Acta Bot. Neerl. 31 (4), August 1982, p. 281–295.

CARBON ASSIMILATION PATTERN IN THE SUBMERGED LEAVES OF THE AQUATIC ANGIOSPERM: VALLISNERIA SPIRALIS L.

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SUMMARY

Segments of leaves of *Vallisneria spiralis* L. were allowed to take up CO_2 and HCO_3-ions and assimilate the inorganic carbon either in the light or in the dark. C4-fixation occurred in the dark producing malate and aspartate as the first compounds, but some residual activity of the Calvin cycle could be detected, too. Both C4 and C3 fixation were stimulated by light, the C4 fixation amounting to 20–25% of total fixation rate under the prevailing conditions. By extending the dark fixation period a label distribution pattern could be obtained which hardly deviated from the pattern found in a light fixation experiment.

Label from aspartate was transferred to other amino acids and to organic acids, citrate-isocitrate being the most apparent one. No detectable activity could be demonstrated in oxalic acid, which is the most abundant acid in *Vallisneria* leaves. In contrast, malate is accumulated and no turn-over could be demonstrated. From preliminary data on label distribution within the malate molecules we deduced that only single-labelled molecules were formed in the dark. The label tended to be distributed evenly among the fourth and first C-atom. However, in the light there was a gradual tendency to uniform labelling, i.e. more C-atoms became labelled.

Transfer of labelled atoms between C3 and C4 products must have occurred in both directions. However, no detectable amounts of labelled PEP, which seemed to be a plausible intermediate in the transfer reactions, could be demonstrated.

The accumulated malic acid may function as a CO_2-reservoir that can be drawn on under conditions of reduced CO_2 supply from the medium, which is, in turn, the results of the highly reduced diffusion of CO_2 in a liquid medium which leads to rapid depletion of the solution contacting the leaves.

1. INTRODUCTION

The long leaves of *Vallisneria spiralis* L. provide excellent material for studies on uptake and symplasmatic translocation (HELDER 1967). Very many experiments have been carried out on the uptake and translocation of C-14 labelled organic substances, using autoradiographic techniques (ARISZ 1966).

These experiments, however, had the disadvantage of demonstrating the distribution of the labelled carbon in the leaves only. In 1971 we therefore decided to study the biochemical aspect of these processes, in particular to find out if metabolic degradation of the substance studied had taken place.

Abbreviations: asp. = aspartic acid; (iso)-citr. = iso-citric and/or citric acid; FDP = fructose di-phosphate; glyc. = glycolic acid; mal. = malic acid; PEP = phospho-enol-pyruvic acid; PGA = phosphoglyceric acid; PK = pyruvate kinase; RuDP = ribulose-di-phosphate; sugar-P = sugar-mono-phosphates; sugar-PP = sugar-di-phosphates.

We soon found that, if labelled serine was administered to leaf segments very little, if any, labelled serine could be recovered from the tissue at the end of the experiments. Most of the label was found to be transferred into two substances i.e. sucrose and malic acid.

This led us into a study of photosynthetic carbon assimilation by these leaves. The experiments provided ready evidence for remarkable production of C4 products as is known for C4 plants. However, in C4 plants this production enhances the water efficiency of the plants, while it is hardly conceivable to have a similar adaptive value in submerged fresh water plants. In fact, we discovered a high rate of photorespiration in *Vallisneria* (HELDER, PRINS & SCHUURMANS 1974) in contrast to its absence in C4 plants.

We have continued this line of biochemical research along with other experimental studies ever since, though in a very spasmodic way. In this paper we give the results of a fixation experiment, in which we used a single batch of plant material in order to study carbon fixation both in the light and in the dark. The results of this experiment summarize many previous results, but allow for a more quantitative comparison between fixation in both light and dark.

Along with the description and discussion some additional data from other experiments have been included. The adaptive value of the malic acid formation in submerged leaves in relation to the restricted carbon supply has been discussed in some detail.

2. MATERIAL AND METHODS

Plants of *Vallisneria spiralis* L. were grown in a sandy soil in large plastic tanks. They were illuminated by high pressure mercury lamps for 16h a day.

Experiments were started by collecting 8 healthy looking leaves from a single tank. After cleaning, the seams of the leaves were cut leaving the middle parts at a constant width of 4 mm. These parts were then sectioned into 8 segments each 5 cm in length. Then 8 sets were composed from the 64 segments obtained in such a way that each set contained a single segment from each leaf.

The 8 segments of each set were then evenly mounted in a perspex support and placed in a flat perspex fixation vessel filled with 20 ml of a 0.25 mM $CaSO_4$ solution. The vessels were placed in front of a pair of fluorescent tubes, providing the leaf material with an illumination of about 1500 Lux. In this way the leaf segments were allowed to recover from the wounding effects and restore their normal uptake capacity.

The next day the $CaSO_4$ solution was replaced by a $KHCO_3$ 10^{-2}M solution, the pH being adjusted to 8.4 by a phosphate buffer. At this pH value the concentration of the molecular CO_2 amounts to 1% of the total inorganic C present, i.e. 10^{-4} molar, which is about 10 times the concentration in air-saturated water. This concentration was high enough to make change in concentration caused by uptake into the plant material and losses to the air negligible. Moreover, the small unavoidable changes that occur could not have any appreciable effect on the rate of fixation as the concentration was hardly limiting at this value.

This unlabelled solution was renewed about one hour prior to the fixation experiment proper in order to approach steady state conditions as much as possible. The fixation experiment was then started by transferring a set from the unlabelled solution into a similar one to which a small amount of $NaH^{14}CO_3$ was added at a concentration of 0.5 mCi/ml at most. If necessary, the activity of the fixation solution was reduced by mixing labelled and unlabelled solution in the appropriate proportion.

The fixation was stopped by dipping the 8 leaf segments into 40 ml of boiling alcohol 80% after a quick rinse in unlabelled solution. However, if the fixation periods were of the order of only a few seconds, this rinse was skipped.

Boiling was continued until about 10 ml of the solution remained. Further extraction was performed at room temperature successively in 30 ml alcohol 70% for 2h, alcohol 40% for 5h and alcohol 20% over night. The extractions were pooled and evaporated to dryness by means of an air stream.

This residue was treated twice with 2 ml chloroform to remove the photosynthetic pigments together with other lipoids. This lipoid fraction was discarded because its activity was only 2% of the total activity. The insoluble fraction of the tissue contained some 6% of the total activity, so that the water soluble fraction accounted for at least 92% of the total carbon assimilated in this experiment.

All activities were estimated by liquid scintillation counting.

Small scale paper chromatography was applied to separate the water soluble fixation products using Whatman no. 1 paper sheets measuring 18 × 18 cm. The solvents used in two-dimensional chromatography were A: propanol-ammonia-water 6:3:1 and B: propyl acetate-formic acid-water 11:5:3.

In some instances paper chromatography was preceded by a separation into an acid, alkaline and neutral fraction with the aid of ion exchange columns (SPLITTSTOESSER 1969). Moreover, thin layer chromatography was used as an alternative. Using the solvents A: butyl acetate-acetic acid-water 3:3:1 and B: pyridine-ammonia-isobutanol 4:2:1, a separation of the amino acids, organic acids and sugars with some further separation of the members of each group was obtained.

Identification of the substances, which appeared on the autoradiograms, was done on the basis of R_f values and co-chromatography. Those substances, which were of crucial importance within the context of this study, were further tested by enzymatic procedures using: phosphatase, malic enzyme and pyruvic acid dikinase. Further details, when necessary, are given along with the description of the results.

3. RESULTS

In order to obtain a general view of the pattern of assimilation products sets of 8 segments from a single batch of *Vallisneria* leaves were allowed to take up and assimilate carbon from a buffered 0.01 M KHC^*O_3 solution, pH 8.4, for 5 minutes both in the light and in the dark.

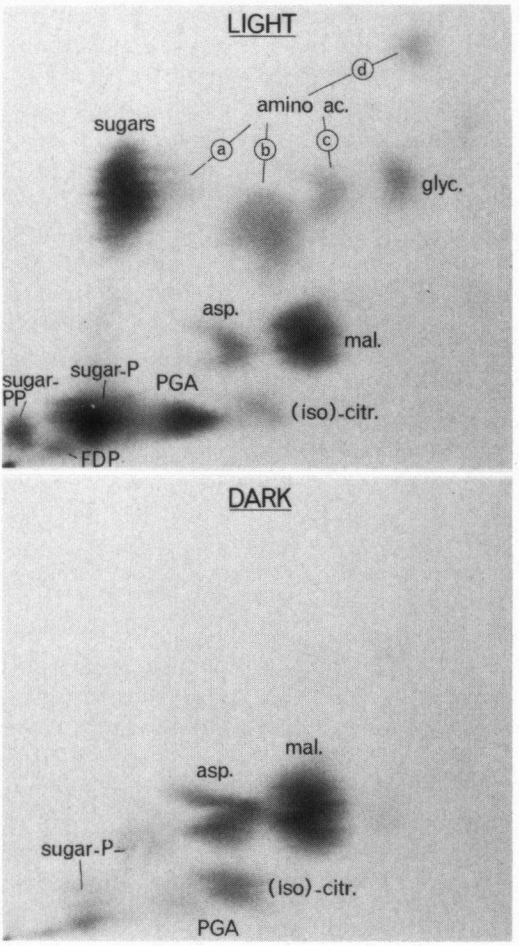


Fig. 1. Autoradiograms of carbon-fixation products in leaf segments of *Vallisneria spiralis* L.
Fixation took place for 5 min. in a 10 mM $KHCO_3$ solution, pH 8.4, containing per ml 0.5 mCi C-14 in the dark and 0.05 mCi in the light.
PGA = phosphoglyceric acid; sugar-P = monophosphorylated sugars; sugar-PP = diphosphorylated sugars; sugars = sucrose, fructose and glucose; mal. = malic acid; glyc. = glycolic acid; asp. = aspartic acid; amino acids: a = asparagine; b = serine, glutamine, glutamic acid; c = proline, alanine; d = phenylanaline, leucine.

As the uptake and assimilation rate is much stronger in the light than in the dark, the activity of the solution used for the light experiment was lowered from 0.5 mCi/ml down to 0.05 mCi/ml, the advantage being that the extracts from the light and dark material could be further manipulated in exactly the same way.

The results for the light experiment are given by the autoradiogram depicted in *fig. 1A*. The experimental period was long enough to produce the full range

of the water soluble compounds. It is only the relative distribution of the compounds which may vary from one experiment to the other, being influenced by internal and external conditions, the duration of the experiment etc.

As the Calvin cycle is operative in the light a great many spots on the autoradiograms could readily be identified as intermediates of this cycle. Moreover, a phosphatase treatment on an aliquot of the extract hydrolysed all phosphate esters. A check by paper chromatography showed that all the corresponding spots had disappeared, whereas darker spots for glyceric acid, glucose and fructose and also sucrose, though less convincingly, could be shown.

In most experiments the triose-phosphates were barely visible, although they must have become labelled too. On the other hand, some dephosphorylation may have occurred spontaneously during the extraction, causing an increase in the normal sugars.

The identification of the other compounds was facilitated by separating the extract in an alkaline, acid and neutral fraction and by an analysis of the average abundancy of the amino acids and organic acids normally present in *Vallisneria* leaves. Simple checks were made by co-chromatography of labelled and unlabelled reference substances. More rigid checks were executed afterwards, if necessary.

Aspartic acid and asparagine are present in *Vallisneria* leaves far in excess (50–60% of total amino acid content) of any other amino acid. They prove also to be produced rapidly in our fixation experiments with labelled carbon. From the amino acid analysis, mentioned above, we also know that glycine and glutamic acid (including glutamine) account for some 10% of total amino acid content each. The abundance of the remaining acids are in the range below 6% (serine, alanine 5–6%; cystine, threonine 3%; leucine, cystine, cysteine 2%). Some of these amino acids are readily labelled too, but their precise identification was limited because of their poor separation on the paper with the solvents used. The spots represent clusters of amino acids rather than separate amino acids, with the exception of aspartic acid.

As to the organic acids, it is not the acid most abundantly present in *Vallisneria* leaves, i.e. oxalic acid (75%), which became apparent on our autoradiograms. It proved to be malic acid and to a lesser extent (iso-) citric acid and glycolic acid. Citric acid and oxalic acid are not well separated by our normal solvents. By applying a one-dimensional separation on the substances isolated from a citric acid spot using another solvent, we were able to exclude oxalic acid as well as tartaric acid by co-chromatography (HARBORNE 1973).

From these intriguing data, which show a rapid production of malate and aspartate from labelled CO_2 in the light, we could not but conclude the presence of a C4-fixation mechanism. This was soon corroborated by the results from dark fixation experiments as is illustrated by *fig. 1B*, the results of which can be directly compared to those of *fig. 1A* as they were done starting from the same batch of plant material under identical conditions except for the light.

Clearly, malic, aspartic, citric and/or iso-citric acid are all produced in the dark as well as in the light, though at a reduced rate. On the other hand aspara-

gine and the other amino acids were absent in the dark. Obviously, the same applied to the intermediates of the Calvin cycle. Still, small amounts of labelled phosphorylated compounds, including PGA, were present.

In a separate experiment we studied the dark fixation for 20 seconds after a variable stay in the dark ranging from 0 to 30 minutes. In all cases labelled PGA was produced, and no significant decline of the PGA production could be demonstrated. This result may point to some residual activity of the RuDP-carboxylase or to a rapid formation of PGA from C4 products. In the latter case one would expect the presence of some labelled PEP, as this compound is a plausable intermediate for the PGA found.

Unfortunately, our chromatographic methods did not separate PEP from citrate, tartarate and oxalate well enough, if we started from leaf extracts. Therefore, it was necessary to demonstrate the presence or absence of labelled PEP in the extracts or in the 'citrate'-spot in another way. This was done by hydrolysing PEP from the extracts with the aid of pyruvate kinase or by boiling with HCl.

We started from 2 ml samples of an unlabelled 0.1 mM PEP solution. These were boiled after the addition of 2 ml HCl 2N. The amount of inorganic phosphate could be determined after the removal of the excess HCl. In this way we found hydrolysis to be completed within 60 minutes.

If a labelled PEP solution of the same concentration was used and paper chromatograms were made from it at the start and at the end of the hydrolysis we found that virtually all labelled PEP had been degraded. However, very little if any pyruvate could be shown instead. In fact, almost all activity had been lost from the solution. This loss could be prevented by the addition of phenyl hydrazine before boiling. Some 90% of the activity was then recovered.

If this acid hydrolysis was applied to an aliquot of the extract and chromatograms before and after hydrolysis had been prepared, no difference could be seen between these chromatograms and no measurable loss of activity could be demonstrated.

In a similar way we studied the hydrolysis with the aid of PK starting from unlabelled and labelled PEP solutions. No loss of activity occurred with this procedure and the formation of labelled pyruvate could easily be shown by chromatography. No labelled pyruvate could be shown, however, if we started from an unlabelled PEP solution to which the activity from the "citrate spot" was added. However, if we used a labelled PEP solution instead of the unlabelled one, pyruvate could be shown. This proved that the negative result mentioned was not due to some interfering factor present in the citrate spot. Such a factor interfering with the enzyme, must have been present in the extract as a whole, as no hydrolysis occurred if extract was added to a labelled PEP solution.

We can only conclude that labelled PEP was absent in the extracts of the dark fixation experiments, in spite of the fact that small quantities of labelled PGA were found. This points to a residual activity of the RuDP-carboxylase and the presence of sufficient RuDP in the dark.

Extending the fixation period will gradually increase the number of labelled

compounds. This relates in particular to the intermediates of the Calvin cycle. The overall result was that dark fixation soon produced a pattern of labelled compounds on the autoradiograms which can hardly be distinguished from that produced by light fixation.

In contrast, even a one hour fixation in the light did not materially change the pattern from that shown in *fig. 1A*. As a matter of fact, the pattern was already obtained after a light fixation period as short as 5–10 seconds and it was only in 1 sec. fixation experiments that we could show PGA to be the first product. After 2–3 sec. malate became discernable too. These results make it very unlikely that the PGA in the light is synthesized from labelled CO_2 originating from labelled malate by decarboxylation as in the case with C4 plants.

From the data on the activity present in the labelled compounds in the experiment illustrated in *figs 1A* and *1B* we calculated that some 600 nmol CO_2 had been fixed in the light in 5 min. and 26 nmol CO_2 in the dark. Therefore, dark fixation amounted to some 4% of the fixation in the light.

The compounds found in the dark are mainly the result of C4 fixation. Their activity may be compared with the activity in the corresponding compounds i.e. malate, aspartate and citrate formed in the light. From this we calculated C4 fixation in the dark to amount to nearly 20% of the C4 fixation occuring in the light.

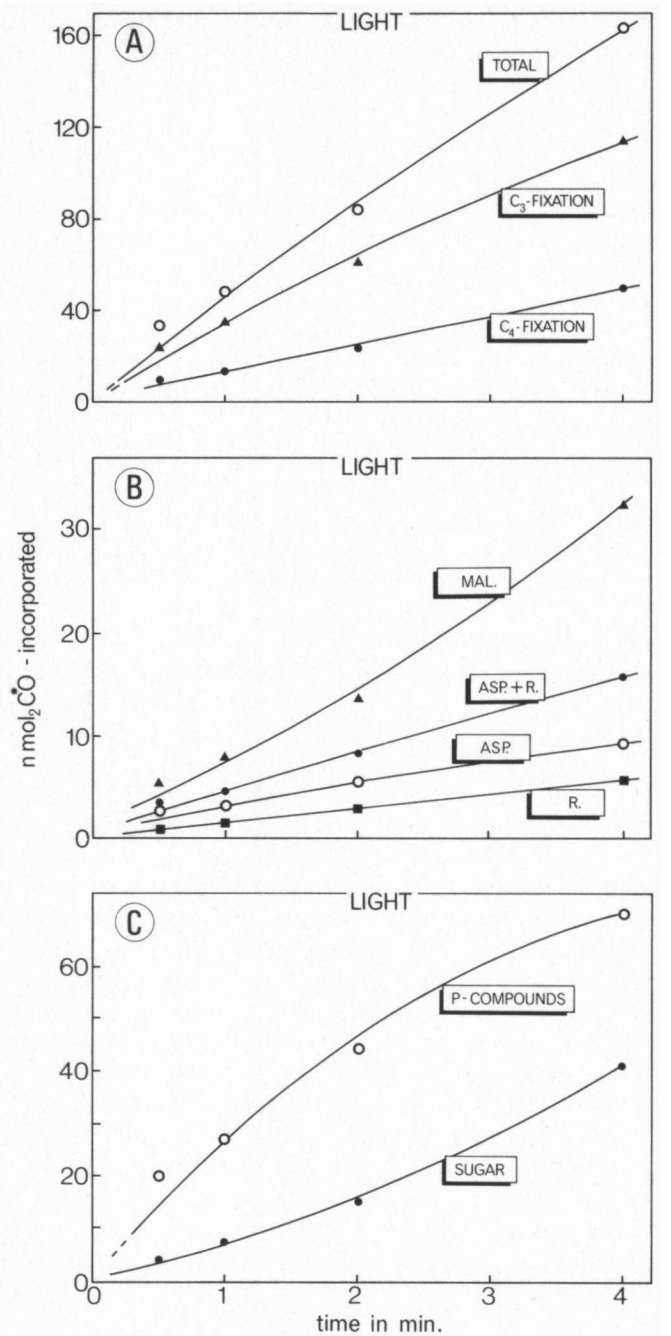
These figures are based on the amounts of labelled CO_2 incorporated. Therefore, they also represent the amount of compounds synthesized, provided a single C atom has been incorporated into each molecule newly formed.

Similar and more detailed information could be drawn from experiments on the time course of the labelled CO_2 assimilation in either the light or the dark. The results are depicted in *figs. 2* and *3*, respectively. The light and the dark experiments were carried out successively with two different batches of plant material, taken however, from the same container. These leaves had a much lower assimilation rate than the leaves of the previous experiment. However, comparison of the results of the light and the dark experiment produced almost the same conclusions arrived at in the former experiment.

We calculated from the initial slopes of the curves a total fixation rate in the light of 43 nmol CO_2 per minute (*fig. 2A*). The dark fixation rate amounted to 7% of this value (*fig. 3A*). Again, dark fixation was almost exclusively by way of the C4-mechanism, but some residual activity of the Calvin cycle was still present. C4 fixation in the dark amounted to about 25% of that found in the light. C4 fixation in the light represented, in turn, 20–25% of total fixation.

The results found for the sugars and phosphorylated compounds formed in the light were quantitatively in accord with a precursor-product relationship (*fig. 2C*). A similar relationship was suggested by the data for aspartate and the remaining labelled compounds, which comprised the other amino acids and organic acids among which citrate/isocitrate was far the most abundant (*figs. 2B, 3B*).

In contrast, malate did not show any turnover in the dark, whereas in the light even an increased rate of carbon incorporation into malate became appar-


A
LIGHT
TOTAL
C_3-FIXATION
C_4-FIXATION
160
120
80
40
0
B
LIGHT
MAL.
ASP.+R.
ASP.
R.
30
20
10
0
n mol $^{14}CO_2$ - incorporated
C
LIGHT
P-COMPOUNDS
SUGAR
60
40
20
0
0
1
2
3
4
time in min.

ent, which may be ascribed to the gradual appearance of two or more labelled C atoms in the malate molecules formed, as will be discussed in greater detail below.

4. DISCUSSION

The present findings corroborate most of the reports from literature on the photosynthetic mechanism of submerged Angiosperms, which has received ever increasing attention during the last few years (BENEDICT & SCOTT 1976, VAN, HALLER & BOWES 1976, DE GROOTE & KENNEDY 1977, HOUGH & WETZEL 1977, BROWSE , DROMGOOLE & BROWN 1977, WINTER 1978, ANDREWS & ABEL 1979, HOLADAY & BOWES 1980, BEER & WETZEL 1981).

The production of significant amounts of C4 acids as observed in our experiments raised the question whether the C4 photosynthetic mechanism present in C4 plants is also involved in submerged aquatic plants. If this applies, malate would be formed prior to PGA. We found, however, that PGA appeared prior to malate on our autoradiograms as was also reported by STANLEY & NAYLOR 1972. However, malate is formed simultaneously and at such a rate that 1 second pulses were necessary to demonstrate PGA to be the very first labelled compound. This makes its formation from labelled malate very unlikely. Nevertheless, we realize that rapid formation of both PGA and malate renders it possible that in some experiments malate may become discernable prior to PGA, especially in plant material where C4 fixation processes are quantitatively important and malate is accumulated in contrast to PGA.

Additional information came from a few pulse-chase experiments not dealt with above. Labelled PGA formed in 1–2 second pulses is completely converted into sugar and malate, whereas no decline in labelled malate has become evident from any of our experiments (ANDREWS & ABEL 1979, HOLADAY & BOWES 1980, BROWSE, BROWN & DROMGOOLE 1980). Moreover, *Vallisneria* leaves show surprisingly high photo- and dark respiration rates (HELDER, PRINS & SCHUURMANS 1974), resulting in high compensation points. In this respect *Vallisneria* resembles most other aquatic Angiosperms so far examined (VAN HALLER & BOWES 1976, JANA & CHOUDHOURI 1979). Consequently, there is now general agreement that the investigated aquatic Angiosperms are basically C3 plants.

We like to stress that external and internal conditions have a marked effect on the quantitative aspects of the results obtained in these experiments. This may lead to conflicting results among various investigators. However, we wish to stress the general pattern, so our present discussion is restricted to those experimental results obtained under conditions of ample carbon supply (10 mM

Fig. 2. Time course of carbon-fixation in the light by leaf segments of *Vallisneria spiralis* L.
The data are based on activity measurements of single or combined labelled compounds obtained by paper chromatography. The results for C3-fixation are given in A and C, for C4-fixation in A and B. R represents all organic and amino acids with the exception of malic acid and aspartic acid. They are supposed to originate from aspartic acid (compare *fig. 3*).

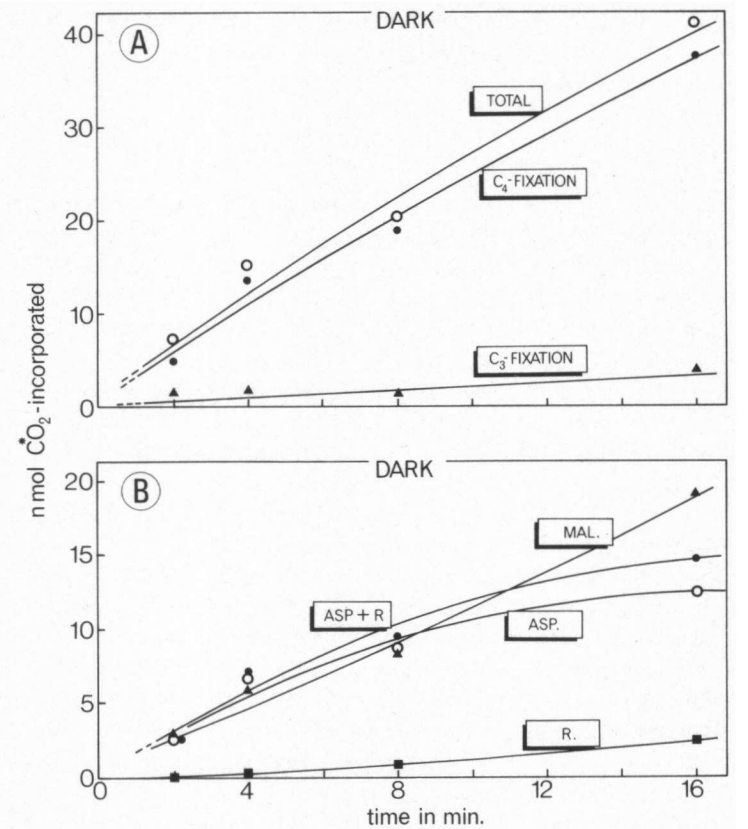


Fig. 3. Time course of carbon-fixation in the dark by leaf segments of *Vallisneria spiralis* L.
The products of C3-fixation have not been split up into sugars and phosphorylated compounds as was done for the light fixation experiment (*fig. 2*) in view of the small amounts involved.
As in *fig. 2*, R represents all organic and amino acids with the exception of malic and aspartic acid. However, here it represents mainly (iso)-citric acid.

$KHCO_3$, pH 8.2). In our light experiments leaf segments were exposed to some 3000 lux fluorescent light and all experiments were carried out at 20–22 °C. We start with a discussion of the results depicted in *figs. 1, 2* and *3* and include some previous results for the sake of completeness.

The Calvin cycle is normally considered to be absent in the dark, due to the absence of light-activation of the enzymes involved. However, in short labelling periods we always found a slight amount of labelled PGA and after longer periods all intermediates from the cycle became apparent (BROWSE, BROWN & DROMGOOLE 1980). We concluded that inactivation was never complete, neither immediately after turning off the light nor after a prolonged stay in the dark. The results rather suggest a continued though much reduced activity of the cycle, the reduction being due to reduced ATP and RuDP levels, increased competi-

tion for the RuDP carboxylase by inorganic ions and organic compounds and inactivation of vital enzymes of the cycle (KELLY, LATZKO & GIBBS 1976).

As phosphorylated hexoses were found soon after the appearance of PGA, whereas the production of sucrose was somewhat retarded, the well-known inactivation of the rate limiting fructose-diphosphate phosphatase may have come into play in our dark experiments.

As was mentioned before, label from compounds of the Calvin cycle, in particular label from PGA, was transferred to malate. The simplest explanation for this transfer is the assumption of a conversion of PGA into PEP, which is subsequently carboxylated into oxaloacetic acid and then reduced to malic acid.

This hypothesis was tested in various ways. First of all by carrying out some rigorous tests for the presence of labelled PEP in our extracts. These tests were necessary as labelled citric acid proved to coincide almost completely with PEP on our autoradiograms. As indicated before, they gave excellent results on labelled reference PEP solutions, but did not produce any positive result for our extracts from both dark and light experiments. Therefore, label must have been derived from secondary C3 fixation products (BROWSE, BROWN & DROMGOOLE 1980, OSMOND 1978).

It remains possible that the steady state level of PEP was too small to be detected. In that case, however, the small amounts of PEP available for C4 fixation would soon have become completely labelled. This would have led to an early production of double labelled malate and aspartate.

A few experiments have been carried out to trace the labelling pattern of malate in time by splitting off the C4 carbon with the aid of malic enzyme (Anneke Bosgraaf, unpublished results). The amount of label in the C4 atom of malate after a 1 minute fixation period in the dark amounted to 83% of total labelling i.e. 17% of the label was present in the C1 atoms of the newly formed malate molecules. This distribution changed slowly until after half an hour an almost constant distribution of 70% against 30% was reached (*fig. 4*).

Starting from a small PEP pool one would expect a much quicker change down to a fifty-fifty ratio, as single labelled malate molecules would be formed in the very beginning of the experiment only. In fact, the results can be explained more easily by the action of fumarase which tends to randomize the label distribution between the C4 and C1 atoms, along with a constant production of new C4 labelled malate by C4 fixation. Unfortunately, the enzymatic analysis does not discriminate between a mixture of single-labelled C1 molecules and single-labelled C4 molecules, and double-labelled C1 and C4 molecules. In principle, mass spectroscopy could settle the matter, but the amount of newly formed malate relative to the vast amounts already present are very small.

Still another piece of evidence for the formation of labelled PEP can be derived from the apparent increase of labelled malate production. This production is normally expressed by the rate at which labelled CO_2 is incorporated. At the start of a labelling experiment each malate molecule formed corresponds to a single-labelled CO_2 molecule. However, as soon as PEP would have become labelled, double-labelled malate molecules will be formed, i.e. two labelled CO_2

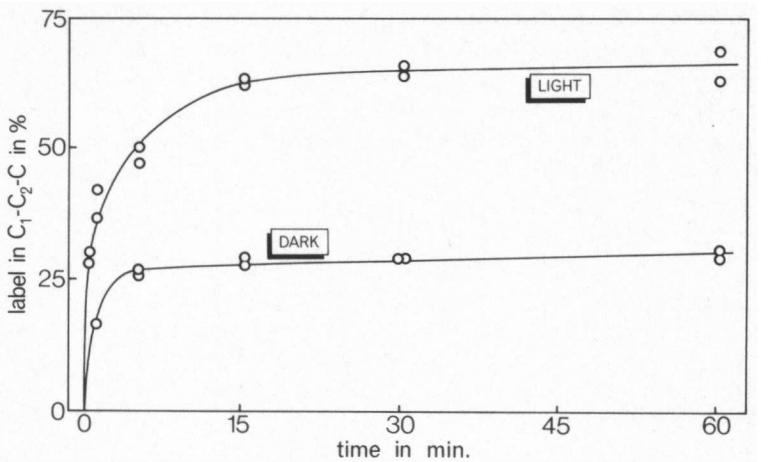


Fig. 4. Time course of label-distribution in malic acid formed by carbon fixation in the light and the dark by leaf segments of *Vallisneria spiralis* L.

The plant material was allowed to take up labelled carbon and fix it. The malate formed was isolated by paper chromatography, added to an unlabelled malate solution and subjected to a malic enzyme treatment. The activity found in the remaining C_1-C_2-C_3 moiety of the labelled malic acid molecule was expressed as a percentage of the initial activity of the intact molecules.

The label will first appear in the C4 atom. However, by the action of fumarase the label will be transferred to the C1-atom, causing a tendency to rise the percentage value from 0 up to 50. If the 50% value is surpassed. the C2 or C3 atom must have become labelled, too, as a result of a more complicated biochemical pathway.

molecules are incorporated in each malate molecule newly formed, suggesting an increasing rate of malate production.

As our experiments were carried out under constant conditions and for short periods only, we may assume steady state conditions to prevail. This involves a constant rate of malate production within the limits of our experiments. In the dark we never saw a gradual apparent increase of malate production. In fact, the rate was constant, indicating that the malate produced was not further metabolized either.

By contrast, in the light there was always an apparent slight increase in the rate of malate production, if it was expressed in labelled CO_2 incorporated. This suggests, that double-labelling did occur gradually. This, in turn, points to the appearance of labelled PEP. However, in view of the assumed smallness of the PEP pool, mentioned above, one would expect a much faster increase up to the final value of twice the initial rate of malate production.

A futher point of scepticism may be derived from the data on the distribution of the label in the malate molecules formed in the light. Most of the label was found to be in the C4 position after short periods: e.g. about 70% after 30 seconds; this percentage decreased in time as occurred in the dark extracts and

reached the 50% value within some 5 minutes. However, the value then further declined to some 30% in two 1 hour-experiments.

This is completely at variance with the assumption of a simple C4 fixation. It rather stresses the conclusion arrived at before that malate is also formed via a longer pathway, which will gradually lead to the production of uniformly labelled malic acid. Participation of the Krebs cycle therein seems a plausible assumption, provided labelled acetyl-groups are furnished to the cycle.

We have excluded aspartate from the reasoning just given, because the increase of labelled aspartate content slowed down in all experiments in contrast to the labelled malate. Obviously, malate is excluded from further metabolization, most likely by being accumulated in the vacuole, whereas asparate is converted into other substances such as amino acids and organic acids, mainly citric acid. The rate of synthesis of aspartate can be estimated from the initial slope of the curves. We can also add amounts of the remaining compounds produced from aspartate to the amount of labelled aspartate. In this case, an almost rectilinear curve can be obtained (compare results with *Egeria* by BROWSE, BROWN & DROMGOOLE 1980), although some decline still remained, most probably due to losses of volatile compounds, in particular respiratory carbon dioxide (KELLY, LATZKO & GIBBS 1976).

Both the loss of carbon dioxide and the delayed formation of citrate point to involvement of mitochondrial activity. It is known that aspartate can be taken up by the mitochondria and then be converted back to oxaloacetic acid which may be degraded to pyruvic acid and carbon dioxide or converted into other compounds of the Krebs cycle. Citrate, being a transport metabolite, will leave the mitochondria and pile up in the vacuoles.

This is in harmony with our analysis of the organic acids accumulated in *Vallisneria* leaves and with the results of experiments in which leaves were allowed to assimilate labelled CO_2 in the presence of K_2SO_4. This salt causes an excess cation uptake which is known to be accompanied by the production of organic acid anions. Both labelled malate and citrate or iso-citrate are then synthesized at an increased rate.

As the synthesis of both malate and aspartate depends on the same fixation product oxaloacetate it is understandable that the production of these compounds is reduced simultaneously in the dark. However, the rate ratio between malate and aspartate production dropped from 1.2 in the light down to 0.6 in the dark. It follows that malate synthesis from oxaloacetate is more sensitive to light than aspartate. One reason might be a lower steady state level of the precursor, owing to a reduced supply of PEP and/or inactivation of the PEP carboxylase. It presupposes a higher K_m value for the dehydrogenase than for the transaminase with respect to oxaloacetic acid. Even more important may be the reduced NADH supply in the dark, to which only malate synthesis is susceptible. In short, various product-precursor relationships involved, can be imagined and requires further investigation (HOLADAY & BOWES 1980).

The reduced production of aspartate in the dark is accompanied by and may be the cause of a reduced conversion of aspartate into a number of labelled

substances. However, many other explanations can be given for this reduction e.g. an enhanced respiratory break-down of the compounds produced.

Summing up, *Vallisneria spiralis* L. is a C3 plant. However, the high level of C4 acid production and the accumulation of malate into the vacuoles are reminiscent of the acid metabolism of Crassulacean plants. Moreover, we have evidence from experiments, which are now in progress, that accumulated malic acid can be drawn on if the plant material is subjected to very low CO_2 concentrations, enabling it to function, to some extent, as a CO_2 reservoir (BEER & WETZEL 1981).

ACKNOWLEDGEMENTS

We owe a great debt to some of our students who were either in at the start or subsequently contributed to our research with *Vallisneria spiralis* L., in particular, Aris Otzen, Koos Hettinga, and Anneke Bosgraaf.

We are grateful to Winy Wierenga for all the preliminary experimental work she undertook during the period 1974–1978, and to Janet O'Brien for reading the text and correcting the English.

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