C_4-Metabolism in Marine Brown Macrophytic Algae

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Marine brown macroalgae including *Macrocystis integrifolia, Nereocystis luetkeana, Lessoniopsis littoralis, Laminaria saccharina, Fucus serratus* and some further representatives of the Laminariales and Fucales (Phaeophyta) have been investigated with respect to their remarkably high potential for β-carboxylation of phosphoenolpyruvate supplementing photosynthetic CO_2 fixation. Kinetic tracer studies indicate that ^{14}C-labelling of C_4 acids such as aspartate and malate is not restricted to dark periods, but also occurs during photosynthesis. Rates of carbon fixation into C_4 compounds are approximately equal in the light and in the dark. Distribution of ^{14}C between C_1 and C_4 atoms of aspartate suggests carbon flow from early occurring photosynthates such as 3-phosphoglycerate to C_4 compounds including aspartate and malate. In brown macroalgae dark carbon fixation *via* β-carboxylation of phosphoenolpyruvate is therefore assumed to be quantitatively and qualitatively integrated into photosynthetic CO_2 assimilation thus yielding appreciable ^{14}C-labelling of C_4 dicarboxylic acids. The underlying reactions and conversions are basically different from C_4 photosynthesis and should preferably be termed as C_4 metabolism.

Introduction

The basic questions surrounding the pathway of inorganic carbon assimilation during photosynthesis have been resolved by experiments mostly using unicellular green algae [1]. The essential features of the reactions and conversions proposed have later been extended to all photoautotrophic organisms. Only one decade after finally establishing the universally operating reductive pentose phosphate cycle providing photosynthetic carbon fixation and reduction, a further carboxylating mechanism involved in light dependent harvesting and assimilation of inorganic carbon was found [2]. A complete biochemical reaction scheme to account for the particular pattern of ^{14}C-labelled C_4 dicarboxylic acids observed in ecologically specialized plant species immediately after exposure to $^{14}CO_2$ has been presented [3]. Although initially discussed as a possibly alternative pathway, the series of metabolic reactions constituting the so-called C_4 pathway of photosynthesis is now widely understood to be superimposed on the indispensible reactions of the reductive pentose phosphate cycle. Since its original description, C_4 photosynthesis basically involving the β-carboxylation of a C_3 acid to C_4 compound(s) is not restricted to terrestrial vascular plants, but was also reported to occur in a seagrass [4], in unicellular prokaryotic and eukaryotic algae [5–7] as well as in seaweeds in general [8].

Among marine algae, particularly brown macrophytes belonging to the orders Laminariales and Fucales were recognized to exhibit notibly high potentials for β-carboxylation of phosphoenolpyruvate. This process has hitherto mostly been quoted as dark or non-photosynthetic carbon fixation [9–12]. It is now accepted that the enzyme performing the respective carboxylation of a C_3-unit to give the C_4-compound oxaloacetate is a phosphoenolpyruvate carboxykinase [13–15]. Temporally and spatially different distribution of enzyme activity attributed to β-carboxylation *in vitro* as well as *in vivo* was encountered with ontogenetically different frond areas, topographically different frond tissues, and various developmental stages in the life cycle of macrophytic brown algae [16–18]. The peculiar interface between β-carboxylation and photorespiration has been characterized for a variety of species [19].

The present investigation has been undertaken to evaluate the problem as to what extent β-carboxylation *via* phosphoenolpyruvate carboxykinase might definitely contribute to total inorganic carbon assimilation. In particular, experiments have been conducted to determine i) whether β-carboxylation is fully independent from photosynthesis and therefore continues in the light without rate depression, ii) which are the minimal rates of β-carboxylation during photosynthesis, and iii) from which source is

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the specific substrate of phosphoenolpyruvate carboxykinase metabolically derived in the light. The findings presented suggest that marine brown algae possess C_4 metabolism rather than C_4 photosynthesis.

Materials and Methods

Organisms

Macrocystis integrifolia Bory, *Nereocystis luetkeana* (Mert.) P. & R., *Lessoniopsis littoralis* (Tild.) Reinke, *Cymathere triplicata* (P. & R.) J. Ag., and *Eisenia arborea* Aresch. (Laminariales, Phaeophyta) were collected from kelp stands in Barkley Sound, Vancouver Island, B. C., Canada near Bamfield Marine Station. *Laminaria digitata* (Huds.) Lamour., *Laminaria saccharina* (L.) Lamour. (Laminariales) as well as *Ascophyllum nodosum* (L.) Le Jol. and *Fucus serratus* L. (Fucales, Phaeophyta) originated from the rocky shores of Helgoland, North Sea, German Bight, where they were collected during low tide. Experiments were performed with carefully checked specimens within 6 – 12 h after collection and maintaining in running seawater at ambient temperature.

CO_2 Fixation

Sections of about 2 cm diameter or 2 × 1 cm edge length were cut from the leafy parts of the fronds, repeatedly rinsed in membrane-filtered seawater and equilibrated in the light (photon flux density 200 μE m^{-2} s^{-1}; 15 min) or in the dark (30 min) at ambient temperature (10 – 15 °C). Following the equilibration period, frond samples were submerged in a $H^{14}CO_3^-$-containing seawater medium (50 μCi/100 ml; specific activity > 55 mCi/mM) and allowed to fix the radioactive bicarbonate for various lenghts of time. The assimilation was terminated by immersion of the samples into liquid N_2. Carbon fixation rates were calculated by measurements of acid-stable ^{14}C incorporation and referred to the total inorganic carbon content (2.7 mM) of the incubation media used.

Enzyme extraction and assays

Frond samples (1 – 5 g fresh weight) were deep frozen in liquid N_2 and homogenized by grinding with quartz sand adding polyvinylpolypyrrolidone, EDTA, D-araboascorbate, $MgCl_2$, DTT and Triton-X-100 to the still frozen mixture. The resulting supernatant obtained after centrifugation at 40 000 × *g* and 4 °C was regarded as crude enzyme extract and used for assays of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) and phosphoenolpyruvate carboxykinase (EC 4.1.1.31). For details on the extraction and assay procedures see Kremer and Küppers [11].

Extraction and chromatography of ^{14}C-labelled compounds

Frond samples ^{14}C-incubated in the light or in the dark were exhaustively extracted in aqueous ethanol (50%) and a mixture of methanol-chloroform-formic acid (6 *N*) = 12:5:1. The combined extracts were partitioned into an aqueous/alcoholic and a chloroform phase and counted for total ^{14}C-activity incorporated. The aqueous phase was evaporated to dryness *in vacuo*, redissolved in 30% ethanol, and further analyzed by thin-layer chromatography on cellulose (MN 300, 200 – 400 μm layer thickness), either directly or following pre-fractionation into neutral, anionic, and cationic compounds by passing through columns of Dowex-50 (H^+) and Dowex-1 (formate) resins. Details on these procedures have already been described in an earlier technical paper [20]. Distribution of radiocarbon between the C_1 and C_4 atoms of aspartate was determined by degradation of the chromatographically isolated compound to β-alanine using a ninhydrin treatment according to ref. [21].

Results

Gross patterns of carbon fixation

Fig. 1 describes the time courses of carbon fixation in the light and in the dark for two macrophytic brown algae including the giant kelp, *Macrocystis integrifolia*, for incubation periods ranging up to 90 min. Several features are noteworthy. All frond regions specifically investigated reveal linearity of CO_2 incorporation. Some depression of the almost constant rates is observed after longer exposure periods exceeding 3 – 5 h (data not included in Fig. 1) thus suggesting presumable depletion of inorganic radiocarbon in the incubation medium. Photosynthetic rates in ontogenetically young (*i.e.* growing) frond areas comprising the meristematic intercalary region of the leafy thallus part account

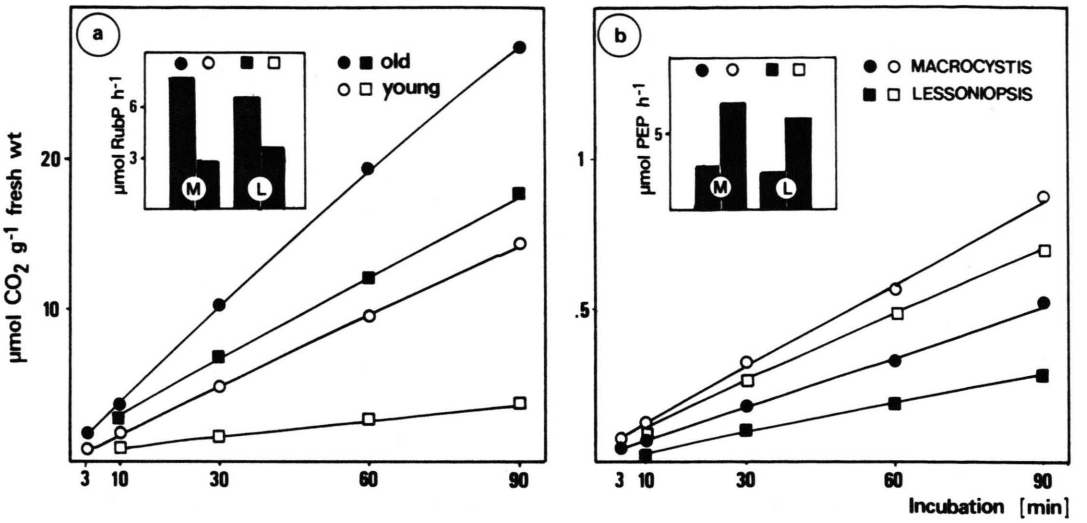

Fig. 1. *Macrocystis integrifolia* (M), *Lessoniopsis littoralis* (L). Rates of photosynthetic (a) and light independent = dark (b) carbon assimilation in growing (= young) and differentiated (= old) frond areas. Inserts: Activity of RubP-C and PEP-CK, respectively, in µmol substrate carboxylated on a fresh weight basis.

for generally less than 60% of those recorded for fully differentiated tissue. It is also obvious from Fig. 1 that the frond samples investigated show inorganic carbon incorporation and fixation into acid-stable organic compounds even in the dark. On the average, in adult frond areas about 5% of maximal carbon assimilation in the light are due to non-photosynthetically performed CO_2 uptake. In growing zones, this proportion is considerably higher. Expressed in terms of relative as well as absolute amounts of radiocarbon incorporated, β-carboxylation as measured by *in vivo* dark carbon assimilation is more significant in growing (young) than in adult, non-growing frond areas.

Activities of the respective enzymes responsible for photosynthetic and dark carbon fixation as determined by *in vitro* carboxylation are inserted in Fig. 1. Again, appreciably higher activity of phosphoenolpyruvate carboxykinase (= PEP-CK below) is confined to meristematic frond regions, whereas more activity of ribulose-1,5-biphosphate carboxylase (further quoted as RubPC) is encountered with the fully differentiated parts. The gross pattern of *in vivo* and *in vitro* carbon fixation is thus basically the same, though the enzymatic carboxylation performed with the isolated system cannot account for the equivalent rates obtained from intact frond samples. Very similar results have been found for all further algal species investigated in this connection.

Assimilates ^{14}C-labelled in the dark

In contrast to photosynthesis, radiocarbon introduced *via* β-carboxylation in the dark is recovered from only a few low-molecular weight assimilates as is shown in the autoradiograph (Fig. 2). Among them, aspartate along with malate and some further compounds including alanine and glutamate as well as citrate and fumarate form the bulk of ^{14}C-labelled material. Traces of other assimilates mostly representing intermediates of the tricarboxylic acid cycle are also encountered. Fig. 3 includes data demonstrating the time course of ^{14}C-labelling in the dark. Kinetics of ^{14}C-incorporation into individual compounds suggest that the C_4 acids aspartate and malate occur among the first labelled metabolites, whereas other dark assimilatory products receive radiocarbon distinctly later. This observation is consistent with the assumption that the reaction product of β-carboxylation *via* PEP-CK is oxaloacetate which then is immediately converted to aspartate by transamination and to malate upon a simple reduction step (*cf.* [10, 12]).

Photosynthetic ^{14}C-labelling of C_4-compounds

Under steady state conditions of photosynthesis, certain amounts of ^{14}C are consistently recovered from aspartate and malate even after short-term exposure of frond samples to $H^{14}CO_3^-$ in the light. Dis-

tribution of photosynthetically fixed ^{14}C among early ^{14}C-labelled metabolites is exemplified in Fig. 4 for growing and fully differentiated frond areas of *Laminaria saccharina* and *Fucus serratus*. It may be seen that percentage of ^{14}C-labelling in organic phosphates including diverse sugar phosphates and 3-phosphoglycerate rapidly decreases from approximately 80% to about 25% of total ^{14}C recovered from soluble photosynthates. Kinetics of ^{14}C-labelling thus show a characteristic negative slope curve. Time dependent shifts in percentage ^{14}C-labelling of C_4-compounds such as aspartate and malate are basically different. Both photosynthates achieve increasing percentage up to 10–30 s after exposure to $H^{14}CO_3^-$. Hence, maximum relative ^{14}C-labelling confined to these assimilates is distinctly delayed as compared to the organic phosphates. Again, it is interesting to note that in young, growing frond areas the initial photosynthetic ^{14}C-labelling of aspartate and malate exceeds that recorded for samples not taken from the intercalary (*Laminaria*) or apical (*Fucus*) growing zones.

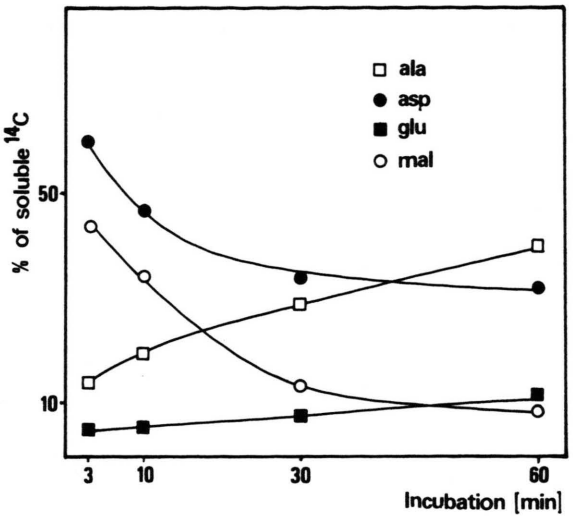

Fig. 3. *Laminaria digitata*. Time dependency of ^{14}C-labelling in single assimilates during dark incubation. For abbreviations see Fig. 2.

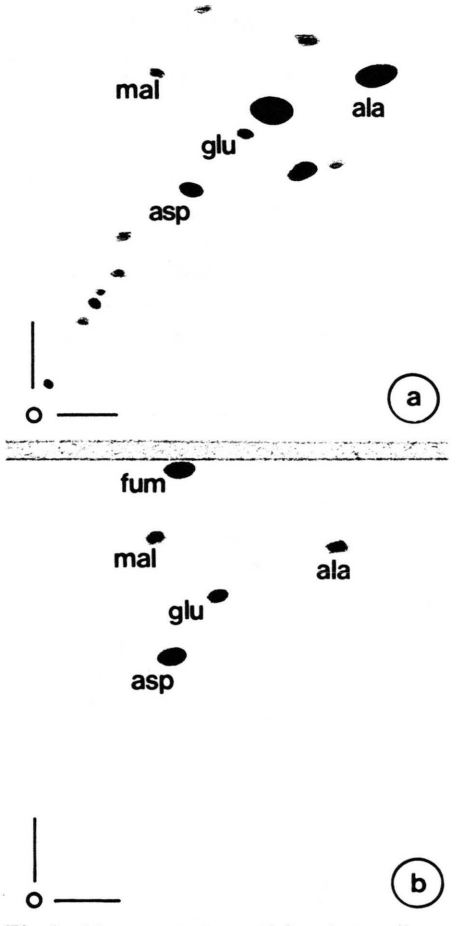

Fig. 2. *Macrocystis integrifolia*. Autoradiographs showing the patterns of photosynthetically (a) and non-photosynthetically (b) ^{14}C-labelled assimilates as demonstrated after two-dimensional TLC: Dark assimilates are qualitatively integrated into pattern of photoassimilatory compounds; asp = aspartate, glu = glutamate, ala = alanine, mal = malate, fum = fumarate. For further details (*cf.* ref. [20]).

Position of radiocarbon in ^{14}C-aspartate

Provided that ^{14}C-labelling of aspartate occurring in the assimilate pattern after photosynthesis and light independent carbon fixation is actually initiated by the carboxylation of phosphoenolpyruvate *via* PEP-CK, then it might be assumed that the distribution of ^{14}C between both carboxyl groups must be helpful for some insight into the metabolic origin of phosphoenolpyruvate required as a carboxylation substrate. The results of appropriate experiments and analyses are shown in Fig. 5. Most of the ^{14}C-activity of aspartate, photosynthetically ^{14}C-labelled during short-term photosynthesis, is released upon ninhydrin-mediated decarboxylation to β-alanine. This indicates preferential location of ^{14}C in the C_1-atom (= α-carboxyl group) of aspartate. After longer photosynthetic incubation exceeding 2–3 min the percent ^{14}C-activity, which can be removed by nin-

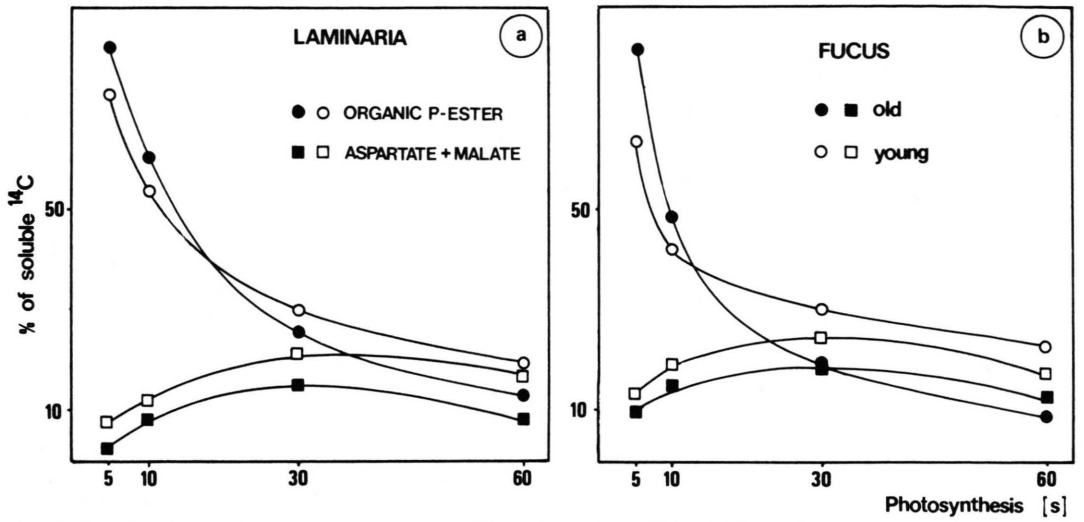

Fig. 4. *Laminaria saccharina, Fucus serratus.* Time dependent ^{14}C-labelling of C_4-acids (aspartate amd malate) and organic phosphates in growing (= young) and differentiated (= old) frond areas after short-time photosynthesis.

hydrin, distinctly decreases to approximate an apparent theoretical level of 50%. This suggests increasing ^{14}C-amounts within the β-carboxyl group of the aspartate molecule which must be due to β-carboxylation. Symmetrical ^{14}C-labelling of the C_1 and C_4-atoms of aspartate therefore suggests β-carboxylation progressing in the light during photosynthesis. From aspartate ^{14}C-labelled in the dark rather low amounts of radiocarbon can be released by ninhydrin treatment. This result indicates prepondering (if not exclusive) ^{14}C-labelling in the C_4-position of aspartate.

Integration of β-carboxylation into photosynthesis

From the results contained in Fig. 5 the question arises as to what extent β-carboxylation of phosphoenolpyruvate contributes to photosynthetic carbon fixation. How much carbon is thus additionally provided via β-carboxylation as compared to photosynthetic CO_2 fixation via RubP-C? More precise information on this problem may be obtained from a calculation of the respective carboxylation rates as based on percentage ^{14}C-labelling of aspartate and malate as well as carbon fixation achieved in the light and the dark. If it is assumed that both carboxyl groups of malate are ^{14}C-labelled in a very similar way to those of aspartate, then photosynthetic and dark CO_2 assimilation may be expressed as amount of carbon fixed into the C_4 of aspartate and malate *via* β-carboxylation. The data compiled in Fig. 6 show the appropriate rates of carbon flow into C_4 acids as calculated for several brown macroalgae. When the values for photosynthetically derived aspartate and malate are reduced by 50% (*cf.* Fig. 5) to negate the contribution of ^{14}C in the

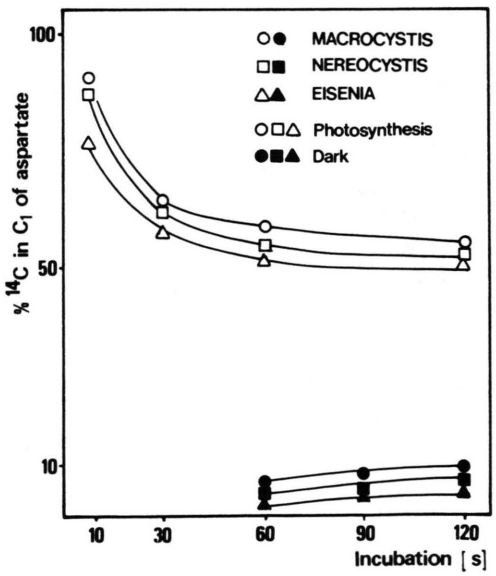

Fig. 5. *Macrocystis integrifolia, Nereocystis luetkeana, Eisenia, arborea.* Percentage ^{14}C-labelling of the C_1-atom (= α-carboxyl group) of aspartate during short-term photosynthesis and in the dark.

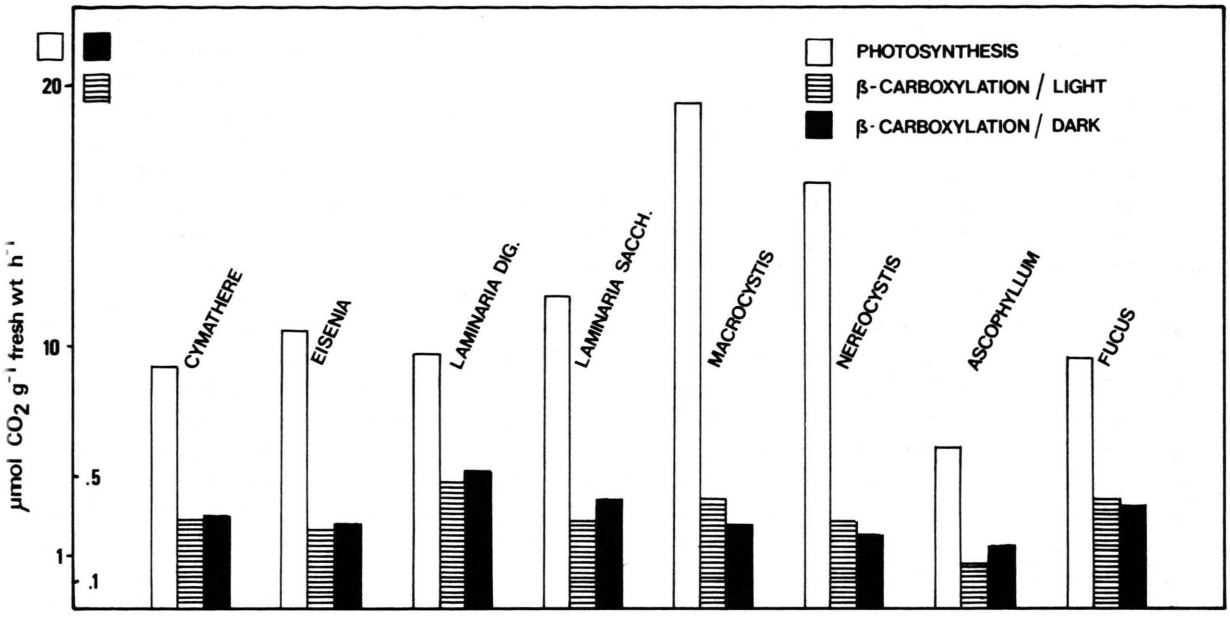

Fig. 6. Rates of β-carboxylation in the light and in the dark as expressed in terms of inorganic carbon fixed into C_4-acids in relation to photosynthesis. Note different ordinate scales.

C_1-position, it becomes possible to approximate the amounts of carbon directly introduced via β-carboxylation in the light. It is obvious then that the quantities of carbon fixed into the β-carboxyl groups in the light and in the dark compare reasonably well. As based on the formation of aspartate and malate, in brown algae β-carboxylation in the light at least equals that achieved in the dark (Fig. 6).

Discussion

Assimilation of inorganic carbon by β-carboxylation *via* phosphoenolpyruvate carboxykinase is a metabolic feature particularly occurring in brown macrophytic algae, whereas its extent is negligible in representatives of the Chlorophyceae and Rhodophyceae. Evidence for the relative efficiency of this process is derived from a comparison of photosynthesis and non-photosynthetic carbon fixation in the dark as well as by measurements of the respective carboxylating enzymes mediating the entrance of CO_2 into organic compounds. The potential for β-carboxylation of phosphoenolpyruvate is particularly high in developping, growing frond (blade) areas, by which species of the Laminariales and Fucales differ from the majority of further algae [10–12, 18, 22] (Fig. 1).

The question arose whether or not β-carboxylation contributes fixed carbon not only in the dark period (when blade growth is initiated), but also during photosynthetic CO_2 reduction. A first indication on the probable participation of β-carboxylation in the light had been obtained from kinetic tracer experiments [12, 23, 24] resulting in relative strong ^{14}C-labelling of typical C_4 acids such as aspartate and malate (Fig. 2 and 4). However, no precise data were hitherto available allowing for a quantification of β-carboxylation progressing in the light.

In this connection it is interesting to take into account the photosynthetic ^{14}C-labelling of aspartate. Chemical degradation of ^{14}C-aspartate demonstrated that an appreciable proportion of radiocarbon is located in the C_1-atom (= α-carboxyl group), particularly following short-term incubation periods less than 60 s (Fig. 5). However, increasing photosynthetically fixed ^{14}C-activity is also seen in the C_4-atom (β-carboxyl group) of aspartate. At a first approach, this finding suggests that β-carboxylation actually takes place in the light and is thus obviously not restricted to dark periods. Another conclusion may be drawn: The substrate of PEP-CK, phosphoenolpyruvate, is an early occurring photosynthate most probably derived from ^{14}C-labelled 3-phosphogly-

cerate. Photosynthetic CO_2 fixation *via* RubP-C yields 3-phosphoglycerate ^{14}C-labelled in the C_1-atom [1]. This compound is presumably not quantitatively fed into the reductive pentose phosphate cycle, but undergoes further conversion to $^{14}C_1$-phosphoenolpyruvate. Upon carboxylation by PEP-CK $^{14}C_{1,4}$-oxaloacetate is readily obtained, which in turn gives rise to the formation of aspartate and malate and thus accounts for the radiocarbon being symmetrically distributed between C_1- and C_4-atoms (Fig. 5). Therefore, photosynthesis preferentially provides the substrate for β-carboxylation in the light. This also explains the delay in ^{14}C-labelling of the C_4-atom as compared to the α-carboxyl group (Fig. 5). Earlier investigation on brown macroalgae provided evidence that in the dark phosphoenolpyruvate is preponderingly derived from mannitol catabolism *via* glycolysis [15].

The position of radiocarbon within the aspartate molecule is not only indicative for β-carboxylation progressing in the light, but also provides a basis for the tentative quantification of this process. Since it is rather likely to assume that the distribution of ^{14}C between the C_1 and C_4-atoms of malate is very similar to the pattern recorded for aspartate, the occurrence of ^{14}C-labelling in the C_4-position may be taken as a measure of β-carboxylation during photosynthesis. A comparison of respective calculations (Fig. 6) suggests that rates of β-carboxylation in the light are not basically different from those observed in the dark (*cf.* Fig. 1). Hence it may be concluded that products arising from β-carboxylation *via* PEP-CK under dark conditions are qualitatively and quantitatively comparable to those provided by the same set of reactions in the light. "Dark" carbon fixation is thus fully integrated into photosynthetic CO_2 assimilation contributing fixed carbon primarily in the form of C_4-compounds. This additional pathway of CO_2 fixation particularly occurring in brown seaweeds [11, 12, 24] at least partly compensates for respiratory carbon loss [15]. It is therefore justified to consider (dark) β-carboxylation for calculations of primary productivity [25].

The well documented occurrence of C_4 acids among the early ^{14}C-labelled photosynthates of marine algae [11, 23, 24] caused considerable confusions and uncertainties with respect to the mechanisms primarily involved in CO_2 fixation. Marine algae in general have been claimed to exhibit C_4 photosynthesis [8]. Similarly, the C_4 pathway of photosynthesis was reported to operate in blue-green algae [5]. Proofs for such generalizing qualifications of algal photosynthetic pathways have mainly been derived from ^{14}C-labelling patterns of short-term assimilates. In essence, however, there is no immediate and clear cut experimental evidence for any algal species strongly suggesting that radiocarbon in the C_4-position of aspartate and/or malate is actually transferred to the C_1-position of 3-phosphoglycerate through decarboxylation and subsequent refixation – metabolic events invariably linked with C_4 photosynthesis in ecologically specialized terrestrial plants [26]. Moreover, findings presented in this contribution indicate that, at least in brown algae, photosynthetically fixed radiocarbon obviously flows in the opposite direction from 3-phosphoglycerate to the C_4-acids (Figs. 3 and 5). Ocurrence of ^{14}C-labelling in aspartate and malate is thus a secondary event which, in the light, clearly depends on primary carbon fixation *via* RubP-C instead of preceeding this essential step.

There is no doubt that, particularly in brown macrophytic algae, C_4 acids are important intermediates of primary metabolism certainly supplementing the universally occurring reactions of the reductive pentose phosphate cycle. Biosynthesis and turnover of these compounds are more intense in brown than in green or red pigmented algae [10–12, 14–18]. However, the participation of β-carboxylation accounting for rapid formation of C_4 acids is basically different from that encountered with vascular plants. Therefore the respective metabolic reactions and conversions involved should be associated with the certainly less ambiguous term C_4 metabolism instead of C_4 photosynthesis [26]. Marine brown algae are thus C_4 plants in a very modified sense (*cf.* ref. [19]). It is now recognized that the high potential for β-carboxylation is an indispensable component of the overall carbon strategy of brown macrophytes [16, 17, 27, 28] providing the metabolic basis for the remarkable seasonality of their growth performance.

Acknowledgements

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