Sodium Chloride Effect on Dark Fixation of CO_2 by Marine & Terrestrial Plants [1, 2]

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The photosynthetic parts of terrestrial plants can fix CO_2 in the dark. Previous experiments carried out in our laboratory (5, 6, 8, 9) on succulent and non-succulent leaves showed that these tissues incorporated $C^{14}O_2$ non-photosynthetically exclusively into organic and amino acids with the predominant amount of radioactivity, 50 to 85 %, appearing in the organic acid fraction. While investigating light and dark $C^{14}O_2$ fixation in cell-free systems from spinach leaves, we observed that in the presence of increasing sodium chloride concentrations, the fraction of activity fixed in the amino acids was considerably enhanced. Holm-Hansen and co-workers (4) observed that the presence of salt not only increases the rate of light and dark $C^{14}O_2$ fixation by intact Chlorella, but considerably affects the distribution pattern of photosynthetically labeled compounds. They, too, observed an increase in the proportion of radioactivity in the amino acid fraction. It became of interest to investigate the pathways of dark CO_2 fixation in a variety of marine plants, which exist in an environment of high salt concentration. The three marine plants investigated readily incorporated $C^{14}O_2$ with a pattern of labeling different from that seen in terrestrial plants and similar to that observed in the cell-free system from spinach in the presence of sodium chloride. We, therefore, returned to the spinach cell-free system to investigate the mechanisms by which the sodium chloride is active. It will be shown in this paper that the effects of sodium chloride can be reversed by adding oxidized or reduced diphosphopyridine nucleotide (DPN^+, DPNH). Two possible mechanisms are advanced to explain the action of salt in directing the fixation pattern from the organic acids to amino acids.

Material & Methods

Three species of marine plants, *Zostera marina,* Morong (angiosperm), *Egregia laevigata,* Setchell (brown alga) and *Gigartina canaliculata,* Harvey (red alga), were collected from Newport Bay, Cal., with the help of the staff of the Marine Biological Laboratories of the California Institute of Technology. Experiments were carried out from June through August 1960. The temperature of the sea water at the time of collection was 15 C. The material was harvested between 11 AM and 1 PM on sunny days and used within 2 hours after harvest. The plants were thoroughly washed in running sea water to remove contaminating epiphytes and washed again in synthetic sea water[6]. Each sample was then blotted with paper towels and 5 g of fresh material weighed and cut into 1 cm squares. The tissue was transferred into 150 ml amber glass bottles made light-tight with aluminum foil and black paper containing 20 ml of synthetic sea water, pH 7.4. The reaction was started by adding 60 to 70 μc of NaH $C^{14}O_3$, specific activity 3.9 μc/m mole. The bottles were shaken continuously for 1 hour at 15 C.

The reaction was stopped by adding sufficient boiling ethanol to make the final concentration 80 % in ethanol; this mixture was left to stand for 24 hours. The alcoholic extract was decanted, and the remaining solids were ground in a mortar with 20 ml of 80 % boiling ethanol to complete the extraction. The residual solids were re-extracted three times with small portions of boiling 80 % ethanol. A 0.1 ml aliquot of the combined filtered extract was acidified and counted to determine the total activity fixed. The total filtrate was then concentrated under re-

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[6] The synthetic sea water was prepared by the formula used by Eppley (1). At the suggestion of Eppley (personal communication) the formula of the artificial sea water was further improved by adding trace elements in the following concentration, NaEDTA 45 μM, $FeSO_4$ 14.9 μM, H_3BO_4 9.75 μM, $MnCl_2$ 2.02 μM, $ZnSO_4 \cdot 7H_2O$ 1.73 μM, $CuSO_4 \cdot 5H_2O$ 0.2 μM, $(NH_4)_2MoO_4$ 0.25 μM.

duced pressure and electrolytically desalted prior to chromatography. Two dimensional paper chromatographic procedures using phenol: water (80: 20 w/v) and butanol: acetic acid: water (74: 19: 50 v/v/v) were used to separate and identify the compounds formed. The identity of each spot was confirmed by color tests as well as by co-chromatography with authentic samples (9). Radioactive compounds were located by exposing the chromatogram to no-screen X-ray film. The spots were then cut out of the chromatogram and counted with a micromil end-window Geiger tube.

The methods used in the study of the dark fixation of CO_2 in intact spinach leaves are the same as those detailed in earlier communications (8, 9). Spinach leaves were obtained commercially and thoroughly washed in distilled water and blotted dry; 1 g of young leaves was placed in an apparatus which permits exposure to the $C^{14}O_2$ in total darkness. After equilibration of the leaves for 5 minutes in the dark to remove any transient reducing compounds of photosynthesis, $C^{14}O_2$ generated from 12 mg of $BaC^{14}O_3$, (specific activity 120 μc/mg), was admitted into the chamber. After 2 hours, the reaction was terminated by homogenization in boiling 80 % ethanol. The ethanol homogenate was filtered, the filtrate extracted with Skellysolve A, and the extract concentrated to a volume of 3 ml under reduced pressure. Concentrated alcoholic extracts were separated by two dimensional paper chromatography in the manner described above.

The spinach cell-free homogenates were prepared from young, healthy looking leaves, purchased at a local market. They were washed in distilled water, blotted dry, and their midribs and petioles removed. Fresh-cut leaf blades were sliced into narrow strips and ground in an ice-cold mortar, in a 4 C room. The leaves were ground using sand with 1 ml per g tissue of NaCl, 0.35 M, tris, 0.05 M, pH 7.8. The concentration in the final homogenate of NaCl was 0.22 M, and the tris, 0.03 M. In order to avoid any dilution effects, a second method of homogenization was employed. The leaf blades were ground without the addition of buffer or sand. Following the grinding, the slurries obtained were strained through four layers of damp cheese cloth and centrifuged 5 minutes at 200 *g* to remove whole cells and wall material. The pH of the homogenates prepared without buffer were in the range of 6.3 to 6.9. The pH of these homogenates was adjusted to 7.8 either with a tris buffer, 1.0 M, pH 8.5 or with 0.02 M KOH. When NaCl effects were studied, a concentrated stock solution was used to obtain the desired concentration. Dilution was minimal. Control homogenates were always run at the same homogenate concentration using water instead of salt solution.

The dark fixation of $C^{14}O_2$ by the homogenates was carried out in light-tight glass bottles. The reaction was started by adding 0.2 ml of NaH $C^{14}O_3$ solution, containing 40 to 60 μc C^{14}, to 5.8 ml of the total homogenate. Temperature was maintained at 18 C with shaking in a Dubnoff metabolic incubator. The reaction was terminated by adding sufficient boiling ethanol to make the final concentration 80 %. The extraction, concentration, and chromatography were carried out in the manner described above. The concentrated extract was desalted prior to chromatography to obtain clean separation.

Results & Discussion

Exposure of the marine plants to $C^{14}O_2$ in the dark for 1 hour resulted in the incorporation of the radioactive carbon into several compounds, principally amino acids. In table I is the percentage radioactivity incorporated per g of tissue as well as the relative amount of each of the compounds formed by the three plants. Although some of the $C^{14}O_2$ incorporated by the marine plants appears in the organic acid fraction, the activity predominantly is found in the amino acids. Even though the compounds incorporating radioactivity are similar in the three marine organisms, distribution of activity in the particular amino acids varies from species to species. Aspartic acid, glutamic acid, glycine, serine, alanine, glutamine, and citrulline are the principal amino acids found.

Despite the extensive literature on dark $C^{14}O_2$ fixation by terrestrial plants and fresh water algae, there is scant work on the marine plants which have a rather different physiological environment. Recently Rho (7) has reported on dark and light $C^{14}O_2$ fixation by marine diatoms. He found that, although

Table I

Products From 1 Hour Dark Fixation of $NaHC^{14}O_3$ by Marine Plants*

Compounds	*Zostera marina* (Angiosperm)	*Egregia laevigata* (Brown alga)	*Gigartina canaliculata* (Red alga)
% Incorporation g tissue	3.52	3.52	5.86
Aspartate	38.0	34.0	32.8
Glutamate	10.3	16.5	14.0
Glycine-serine	17.7	0.4	2.2
Alanine	1.8	13.3	1.6
Glutamine	Trace	11.0	4.1
Asparagine	Trace	Trace	25.1
Citrulline			10.0
Phenylalanine	3.6	0.2	
Leucine-isoleucine	1.7	4.1	
Homoserine			4.0
Amino acids	73.1	79.5	93.8
Malate	13.0	Trace	1.5
Citrate-isocitrate	3.1	Trace	2.9
Succinate	6.4	10.5	Trace
Fumarate	Trace	7.1	Trace
Organic acids	22.5	17.6	5.9

* Activities are expressed as percentage of total activity counted on the chromatogram. Those compounds indicated as "Trace" had less than 0.1 % of the total activity.

the diatoms in light produce similar compounds as land plants, the products of dark fixation are strikingly different. There was little activity seen in malic acid and surprisingly large amounts of activity spread through a group of amino acids including ornithine, citrulline, histidine, and lysine. Holm-Hansen et al. (4), while studying effects of mineral salts on short term incorporation of $C^{14}O_2$ by Chlorella, found that salt greatly stimulates $C^{14}O_2$ incorporation both in the light and the dark. The increased activity due to the presence of salt during the photosynthetic fixation can be accounted for primarily by the increase of activity in the amino acid fraction. The presence of $MgSO_4$, KNO_3, or K_2HPO_4 in the medium increased radioactivity in the amino acids with a decrease in relative amounts of radioactivity in the sugar phosphates. Holm-Hansen et al. further found that adding ammonium chloride increases the radioactivity in the amino acids from 9.9 % in the controls to 57 % in the presence of salt. The radioactivity in the sugar phosphates fell from 64 % to 7.1 %. They attribute the effect of ammonium chloride to the direct participation of the ammonium ion in amino acid synthesis. It is important, however, that all salts tested enhanced both dark and light fixation. No details regarding the distribution of radioactivity were presented for the dark fixation.

Table II

Comparison of Activity in Products From Dark Fixation of $C^{14}O_2$ by Intact Spinach Leaf & Spinach Leaf Homogenate Systems

Compounds	Intact spinach leaf	Spinach homogenate	Spinach homogenate in NaCl-tris buffer*	Dilute spinach homogenate ground in NaCl-tris buffer*
% Incorporation g tissue	3.85	1.29	1.04	0.61
Aspartate	8.1	46.8	85.0	86.0
Glutamate	12.4	0.2	3.7	0.1
Glycine-serine	6.3	2.3	4.7	0.7
Alanine	4.3	0.3	0.5	0.3
Glutamine	2.8	Trace	0.1	0.6
Asparagine	1.0	0.1	0.4	0.4
Histidine	Trace	...	Trace	Trace
Leucine-isoleucine	0.2	Trace	Trace	0.3
Phenylalanine	0.6	Trace	Trace	0.4
Valine	0.2	Trace	Trace	Trace
Homoserine	...	2.8	Trace	...
Amino acids	35.9	52.7	94.4	88.8
Malate	15.7	27.5	1.8	6.3
Citrate-isocitrate	40.6	32.3	0.6	0.4
Succinate	Trace	Trace	Trace	1.3
Fumarate	Trace	1.8	0.9	Trace
Glycollate	0.3	...	Trace	1.3
Glycerate	...	0.6	Trace	...
Organic acids	56.6	42.2	3.3	9.3
Phosphoglycerate	3.0	4.0	0.9	Trace
Phosphoenolpyruvate	2.1	...	Trace	Trace

* NaCl concentration in both experiments was 0.22 M and tris was 0.03 M. Time was 2 hours.

In table II are the results of a 2 hour dark $C^{14}O_2$ fixation by both intact spinach leaves and the homogenate systems. The intact spinach leaf, like all other land plants examined, incorporates most of the $C^{14}O_2$ into organic acids. The spinach homogenate prepared in the absence of NaCl-tris, but adjusted to pH 7.8 with KOH, exhibits a pattern of fixation similar to the intact leaf. However, the homogenate adjusted to pH 7.8 with NaCl-tris incorporates more than 94 % of the activity into the amino acid fraction; aspartic acid alone is responsible for 85 %. Since many investigators prepare their leaf homogenates by grinding with sand in the presence of an equal volume of buffer and salt solution, such a preparation was compared with the undiluted and salt-free homogenates. Both the qualitative and quantitative distributions of $C^{14}O_2$ incorporated by leaf homogenates in NaCl-tris buffer are similar to those observed for marine plants; this fact suggests that salt concentration may play a role in the regulation of amino and organic acid metabolism. No striking effect of dilution was observed. Recently, Hiller and Walker (3) using Kalanchoe leaf extracts reported the formation of labeled aspartic acid during the dark $C^{14}O_2$ fixation by a process of exchange transamination. It is possible that a large pool of labeled aspartic acid in the cell-free homogenates may arise by such a mechanism. No observations on the effect of salts in the transamination system were reported.

There is competition for a common pool of oxaloacetate, the first product of the dark fixation of CO_2 (8), between malic and aspartic acids. Thus, in the presence of low concentrations of reduced pyridine nucleotide, the synthesis of malic acid would be inhibited and the oxaloacetate would be more favorably shunted into the aspartic acid. To test this hypothesis both DPNH and DPN^+ were added to the undiluted NaCl-tris buffer homogenate of spinach leaves, and the pattern of fixation observed. From table III, it is clear that adding DPNH and DPN^+ to the spinach homogenate system has altered considerably the pattern of fixation in favor of the formation of malic acid. The activity lost from aspartic acid now appears in the organic acid fraction. However, the dominant position of citric and isocitric acids is not re-established in the homogenate system, even though the activity of the organic acids is greatly enhanced.

The studies of Hiatt and Evans (2) on isolated malic dehydrogenase from spinach leaves indicate the striking inhibition of the enzyme both in low and high salt concentration. It is therefore possible that the altered pattern of labeling is caused by an inhibition of the malic dehydrogenase system independent of the general level of DPNH present in the cell. Thus, at present, there are two possible explanations

Table III

Effect of Pyridine Nucleotides on Spinach Homogenate Dark $C^{14}O_2$ Fixation*

Compounds	Homogenate	Homogenate + DPN+ 6.7×10^{-4} M	Homogenate + DPNH 6.7×10^{-4} M
% Incorporation g tissue	1.04	1.44	1.17
Aspartate	85.0	65.8	53.8
Glutamate	3.7	1.6	1.3
Glycine-serine	4.7	1.9	1.2
Alanine	0.5	0.1	0.2
Glutamine	0.1	0.1	0.2
Asparagine	0.4	0.9	10.3
Homoserine	Trace	Trace	6.0
Leucine-isoleucine	Trace	Trace	Trace
Phenylalanine	Trace	Trace	Trace
Valine	Trace	0.1	Trace
Amino acids	94.4	70.5	73.0
Malate	1.8	23.6	19.3
Citrate-isocitrate	0.6	2.0	2.9
Succinate	Trace	2.5	2.5
Fumarate	0.9	0.5	Trace
Organic acids	3.3	28.6	24.7
Phosphoglycerate	0.9	0.3	0.6

* Expressed in terms of percentage of total counts on paper. Homogenate is 0.032 M tris, 0.22 M NaCl, pH 7.8. Time: 2 hours.

for the patterns of dark CO_2 fixation observed in marine plants and sodium chloride plant extracts. High NaCl concentration could (1) activate the oxaloacetate-aspartate and other transaminase enzymes (2) inhibit malic dehydrogenase by altering the affinity of the coenzyme for the enzyme. At present we are seeking to define more clearly the site of the NaCl action.

An overall summary of the distribution of $C^{14}O_2$ after dark fixation in both terrestrial and marine intact plants and the cell-free spinach homogenate in the presence of salt is presented in table IV. The succulents tested show relatively little activity incorporated into the amino acids when compared with the non-succulents. The large fraction of activity in the amino acids in the marine plants tested seems to be a characteristic feature.

Table IV

Effect of Saline Environment on Dark Fixation of $C^{14}O_2$

		Amino acids	Organic acids
Non-saline environment	Bryophyllum leaf	7.3	89.7
	Kalanchoe leaf	15.7	76.3
	Tobacco leaf	25.1	72.3
	Spinach leaf	35.9	56.6
	Spinach homogenate	52.7	42.2
Saline environment	Spinach homogenate NaCl-tris buffer	94.4	3.3
	Spinach dilute homogenate NaCl-tris buffer	88.8	9.3
	Zostera	73.1	22.5
	Egregia	79.5	17.6
	Gigartina	93.8	5.9

Summary

I. From the pattern of the dark incorporation of $C^{14}O_2$ into the 80 % alcohol soluble fraction of three marine plants: *Zostera marina,* Morong (angiosperm), *Egregia laevigata,* Setchell (brown alga), and *Gigartina canaliculata,* Harvey (red alga) it can be shown that the predominant fraction of activity is in the amino acids.

II. Cell-free homogenates of spinach leaves can fix $C^{14}O_2$ in the dark with a pattern similar to that of the intact leaf. In the presence of salt, however, this pattern is altered to one which more closely resembles that observed for intact marine plants.

III. Adding DPNH and DPN+ to the NaCl-tris spinach homogenate caused a shift in the pattern of labeling toward that observed in the intact leaf.

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