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# $^{14}CO_2$ Fixation and Assimilate Transformation in Barley Leaves

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# 보리 葉의 $^{14}CO_2$ 固定과 同化産物의 轉換

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## ABSTRACT

Young barley plants (*Hordeum vulgare*) were exposed to $^{14}Co_2$ for 10 and 30 minutes. Leaves were harvested and extracted for aqueous soluble metabolites which were fractionated into amino acids, sugars, sugar phosphates and organic acids. It was found that both 10 and 30 minute incubation periods had similar quantities of label but differed in the quality and quantity. It was also found that the neutral fraction(sugars, phosphate esters and organic acids) had 4.5 times more label than the amino acid fraction at 10 minutes and only about 1.7 times at 30 minutes. Label increased moderately from 10 minutes in the sugar fraction where there was a large increase in the amino acid fraction, both qualitatively and quantitatively.

Photosynthetic rate was found to be quite different when the 10 minute incubation and the 30 minute incubation were compared (0.125 vs 0.034 and 0.042 μmole $co_2$/mg chlorophy 11/min respectively).


## INTRODUCTION

Photosynthesis, illustrated in its simplest form, comprises of two basic phases. The first phase is the light reaction where photons of light are captured by light harvesting pigments in the cell, specifically the choroplast. In photosystem II, this energy is used to split water and 2 electrons are transferred to an electron transport chain to form ATP. The electrons are then further passed on the photosystem I and generates reducing power in the form of $NADPH_2$. It is the ATP and $NADPH_2$ that is used in the dark reaction to fix $CO_2$.

In the dark reaction, $CO_2$ is reduced by first combining with a five carbon sugar to from 2 molecules of 3-PGA. This molecule is sequentially phosphorylated to 1, 3-PGA and then reduced to 3-PGAld by $NADPH_2$. It is the reduction to 3-PGAld which stores the energy garnered by the light reaction of photosynthesis(Goodwin and Mercer '83).

As the reduced carbon spends more time in the plant, it becomes part of many types of compounds. It goes into carbohydrates and can remain as such or can be transformed into organic acids and amino

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acids which in turn form other plant constituents.

By using radiolabelled $CO_2$, one is able to follow the reduced carbon into various compounds. In the present study, $NaH^{14}CO_3$ is used to study the incorporation of $^{14}CO_2$ into extractable assimilates in young barley plants.

## MATERIALS AND METHODS

Plant material : Barley (*Hordeum vulgare*) seeds were planted in 12cm pots containing regular potting soil. The plants were placed in the greenhouse, under greenhouse lighting and watered daily. Plants of approximately 10 to 12 days old were used for the experiment.

Radioactive labelling : The barley plants were first watered with 100 ml of tapwater. A small vial was placed securely in the soil and 15 $\mu$ci of $NaH^{14}CO_3$ (7.0 mCi/mmol) was placed inside. A transparent bag was then placed over top of the plant so as not to touch the tops of the leaves but not leaving too much dead volume. The bottom of the bag was sealed securely around the brim of the pot to generate and airtight seal. At this point, the experiment was continued in a fume hood.

The plants were illuminated with 4 foot Sylvania Gro-Lux WS fluorescent tubes at an approximate fluence flux density of 70-90 $\mu E/m^2/s$ (photosynthetically active radiation, PAR). At time zero, 2ml of lactic acid was injected into the vial by piercing the bag with a syringe and needle. Immediately after the acid injection (liberating $^{14}CO_2$) the hole was sealed with masking tape.

The plants were allowed to photosynthesize for 10 and 30 minutes (2 different groups) at 23℃ after which the bags were opened and the residual labelled gas was allowed to disperse in the fumehood for 2 minutes.

Extraction : Two grams of leaf tissues (fresh weight) was weighted out and ground with fine sand and a minimum amount of hot 80% ethanol for about 10 minutes. The slurry was then transferred to a Buechner funnel with filter paper (Whatman No. 1) mounted on a 250ml vacuum erlenmeyer flask. The mortar and pestle were rinsed with the extraction ethanol. The solid residue was rinsed with approximately 50ml of extraction ethanol.

The green filtrate was collected into a round bottomed flask and flash evaporated. Twenty ml of water and 20ml of chloroform was added to the dry residue after evaporation. The mixture was shaken to resolve all the constituents and 5ml of the aqueous layer was pipetted into a 50ml erlenmeyer flask and stoppered with a rubber serum cap. A 100$\mu$l aliquot of this crude extract was used for scintillation counting. The crude extract was also used for fractionation.

FRACTIONATION : One ml of crude extract was loaded onto a cation exchange column (Dowex 50, $H^+$ form). The column was washed with 6ml of distilled water and the eluant collected in a round bottom flask, flash evaporated and resolved in 500 $\mu$l of distilled water, (this fraction contained sugars, phohphate esters and organic acids). The amino acids were eluted from the column by the addition of 2N $NH_4OH$, after which they are also flash evaporated and resolved in 500$\mu$l of distilled water.

One hundred $\mu$l of each fraction was transferred to 10ml of Picofluor-15 (Packard Technologies Ltd) in a scintillation vial and measured in a liquid scintillation spectrometer (Minaxi, Tricarb 4000 series, Packard United Technologies).

THIN LAYER CHROMATOGRAPHY : Aliquots containing about 5000 DPM for the amino acid fraction and 10,000 DPM for the neutral fraction were spotted on precoated microcrystalline cellulose, 250$\mu$m thickness (Whatman) 2cm away from each edge. A hairdryer was used for intermittent drying of the applied spots. The neutral fraction was separated two-dimensionally with 5 runs in 3 different solvent systems as follows :

*SOLVENT* I

200ml isobutyric acid
6ml n-butanol
6ml iso-propanol
28ml n-propanol
76ml water
100mg EDTA

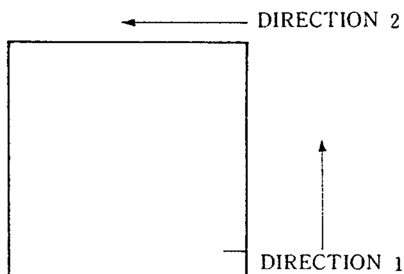
*SOLVENT* II
80ml n-butanol
35ml n-propanol
57ml n-propionic acid
75ml water
*SOLVENT* III
200ml n-butanol
40ml acetic acid (glacial)
160ml water

1. Run in direction 1 in Solvent I about 12cm (4-5 hours)
2. Run in direction 2 in Solvent 2 about 12cm (3-4 hours)
3. Run in direction 2 in Solvent 3 about 12cm (3-4 hours)
4. Run in direction 2 in Solvent 3 about 12cm (3-4 hours)
5. Run in direction 1 in Solvent 1 about 12cm (4-5 hours)

Amino acids were separated two dimensionally with 2 runs in 2 different solvent systems as follows:

*SOLVENT* I
60ml n-butanol
60ml acetone
12 diethylamine
30ml water
(pH 11.5, Direction I)
*SOLVENT* II
120ml iso-propanol
6ml formic acid
30ml water
(pH 2.7, Direction II)

AUTORADIOGRAPHY: After the TLC plates were developed in their respective solvent systems and dried, radioactive ink spots were put in two




corners of the plate. An x-ray film (emulsion on both sides) was then placed on the TLC plate and a glass plate over top of this and fixed in place with masking tape. The sandwich was then wrapped with aluminum foil to exclude light.

After 21 days, the x-ray film was developed and the black spots identified. Tracing paper was used to trace the labelled spots and ink spots on the x-ray film. The tracing paper was then oriented, with the aid of the ink spots, to the TLC plate where the spots were redrawn, leaving indentations on the cellulose layer. The indicated spots on the TLC plate were scraped off and transferred into liquid scintillation vials. One ml of distilled water was added to each vial and shaken to resolve labelled metabolites from the cellulose. Ten ml of Picofluor -15 was added and allowed to dark adapt for 1 hour.

The data obtained was calculated on a DPM per labelled metabolite per gram fresh weight basis. Calculations are based on corrected values of total recovered DPM's from TLC plates. A correction factor was calculated by generating a ratio of total recovered label between parallels.

Example: 2700/2300=1.174

Each metabolite from the 2300 DPM plate would then be multiplied by the correction factor so comparison between the parallels can be acheived. An additional 20% was added to averages to correct for reasonable, acceptable losses.

## RESULTS

The incorporation of $^{14}CO_2$ into identified radioactive assimilates is represented in table 1. Most noticable differences in the list will be discussed in the discussion section. It was found that there were noticable differences in the quantity and quality of lable found in the two different incubation periods. Fructose and glucose contained the greatest amount of label in both the 10 and 30 minute incubations. It was found that the 30 minute glucose and fructose fractions had more label than the 10 minute fractions. Also noteworthy in the sugar

**Table 1.** The incorporation of $^{14}CO_2$ into indentified radioactive assimiliates of young barley leaves.

| Compound | DPM/ Gram fresh weight | |
|---|---|---|
| | 10 min | 30 min |
| GLYCOALDEHYDE | 1860 | 2170 |
| FRUCTOSE | 23040 | 34420 |
| GLUCOSE | 42790 | 46760 |
| SUCROSE | 2740 | ...... |
| MALT./TREHALOSE | 5220 | ...... |
| FRUCTOSE-PHOSPHATE | 2870 | 1980 |
| GLUCOSE-1-PHOSPHATE | 1580 | 900 |
| GLUCOSE-6-PHOSPHATE | 1490 | 1220 |
| GLYCERATE | 2830 | 1420 |
| MALATE | 4100 | 3670 |
| CITRATE | 2510 | 710 |
| PEP | 6310 | 2050 |
| PGA | 4220 | 2140 |
| ORIGIN | 2980 | 2460 |
| TOTAL | 101380 | 99900 |
| SERINE | 11390 | 19370 |
| ASP(N) | 3550 | 840 |
| ALANINE | 11640 | 16030 |
| GLYCINE | 1140 | 2110 |
| GLUTAMATE | 2520 | 5570 |
| GLUTAMINE | 1720 | 12830 |
| ORIGIN | 100 | 190 |
| TOTAL | 32060 | 56940 |

fractions was the fact that sucrose and maltose/ trehalose spots were present in the ten minute fractions but absent in the thirty minute fractions. There is relatively little difference between the two incubation time periods in as far as the sugar phosphates are concerned. Quantity of label in malate remains about the same for both time periods whereas label citrate decreases about 3 fold in the 30 minute incubation. PEP and PGA label decreases in the 30 minute incubation as compared to the 10 minute incubation. Label in the amino acid fraction is as expected.

All, (except ASP(N), probably experimental error) of amino acids have a large increase in quantity of label going from the 10 minute to the 30 minute incubation period.

**Table 2.** Photosynthetic rates of young barley leaves in relation to incubation time.

| | Photosynthetic rates ($\mu$moles $CO_2$ fixed/mg chlorophyll/ minute) |
|---|---|
| GROUP 1. (10 Minutes) | .125 |
| GROUP 2. (10 Minutes) | .125 |
| GROUP 3. (30 Minutes) | .034 |
| GROUP 4. (30 Minutes) | .042 |

Photosynthetic rates are represented in table 2 with the ten minute incubation calculated at 0.125 $\mu$moles $CO_2$ fixed per mg chlorophyll per minute whereas the thirty minute incubation was calculated to be 0.034 and 0.042 for the parallels.

## DISCUSSION

Photosynthesis has elucidated by the use of radiolabelled compounds, specifically $^{14}CO_2$. By feeding $^{14}CO_2$ to plants we can follow the fate of the fixed carbon into various metabolic pathways. The reduction of $CO_2$ occurs in the chloroplasts where triose phosphates are produced. The triose phosphates then have two fates. They can produce glucose which is turned into the storge carbohydrate starch, or they can be transported out of the chloroplast via the phosphate translocator into a pool of triose phosphates. The fate of this pool is highly variable and depends largely on the metabolic status, age and energy charge of the cell. Ultimately, the triose phosphates can be used to

produce cell walls, lipids, pigments, amino aceds, proteins, energy, etc.

In this experiment we isolataed a portion of labelled compounds from barley leaves. The highest amount of label was found in fructose and glucose at 10 and 30 minutes incubation. Glucose is a common product of photosynthesis and, therefore, was expected to be present in large amounts. The large amount of fructose is possibly because it is the additional component of the common export sugar, sucrose. Another reason is that barley has been found to accumulate. fructosans in the leaves (Gordon et al '79) and, therefore, needs the fructose moieties to form the fructosans. The former would be true if more label was found in the sucrose fractions. This is not the case, though, because we find little label in the 10 minute fraction and none in the 30 minute fraction. This particular anomally brings up the points of energy status of the cell and source-sink relations(Herald '80, Huber and Israel '82). Sucrose is supposedly the main storage product in barley (Gordon et al '80).

Assuming this is true, we should find more label from $^{14}CO_2$ (the source) in the sucrose fraction (the sink). We didn't find this and therfore, secondary source-sink relations should be considered. In Gordon et al (1980), they illuminated barley plants for extended periods of time with $^{14}CO_2$ and found that there was an initial lag of sucrose and starch accumulation. They were using plants freshly out of a dark phase and therefore, their sinks were depleted. This is why they found that these metabolites accumulated before being transported. In our case, the plants that we used had been in the light for approximately two hours before the experiment commenced. Our plants then, had a chance to reestablish physiological pool sizes to the capacity where they would start transporting assimilates. This is probably why we see just a little babel in sucrose at 10 minutes, and none at 30 minutes.

Any assimilate that was stored in starch would not have been detected because starch is insolubel and can only be solubilized by perchloric acid of enzymatic degradation.

It is, therefore, feasible to assume that label assimilated in the 30 minute treatment has been either incorporated into lipids, pigments and insoluble material (which was not analyzed) (Bassham '71) or it was transported to other parts of the plant such as the hypocotyls and roots. The latter is possible because this has been seen in the Gramineae (Gordon et al '80) in which a large percentage of total label was transported (Gordon et al '77, '79, '80). This is based on the fact that the 30 minute treatment doesn't have three times as much label as does the 10 minute treatment.

Other metabolites such as citrate, PEP and PGA decrease in $^{14}C$ incorporation from 10 to 30 minute treatments. The possible reason for this is that their turnover rate is faster and, therefore, the don't have as much as the 10 minute treatment.

Amino acids as expected, contain more label in the 30 minute treatment than the 10 minute treatment. This is expected because the longer an assimilate remains in a cell, the better chance it has of turning into a metabolite other than carbohydrate, or in this case, an amino acid. Serine, alanine, glycine, glutamate and glycine are labeled because they are the simplest, derived from 2 and 3 carbon precursors from the triose phosphate pool (eg. pyruvate forms alanine, 3-PGA forms serine which in turn forms glycerine) or form the TCA cycle intermediate alphaketoglutarate which forms glutamate and in turn glutamine(Stryer '81) (serine and glycine may also come from photorespiration, Goodwin and Mercer, '83, Salisbury and Ross '85). Glutamine, which is important as an amino transfer agent has greatly increased in label content over the 10 minute treatment. This also follows the general scheme in that the young plants are actively growing and therefore have high demand for nitrogen of which glutamine is one of the main supplyers (Bassham '71).

Considerations other than label incorporation into identifiable compounds are losses such as those found with photorespiration and respiration. It is likely that both are occuring and are synergistic to fixed $CO_2$ losses. Photorespiration is not obvious because we don't find any labelled glycollic acid or

glyoxalate. Respiration is also difficult to discern in this experiment because it releases $CO_2$ which may or may not be fixed again. It has been shown that losses as great as 48% of total fixed carbon were lost in short term labelling in Graminaceous plants (Gordon et al '77, '79, '80).

When considering the photosynthetic rate, one should keep in mind several points since the two treatments very dramatically (10 minutes= 0.125 : 30 minutes=0.034 and 0.042). Photosynthetic rate is not the ture rate because on the one hand we have reduction of $CO_2$ into assimilates and on the other hand we have photorespiration and respiration. Therefore, the calculated photosynthetic rate is more realistically called the "net photosynthetic rate", Secondly, the qeneral calculation is based on total fixed carbon throughout the plant at time of harvest. In our experiment, we only measured the ethanol soluble portion of the leaves. to have properly calculated the net photosynthetic rate we would have had to extract total fixed carbon from the entire plant.

The total $CO_2$ taken up in the leaves seems to be in a steady state because it is similiar in both treatments. This implies, then, as mentioned above, the assimilates are being transported to the roots due to a secondary sink attraction from the leaf source. It is assumed that if the entire plant was extracted in the two treatments, the net phothosynthetic rates would be similar.

## 적　　요


보리(***Hordeum vulgare***)의 유묘에 $^{14}CO_2$를 10분 및 30분간 처리한 후 잎을 채취하여 아미노산, 당, 인산염 및 유기산과 같은 수용성 대사물질을 분석하였다.

배양기간에 따른 label 정도는 비슷하였으나 종류와 양에 있어서는 차이가 있었다. 10분 처리시에는 아미노산 분획보다 중성분획(당, 인산염 에스테르 및 유기산)에서 label 양이 4.5배 증가하였으나 30분 처리시에는 1.7배로 감소하였다. 당분획에 있어서는 처리 시간이 길어짐에 따라 label 양이 약간 증가하였으나 아미노산 분획에 있어서는 양과 질적으로 label 양이 현저히 증가하였다.

탄소 동화율은 10분에서 30분으로 길어질수록 현저히 감소하였다(0.125 대 0.034와 0.042 mole $CO_2$/mg 엽록소/분).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