formed a pellet at the bottom of the centrifuge tube, whilst the broken fragments and other debris remained in a layer at the interface between the resuspension medium and the 40% Percoll medium. After carefully removing these layers with a Pasteur pipette, the surfaces of the chloroplast pellets were rinsed once with resuspension medium. Finally, the chloroplasts were resuspended in the resuspension medium (3 mL) and stored on ice. The above procedures were carried out on ice using previously chilled equipment. All parameters (density, yield, photosynthetic activity) describing chloroplasts were related to the chlorophyll content which was measured according to the procedure below.

Chloroplast isolation is a technique which can give highly variable results. Its success depends to a large extent on the quality of the leaf material used and the experience of the operator in recognizing the many pitfalls which can arise. The two most commonly used plant species, spinach and pea were both used in this study and although peas were easier to grow to a consistent quality throughout the year, their photosynthetic activity was always lower than that of spinach. The seasonal variability of spinach leaves requires careful management. Being a "long day" plant, spinach produces very little vegetative growth once the day length exceeds a critical value and instead turns to reproductive growth and produces flowers and seeds before quickly dying. Thus during the long, hot days of summer the leaf quality is poor unless due attention is paid to the provision of artificially regulated daylength and temperature conditions. During the winter months when suitable glasshouse

facilities may provide adequate temperature conditions in spite of the low ambient temperature, the light level and day length are too short. Under these conditions much higher levels of chlorophyll are produced in an effort to compensate for the low light and chloroplast activity expressed "per mg chlorophyll" is greatly decreased. Satisfactory experiments may be conducted with such "winter grown" spinach, although it must be recognized that the activity expressed per mg of chlorophyll will be significantly lower than that achieved from chloroplasts prepared from spinach grown in the spring or autumn. The yield of isolated chloroplasts depended on the amount of leaf material used and the quality of the leaves and ranged from 3 mg chlorophyll from 25 g leaves to 8 mg using 65 g leaves.

Techniques for the isolation of chloroplasts that involved the silica-sol gradient, Percoll, greatly increased the ease and reliability with which highly intact and active chloroplast preparations were achieved. Perversely, the Percoll itself could be a cause of failed preparations if it was too old and thus could be a source of confusion in the hands of an inexperienced operator. Autoclaving the Percoll was ineffective at preserving the efficiency of separation of the broken chloroplast fragments from the intact organelles. Once Percoll was too old, dialysis did not restore its effectiveness and it was therefore best to maintain fresh supplies. Provided the quality of the leaf material was satisfactory and the Percoll was fresh, the method never failed to produce a high yield of highly intact chloroplasts which were always better than 95 % intact when measured by the method described in section 3.4.2 below.

**(iii) Chlorophyll assay**

The chlorophyll content of isolated chloroplast suspensions was estimated according to the method of Arnon (Arnon, 1949). Aliquots (50 µL) of the isolated chloroplast suspension were added to 80% acetone (5 mL) and mixed. The suspension was then centrifuged for 5 min at 5,000 x g in a Sorvall SS34 rotor and the absorbance (A) of the supernatant fluid was measured at 645 and 663 nm against an 80% acetone blank in a Gilford 2600 single beam spectrophotometer.

The chlorophyll concentration was estimated using the following relationship:

$$\text{Chlorophyll (µg chlorophyll mL}^{-1}) = [(20.2 \times A_{645}) - (8.08 \times A_{663})] \times 101$$

The chloroplast suspension was then made to a density of 1 mg chlorophyll per mL with resuspension medium.

### *3.4.2 Evaluation of the isolated chloroplast preparation*

**(i) Measurement of chloroplast intactness**

The intactness of chloroplasts was measured by the ferricyanide reduction method (Lilley et al., 1975) and was determined using the following equation which relates the rate of ferricyanide reduction in intact chloroplasts to the rate in chloroplasts which have been ruptured by osmotic shock:

$$\% \text{ intactness} = 100 - \left[\frac{\text{rate "intact"}}{\text{rate "shocked"}} \times 100\right]$$

The two rates of ferricyanide reduction were measured by the rate of oxygen evolution in an oxygen electrode using two separate incubations. The assay medium for these incubations contained 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES, 0.5 mM Pi, 5 mM PPi, 500 units of catalase, 10 mM D,L-glyceraldehyde, 1.5 mM $K_3Fe(CN)_6$ and chloroplast suspension equivalent to 80 µg of chlorophyll and made to a volume of 2 mL with water.

In the incubation for the measurement of the rate of ferricyanide reduction in osmotically shocked chloroplasts, the chloroplast suspension was added to water 1 min prior to the addition of double strength assay medium to ensure lysis of the chloroplasts. For assays of intact chloroplasts the chloroplast suspension was added to the isotonic solution. For both intact and lysed chloroplasts, electron transport was uncoupled from photophosphorylation after 60 seconds of illumination by the addition of ammonium chloride to a final concentration of 2.5 mM. Illumination was continued and a linear rate of oxygen was maintained for several minutes. The oxygen evolution was recorded on a Goerz Metrawatt SE 120 chart recorder and the linear portion of the trace was used to obtain the rate measurements for the intactness calculation above.

**(ii) Measurement of chloroplast activity**

(a) Chloroplasts in suspension.

The activity of the isolated chloroplast suspensions was measured polarographically using a Clark oxygen electrode. The $CO_2$-dependent $O_2$ evolution of isolated chloroplast suspensions illuminated in an assay medium at 23 °C was recorded using a Goerz Metrawatt SE 120 chart recorder and the rate of oxygen evolution was calculated from the linear portion of the trace. The chloroplasts were illuminated at an intensity of 1200 $\mu E\ m^{-2}\ s^{-1}$ using the quartz-halogen light from a slide projector. The assay medium contained 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 50 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), 0.5 mM Pi, 5 mM PPi, 500 units of catalase, 10 mM $NaHCO_3$, and chloroplast suspension equivalent to 40 $\mu g$ of chlorophyll in a volume of 2 mL and at a pH of 7.9.

(b) Chloroplasts on filter membranes

The activity of the isolated chloroplasts mounted on a filter membrane was measured polarographically using a Clark oxygen electrode adapted for

measurement of the oxygen evolution from leaf-discs (Delieu et al., 1981). Methods for the preparation of chloroplasts for use in the leaf-disc oxygen electrode were based on those previously described (Cerovic et al., 1987). Chloroplasts were mounted on a filter membrane by filtering a suspension of chloroplasts through a cellulose nitrate membrane (Sartorius, 50 mm diameter, pore size 8 μm). This was accomplished using a Millipore solvent clarification apparatus fitted with a 100 mL funnel. A suspension of chloroplasts equivalent to 250 μg chlorophyll in 40 mL resuspension medium (section 3.4.1.(ii)) was allowed to filter through the membrane under gravity or with a very light suction so that the chloroplasts became trapped on the surface of the membrane in a thin film of resuspension medium. The chloroplasts were washed with a further 10 mL of the resuspension medium and then given a final wash with 3 mL of assay medium containing 6 mM inorganic phosphate, 22.5 U $mL^{-1}$ alkaline phosphatase (Sigma, human placenta) and 1000 U $mL^{-1}$ catalase in resuspension medium. A disc of 1018 $mm^2$ (representing 94% of the chloroplasts) was cut from the membrane and mounted in a leaf-disc oxygen electrode (Hansatech). Once assembled, the leaf-disc oxygen electrode was charged with an atmosphere of 1% $CO_2$ in $N_2$ at atmospheric pressure and sealed. The chloroplasts were exposed to incandescent light from a pair of bifurcated fibre optic light sources at an intensity of 1200 $\mu Em^{-2}s^{-1}$ measured at the underside of the leaf-disc electrode light window. The temperature of the water bath supplying the water jacket which regulated the oxygen electrode temperature was maintained at 23 °C. After a lag period of approximately 1.5 minutes, the evolution of oxygen could be monitored as a digital voltage on the oxygen electrode control box and was also recorded on a Goerz Metrawatt SE 120 chart recorder. The rate of oxygen evolution reached a linear and maximum rate at approximately four minutes after switching the lights on. The rate of oxygen evolution was calculated from the linear portion of the trace.

### *3.4.3 Photosynthesis experiments*

Because the photosynthesis apparatus had no provision for the measurement of oxygen evolution, the photosynthetic activity of the chloroplasts could not be established once they were mounted in the photosynthesis apparatus. In addition, the short life of the isolated chloroplast preparations (2 hours) prevented the prior measurement of photosynthetic activity in the leaf-disc oxygen electrode. The only way to ascertain chloroplast activity in the apparatus was to conduct a $^{13}CO_2$ photosynthesis experiment and to measure by GC/MS the incorporation of $^{13}C$ into the PCR pathway intermediates. Early attempts to demonstrate chloroplast activity in the apparatus failed and in an attempt to understand the reasons for this failure, experiments were conducted in the leaf disc oxygen electrode where the chloroplast activity could be established prior to the commencement of the $^{13}CO_2$ photosynthesis experiment.

**(i) Experiments in the leaf-disc oxygen electrode**

Chloroplasts were prepared for experiments in the leaf disc oxygen electrode as discussed for the assay of chloroplast activity in that equipment (section 3.4.2.(ii)(b)) and a linear rate of oxygen evolution was established. At this time, a volume (60 mL) of 1% $^{13}CO_2$ in $N_2$ was flushed through the 6 mL chamber of the oxygen electrode that contained the membrane-mounted chloroplasts and after resealing the chamber, the chloroplasts were allowed to photosynthesize in the $^{13}C$-enriched atmosphere for a prescribed period of time before the reactions were terminated using the quenching methods described below. Experiments were conducted for 0, 30 and 45 seconds exposure to 1% $^{13}CO_2$ in $N_2$ and the "control" experiment was conducted with chloroplasts exposed to 1% $^{13}CO_2$ in $N_2$ in the dark. In additiion, a "blank" experiment was conducted in which the full extraction procedure was followed in the absence of chloroplasts to measure the extent of any contaminants that

may interfere with the analysis. Five repetitions of each experiment were carried out using the same chloroplast preparation.

*Quenching photosynthesis reactions*

Reactions conducted in the leaf-disc oxygen electrode were terminated by disassembling the apparatus as quickly as possible and plunging the chloroplast-containing membrane into a beaker of liquid nitrogen. It was estimated that this procedure took no more than 2.5 seconds. The beaker containing the membrane was then stored in a freezer at -20 °C where the liquid nitrogen was allowed to evaporate and the temperature rose to -20 °C.

After overnight storage at -20 °C the enzymes were denatured and the metabolites were extracted by removing the beaker containing the membranes from the freezer and immediately adding boiling 80% ethanol (75 mL) and boiling for a further 5 minutes. The membranes were then removed from the extract and thoroughly rinsed with water, adding the washings to the extract. After cooling, the pooled extracts were then evaporated to dryness at 37 °C under a stream of dry nitrogen.

**(ii) Experiments in the photosynthesis apparatus**

Chloroplasts were prepared on a filter membrane as described for the experiments in the oxygen electrode. The loaded membrane was mounted intact in the photosynthesis apparatus (illustrated in Figures 3.2 and 3.3). A gas mixture consisting of 1 % $^{12}CO_2$ in nitrogen was applied to the two gas inlet ports via an electrically operated valve connected to a specially made electro-mechanical timer unit. The gas stream (at a flow rate of 200 $cm^3$ $min^{-1}$) was humidified by bubbling it through a tube of water before entering the valve. The chloroplasts were illuminated through the transparent window in the apparatus by a pair of 150 W tungsten lamps, each directed through a bifurcated fibre optic light guide. The gas mixture was supplied for 4 min before the timer operated the valves supplying the gas and switched to the

source supplying 1 % $^{13}CO_2$ in $N_2$ for the prescribed period. Experiments were conducted for 0, 6, and 30 seconds exposure to 1% $^{13}CO_2$ in $N_2$ and the "control" experiment was conducted with chloroplasts exposed to 1% $^{13}CO_2$ in $N_2$ in the dark for 30 sec. The results of these $^{13}C$ incorporation studies are discussed in Chapter 4.

*Quenching photosynthesis reactions*

For experiments conducted in the photosynthesis apparatus, the reactions were terminated and the metabolites were extracted by injecting hot 80% ethanol (5 mL) into the apparatus and allowing the chloroplasts to stand in the solution for ten seconds. Although the 80 % ethanol was at boiling point, the act of loading this into a 50 mL syringe and assembling the syringe in place above the photosynthesis apparatus meant that the temperature was considerably reduced by the time it came in contact with the chloroplasts. The ethanolic chloroplast extract was then drawn through the membrane using gentle suction into a flask that contained boiling 80% ethanol (30 mL). The extraction process was repeated with a further volume of boiling 20% ethanol (5 mL) followed by boiling water (5 mL). The combined extract was then boiled for five minutes and allowed to cool. After cooling, the extract was evaporated to dryness at 37 °C using a stream of dry nitrogen.

### *3.4.4 Sample handling*

The dried samples from both (*i*) and (*ii*) above, were redissolved in water (2 mL) and then processed for GC/MS analysis according to the methods described in section 2.2.5 (Chapter 2) and illustrated in the flow diagram (Fig. 3.4).

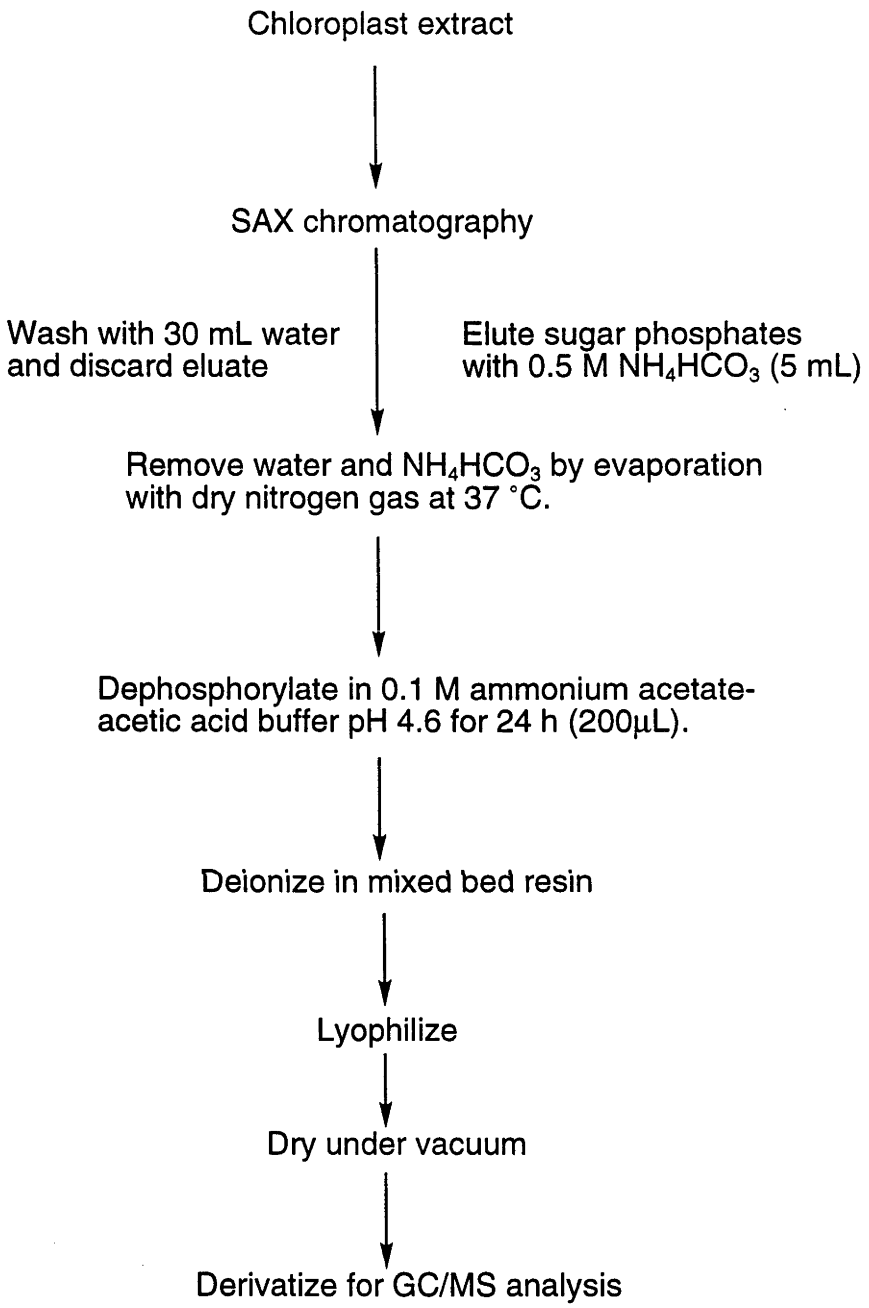


**Figure 3.4.** Flow diagram of the sample workup for GC/MS analysis. (See Section 2.2.5 in Chapter 2 for full experimental details).

### *3.4.5 Identification and quantification of metabolites*

**(i) GC retention times of derivatized sugars**

The GC retention times of Mox- or Eox-TMS derivatives of sugars were determined after at least three consecutive injections of 50 -100 pmol of the individual sugar derivative. The retention times varied with the condition and length of the column and typical values of both the Mox- and Eox-TMS derivatives of relevant sugars are given in Table 3.1 (Results section).

**(ii) Pentose resolution**

Improved separation of the pentoses was achieved using a column having a more polar stationary phase (14 % cyanopropylphenyl methylpolysiloxane DB-1701 30 m x 0.244 mm x 0.25 µm film thickness, J&W Scientific, Folsom, California, 95630) than the non-polar HP-1 column used for routine analyses. For the elution of the derivatized pentose sugars the initial temperature of 120 °C was maintained for 12 min. then the column temperature increased to 200 °C at 15 °C per min. This temperature was maintained for a further 2.67 min. for a total run time of 20 min. This column was unsuitable, however, for the complete analysis of all of the C4 to C8 sugars.

**(iii) Identification of chloroplast metabolites**

Chloroplast metabolites were identified when both the retention time and mass spectrum corresponded with those of the authentic standard. In critical cases, such as in the identification of the octulose, when sufficient material permitted, the extract was spiked with a small amount of the authentic compound to confirm the identification.

**(iv) Calibration of sugars against the internal standard**

Sugars which were available in the free form with a high degree of purity were dried for three days over phosphorous pentoxide under a low

vacuum. Approximately 0.02 g of the dried powder or syrup (ketopentoses) was weighed to five decimal places to prepare standard solutions of each sugar. Appropriate dilutions of these were taken to prepare a set of 0.5 mM standard solutions.

Some sugars were only available as the phosphate esters and frequently in the presence of various amounts of buffer salts. In these cases, after weighing the dried powders and preparing standard solutions, they were assayed enzymically to establish their concentrations prior to use. Sedoheptulose, D-*glycero* D-*ido*- and D-*glycero* D-*altro*-octuloses were assayed as their bisphosphates using an aldolase-based assay (Bergmeyer, 1974). Erythrose was assayed as the monophosphate ester using a transketolase-based assay (Paoletti et al., 1979b). Sugar phosphates (3 μmol) were then evaporated to dryness in a rotary film evaporator and dephosphorylated in a solution (0.5 mL) which contained citrate buffer 50 μmol, pH 5.4) and human prostatic acid phosphatase (1.25 units). After allowing the dephosphorylation to proceed for 4 h at 37 °C, the reaction was terminated by heating at 100 °C for 2 min. The samples were then treated with mixed bed resin according to section 2.2.5.3. and lyophilized.

Recovery from the dephosphorylation step was assessed by carrying out parallel dephosphorylations of glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate, all of which were assayed enzymically as the free sugars.

Appropriate dilutions of the lyophilized sugars were then prepared to produce a set of 0.5 mM solutions for use as standards. Two groups of sugar mixtures were prepared, each containing 500 μL of the 0.5 mM standard solution of each of four sugars. Each sugar was thus present in a volume of 2 mL and at a concentration of 0.125 mM. The first group contained erythrose, fructose and D-*glycero* D-*ido*-octulose. The second group contained glucose, sedoheptulose and D-*glycero*-D-*altro*-octulose. Pentoses were initially calibrated separately as the ethoxime-TMS derivatives, and later as the

methoxime-TMS derivatives when the ethoximes were not used. A series of dilutions of the standard mixtures containing 0.025, 0.125, 1, 2, 3, 4 or 5 nmol of each sugar together with 5 nmol of the erythritol standard were added to separate Reacti Vials.

The mixtures were then first dried over phosphorous pentoxide, then under high vacuum and derivatized as the methoximes according to section 2.2.5.4. This gave solutions which contained 0.5, 2.5, 20, 40, 60, 80, or 100 pmol of each sugar together with 100 pmol of the erythritol standard in each µL of the derivatized solution.

GC/MS analysis was carried out by the measurement of the peak areas obtained for the *m/z* 147 ion each sugar using single ion monitoring. Three injections were made at each sugar level and the average ratio of sugar to erythritol peak area was determined. A graph plotting the measured ratio of each sugar *m/z* 147 ion area to the erythritol *m/z* 147 ion area against the actual ratio of the sugar to erythritol for the series of sugar concentrations was then plotted. The slopes of these graphs then represent the calibration factors relating the sugars to the internal standard, erythritol, and were determined as the slope of the least squares line of regression of measured area ratio on actual ratio.

**(v) Recovery of extracts during the initial workup**

For metabolite quantification purposes, the internal standard should ideally be added to the chloroplasts during the reaction quenching process. In this investigation such a procedure was unable to be adopted because the neutral erythritol selected as the internal standard would be lost during the column washing at the subsequent strong anion exchange (SAX) chromatography step (Figure 3.4) whilst the sugar phosphates extracted from the chloroplasts are retained on the resin. It was therefore necessary to add the internal standard after the sugar phosphates had been eluted from the SAX column. The sample recovery for the initial sample workup steps prior to

SAX chromatography was therefore estimated using radioactivity tracing. As it is possible that mono- and bisphosphates suffer unequal adsorption losses, equal radioactivities of a mono- and a bisphosphate were used in the recovery test.

The recovery of metabolites from chloroplast extracts was estimated in separate experiments using $^{14}C$-labelled sugar phosphates. [1-$^{14}C$]fructose 6-phosphate (0.009 μCi) and [4-$^{14}C$]D-*g* D-*a*-octulose 1,8-bisphosphate (0.009 μCi) were added to chloroplasts (250 μg) mounted on a membrane in the photosynthesis apparatus. The extraction and processing of the sample was carried out according to the methods described in sections 3.4.3(ii) and 2.2.5. Triplicate samples (200 μL) taken from the eluate of the SAX column were mixed with Ready Safe liquid scintillation cocktail (3 mL) and measured for radioactivity using a Beckman liquid scintillation counter in the Auto DPM mode after autocalibration. The recovery factor was determined from the average recovery for the three replicate samples taken from a single recovery experiment. The recovery of individual sugars in a chloroplast extract may vary greatly depending on the concentration of the particular sugar in the extract. The less abundant sugars are much more strongly affected by the adsorption losses than the more abundant sugars which benefit from a greater "carrier effect". Consequently the concentrations determined for the individual sugars can be regarded as minimum estimates only.

**(vi) Quantification of chloroplast metabolites**

The concentration of metabolites in isolated chloroplast suspensions was estimated by measuring the ratio of the area of the m/z 147 ion chromatogram for the sugar to that of the internal standard, erythritol for three consecutive injections of 1 μL of the sample. The measured ratio was then divided by the calibration factor from (iv) above and then divided by the recovery factor determined in (v) above and by the amount of chloroplast material from which the extracts were obtained (1.25 mg chlorophyll for 30

sec. photosynthesis apparatus experiment; 0.25 mg chlorophyll for 6 sec. photosynthesis apparatus experiment; 1.18 mg for experiments using the leaf disc oxygen electrode). The chloroplast extracts revealed the presence of some sugars which were unexpected for which the calibration factors had not been determined. The concentrations of these sugars were estimated from the calibration factors of the most closely related sugar for which calibration factors were available and are thus regarded as approximations.

## 3.5 Results and discussion

### (i) Design of photosynthesis apparatus

The design of the photosynthesis apparatus was shown to be satisfactory as a prototype for further development. The initial failure to observe chloroplast metabolites in the GC/MS analysis of extracts from experiments conducted in the apparatus proved to be due to the decomposition and adsorption of sugar phosphates on the glass surfaces used in sample processing. These problems were largely overcome by the switch to non-glass materials for sample handling and the pooling of the extracts from five experiments to obtain sufficient material for the less abundant sugars to be analyzed. Thus the major design deficiency was that the apparatus proved to be too small and any future work would benefit from the design of larger apparatus. In that case, the adoption of a non-glass means of reducing the volume of the space beneath the filter membrane would be beneficial.

Experiments conducted in the leaf-disc oxygen electrode had the advantage that the activity of the chloroplast preparation could be verified prior to the introduction of the $^{13}CO_2$ and the commencement of the experiment. This assisted in the identification of problems experienced with early experiments in the photosynthesis apparatus. Experiments conducted in the leaf-disc oxygen electrode suffer, however, from the disadvantage that the placement of the gas entry and exit ports is unfavourable for the efficient

replacement of $^{12}CO_2$ with $^{13}CO_2$. This led to a lower enrichment than was expected with the specially designed apparatus. Leaf-disc oxygen electrode experiments had the disadvantage that the equipment had to be disassembled to terminate the photosynthesis reactions, thereby exposing the chloroplasts briefly to low light and atmospheric (unlabelled) $CO_2$.

It is also possible that further improvements could be made to sample recoveries, and this aspect of the experimental protocols deserves further investigation.

**(ii) Chloroplast activity**

The activity of spinach chloroplasts in suspension was greater than 100 μmol $h^{-1}$ mg $chl^{-1}$ (18 preparations) whilst the maximum achieved for pea was 80 μmol $h^{-1}$ mg $chl^{-1}$ (6 preparations).

Some difficulty was experienced in achieving photosynthetic activity from chloroplasts mounted on a filter membrane. The source of alkaline phosphatase was not specified in the original publication of the method (Cerovic et al., 1987). Five different alkaline phosphatases were tested and activity could be obtained from chloroplasts only when the alkaline phosphatase from human placenta was used. Other sources of alkaline phosphatase tested were calf intestine and bovine intestine (3 types, including an affinity chromatography purified product), but all of these completely inhibited oxygen evolution in the leaf-disc oxygen electrode. Neither purification of the enzyme using Sephadex G25 nor dialysis removed the inhibition and no explanation for the inhibitory effect could be found.

The activity of spinach chloroplasts on a filter membrane was greater than 65 μmol $h^{-1}$ mg $chl^{-1}$. The reaction medium for isolated chloroplasts normally contains bicarbonate as a source of $CO_2$. However, bicarbonate had to be omitted from the reaction medium for chloroplasts used in $^{13}CO_2$ incorporation experiments in order to maximize the enrichment of $^{13}CO_2$ supplied to the chloroplasts. It was therefore appropriate that bicarbonate was

also omitted from the medium for the assay of chloroplast activity in the leaf-disc oxygen electrode. This produced little decrease the chloroplast activity but reduced the time during which the chloroplasts remained sufficiently active. *Therefore all experiments conducted with chloroplasts mounted on a filter membrane had to be completed within two hours.*

No activity could be obtained using pea chloroplasts under the same conditions as those used for spinach. Only when alkaline phosphatase was omitted from the assay medium could any activity be obtained using pea chloroplasts, and in these cases the activity was always less than 27 μmol $h^{-1}$ mg $chl^{-1}$. Pea chloroplasts were therefore not used for any of the $^{13}CO_2$ incorporation experiments.

Spinach is the most commonly used plant for the conduct of photosynthetic studies using isolated chloroplasts. In this study spinach proved to be a very suitable species from which to isolate highly intact and reasonably active chloroplasts with high reliability once the pitfalls were understood and controlled. Peas proved to be unsuitable as conditions could not be found under which pea chloroplasts showed any activity on the filter membrane in the presence of alkaline phosphatase.

**(iii) Photosynthesis experiments**

In early experiments conducted with the photosynthesis apparatus the expected chloroplast metabolites were not observed during GC/MS analysis. Instead, a variety of other unidentified products were observed, some of whose mass spectra exhibited sugar-like fragment ions. Possible sources of these products that were investigated were: extractables from the cellulose-nitrate membranes used to support the chloroplasts; extractables from the various ion exchange resins used to purify the extract; and impurities in the sorbitol osmoticum and the buffers used in the chloroplast assay medium and in the dephosphorylation reactions. A variety of alternative membranes were tested, including cellulose acetate, mixed cellulose esters, nylon and glass

fibre. However chloroplasts were active only on the cellulose nitrate membranes and these did not contribute any significant impurities. The use of analytical grade cation exchange resin (AG 50) rather than Dowex 50 brought about some reduction in the levels of the impurities but the resin was shown not to be the major source of impurities. Citrate buffer previously used in the dephosphorylation of the sugar phosphates prior to GC/MS analysis (Irvine et al., 1992) contributed some impurities and was replaced by ammonium bicarbonate. However citrate was also shown not to be the principal source of the sugar-like impurities observed during the analysis of the extracts. These impurities were eventually found to be decomposition products that were formed when the sugars were degraded on the glass surfaces of the equipment used during the sample handling procedures. This finding led to the use of non-glass vessels and equipment for all steps in the sample processing. Only after this change was it possible to observe the expected chloroplast metabolites free from the impurities previously seen. In addition, glass beads, which were previously used in the lower part of the photosynthesis chamber to reduce the "dead volume" of $^{12}CO_2$ were omitted. This was at the expense of a possible reduced enrichment with $^{13}CO_2$ in the atmosphere to which the chloroplasts were exposed.

In addition to the decomposition of sugars by glass surfaces, the adsorption of sugars onto glass and non-glass surfaces led to a much lower recovery of chloroplast metabolites than had been anticipated in the design of the experiments. To overcome this problem it was necessary to combine the extracts from five experiments in order to obtain sufficient material for the analysis of those sugars which are present in the extract in low concentrations. It was therefore possible to obtain only one sample from each chloroplast preparation before the useful life of the chloroplasts was exceeded (2 hours). Tracer studies using $^{14}CO_2$ have traditionally been able to overcome problems of adsorptive losses when working with very small samples by the inclusion of unlabelled "carrier" compounds in the sample processing, an option which

was not available in this $^{13}CO_2$ study. The inability to conduct more than one experiment per chloroplast preparation therefore greatly reduced both the number of replicates that could be obtained and the number of time points which could be analyzed in this study. Nevertheless, each sample obtained represents an average of five experiments and therefore provides better data than that from a single experiment conducted with one membrane. The results of photosynthesis experiments conducted with chloroplasts exposed to a $^{13}C$-enriched atmosphere are discussed in Chapter 4.

Although it was possible to devise procedures which eliminated the use of glass during sample processing in aqueous solutions, the derivatization steps involved reations in organic solvents at 80 °C and were best carried out in glass Reacti Vials. The differential adsorption of the derivatives of high molecular weight sugars was clearly evident when dilutions of the main derivatized sample exposed the extract to fresh glass surfaces. This effect reduced the accuracy of the quantitative measurements even though an internal standard was used.

Experiments involving isolated chloroplasts were carried out in a medium containing 330 mM sorbitol. It was not possible to remove the sorbitol completely from the SAX column, even with extensive washing. A 30 mL wash was, however, usually sufficient to ensure that any residual sorbitol in the sample was reduced to the level at which it did not interfere with the analysis, since the retention time of its TMS derivative did not coincide with that of any of the labelled metabolites.

The experimental procedures reported in section 3.4.3 were chosen because they were best able to meet the many requirements of the investigation. Alternative approaches were considered. If alkaline phosphatase included in the reaction medium is not capable of dephosphorylating all exported sugar phosphates at a sufficient rate, there could be distortions introduced into the observed labelling levels and patterns by the residual exogenous sugar phosphates. An alternative would have been

to inject a "wash" with fresh assay medium onto the membrane-supported chloroplasts to remove the exogenous sugar phosphates prior to the injection of the quenching solution. However, it was observed during the loading of chloroplasts onto the filter membrane that the organelles were very susceptible to rupturing if there was more than the most gentle suction applied to the underside of the membrane. It would have been essential for the "wash" to be rapidly removed in order to minimize the effects of the disturbance of the steady state of photosynthetic metabolism that this would cause, even if the "wash" medium was pre-saturated with $H^{13}CO_3^-$ prior to injection. It became evident that any rapid removal of the "wash" fluid would result in the rupturing of the chloroplasts and the chloroplast metabolites would be lost. This approach was therefore not used and the chosen approach relied on the effectiveness of the human placental alkaline phosphatase to dephosphorylate the exogenous sugar phosphates, with its possible limitations.

The results reported in Chapter 4 are obtained from the areas of the selected ion chromatograms from three separate injections. When the ion cluster was small, ion intensity data were used instead and in these cases corrections were applied for any contributions from background ions.

**(iv) Identification of sugars**

Figure 3.5 shows the total ion chromatogram for 45 second experiment no.1 in the leaf disc oxygen electrode. Sugars were identified by their retention times (Table 3.1) and their characteristic mass spectra (Chapter 2, Figures 2.10 to 2.16). In some cases the *syn*- and *anti*-isomers were unresolved.

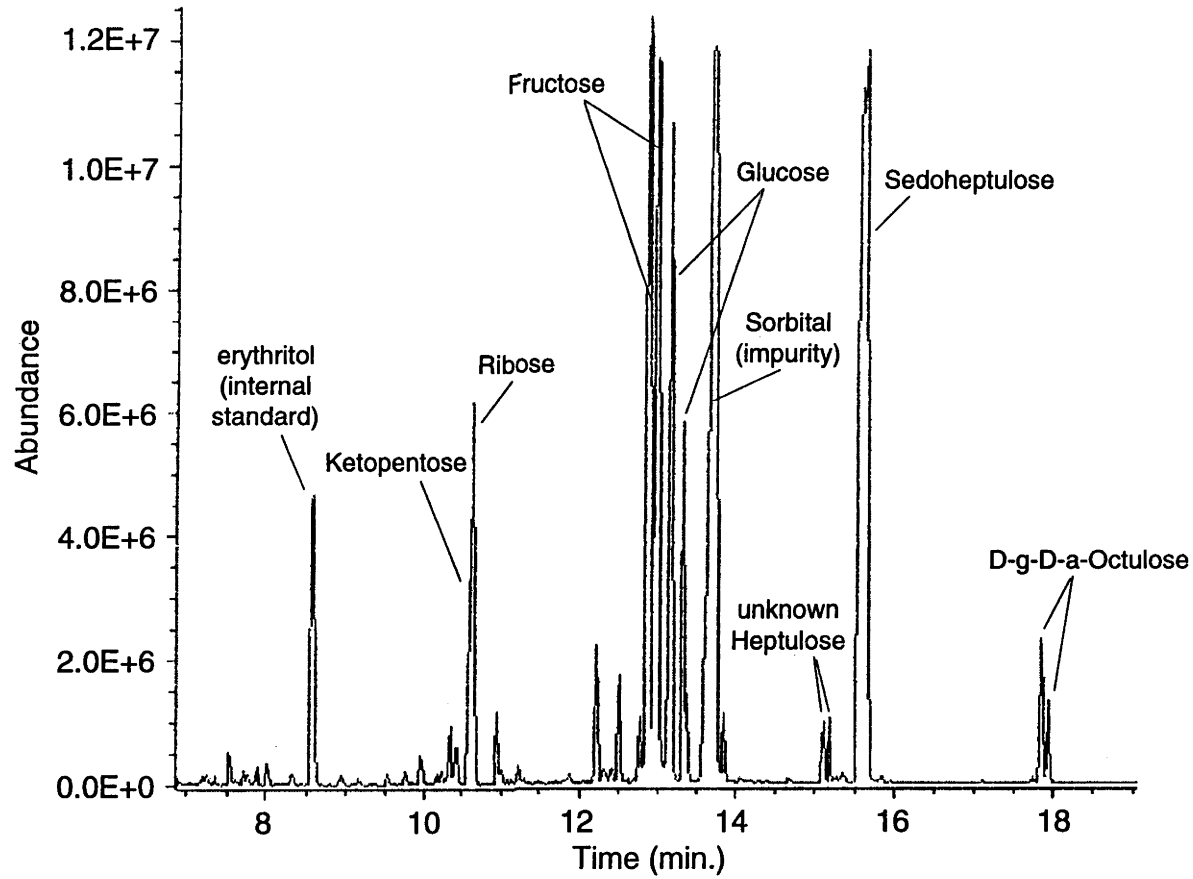


**Figure 3.5.** Total ion chromatogram (TIC) of Mox-TMS derivative of extract from 45 sec exposure of intact chloroplasts to $^{13}CO_2$ in leaf-disc $O_2$ electrode.

**Table 3.1.** GC retention times for the Mox- and Eox-TMS derivatives of authentic sugars on the HP-1 capillary column.

| Sugar | Retention time (min) | | | |
|---|---|---|---|---|
| | Mox-TMS | | Eox-TMS | |
| | 1 | 2 | 1 | 2 |
| **Erythrose** | 5.571 | 5.736 | 6.252 | 6.397 |
| **Xylose** | 8.089 | 8.187 | | 8.764 |
| **Lyxose** | 8.133 | 8.263 | 8.739 | 8.841 |
| **Arabinose** | 8.043 | 8.282 | 8.667 | 8.857 |
| **Xylulose** | 8.421 | | 8.902 | |
| **Ribulose** | 8.426 | 8.552 | 8.874 | 9.070 |
| **Ribose** | 8.460 | | 9.033 | |
| **Fructose** | 10.660 | 10.740 | 11.085 | |
| **Glucose** | 10.900 | 11.078 | 11.366 | 11.625 |
| **Mannoheptulose** | 13.079 | | 13.417 | 13.555 |
| **Sedoheptulose** | 13.283 | 13.350 | 13.585 | 13.785 |
| **D-*g* D-*a*-octulose** | 15.511 | 15.582 | 15.925 | 15.999 |
| **D-*g* D-*i*-octulose** | 15.348 | 15.443 | 15.648 | 15.925 |

1, leading isomer; 2, trailing isomer.

The D-*glycero* D-*altro*-octulose was identified in the extract by the later addition of authentic D-*glycero*-D-*ido*-octulose which eluted after the endogenous *altro*-isomer, confirming that the octulose found in the extract was the *altro*-epimer and not the expected *ido*-epimer. A compound eluting in the C4 region was tentatively identified as erythrose on the basis of its retention time and partial mass spectrum. However, the concentration of this compound was sufficient to see only the most intense ions of the spectrum and in the presence of other unidentified compounds its identity could not be confirmed.

Other unexpected compounds were also observed. In particular, an as yet unidentified heptulose was found to have a high incorporation of $^{13}C$ (as discussed in Chapter 4). Small amounts of xylose and either arabinose and/or lyxose were also found. Since arabinose was an expected intermediate of the alternative PCR pathway (Figure 1.3) the possibility that it could become converted by a transketolase reaction into mannoheptulose, which may be the unidentified heptulose, was investigated. Derivatized

authentic mannoheptulose, however, had a retention time intermediate between those of the unidentified heptulose and sedoheptulose and this finding excluded the above possibility. It is therefore possible that the unidentified heptulose may be either D-*ido*-heptulose or D-*talo*-heptulose and is thus stereochemically related to the D-xylose or D-lyxose, respectively, but this could not be confirmed. D-Xylose and D-lyxose showed lower $^{13}C$ incorporations than the unidentified heptulose and would therefore appear to be products rather than precursors of the unidentified heptulose. At this stage there is insufficient evidence to propose an alternative reaction scheme involving these sugars which have not previously been implicated in the path of carbon reactions.

An experiment was carried out using the leaf disc oxygen electrode in which chloroplasts were exposed to $^{13}CO_2$ for 45 seconds in the dark. This experiment showed that although many sugars were found (Table 3.4), no $^{13}C$ incorporation took place. This finding ruled out the possibility of non-photosynthetic processes giving rise to the high labelling found in the octulose and other novel sugars. In addition, blank experiments were carried out in both the photosynthetic apparatus and the leaf disc oxygen electrode in which all components of the reaction mixture were present except for the chloroplasts. The analysis of the extracts from these experiments showed that there were traces of some sugars e.g. glucose, fructose and ribose (Table 3.4) but these were not present in sufficient quantity to affect the $^{13}C$ incorporation results to any significant degree. The origin of these contaminants is not known.

**(v) Pentose resolution**

The 12.5 m HP-1 methyl silicone column chosen for the routine analyses did not provide satisfactory resolution of the pentose methyl- or ethyloxime-TMS derivatives under temperature gradient or isothermal conditions. When a 25 m HP-1 column was used with a temperature gradient

of 2 °C per minute, the resolution of the ethoxime-TMS derivatives was improved. The best resolution achieved, however, was with a DB-1701 column. With that column, the ethoxime-TMS derivatives had the retention times shown in Table 3.2, although baseline resolution was still not obtained. The resolution between compounds using GC/MS using full scan acquisitions is not as high as it is when using selected ion monitoring (SIM). The GC resolution is the same in both cases, but the effective resolution is improved using SIM acquisitions because of the greatly increased sampling frequency under these conditions. Investigations aimed at optimizing the GC conditions for the resolution of the Eox- or Mox-TMS derivatives of the pentoses and pentuloses were therefore carried out using SIM.

**Table 3.2.** Retention times of the Eox-TMS derivatives of the pentoses on the DB-1701 column.

| Sugar Eox-TMS | Retention time (Min.) |
|---|---|
| Arabinose isomer 1 (major) | 15.215 |
| Arabinose isomer 2 (minor) | 15.402 |
| Xylulose isomer 1 and 2 | 15.402 |
| Ribulose 1 | 15.585 |
| Ribulose 2 | 15.668 |
| Ribose 1 (major) | 15.866 |
| Ribose 2 (minor) | 16.085 |

Although these conditions offered better resolution for the pentoses than could be obtained on the HP-1 column, they were unable to be used because the limited amount of $^{13}C$-labelled chloroplast extract did not permit another series of acquisitions using the second column.

### (vi) Calibration of sugars

Experiments were conducted with glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate in order to assess their recovery from the dephosphorylation steps. Dephosphorylation was a necessary step for the preparation of some standards and showed that the recovery of the monophosphates was 88 % and that of the bisphosphates was 78 %.

**Table 3.3.** Sugar calibration factors

| Sugar | Intercept | Slope | Coefficient of correlation (r) |
|---|---|---|---|
| Erythrose | -0.017 | 1.068 | 0.999 |
| Ribose/Arabinose | 0.001 | 0.625 | 0.998 |
| Xylulose/Ribulose | -0.010 | 1.077 | 0.999 |
| Fructose | -0.008 | 0.305 | 0.995 |
| Glucose | -0.011 | 0.524 | 0.998 |
| Sedoheptulose | -0.013 | 0.678 | 0.998 |
| D-g D-a octulose | -0.009 | 0.318 | 0.996 |
| D-g D-i octulose | -0.005 | 0.177 | 0.992 |

**Notes:**

1. The above slope values, are the calibration factors for the sugars with respect to erythritol which was used as an internal standard. The values are the coefficients of regression for the line representing the least squares line of regression of Y on X, where Y is the measured ratio of sugar to 5 nmol of erythritol and X is the actual ratio of sugar to 5 nmol of erythritol.

2. To use the calibration factor, the measured ratio of sugar to erythritol is divided by the calibration factor to give the actual amount of sugar present.

Although these recoveries were not necessarily applicable to other phosphorylated sugars, the use of these figures was a useful the estimate of the recoveries of sugars that could not be enzymically assayed in the non-phosphorylated form. These recovery percentages were therefore used as corrections in the preparation of standard sugar solutions in section 3.4.5(iv).

The calibration factors measured for the sugars of interest in this investigation are shown in Table 3.3.

**(vii) Quantification of sugars**

The recovery of the sample was estimated by the measurement of radioisotope tracer added to the chloroplasts mounted on a membrane in the specially designed apparatus and was found to be 73%. This figure represents the minimum recovery values because the method eventually used involved the pooling of the extracts from five membranes which provides an increased "carrier effect" in the recovery of metabolites. Estimates of the recovery from the experiments conducted in the oxygen electrode were not made and the results above can only be considered to be an approximation for these experiments.

Table 3.4 shows the estimates of chloroplast metabolite concentrations made using data from the experiments reported in this investigation. The concentrations of most sugars is seen to increase with increased duration of the experiment. This may imply that a photosynthetic steady state of photosynthesis was not achieved in the chloroplasts under these conditions. Alternatively, it may be that the activity of the alkaline phosphatase added to the medium was insufficient to match the rate at which the sugar phosphates are exported from the chloroplasts thereby permitting the build up of some sugar phosphates in the external medium. The concentrations of some of the other sugars of interest, however, was low. This factor, together with the disproportionately high adsorption losses for such

**Table 3.4.** (a) Chloroplast metabolite concentrations (nmol (mg chl)$^{-1}$)

| Equipment for experiment | Apparatus | | Leaf disc oxygen electrode | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Exposure to $^{13}CO_2$ (sec) | 0 | 30 | 0 | 45 | 0 | 30 | | 45 | |
| Compound | Blank[a] | | Blank[a] | Control[b] | | No 1 | No 2 | No 1 | No 2 |
| Erythrose[c] | 0.00 | 0.00 | 0.00 | 0.00 | 0.28 | 0.14 | 0.55 | 0.79 | 0.35 |
| Xylose | 0.00 | 0.39 | 0.00 | 0.00 | 0.00 | 0.00 | 0.86 | 1.10 | 0.61 |
| Lyxose/Arabinose | 0.00 | 0.63 | 0.00 | 0.00 | 0.00 | 0.00 | 1.05 | 0.68 | 0.81 |
| Ribose/Ribulose/Xylulose | 0.20 | 5.74 | 0.21 | 1.86 | 4.70 | 2.54 | 9.79 | 14.87 | 6.61 |
| Fructose | 0.29 | 33.71 | 0.31 | 2.67 | 29.66 | 23.48 | 42.06 | 117.6 | 58.43 |
| Glucose | 0.33 | 10.55 | 0.35 | 6.71 | 8.21 | 3.60 | 15.63 | 39.38 | 16.18 |
| Unidentified heptulose | 0.00 | 0.00 | 0.00 | 0.00 | 0.29 | 0.00 | 0.85 | 1.04 | 0.84 |
| Sedoheptulose | 0.01 | 9.04 | 0.01 | 1.06 | 12.97 | 1.17 | 21.95 | 38.90 | 16.26 |
| Octulose | 0.000 | 0.17 | 0.00 | 0.28 | 0.40 | 0.00 | 1.53 | 3.53 | - e |

**Table 3.4.** (b) Chloroplast metabolite concentrations (μM)[d]

| Equipment for experiment | Apparatus | | Leaf disc oxygen electrode | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Exposure to $^{13}CO_2$ (sec) | 0 | 30 | 0 | 45 | 0 | 30 | | 45 | |
| Compound | Blank[a] | | Blank[a] | Control[b] | | No 1 | No 2 | No 1 | No 2 |
| Erythrose[c] | 0.0 | 0.0 | 0.0 | 10.6 | 0.0 | 5.4 | 21.2 | 30.5 | 13.4 |
| Xylose | 0.0 | 0.0 | 0.0 | 0.0 | 15.1 | 0.0 | 32.9 | 42.4 | 23.5 |
| Lyxose/Arabinose | 0.0 | 0.0 | 0.0 | 0.0 | 24.3 | 0.0 | 40.4 | 26.1 | 31.2 |
| Ribose/Ribulose/Xylulose | 7.5 | 8.0 | 71.4 | 180.9 | 220.7 | 97.9 | 376.6 | 571.7 | 254.4 |
| Fructose | 11.1 | 11.8 | 102.6 | 1140.9 | 1296.5 | 902.9 | 1617.8 | 4522.9 | 2247.2 |
| Glucose | 12.7 | 13.5 | 258.0 | 315.7 | 405.8 | 138.3 | 601.1 | 1514.8 | 622.3 |
| Unidentified heptulose | 0.0 | 0.0 | 0.0 | 11.3 | 0.0 | 0.0 | 32.6 | 40.0 | 32.3 |
| Sedoheptulose | 0.3 | 0.3 | 40.6 | 498.9 | 347.8 | 45.1 | 844.2 | 1495.3 | 625.4 |
| Octulose | 0.0 | 0.0 | 10.6 | 15.5 | 6.4 | 0.0 | 58.9 | 135.8 | - e |

[a], extract containing chloroplast medium without chloroplasts; [b], 45 sec exposure to $^{13}CO_2$ in the dark.
[c], tentatively identified; [d], based on the literature value for chloroplast stromal volume of 26 μL per mg chlorophyll (Schäfer et al, 1977); [e], 147 ion not monitored.

sugars during sample workup compared with the more abundant sugars has led to the need to use five times the amount of chloroplast material in the extracts than was originally planned. Consequently the concentrations of the more abundant sugars is very high and greatly exceeds the concentration range over which the sugars were calibrated. It is to be expected, therefore, that the accuracy of the measurements for the concentrations of the more abundant sugars was reduced.

# CHAPTER 4

## ANALYSIS OF PHOTOSYNTHESIS EXPERIMENTS

## Introduction

Most of the material reported in this thesis has been concerned with the development of methods which would permit the conduct of experiments in which isolated intact chloroplasts would be exposed to an atmosphere containing $^{13}CO_2$. The consequences of the time course of the photosynthetic incorporation of $^{13}C$ into sugar phosphates could then be analyzed by GC/MS. In the time available, these methods were not fully completed. The photosynthetic experiments were intended to be conducted in a specially designed apparatus (Figures 3.2 and 3.3, Chapter 3) but the recoveries from sample processing were initially low and it was unclear whether photosynthesis was taking place in the apparatus. In the course of solving this and other problems, most of the experiments had to be conducted in a leaf-disc oxygen electrode (Figure 3.1, Chapter 3) in order that active photosynthesis could be verified before the $^{13}CO_2$ incorporation experiments were carried out. Even using this apparatus, the amount of material available for GC/MS analysis from a single filter membrane containing chloroplasts, equivalent to 250 μg chlorophyll, was insufficient to allow for the significant adsorption losses which occurred during sample processing, particularly for the analysis of the less abundant sugars. It was therefore necessary for the pooled extracts from five experiments to be used before sufficient extracted material was available for analysis. However, this still proved to be inadequate to carry out a comprehensive analysis of all of the sugars of interest, e.g. it was not possible to carry out separate analyses of the pentoses as was originally planned (as the ethoxime-TMS derivatives on the more polar DB-1701 column) after the main analyses of the other sugars as the methoxime-TMS derivatives on the non-polar HP-1 column had been conducted. This also meant that only a few replicate experiments were performed. Investigations using the leaf-disc oxygen electrode rather than the specially designed apparatus reduced the degree of $^{13}C$ enrichment of the $^{13}CO_2$ in the

atmosphere surrounding the chloroplasts because the geometry of the leaf-disc oxygen electrode chamber was less satisfactory for the exchange of the $CO_2$ gas. Thus the results of the experiments reported in this thesis are considered to be preliminary. They have allowed the identification of the limitations of the methods and of the size of a new apparatus as well as the manner in which a more definitive set of experiments could be carried out. There are, nevertheless, a number of positive conclusions that can be drawn from the experiments carried out and these are discussed later in this chapter.

The GC/MS criteria against which this investigation was conducted have been stated previously and are given here in summary form, together with some additional criteria which relate to the accuracy of measurements and the physiological significance of the sugars and carbons studied.

1. The sugars of particular interest are those indicated in Figures 1.5 and 1.6 (Chapter 1).

2. The carbons of interest in these sugars are those not influenced by exchange reactions, as shown in Figures 1.5 and 1.6 and identified by the letters a to d and e to i respectively.

3. For a reliable determination of the $^{13}C$ enrichment in these carbons as a result of photosynthetic $^{13}CO_2$ incorporation, those ions which have been shown by $^{13}C$ labelling studies to arise almost exclusively (>95%) from a single origin are the most useful (Chapter 2, Table 2.4). However in some instances ions of less than 95% "purity" were used when it was necessary to obtain information about critical carbons e.g. C4 of fructose using the ion at *m/z* 263. In addition, in the absence of data from $^{13}C$-labelled, isotopomers some ions in particular sugars were assumed to have a single origin e.g. *m/z* 307 in sedoheptulose, on the basis of results of studies with closely related sugars (fructose and octulose) and these were used in the enrichment

**Table 4.1** Ions found to have a single origin in key PCR pathway intermediates.

| m/z | Calvin branch | | | | Alternative branch | | |
|---|---|---|---|---|---|---|---|
| | Fructose | Erythrose | Sedohept-ulose | Ribose | Glucose | Octulose * | Arabinose |
| 160 | | | | yes | yes | | yes |
| 161 | | yes | | | | | |
| 205 | | yes | yes | | yes | yes | |
| 259 | | | | | | yes | |
| 262 | | yes | yes | | | yes | |
| 263 | yes | | | | | | |
| 307 | yes | | assumed | assumed | | yes | assumed |
| 319 | | | yes | | yes | yes | |
| 344 | | | | | | yes | |
| 350 | | yes | | | | | |
| 364 | yes | | assumed | | yes | yes | |
| 376 | | | assumed | | | yes | |
| 421 | | | | | | yes | |
| 466 | | | assumed | | | yes | |
| 568 | | | | | | yes | |

* Irvine, 1992.

calculations. The data of Table 2.4 are summarized below in Table 4.1 and incorporate these assumptions.

4. The ions of interest should be of sufficient intensity to minimize interference from background ions which arise mainly from column bleed e.g. *m/z* 207. When ions are of low intensity, background ions can be subtracted, but it may also happen that the passage of the analyte through the GC column can carry with it an increase in the amount of column bleed. Therefore background correction does not always fully correct for this interference. When the ions to be monitored are small, the sample should be concentrated where possible to help reduce errors that background ions may introduce.

5. Where possible, the ions to be monitored should be free from interference from neighbouring ions in the mass spectrum i.e. those within one or two mass units. This can be corrected for when the origin and "purity" of the neighbouring ion(s) are the same as that of the ion(s) being monitored, but can seriously reduce the usefulness of such ions if this is not the case.

## Results and Discussion

The $^{13}C$ enrichments of the sugar fragment ions monitored for four experiments conducted in the leaf disc oxygen electrode are shown in Tables 4.2 to 4.5. The results for two experiments conducted in the photosynthesis apparatus are shown in Tables 4.6 to 4.7. The results of the "six second" experiment in the photosynthesis apparatus are derived from the extract of a single filter membrane whilst the results of all other experiments are from the pooled extracts from five membranes. Using data on the $^{13}C$ enrichments of the fragment ions listed in Tables 4.2 to 4.7, the $^{13}C$ enrichments for many of the individual carbons can be calculated. Appendix 1 shows how individual carbon $^{13}C$ enrichments can be derived from ion enrichment data such as that in Tables 4.2 to 4.7.

**Table 4.2.** Enrichment results[a] for 45 second experiment No 1 in the leaf disc oxygen electrode.

| Sugar | *m/z* | Carbons | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ | $^{13}C_3$ | $^{13}C_4$ | $^{13}C_5$ |
|---|---|---|---|---|---|---|---|---|
| Erythrose | 205 | C3-4 | 94.2 | 3.9 | 1.9 | | | |
| | 262 | C1-3 | 85.7 | 9.1 | 4.3 | 1.0 | | |
| | 365 | C1-4 | 74.1 | 13.7 | 9.8 | 2.4 | | |
| Xylose | 205 | C4-5 | 73.5 | 21.9 | 4.6 | | | |
| | 307 | C3-5 | 66.6 | 23.2 | 7.8 | 2.3 | | |
| Lyxose/ | 205 | C4-5 | 91.2 | 8.8 | | | | |
| Arabinose[b] | 307 | C3-5 | 65.6 | 24.0 | 8.2 | 2.2 | | |
| Xylulose | 263 | C1-3 | 58.1 | 29.1 | 10.2 | 2.5 | | |
| | 364 | C1-4 | 57.5 | 27.4 | 11.3 | 3.8 | 0.0 | |
| Ribose/ | 205 | C4-5 | 84.4 | 9.4 | 6.2 | | | |
| Ribulose[c] | 307 | C3-5 | 70.9 | 20.8 | 6.7 | 1.6 | | |
| Fructose | 205 | C5-6 | 89.4 | 10.6 | | | | |
| | 307 | C4-6 | 71.1 | 19.2 | 7.7 | 2.0 | | |
| Glucose | 160 | C1-2 | 72.0 | 21.2 | 6.8 | | | |
| | 205 | C5-6 | 75.6 | 16.4 | 8.0 | | | |
| | 319 | C3-6 | 35.1 | 28.9 | 24.2 | 9.4 | 2.4 | |
| | 364 | C1-4 | 31.7 | 34.9 | 22.4 | 8.7 | 2.3 | |
| Unidentified | 205 | C6-7 | 74.2 | 21.0 | 4.8 | | | |
| heptulose | 319 | C4-7 | 26.4 | 34.3 | 26.8 | 9.8 | 2.6 | |
| Sedo- | 205 | C6-7 | 72.7 | 20.4 | 6.9 | | | |
| heptulose | 262 | C1-3 | 37.4 | 36.0 | 18.9 | 7.7 | | |
| | 319 | C4-7 | 26.1 | 32.9 | 26.6 | 11.4 | 2.9 | |
| | 364 | C1-4 | 34.1 | 34.4 | 21.4 | 8.1 | 1.9 | |
| | 376 | C1-5 | 19.9 | 27.0 | 27.4 | 17.7 | 8.0 | |
| | 466 | C1-5 | 18.2 | 28.1 | 29.3 | 17.8 | 5.7 | 0.9 |
| Octulose | 205 | C7-8 | 83.6 | 15.8 | 0.7 | | | |
| | 262 | C1-3 | 60.9 | 27.4 | 8.0 | 3.7 | | |
| | 319 | C5-8 | 47.2 | 30.1 | 16.7 | 4.7 | 1.3 | |
| | 331 | C4-8 | 36.6 | 25.2 | 20.7 | 10.9 | 4.5 | 2.1 |
| | 466 | C1-5 | 44.8 | 28.0 | 18.9 | 7.4 | 0.9 | 0.0 |

[a] After correction for natural abundance of $^{2}H$, $^{13}C$, $^{18}O$, $^{29,30}Si$, $^{15}N$ and any contributions from neighbouring ions. Results represent an average of at least three consecutive runs. Standard deviations for most sugars were less than 1% and for the less abundant sugars (erythrose, xylose, arabinose/lyxose, octulose and unidentified heptulose) standard deviations were less than 2%. When significantly different enrichments were measured for *syn* and *anti* methoxime isomers it was assumed that some interference was present causing the higher result and the one showing the lower enrichment was used; otherwise both *syn* and *anti* isomers were used in the calculations.

[b] Lyxose/Arabinose refers to either one or both of the sugars which could not be distinguished with the capillary GC column used for these analyses.

[c] Ribose/Ribulose refers to both sugars, which, although they were not resolved on the capillary GC column used in these analyses, were both shown to be present from their characteristic mass spectra.

**Table 4.3.** Enrichment results[a] for 45 second experiment No 2 in the leaf disc oxygen electrode.

| Sugar | m/z | Carbons | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ | $^{13}C_3$ | $^{13}C_4$ | $^{13}C_5$ |
|---|---|---|---|---|---|---|---|---|
| Xylose | 160 | C1-2 | 69.3 | 25.1 | 5.6 | | | |
| | 205 | C4-5 | 79.2 | 16.1 | 4.8 | | | |
| | 262 | C1-3 | 56.8 | 29.3 | 9.2 | 4.5 | | |
| | 307 | C3-5 | 68.3 | 24.0 | 7.1 | 0.5 | | |
| | 452 | C1-5 | 56.6 | 27.7 | 12.8 | 2.3 | 0.4 | |
| Lyxose/ Arabinose[b] | 160 | C1-2 | 68.1 | 26.1 | 5.8 | | | |
| | 205 | C4-5 | 92.2 | 7.8 | | | | |
| | 262 | C1-3 | 59.6 | 33.0 | 7.4 | | | |
| | 307 | C3-5 | 70.4 | 20.9 | 7.9 | 0.8 | | |
| Xylulose | 205 | C4-5 | 92.0 | 6.0 | 2.0 | | | |
| | 263 | C1-3 | 60.7 | 29.4 | 8.5 | 1.4 | | |
| | 307 | C3-5 | 81.6 | 16.1 | 2.3 | 0.0 | | |
| | 319 | C2-5 | 76.0 | 23.2 | 0.5 | 0.3 | | |
| | 364 | C1-4 | 59.8 | 28.4 | 9.5 | 2.3 | 0.0 | |
| | 421 | C1-5 | 52.4 | 31.4 | 11.4 | 3.9 | 0.7 | 0.2 |
| Ribose/ Ribulose[c] | 160 | C1-2 | 69.6 | 25.0 | 5.4 | | | |
| | 205 | C4-5 | 86.6 | 9.0 | 4.4 | | | |
| | 262 | C1-3 | 64.9 | 25.4 | 8.4 | 1.3 | | |
| | 307 | C3-5 | 72.8 | 19.5 | 6.5 | 1.1 | | |
| | 319 | C2-5 | 59.7 | 28.6 | 8.8 | 2.5 | 0.4 | |
| | 364 | C1-4 | 68.7 | 22.3 | 6.7 | 2.1 | 0.1 | |
| | 421 | C1-5 | 58.7 | 25.7 | 10.4 | 3.6 | 1.1 | 0.5 |
| Fructose | 263 | C1-3 | 70.4 | 19.4 | 8.8 | 1.4 | | |
| | 307 | C4-6 | 75.6 | 18.2 | 5.5 | 0.8 | | |
| | 319 | C3-6 | 68.1 | 22.9 | 6.7 | 1.8 | 0.5 | |
| | 364 | C1-4 | 59.8 | 26.5 | 10.7 | 2.7 | 0.4 | |
| Glucose | 160 | C1-2 | 75.6 | 19.7 | 4.7 | | | |
| | 205 | C5-6 | 80.0 | 15.7 | 4.4 | | | |
| | 307 | C4-6 | 68.3 | 24.1 | 6.0 | 1.6 | | |
| | 319 | C3-6 | 45.4 | 28.6 | 18.8 | 6.1 | 1.1 | |
| | 364 | C1-4 | 43.4 | 34.0 | 16.6 | 5.2 | 0.8 | |
| Unidentified heptulose | 307 | C5-7 | 64.9 | 25.7 | 7.6 | 1.8 | | |
| | 319 | C4-7 | 34.0 | 34.9 | 22.4 | 7.4 | 1.3 | |
| | 421 | C3-7 | 32.3 | 32.6 | 21.8 | 9.8 | 3.3 | 0.2 |
| Sedo- heptulose | 205 | C6-7 | 75.1 | 20.5 | 4.4 | | | |
| | 262 | C1-3 | 48.3 | 34.6 | 12.6 | 4.5 | | |
| | 307 | C5-7 | 59.3 | 29.1 | 9.1 | 2.5 | | |
| | 319 | C4-7 | 33.3 | 34.1 | 22.8 | 8.4 | 1.4 | |
| | 364 | C1-4 | 39.4 | 36.3 | 17.8 | 5.5 | 1.0 | |
| | 421 | C3-7 | 28.2 | 30.7 | 22.8 | 12.5 | 4.9 | 0.8 |
| | 466 | C1-5 | 25.6 | 32.5 | 26.1 | 12.0 | 3.3 | 0.5 |
| Octulose | 205 | C7-8 | 81.5 | 16.2 | 2.3 | | | |
| | 307 | C6-8 | 66.2 | 25.8 | 6.7 | 1.4 | | |
| | 319 | C5-8 | 50.9 | 31.2 | 14.0 | 3.5 | 0.3 | |
| | 331 | C4-8 | 39.8 | 27.7 | 18.6 | 8.5 | 3.7 | 1.6 |
| | 421 | C4-8 | 45.4 | 29.7 | 16.9 | 6.4 | 1.5 | 0.1 |

[a] For explanation of footnotes a,b,c, see Table 4.2.

**Table 4.4.** Enrichment results[a] for 30 second experiment No 1 in the leaf disc oxygen electrode.

| Sugar | m/z | Carbons | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ | $^{13}C_3$ | $^{13}C_4$ | $^{13}C_5$ |
|---|---|---|---|---|---|---|---|---|
| Ribose/ | 205 | C4-5 | 89.4 | 10.6 | | | | |
| Ribulose[c] | 307 | C3-5 | 87.0 | 9.7 | 3.0 | 0.3 | | |
| Fructose | 205 | C5-6 | 90.6 | 9.4 | | | | |
| | 263 | C1-3 | 70.9 | 19.6 | 8.0 | 1.4 | | |
| | 307 | C4-6 | 69.5 | 21.2 | 7.8 | 1.5 | | |
| | 364 | C1-4 | 56.5 | 29.4 | 10.9 | 3.0 | 0.3 | |
| Glucose | 160 | C1-2 | 82.1 | 14.5 | 3.4 | | | |
| | 205 | C5-6 | 85.7 | 10.5 | 3.8 | | | |
| | 319 | C3-6 | 62.4 | 19.9 | 12.7 | 4.1 | 0.7 | |
| | 364 | C1-4 | 57.6 | 28.7 | 9.6 | 3.4 | 0.7 | |
| Sedoheptulose | 205 | C6-7 | 78.2 | 18.1 | 3.8 | | | |
| | 319 | C4-7 | 36.4 | 33.3 | 21.5 | 7.5 | 1.4 | |

[a] For explanation of footnotes a, c see Table 4.2.

**Table 4.5.** Enrichment results[a] for 30 second experiment No 2 in the leaf disc oxygen electrode.

| Sugar | m/z | Carbons | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ | $^{13}C_3$ | $^{13}C_4$ | $^{13}C_5$ |
|---|---|---|---|---|---|---|---|---|
| Xylose | 160 | C1-2 | 72.3 | 22.3 | 5.3 | | | |
| | 205 | C4-5 | 89.6 | 7.5 | 2.9 | | | |
| | 262 | C1-3 | 58.7 | 29.9 | 6.8 | 4.2 | 0.4 | |
| | 307 | C3-5 | 72.7 | 21.3 | 4.8 | 1.2 | | |
| | 364 | C1-4 | 86.5 | 10.5 | 2.4 | 0.6 | 0.0 | |
| | 452 | C1-5 | 63.6 | 24.6 | 9.2 | 1.4 | 1.2 | |
| Lyxose | 205 | C4-5 | 88.7 | 6.7 | 4.5 | | | |
| | 307 | C3-5 | 71.6 | 20.5 | 6.6 | 1.2 | | |
| Ribose/ | 160 | C1-2 | 69.8 | 24.5 | 5.7 | | | |
| Ribulose[c] | 205 | C4-5 | 89.1 | 6.8 | 4.1 | | | |
| | 263 | C1-3 | 62.0 | 26.4 | 9.7 | 2.0 | | |
| | 307 | C3-5 | 75.3 | 18.5 | 5.0 | 1.3 | | |
| Fructose | 205 | C5-6 | 91.9 | 8.1 | | | | |
| | 307 | C4-6 | 71.5 | 19.7 | 7.3 | 1.6 | | |
| Glucose | 205 | C5-6 | 80.2 | 13.1 | 6.7 | | | |
| | 319 | C3-6 | 41.5 | 29.3 | 21.5 | 6.5 | 1.3 | |
| Sedoheptulose | 205 | C6-7 | 77.5 | 18.8 | 3.8 | | | |
| | 262 | C1-3 | 46.0 | 34.7 | 13.6 | 5.8 | | |
| | 319 | C4-7 | 28.1 | 35.1 | 25.8 | 9.1 | 1.9 | |
| | 364 | C1-4 | 37.0 | 36.4 | 19.6 | 5.8 | 1.2 | |
| | 376 | C1-5 | 20.3 | 26.3 | 24.2 | 18.8 | 10.4 | |
| | 466 | C1-5 | 21.4 | 31.9 | 29.3 | 13.7 | 3.2 | 0.5 |
| Octulose | 205 | C7-8 | 87.4 | 12.6 | 0.0 | | | |
| | 262 | C1-3 | 68.8 | 23.6 | 5.3 | 2.4 | | |
| | 319 | C5-8 | 55.1 | 28.8 | 12.9 | 3.1 | 0.1 | |
| | 331 | C4-8 | 45.5 | 24.4 | 17.1 | 8.1 | 3.3 | 1.6 |
| | 466 | C1-5 | 54.5 | 26.7 | 13.4 | 4.5 | 0.7 | 0.1 |

[a] For explanation of footnotes a,b,c, see Table 4.2.

**Table 4.6.** Enrichment results[a] for 30 second experiment No 1 in the photosynthesis apparatus.

| Sugar | m/z | Carbons | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ | $^{13}C_3$ | $^{13}C_4$ | $^{13}C_5$ |
|---|---|---|---|---|---|---|---|---|
| Fructose | 205 | C5-6 | 100.0 | 0.0 | | | | |
| | 263 | C1-3 | 99.4 | 0.6 | | | | |
| | 307 | C4-6 | 93.2 | 4.9 | 2.0 | 0.0 | | |
| | 364 | C1-4 | 94.6 | 4.7 | 0.7 | 0.0 | 0.0 | |
| Glucose | 160 | C1-2 | 93.8 | 6.1 | 0.1 | | | |
| | 205 | C5-6 | 100.0 | 0.0 | | | | |
| | 319 | C3-6 | 98.7 | 0.8 | 0.4 | 0.0 | 0.0 | |
| | 364 | C1-4 | 85.2 | 14.8 | 0.0 | 0.0 | 0.0 | |
| Sedoheptulose | 205 | C6-7 | 100.0 | 0.0 | | | | |
| | 319 | C4-7 | 95.4 | 3.7 | 0.9 | 0.0 | 0.0 | |

[a] For explanation see Table 4.2.

**Table 4.7.** Enrichment results[a] for 30 second experiment No 2 in the photosynthesis apparatus

| Sugar | m/z | Carbons | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ | $^{13}C_3$ | $^{13}C_4$ | $^{13}C_5$ |
|---|---|---|---|---|---|---|---|---|
| Xylose | 205 | C4-5 | 97.4 | 2.6 | | | | |
| | 307 | C3-5 | 89.5 | 8.2 | 1.7 | 0.6 | | |
| Lyxose/ | 205 | C4-5 | 97.4 | 2.6 | | | | |
| Arabinose[b] | 307 | C3-5 | 89.0 | 8.2 | 2.2 | 0.5 | | |
| Xylulose | 263 | C1-3 | 83.8 | 12.3 | 3.1 | 0.8 | | |
| | 364 | C1-4 | 86.4 | 10.1 | 2.6 | 0.8 | 0.2 | |
| Ribose/ | 307 | C3-5 | 91.2 | 6.8 | 1.8 | 0.3 | | |
| Ribulose[c] | 452 | C1-5 | 90.8 | 6.0 | 1.9 | 1.3 | 0.0 | |
| Fructose | 205 | C5-6 | 98.2 | 1.8 | | | | |
| | 263 | C1-3 | 89.1 | 6.0 | 4.9 | | | |
| | 307 | C4-6 | 91.0 | 6.7 | 1.9 | 0.3 | | |
| | 364 | C1-4 | 83.0 | 13.2 | 3.0 | 0.7 | 0.2 | |
| Glucose | 160 | C1-2 | 91.3 | 7.9 | 0.8 | | | |
| | 205 | C5-6 | 95.4 | 4.6 | | | | |
| | 307 | C4-6 | 93.9 | 6.0 | 0.1 | | | |
| | 319 | C3-6 | 83.3 | 10.6 | 5.0 | 0.9 | 0.2 | |
| | 364 | C1-4 | 67.3 | 27.4 | 4.0 | 1.1 | 0.3 | |
| Unidentified | 307 | C5-7 | 84.2 | 15.1 | 0.7 | | | |
| heptulose | 319 | C4-7 | 62.4 | 20.8 | 11.9 | 3.7 | 1.2 | |
| | 421 | C3-7 | 61.8 | 18.1 | 10.3 | 8.0 | 1.8 | 0.1 |
| Sedoheptulose | 205 | C6-7 | 90.1 | 7.9 | 2.0 | | | |
| | 262 | C1-3 | 68.3 | 21.7 | 5.5 | 4.4 | | |
| | 307 | C5-7 | 77.4 | 17.7 | 3.2 | 1.7 | | |
| | 319 | C4-7 | 61.8 | 20.8 | 12.1 | 4.1 | 1.2 | |
| | 364 | C1-4 | 64.9 | 21.5 | 9.8 | 2.9 | 0.9 | |
| | 376 | C1-5 | 55.8 | 21.5 | 12.7 | 7.7 | 2.4 | 2.0 |
| | 364 | C1-4 | 64.9 | 21.5 | 9.8 | 2.9 | 0.9 | |
| | 466 | C1-5 | 56.8 | 22.1 | 13.1 | 5.4 | 1.6 | 1.0 |
| Octulose | 262 | C1-3 | 86.2 | 11.6 | 2.3 | 0.0 | | |
| | 307 | C6-8 | 87.6 | 11.6 | 0.6 | 0.3 | | |
| | 319 | C5-8 | 77.2 | 17.2 | 4.4 | 1.2 | 0.1 | |
| | 331 | C4-8 | 65.7 | 15.7 | 11.1 | 4.3 | 1.9 | 1.4 |
| | 421 | C4-8 | 73.3 | 17.4 | 6.3 | 2.3 | 0.7 | 0.1 |
| | 466 | C1-5 | 78.5 | 14.1 | 7.4 | | | |

[a] For explanation of footnotes a,b,c, see Table 4.2.

It was not possible to determine the $^{13}C$ enrichments for all individual carbons. This was due to either the absence of a suitable ion, or because the ion when present was not sufficiently intense, or because of interference from neighbouring ions within one or two mass units of the ion of interest. Figures 4.1 to 4.6 show data for the enrichments calculated for individual carbons or groups of two or three adjacent carbons of the sugars of principal interest for each of the photosynthesis experiments reported.

In some instances it was possible to arrive at an enrichment value for a particular carbon atom or group of carbon atoms in two different ways. When this was carried out, there was often reasonable agreement between the two values obtained. For example, in calculating the $^{13}C$ enrichment of the g carbon (C3) for arabinose and/or lyxose in Figure 4.2 from the *m/z* 262 (C1-3) minus the *m/z* 160 (C1-2) ion using the data in Table 4.3 the resulting enrichment was 17.5 %. The enrichment of the same carbon atom calculated from *m/z* 307 (C3-5) minus *m/z* 205 (C4-5) gave a value of 14.5 %. In such cases the two values were averaged to obtain the value of 16 % as shown in Figure 4.2. On other occasions the two values obtained were not as close, as for the b carbon (C5) of sedoheptulose in the same figure, in which *m/z* 307 (C5-7) minus *m/z* 205 (C6-7) gave 17.8 % and *m/z* 466 (C1-5) minus *m/z* 364 (C1-4) gave 25.8 %. In these cases the two values obtained were not averaged but both results are provided. The *m/z* 466 and *m/z* 364 ions of sedoheptulose are both very small (less than 0.5% of the base ion intensity) and may therefore provide less reliable results; on the other hand the *m/z* 307 ion is also small and suffers to an unknown degree from interference from the *m/z* 305 ion which may also introduce an error into the calculation. In the absence of corroborating evidence for either value both results are provided.

The number of ions which can be monitored in these experiments was limited by the data system. Where more than one ion in the mass spectrum arose from the same set of carbon atoms but differed by 90 mass units (loss of TMSOH), the one that gave the most reliable results was

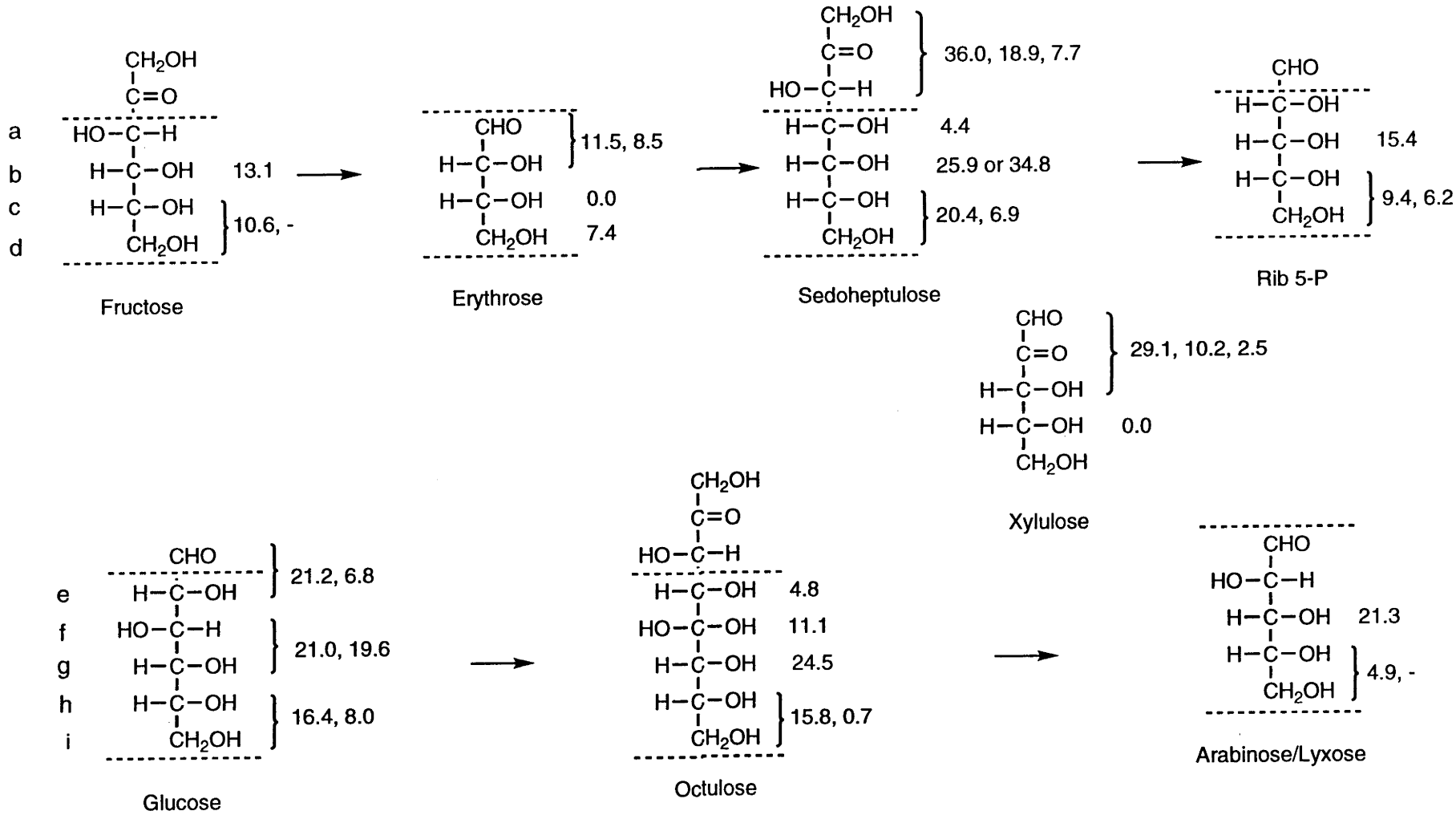


**Figure 4.1** Enrichment results for the 45 sec experiment no 1 in the leaf disc oxygen electrode.

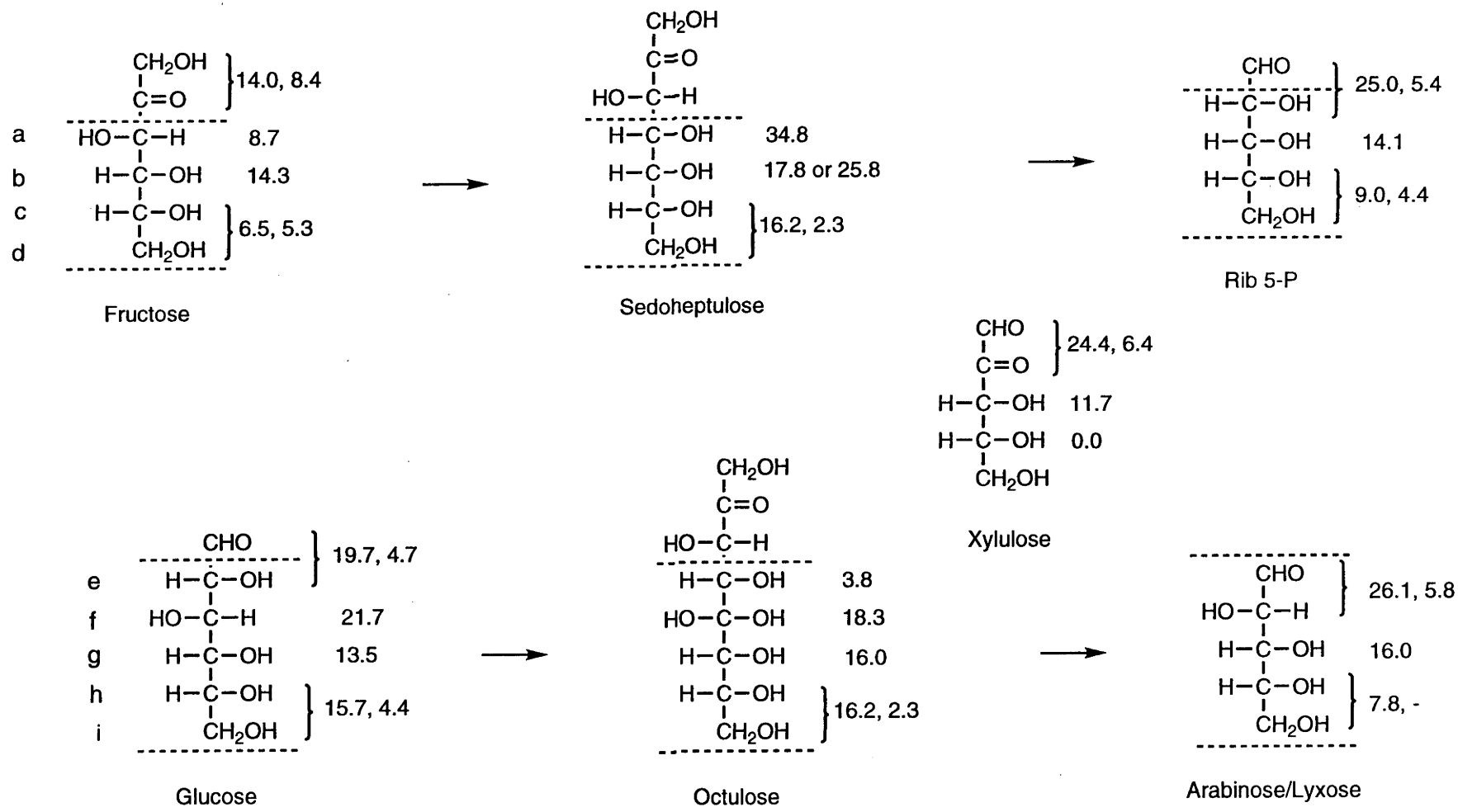


**Figure 4.2** Enrichment results for 45 sec experiment no 2 in the leaf disc oxygen electrode.

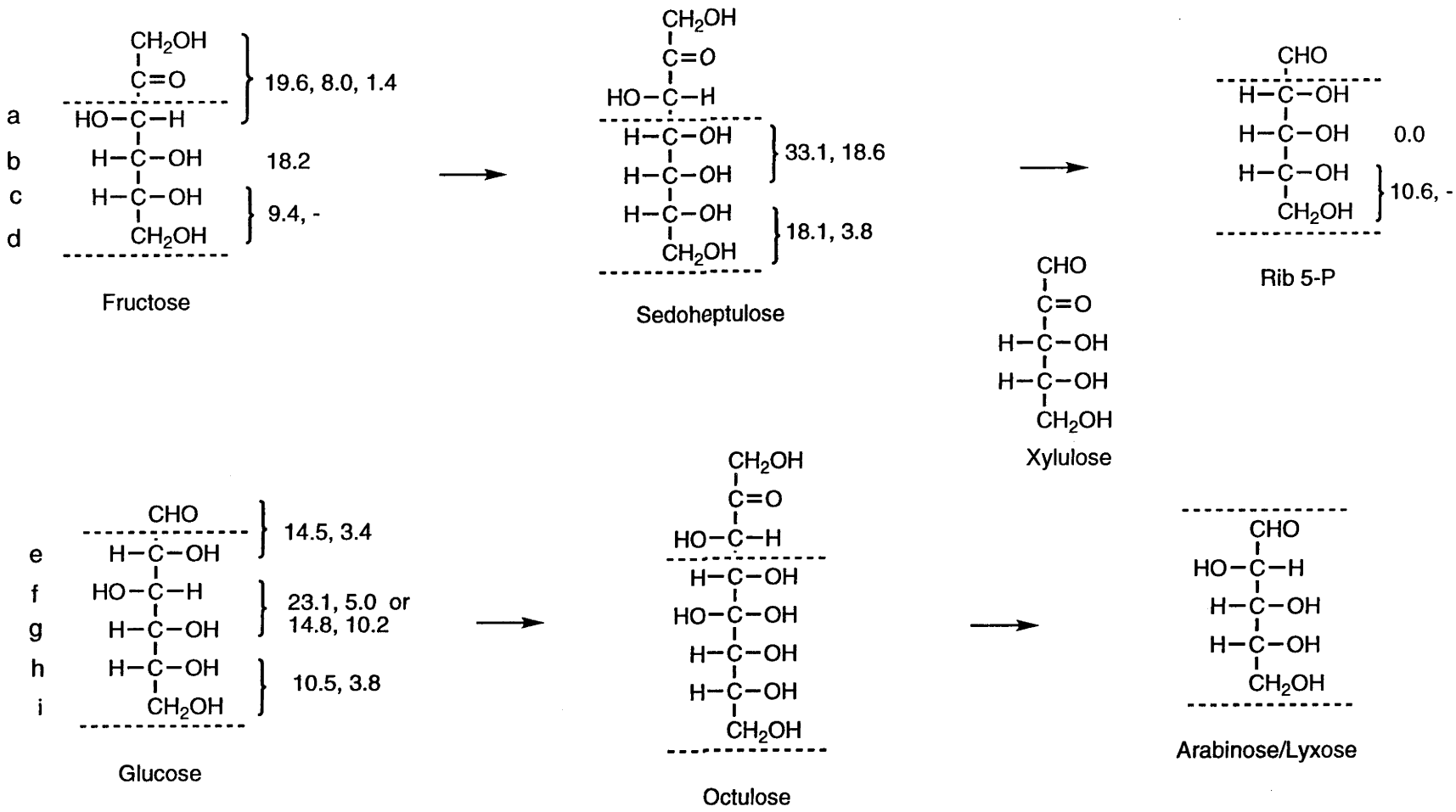


**Figure 4.3** Enrichment results for 30 second experiment no 1 in the leaf disc oxygen electrode.

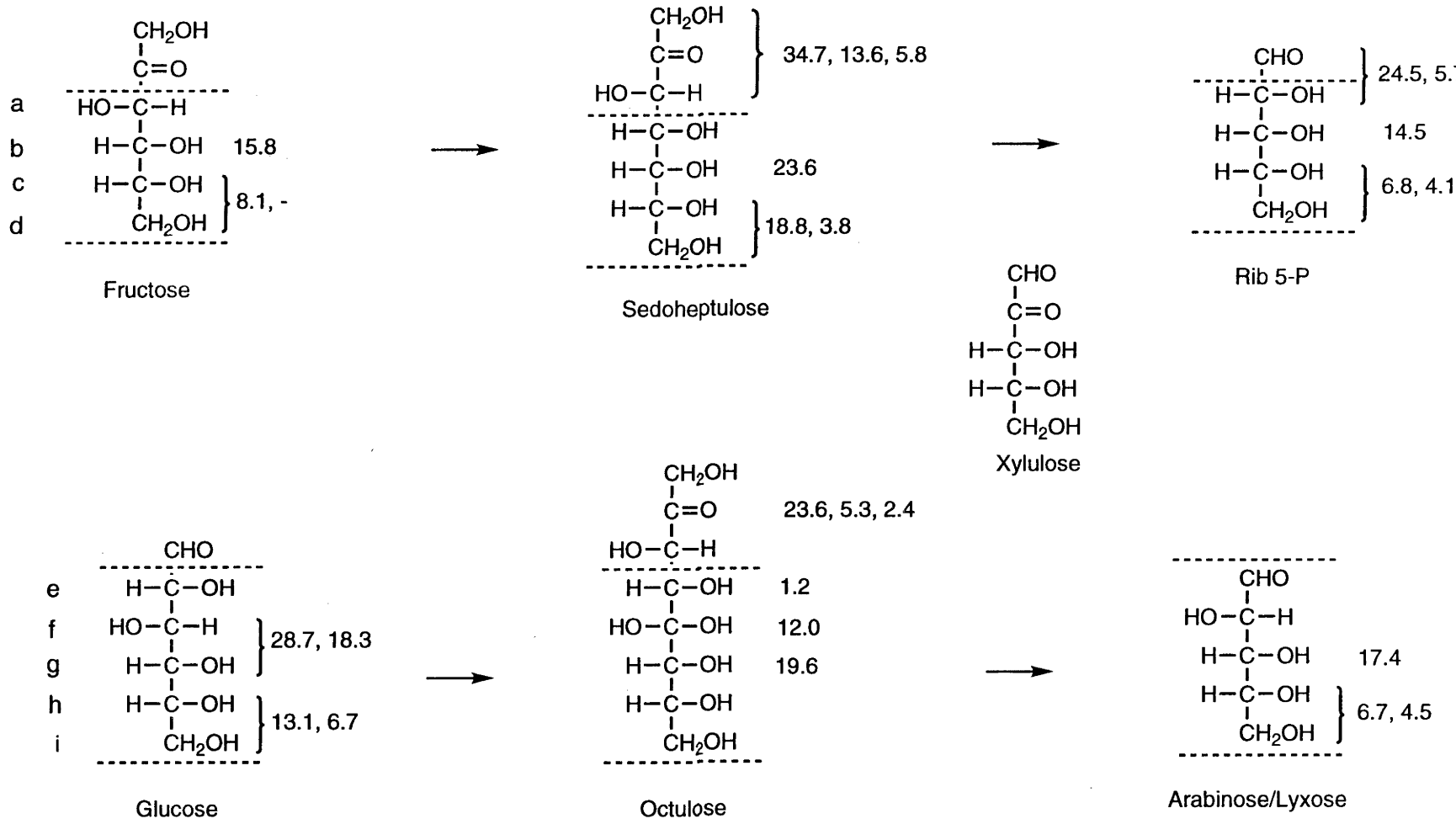


**Figure 4.4** Enrichment results for the 30 sec experiment no 2 in the leaf disc oxygen electrode

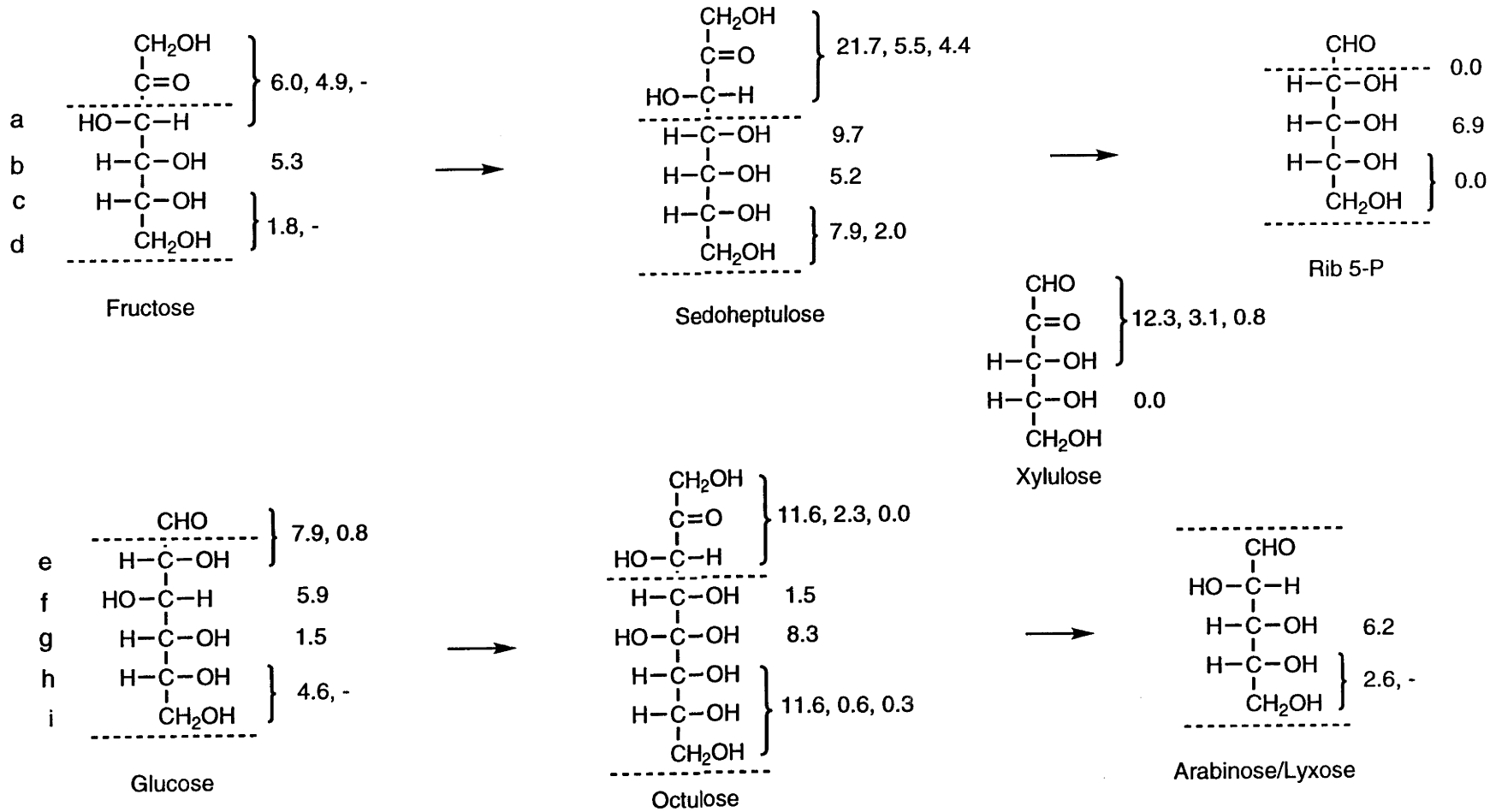


**Figure 4.5** Enrichment results for the 30 sec experiment in the photosynthesis apparatus

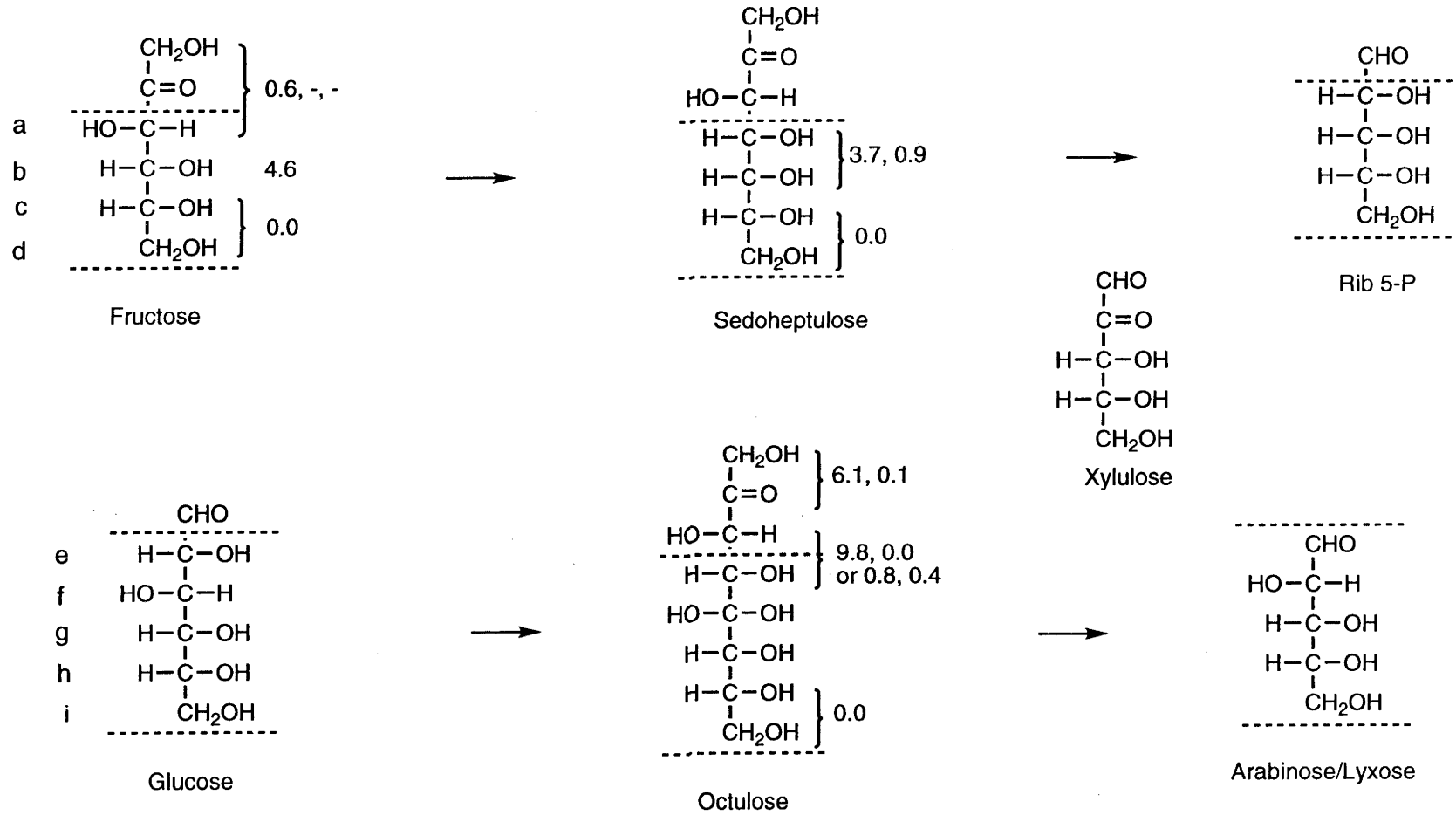


**Figure 4.6** Enrichment results for the 6 sec experiment in the photosynthesis apparatus

selected in subsequent experiments. It was established, for example, that the *m/z* 331 ion of octulose representing C4-8, sometimes but not always, suffered from interference from a *m/z* 333 ion and it proved preferable to monitor the *m/z* 421 ion, even though it was less intense than *m/z* 331, because it was free of such interference and represented the same set of carbons.

For an analysis of the results of the $^{13}CO_2$ incorporation experiments it is of primary interest to discover into which compounds the label is most heavily incorporated. A simple ranking of compounds is therefore provided in the tables below for the carbons for which individual enrichments can be calculated. The values refer to enrichments in carbons that are common to all of the sugars i.e. carbons a and f or carbons b and g in Figures 4.1 to 4.6. Reference to Figures 1.1 and 1.3 (Chapter 1) also indicates that these carbons are, of the carbons unaffected by exchange reactions, the ones which are expected to incorporate label most heavily. The sugars in the tables below have been placed in decreasing order of enrichment as measured by the percentage of unlabelled ion present. Thus in Table 4.8 octulose is seen to have the higher level of $^{13}C$ incorporation in the specified carbon atom.

**Individual carbon enrichments calculated for sugars from 45 second experiment No 1 in the leaf disc oxygen electrode:**

For the first of the 45 second experiments in the leaf disc oxygen electrode the data are not available for all sugars. For those sugars for which data are available, the results for the a and f carbons are shown in Table 4.8 and the results for the b and g carbons are shown in Table 4.9.

**Table 4.8.** Enrichments measured for the b and g carbons of sugars

| Sugar | $^{13}C_0$ | $^{13}C_1$ |
|---|---|---|
| Octulose | 88.9 | 11.1 |
| Sedoheptulose | 95.6 | 4.4 |

**Table 4.9.** Enrichments measured for the b and g carbons of sugars.

| Sugar | $^{13}C_0$ | $^{13}C_1$ |
|---|---|---|
| Sedoheptulose | 65.2 | 34.8 |
| Sedoheptulose | 74.1 | 25.9 |
| Octulose | 74.7 | 25.3 |
| Lyxose/arabinose | 78.7 | 21.3 |
| Ribose/Ribulose | 84.6 | 15.4 |
| Fructose | 86.9 | 13.1 |
| Xylose | 95.1 | 4.9 |

**Individual carbon enrichments calculated for sugars from 45 second experiment No 2 in the leaf disc oxygen electrode:**

For the second and more meaningful of the 45 second experiments in the leaf disc oxygen electrode the results for the a and f carbons are shown in Table 4.10 and the results for the b and g carbons are shown in Table 4.11.

**Table 4.10.** Enrichments measured for the a and f carbons of sugars in 45 second experiment no. 2

| Sugar | $^{13}C_0$ | $^{13}C_1$ |
|---|---|---|
| Unidentified heptulose | 61.3 | 38.7 |
| Sedoheptulose | 65.2 | 34.8 |
| Glucose | 78.3 | 21.7 |
| Octulose | 81.7 | 18.3 |
| Fructose | 91.3 | 8.7 |

**Table 4.11.** Enrichments measured for the b and g carbons of sugars 45 second experiment no 2.

| Sugar | $^{13}C_0$ | $^{13}C_1$ |
|---|---|---|
| Sedoheptulose | 74.2 | 25.8 |
| Sedoheptulose | 82.2 | 17.8 |
| Octulose | 84.0 | 16.0 |
| Lyxose/Arabinose | 84.0 | 16.0 |
| Fructose | 85.7 | 14.3 |
| Ribose/Ribulose | 85.9 | 14.1 |
| Glucose | 86.5 | 13.5 |
| Xylulose | 88.3 | 11.7 |

**Individual carbon enrichments calculated for sugars from 30 second experiments in the leaf disc oxygen electrode:**

For the first of the 30 second experiments in the leaf disc oxygen electrode there were no individual carbon enrichments which could be compared in different sugars. For the second of the 30 second experiments in the leaf disc oxygen electrode only the enrichments for the b and g carbons could be calculated and these are shown in Table 4.12.

**Table 4.12.** Enrichments measured for the b and g carbons of sugars in the second 30 second experiment in the leaf disc oxygen electrode.

| Sugar | $^{13}C_0$ | $^{13}C_1$ |
|---|---|---|
| **Sedoheptulose *** | 66.5 | 33.5 |
| **Sedoheptulose *** | 76.4 | 23.6 |
| **Octulose** | 80.4 | 19.6 |
| **Lyxose/Arabinose** | 82.6 | 17.4 |
| **Xylose** | 83.0 | 17.0 |
| **Fructose** | 84.2 | 15.8 |
| **Ribose/Ribulose** | 85.7 | 14.3 |

* The enrichment for sedoheptulose can be calculated in two different ways

**Individual carbon enrichments calculated for sugars from 30 second experiments in the photosynthesis apparatus:**

Enrichments for the 30 second experiment conducted using the specially constructed photosynthesis apparatus were lower than for the corresponding experiments conducted using the leaf disc oxygen electrode.

**Table 4.13.** Enrichments measured for the a and f carbons of sugars in the 30 second experiment in the photosynthesis apparatus.

| Sugar | $^{13}C_0$ | $^{13}C_1$ |
|---|---|---|
| **Unidentified heptulose** | 86.7 | 13.3 |
| **Sedoheptulose *** | 90.3 | 9.7 |
| **Octulose** | 91.7 | 8.3 |
| **Glucose** | 94.1 | 5.9 |
| **Sedoheptulose *** | 98.8 | 1.2 |

* The enrichment for sedoheptulose can be calculated in two different ways

**Table 4.14.** Enrichments measured for the b and g carbons of sugars in the 30 second experiment in the photosynthesis apparatus.

| Sugar | $^{13}C_0$ | $^{13}C_1$ |
|---|---|---|
| **Ribose/Ribulose** | 93.1 | 6.9 |
| **Lyxose/Arabinose** | 93.8 | 6.2 |
| **Xylose** | 93.9 | 6.1 |
| **Fructose** | 94.7 | 5.3 |
| **Sedoheptulose** | 94.8 | 5.2 |
| **Glucose** | 98.5 | 1.5 |

For the 6 second experiment in the photosynthesis apparatus there were no individual carbon enrichments which could be compared in different sugars.

The most striking feature of the above tables is that sedoheptulose, octulose and the unidentified heptulose are the sugars which have most heavily incorporated label into the two carbon atoms examined. Only for experiments conducted in the photosynthesis apparatus (Tables 4.13 and 4.14) was sedoheptulose not amongst the most heavily labelled. It is surprising to find that apart from sedoheptulose, the sugars of the classical version of the PCR pathway, fructose, ribose and xylulose usually have incorporated the least label. If the classical view of the PCR reaction pathway is correct, these sugars are expected to have more highly enriched carbons than any sugars which are secondarily labelled. Only in the case of the experiments carried out in the specially constructed apparatus do the classical Calvin pathway intermediates sometimes appear more heavily labelled and this is an inconsistency which requires further consideration.

It is possible that significant errors could arise through a compounding of errors present in the original data (Tables 4.2 - 4.7) when enrichments of individual carbon atoms are calculated, thereby giving rise to the unexpected results above. It is therefore instructive to compare any ions common to all compounds without the complications introduced by such calculations. The *m/z* 307 ion (representing the b, c and d carbons of the

Calvin branch of the PCR pathway in Figure 1.5 and the g, h, and i carbons of the alternative branch in Figure 1.6) and *m/z* 205 (representing the c and d carbons of the "Calvin branch" of the PCR pathway in Figure 1.5 and the h, and i carbons of the "alternative branch" in Figure 1.6) are common to all the sugars, although in some the *m/z* 307 ion is of low intensity. Where available, the enrichments for these ions for all of the sugars in the two branches, together with other sugars monitored are given in the following tables. These sugars are again listed in a decreasing order of enrichment of the ion examined.

**Results from 45 second experiment No 1 in the leaf disc oxygen electrode:**

**Table 4.15.** Enrichments measured for the *m/z* 307 ion of sugars from Table 4.2.

| Sugar | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ | $^{13}C_3$ |
|---|---|---|---|---|
| **Lyxose/arabinose** | 65.6 | 24.0 | 8.2 | 2.2 |
| **Xylose** | 66.6 | 23.2 | 7.8 | 2.3 |
| **Ribose/Ribulose** | 70.9 | 20.8 | 6.7 | 1.6 |
| **Fructose** | 71.1 | 19.2 | 7.7 | 2.0 |

**Table 4.16.** Enrichments measured for the *m/z* 205 ion of sugars from Table 4.2.

| Sugar | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ |
|---|---|---|---|
| **Sedoheptulose** | 72.7 | 20.4 | 6.9 |
| **Xylose** | 73.5 | 21.9 | 4.6 |
| **Unidentified heptulose** | 74.2 | 21.0 | 4.8 |
| **Glucose** | 75.6 | 16.4 | 8.0 |
| **Octulose** | 83.6 | 15.8 | 0.7 |
| **Ribose/Ribulose** | 84.4 | 9.4 | 6.2 |
| **Fructose** | 89.4 | 10.6 | * |
| **Lyxose/arabinose** | 91.2 | 8.8 | * |
| **Erythrose** | 94.2 | 3.9 | 1.9 |

* interference from *m/z* 207 (column bleed).

**Results from 45 second experiment No 2 in the leaf disc oxygen electrode:**

**Table 4.17.** Enrichments measured for the *m/z* 307 ion of sugars from Table 4.3.

| Sugar | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ | $^{13}C_3$ |
|---|---|---|---|---|
| Sedoheptulose | 59.3 | 29.1 | 9.1 | 2.5 |
| Unidentified heptulose | 64.9 | 25.7 | 7.6 | 1.8 |
| Octulose | 66.2 | 25.8 | 6.7 | 1.4 |
| Xylose | 68.3 | 24.0 | 7.1 | 0.5 |
| Glucose | 68.3 | 24.1 | 6.0 | 1.6 |
| Lyxose/Arabinose | 70.4 | 20.9 | 7.9 | 0.8 |
| Ribose/Ribulose | 72.8 | 19.5 | 6.5 | 1.1 |
| Fructose | 75.6 | 18.2 | 5.5 | 0.8 |
| Xylulose | 81.6 | 16.1 | 2.3 | 0.0 |

**Table 4.18.** Enrichments measured for the *m/z* 205 ion in sugars from Table 4.3.

| Sugar | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ |
|---|---|---|---|
| Sedoheptulose | 75.1 | 20.5 | 4.4 |
| Xylose | 79.2 | 16.1 | 4.8 |
| Glucose | 80.0 | 15.7 | 4.4 |
| Octulose | 81.5 | 16.2 | 2.3 |
| Ribose/Ribulose | 86.6 | 9.0 | 4.4 |
| Xylulose | 92.0 | 6.0 | 2.0 |
| Lyxose/Arabinose | 92.2 | 7.8 | * |

* interference from *m/z* 207 (column bleed).

**Results from 30 second experiment No 1 in the leaf disc oxygen electrode:**

**Table 4.19.** Enrichments measured for the *m/z* 307 ion of sugars from Table 4.4.

| Sugar | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ | $^{13}C_3$ |
|---|---|---|---|---|
| Fructose | 69.5 | 21.2 | 7.8 | 1.5 |
| Ribose/Ribulose | 87 | 9.7 | 3 | 0.3 |

**Table 4.20.** Enrichments measured for the *m/z* 205 ion of sugars from Table 4.4.

| Sugar | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ |
|---|---|---|---|
| **Sedoheptulose** | 78.2 | 18.1 | 3.8 |
| **Glucose** | 82.1 | 14.5 | 3.4 |
| **Ribose/Ribulose** | 89.4 | 10.6 | * |
| **Fructose** | 90.6 | 9.4 | * |

* interference from *m/z* 207

**Results from 30 second experiment No 2 in the leaf disc oxygen electrode:**

**Table 4.21.** Enrichments measured for the *m/z* 307 ion of sugars from Table 4.5.

| Sugar | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ | $^{13}C_3$ |
|---|---|---|---|---|
| **Fructose** | 71.5 | 19.7 | 7.3 | 1.6 |
| **Lyxose/Arabinose** | 71.6 | 20.5 | 6.6 | 1.2 |
| **Xylose** | 72.7 | 21.3 | 4.8 | 1.2 |
| **Ribose/Ribulose** | 75.3 | 18.5 | 5 | 1.3 |

**Table 4.22.** Enrichments measured for the *m/z* 205 ion of sugars from Table 4.5.

| Sugar | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ |
|---|---|---|---|
| **Sedoheptulose** | 77.5 | 18.8 | 3.8 |
| **Glucose** | 80.2 | 13.1 | 6.7 |
| **Octulose** | 87.4 | 12.6 | 0 |
| **Lyxose/arabinose** | 88.7 | 6.7 | 4.5 |
| **Ribose/Ribulose** | 89.1 | 6.8 | 4.1 |
| **Xylose** | 89.6 | 7.5 | 2.9 |
| **Fructose** | 91.9 | 8.1 | * |

* interference from *m/z* 207 (column bleed)

**Results from the experiments in the photosynthesis apparatus:**

**Table 4.23.** Enrichments measured for the *m/z* 307 ion of sugars from Table 4.6

| Sugar | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ | $^{13}C_3$ |
|---|---|---|---|---|
| Sedoheptulose | 77.4 | 17.7 | 3.2 | 1.7 |
| Unidentified heptulose | 84.2 | 15.1 | 0.7 | |
| Octulose | 87.6 | 11.6 | 0.6 | 0.3 |
| Lyxose/Arabinose | 89.0 | 8.2 | 2.2 | 0.5 |
| Xylose | 89.5 | 8.2 | 1.7 | 0.6 |
| Fructose | 91.0 | 6.7 | 1.9 | 0.3 |
| Ribose/Ribulose | 91.2 | 6.8 | 1.8 | 0.3 |
| Glucose | 93.9 | 6.0 | 0.1 | |

**Table 4.24** Enrichments measured for the *m/z* 205 ion of sugars in the 30 second experiment in the photosynthesis apparatus.

| Sugar | $^{13}C_0$ | $^{13}C_1$ | $^{13}C_2$ |
|---|---|---|---|
| Sedoheptulose | 90.1 | 7.9 | 2.0 |
| Glucose | 95.4 | 4.6 | * |
| Xylose | 97.4 | 2.6 | * |
| Lyxose/Arabinose | 97.4 | 2.6 | * |
| Fructose | 98.2 | 1.8 | * |

* interference from *m/z* 207

For the 6 second experiment carried out in the photosynthesis apparatus, only the data for the *m/z* 205 ion are available and these all showed no enrichment of the *m/z* 205 ion in any of the sugars as would be expected since the two carbon atoms which constitute the ion are not labelled during the first turn of the cycle as seen in Figures 1.1 and 1.3 of Chapter 1.

Of the sugars with the highest labelling in the *m/z* 307 and *m/z* 205 ions in Tables 4.8 to 4.14, only sedoheptulose is represented amongst those from the Calvin pathway. Without exception, the other components of the classical Calvin version of the PCR pathway, fructose, xylulose and ribose incorporated label less heavily than did octulose, the unidentified heptulose and in many cases, the other previously unreported compounds, such as

xylose. The *m/z* 205 ion contains two of the three carbons which are encompassed by the *m/z* 307 ion and it is therefore not surprising to find that there is a similar pattern of enrichment in both ions. The less abundant pentoses, xylose and lyxose/arabinose sometimes showed a significant change in the order of labelling compared with the results of Tables 4.8 to 4.14 in which individual carbon enrichments were calculated. This may be due to the low concentration of the sugars, together with low contribution that the *m/z* 205 ion makes to the mass spectrum of some sugars.

Tables 4.15 to 4.24 above showing the results from a single ion cluster therefore show the same general trends as was found for Tables 4.8 to 4.14 in which the enrichments of individual ions were calculated from more than one ion cluster. Thus it is confirmed that the unexpectedly lower label incorporation in some classical Calvin pathway intermediates compared with other sugars present is not an artefact due to the compounding of errors which exist in the original data through the calculation involved in determining the individual ion enrichments. Sedoheptulose, octulose and the unidentified heptulose are therefore shown to be amongst the compounds which most heavily incorporated label, whilst the classical Calvin pathway sugars fructose, ribose/ribulose and xylulose were amongst the sugars which incorporated the least label. These are unexpected results which provide evidence of the operation of a quite different pathway involving both an unidentified heptulose- and D-*glycero* D-*altro*-octulose phosphates together with one or more of the phosphates of the aldopentoses, xylose, lyxose and arabinose.

One further difference between the results for experiments carried out in the leaf disc oxygen electrode and experiments carried out in the photosynthesis apparatus is apparent. In Tables 4.15 to 4.24 showing the enrichments for *m/z* 307 and *m/z* 205, which are uncomplicated by the possible compounding of errors in calculated results, glucose was more heavily labelled than the Calvin pathway sugars fructose, ribose/ribulose and xylulose in all experiments carried out in the oxygen electrode. In experiments

carried out in the photosynthesis apparatus, fructose and ribose/ribulose were more heavily labelled than glucose. Although the labelling differences were small in this case (Table 4.23), it could be argued that this reversal in the labelling orders of these sugars is indicative of another possible explanation for some of the unexpected labelling patterns reported in this investigation. For this possible explanation, attention is drawn to the different methods of quenching the photosynthetic reactions in these two experimental systems.

In the experiments carried out in the leaf disc oxygen electrode the reactions were terminated by rapidly removing the chloroplast-containing membrane from the electrode chamber and plunging it into liquid nitrogen. During this time the chloroplasts were exposed briefly to the atmosphere before they entered the liquid nitrogen where quenching took place. It is probable that some degree of "washing out" of the $^{13}C$ isotope could have occurred through the continuation of photosynthesis during this exposure to the unenriched atmosphere. In this event, the sugars most closely involved in the path of carbon reactions are those expected to suffer a rapid reduction in the degree of label incorporation. The period of exposure of the chloroplasts to the atmosphere was not more than 2.5 seconds, but the cycle turnover is fast and some washout may have occurred. Experiments conducted in the specially designed apparatus used hot ethanol to quench the photosynthesis reactions. The chloroplasts were therefore not exposed to the unenriched atmosphere prior to quenching and the "washout" of label could not occur. The higher label incorporation in *m/z* 307 of fructose and ribose than in glucose in the 30 second apparatus experiment is consistent with this explanation which is a reversal of the position found in the experiments conducted in the leaf disc oxygen electrode. The *m/z* 205 ion in the 30 second apparatus experiment does not show this reversal of the order of label incorporation that the *m/z* 307 ion shows. However this may be because the *m/z* 205 ion does not contain any of the carbons which become labelled in the first turn of the cycle. The *m/z* 307 ion by contrast, contains carbon 4 of the hexoses and carbon 3 of the

pentoses, which, by reference to Figures 1.1 and 1.3 (Chapter 1) are shown to be carbons which become labelled during the first turn of the cycle. The *m/z* 307 ion would therefore show the effects of washout before the *m/z* 205 ion.

Although the data are incomplete, the relative differences in the magnitude of the $^{13}C$ enrichment in glucose, fructose and ribose/ribulose between the oxygen electrode and apparatus experiments are generally consistent with the proposition that the liquid nitrogen-quenched reactions show a "washing out" of label from the sugars most closely associated with the photosynthetic pathway compared with sugars present as a consequence of side-reactions. However, this does not explain the fact that the octulose, xylose and the unidentified heptulose have a consistently higher label incorporation than any of the Calvin pathway intermediates except sedoheptulose regardless of the method of quenching the reactions. The higher label incorporation in these compounds compared with the traditionally recognized photosynthetic intermediates from which they are presumed to have been formed is not expected of compounds that are only present as a consequence of side reactions. In the one experiment in which evidence for the possible "washout" of label exists, (30 second apparatus, Table 4.22), the enrichments of fructose, ribose and glucose are very close (+/- 1.5%) and entirely within the bounds of experimental error. It can therefore be equally argued that this reversal in labelling order of these sugars arises from normal experimental variability, especially since glucose was also seen to have lower label incorporation from calculated data in one of the oxygen electrode experiments (Table 4.11).

The possibility that the heavy labelling of the non-Calvin sugars may have been due to non-photosynthetic reactions was considered unlikely since an experiment in which chloroplasts were exposed for 45 seconds to $^{13}CO_2$ in the dark (Table 3.4) showed no labelling in any of the compounds monitored.

## Conclusion

The equipment and experimental procedures developed for this investigation have enabled the specific isotopic enrichments to be determined for most of the intermediates implicated in the photosynthetic carbon reduction pathway after a period of exposure to $^{13}CO_2$. The conceptual advantages of these methods over those previously used were discussed in chapter 1 and have been generally confirmed by the results of this study.

In this investigation, experiments were conducted on isolated, intact chloroplasts and the procedure for mounting chloroplasts on the filter membranes involved sufficient washing of the chloroplasts to ensure that the "carry through" of exogenous metabolites with the chloroplasts during isolation was minimized before the experiments commenced. The attempt to reduce the distorting effects that photosynthetically produced metabolites exported from the chloroplasts may have on labelling patterns was made by including in the reaction medium a phosphatase designed to dephosphorylate the metabolites as they are exported. It is not known how effective this was but the quantification results do show an increase in the concentrations of some metabolites with increasing duration of the experiments (Chapter 3). Only phosphoglyceric acid, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate are freely exported across the chloroplast membranes, whilst pentose, hexose and heptulose phosphates are largely retained within the chloroplast (Lilley et al., 1977). Nevertheless, small amounts of the C5 and C6 sugar phosphates do cross the chloroplast membrane and it is possible that some of these were not dephosphorylated and accumulated in the external medium. Alternatively, the increase in metabolite concentrations observed in this investigation may indicate non-steady-state photosynthesis. This is an aspect of the procedures reported here which would need to be considered in any future work.

A more complete set of results than was possible during this investigation may be obtained by the use of a photosynthesis apparatus that has a filter membrane area at least five times the size of that used here. This may permit a more comprehensive analysis of the pentoses and it should allow the resolution of the extract into mono- and bisphosphate fractions (perhaps by HPLC) which would assist in the interpretation of the labelling results. Amongst the issues which should be addressed in any future study is the lower $^{13}C$ incorporation obtained by chloroplasts in the specially designed photosynthesis apparatus than in the leaf disc oxygen electrode. The most likely explanation for this is the large "dead volume" of atmosphere containing $^{12}CO_2$ in the space beneath the filter membrane of the photosynthesis apparatus after the removal of the glass beads as was mentioned in section 3.5(iii). This dead volume is larger than the volume of the oxygen electrode chamber and would easily account for the reduced labelling of the sugars in experiments carried out in the photosynthesis apparatus.

The GC/MS analyses of the $^{13}C$ enrichments of sugars which incorporated $^{13}C$ during photosynthesis experiments were generally reproducible between different experiments although some inconsistencies were noted. Where there were significant differences between experiments in the enrichment values obtained for a particular carbon the differences usually arose as a result of the use of different ions to calculate the two values. In a worst case example, C4 of sedoheptulose in the two 45 second exposure experiments using the leaf disc oxygen electrode (Figures 4.1 and 4.2) gave notably different results. In 45 sec experiment No 1, the value of 4.4 % enrichment was obtained using the *m/z* 364 - *m/z* 262 calculation, and this is judged to be less reliable than the *m/z* 319 - *m/z* 307 calculation which was subsequently used to calculate for the result of 34.8% in the 45 sec. experiment No 2. Therefore more experimental work needs to be directed towards the identification of the sources of these inconsistencies. Notwithstanding these differences, the overall pattern of labelling is consistent

with the labelling patterns predicted by the two pathways of Figures 1.1 and 1.3 as far as it is possible to discern from the incomplete results shown in Figures 4.1 to 4.6.

The most notable of the findings from this investigation are the data for the phosphate esters of D-*glycero*-D-*altro*-octulose, and an as yet unidentified heptulose together with sedoheptulose which were the sugars most heavily $^{13}C$-labelled. They incorporated $^{13}C$ label more than the conventionally accepted intermediates of the PCR pathway, the phosphate esters of fructose, ribose/ribulose and xylulose. The possibility that this effect was due to the "washout" of $^{13}C$ label as a result of continued photosynthesis during the quenching of the reactions was considered. It was found that the evidence for this effect was inadequate and did not account for the higher labelling of octulose, the unidentified heptulose and sedoheptulose in the 30 sec experiment carried out in the photosynthesis apparatus, in which the "washout" effect was thought not to occur. A control experiment in which chloroplasts were exposed to $^{13}CO_2$ for 45 seconds in the dark showed no $^{13}C$ incorporation into any of the sugars, thereby demonstrating that the high incorporation into the above sugars did not arise as a consequence of non-photosynthetic reactions.

There are only three known reaction schemes in biochemistry by which octulose can be formed. These are the group transferring reactions shown in Figure 1.4. Although these reactions relate to the synthesis of D-*glycero*-D-*ido*-octuloses, the replacement of arabinose 5-phosphate with ribose 5-phosphate in Figure 1.4 (i) and (ii) and glucose 6-phosphate with allose 6-phosphate in Figure 1.4 (iii) give rise to the corresponding D-*glycero*-D-*altro*-octuloses found in this investigation. Allose 6-phosphate is not known to be metabolized by higher plants and was not observed in this investigation although its presence can not be excluded. Ribose 5-phosphate is therefore the most likely precursor of D-*glycero*-D-*altro*-octuloses via the aldolase catalyzed condensation with a dihydroxyacetone phosphate moiety. The

results of the experiments carried out using both the photosynthesis apparatus and the leaf disc oxygen electrode show that the octulose is much more heavily labelled than its presumed precursor ribose. The possibility that ribulose present in the GC chromatographic peak representing ribose was diluting the enrichment monitored in the *m/z* 307 ion for ribose is not thought to be a likely explanation because of the low intensity of the *m/z* 307 ion in ribulose. Therefore the finding that D-*glycero*-D-*altro*-octulose contained a higher enrichment than its only known precursors remains an unexplained and intriguing feature of this investigation.

Notwithstanding the enigma provided by the low label incorporation found in the octulose precursors, certain conclusions may be made with regard to the octulose fount in this study: 1) octulose is present in the extracts of isolated, intact chloroplasts; 2) it is amongst the sugars which incorporates $^{13}C$ label most heavily; and 3) the position of the heaviest label within the octulose molecule is generally consistent with that predicted in the alternative pathway of Figure 1.3. The results reported here therefore support previous conclusions that the Calvin version of the PCR pathway may be an incomplete description of the path of carbon in photosynthesis. The alternative scheme for the PCR pathway proposed the involvement of D-*glycero* D-*ido*-octulose but the octulose found in this investigation was the D-*glycero* D-*altro*-epimer and the alternative scheme proposed no role for an alternative heptulose. Thus the path of carbon reactions may be more complex than either of the proposals presently available. The implication of a close involvement of an octulose in the pathway, however, may mean that the present alternative pathway is closer to the true reactions than that proposed by Calvin. An intermixing and/or favouring of one of the octulose phosphates is mechanistically possible using the aldolase exchange catalysis shown in the following equation.

$$\text{D-}g\text{-D-}a\text{-oct 1,8-P}_2 + \text{Ara 5-P} \rightleftharpoons \text{D-}g\text{-D-}i\text{-oct 1,8-P}_2 + \text{Rib 5-P.}$$

Evidence for this aldolase exchange reaction has been published (Bartlett et. al, 1989). It is of note that Kapuscinski, using shorter term $^{14}C$ experiments only found D-*glycero*-D-*ido*-octulose (Kapuscinski, 1985).

The methodology developed for this investigation has laid a foundation for future, perhaps more definitive experimental studies of the path of carbon in photosynthesis and has identified a number of issues which should be addressed in order to further improve the suitability of the methods.

# Appendix 1.

## Method for calculating $^{13}C$ enrichment of ions monitored by SIM using GC-MS.

For a one-carbon fragment (20% enriched with $^{13}C$) and another one-carbon fragment (40% enriched with $^{13}C$) the following notation can be used to describe them, the superscript denoting whether the carbon is labelled or not:-

| Carbon 1 | $^0C_1$ | $^1C_1$ | Total |
|---|---|---|---|
| % in each category | 80 | 20 | 100 |
| Carbon 2 | $^0C_2$ | $^1C_2$ | Total |
| % in each category | 60 | 40 | 100 |

If equal numbers of these one-carbon fragments are combined at random into two-carbon fragments, then they can be combined in the following ways:-

$^0C_1{}^0C_2$ $^0C_1{}^1C_2$ $^1C_1{}^0C_2$ $^1C_1{}^1C_2$

The task is to determine the proportions of the total two-carbon fragments which are unlabelled, singly or doubly labelled, denoted z, y and x respectively.

This is a simple problem of probability which can be written as:-

$$\text{Let } Pr(z) = Pr(^0C_1{}^0C_2) = Pr(^0C_1) \times Pr(^0C_2)$$

$$\text{Let } Pr(y) = Pr(^0C_1{}^1C_2 + {}^1C_1{}^0C_2) = Pr(^0C_1{}^1C_2) + Pr(^1C_1{}^0C_2)$$

$$= Pr(^0C_1) \times Pr(^1C_2) + Pr(^1C_1) \times Pr(^0C_2)$$

$$\text{Let } Pr(x) = Pr(^1C_1{}^1C_2) = Pr(^1C_1) \times Pr(^1C_2)$$

These equations can now be used in the solution of the above example.

$$Pr(z) = Pr(^0C_1) \times Pr(^0C_2)$$

$$\frac{z}{100} = \frac{80}{100} \times \frac{60}{100} = 0.8 \times 0.6 = 0.48$$

$$z = 0.48 \times 100$$

$$z = 48\%$$

$$Pr(y) = Pr(^0C_1) \times Pr(^1C_2) + Pr(^1C_1) \times Pr(^0C_2)$$

$$\frac{y}{100} = \frac{80}{100} \times \frac{40}{100} + \frac{20}{100} \times \frac{60}{100}$$

$$\frac{y}{100} = (0.8 \times 0.4) + (0.2 \times 0.6) = 0.32 + 0.12$$

$$y = 0.44 \times 100$$

$$y = 44\%$$

$$Pr(x) = Pr(^1C_1) \times Pr(^1C_2)$$

$$\frac{x}{100} = \frac{20}{100} \times \frac{40}{100} = 0.2 \times 0.4$$

$$x = 0.08 \times 100$$

$$x = 8\%$$

These relationships so far can be summarized as follows:-

| | | | |
|---|---|---|---|
| % of fragments unlabelled | z | $Pr(^0C_{1-2})$ | 48 |
| % of fragments singly labelled | y | $Pr(^1C_{1-2})$ | 44 |
| % of fragments doubly labelled | x | $Pr(^2C_{1-2})$ | 8 |
| Total | | | 100 |

The superscript now refers to the number of labelled carbons in the carbon 1-2 fragment and the notation can be extended for a 3 or more-carbon fragment as required:-

| | | |
|---|---|---|
| % of fragments triply labelled | w | $Pr(^3C_{1-3})$ |

etc.

If the above relationships are the outcome of a GC-MS analysis and the analysis also showed a three carbon fragment with the following composition:-

| | | | |
|---|---|---|---|
| % of fragments unlabelled | z | $Pr(^0C_{1-3})$ | 33.6 |
| % of fragments singly labelled | y | $Pr(^1C_{1-3})$ | 45.2 |
| % of fragments doubly labelled | x | $Pr(^2C_{1-3})$ | 18.8 |
| % of fragments triply labelled | w | $Pr(^3C_{1-3})$ | 2.4 |
| Total | | | 100 |

then the enrichment of carbon 3 can be determined.

For convenience this problem can be presented as:-

| Fragment | Number of carbons labelled | | | | Total |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | (%) |
| $C_{1-3}$ | 33.6 (=z) | 45.2 (=y) | 18.8 (=x) | 2.4 (=w) | 100 |
| $C_{1-2}$ | 48.0 ($=^0C_2$) | 44.0 ($=^1C_2$) | 8.0 ($=^2C_2$) | | 100 |
| $C_3$ | ($^0C_1$) | ($^1C_1$) | | | 100 |

Since the values shown for the fragment $C_{1-3}$ are the resultant values upon the addition of an extra carbon to the $C_{1-2}$ fragment, the same equations above can be applied to find values for the enrichment of $C_3$; i.e. values for $^0C_1$ and $^1C_1$. The $C_{1-2}$ fragment may be regarded as a single entity.

$$Pr(z) = Pr(^0C_1) \times Pr(^0C_2)$$

$$\frac{33.6}{100} = \frac{^0C_1}{100} \times \frac{48}{100}$$

$$\frac{33.6}{100} \times \frac{100}{48} = \frac{^0C_1}{100}$$

$$^0C_1 = \frac{33.6}{100} \times \frac{100}{48} \times 100 = 0.7 \times 100$$

$^0C_1$ = 70% which is inserted in the table in the position for unlabelled $C_3$

| Fragment | Number of carbons labelled | | | | Total |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | (%) |
| $C_{1-3}$ | 33.6 | 45.2 | 18.8 | 2.4 | 100 |
| $C_{1-2}$ | 48.0 | 44.0 | 8.0 | | 100 |
| $C_3$ | 70.0 | $^1C_1$ | | | 100 |

$^1C_1$ may then be found by subtraction, $^1C_1 = 100 - 70 = 30$

But $^1C_1$ may also be found by applying the equation for Pr(y) above.

$$Pr(y) = Pr(^0C_1) \times Pr(^1C_2) + Pr(^1C_1) \times Pr(^0C_2)$$

$$\frac{45.2}{100} = \frac{70}{100} \times \frac{44}{100} + \frac{^1C_1}{100} \times \frac{48}{100}$$

$$\frac{45.2}{100} - \frac{70}{100} \times \frac{44}{100} = \frac{^1C_1}{100} \times \frac{48}{100}$$

$$0.452 - 0.7 \times 0.44 = \frac{^1C_1}{100} \times \frac{48}{100}$$

$$0.452 - 0.308 = \frac{^1C_1}{100} \times \frac{48}{100}$$

$$0.144 \times \frac{100}{48} \times 100 = {}^1C_1$$

$$^1C_1 = 30\%$$

which equals the value found by subtraction and the value can be inserted to complete the table.

| Fragment | Number of carbons labelled | | | | | Total |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | (%) |
| $C_{1-3}$ | 33.6 | 45.2 | 18.8 | 2.4 | | 100 |
| $C_{1-2}$ | 48.0 | 44.0 | 8.0 | | | 100 |
| $C_3$ | 70.0 | 30.0 | | | | 100 |

The same procedure can be used to find a $C_{3-4}$ fragment if we have the labelling values for the $C_{1-2}$ and the $C_{1-4}$ fragment as a two-carbon fragment may be regarded as a single entity.

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