THE DARK FIXATION OF CO_2 BY SUCCULENT LEAVES: METABOLIC CHANGES SUBSEQUENT TO INITIAL FIXATION[1,2]

PAUL SALTMAN, VICTORIA H. LYNCH,[3] GEORGE M. KUNITAKE, CLYDE STITT AND HERBERT SPOLTER

DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, SCHOOL OF MEDICINE, UNIVERSITY OF SOUTHERN CALIFORNIA, LOS ANGELES 7, CALIFORNIA

Succulent plants are characterized by a diurnal rhythm of dark acidification and light deacidification. Concomitant with the acidification there is a loss of stored carbohydrate while in the light, organic acids are converted to carbohydrate (3). That CO_2 fixation in the dark is directly linked with the formation of acids was conclusively demonstrated by Thurlow and Bonner (20) using $C^{14}O_2$ and excised leaves of *Bryophyllum crenatum*. Thomas and his co-workers (19) have confirmed the relationship of CO_2 fixation and acid synthesis by elegant physiological experiments in which CO_2 uptake was correlated with increased acid formation.

Analysis of several compounds synthesized during the dark fixation has been made by Varner and Burrell (22). Excised *B. calycinum* leaves were exposed to $C^{14}O_2$ for 2.5 hours in total darkness, and the organic acids and carbohydrates isolated by chromatography on silica gel columns. The acids isolated were malic, succinic, oxalic, citric, and iso-citric—the same acids which had been identified chemically by Pucher (11). Stutz and Burris (18) extended the work of Varner and Burrell by exposing a wide variety of leaves from higher plants to $C^{14}O_2$ in the dark, separating the acids by column chromatography and measuring their specific activities. Ranson (13) employed the technique of paper chromatography and radioautography to identify aspartate and glutamate as products of 14-hour dark $C^{14}O_2$ fixation of Kalanchoë leaves. Bradbeer (4) has reported the incorporation of $C^{14}O_2$ label into a α-ketoglutarate, pyruvate, and oxaloacetate.

In order to gain further insight into the pathways of $C^{14}O_2$ during the dark metabolism of succulents, the techniques of Calvin, Benson, and co-workers (2) were adapted to this problem. By exposing *B. calycinum* leaves to $C^{14}O_2$ in the dark for varying periods of time and isolating and identifying the products formed, one should be able to elucidate the metabolic interconversions of the organic and amino acids. This paper will describe experiments in which the compounds incorporating radioactivity have been identified and their activities determined during periods of dark fixation from 1 minute to 2 hours. The pattern of compounds isolated suggests the operation of the enzymes of the Krebs tricarboxylic acid cycle as well as the participation of transamination reactions leading to the formation of amino acids from the α-keto acids of the cycle.

METHODS

The methods used in these longer periods of dark fixation of $C^{14}O_2$ are the same as those described in detail in a previous communication (15). A specially constructed apparatus permits excised leaves from *B. calycinum* to be exposed to $C^{14}O_2$ in total darkness. Approximately one gm of young leaves taken from the apex of the plants immediately before use was placed in the apparatus, $C^{14}O_2$ generated from 4 to 5 mg of $BaC^{14}O_3$ (specific activity 0.0282 mc/mg) was admitted, and the reaction terminated at the desired time by homogenization in boiling 80 % ethanol. The extract was filtered, extracted with Skellysolv A (Pentane), and the ethanolic extract concentrated to a volume of 2.0 ml under reduced pressure. Two dimensional chromatography of an aliquot (0.1 to 0.2 ml) of the concentrated extract in 80 % phenol–20 % water (w/w) in the first direction and (79) butanol–(19) acetic acid–(50) water (v/v/v) in the second, was used to separate the compounds. Radio-autographs were made by exposing "no-screen" x-ray film to the chromatogram. Identification of radioactive compounds was made by elution and subsequent cochromatography with known compounds. The activity of each compound was measured directly on the paper with an end window Geiger tube. Derivatives of the α-keto acids were made by disrupting the tissue with 30 ml of 5 *N* H_2SO_4, adding 30 ml of 5 *N* H_2SO_4 saturated with 2,4-dinitrophenyl hydrazine, and forming the hydrazone derivatives as described by Ranson (personal communication). The mixture was allowed to stand for one hour at room temperature and then filtered. The filtrate was extracted three times with 25-ml portions of ethyl acetate. The ethyl acetate was then extracted three times with 25-ml portions of 10 % Na_2CO_3. The Na_2CO_3 solution was acidified to pH 1.0 with cold 5 *N* H_2SO_4 and the hydrazones extracted into ethyl acetate. The ethyl acetate fraction was dried over anhydrous Na_2SO_4 and then concentrated to 2.0 ml under reduced pressure. The hydrazones were separated by paper chromatography by the method of Isherwood and Cruickshank (9), and were eluted and identified by cochromatography with authentic derivatives of the keto-acids.

[1] Received October 30, 1956.

[2] This investigation was supported in part by a research grant from the National Science Foundation, and in part by the Tobacco Industry Research Committee. The facilities of the Allan Hancock Foundation were generously provided.

[3] Present address: Carnegie Institution of Washington, Stanford, California.

Results and Discussion

A radio-autograph for a 1-hour dark fixation is presented in figure 1. The compounds identified conclusively are: malate, citrate, isocitrate, succinate, fumarate, aspartate, asparagine, glutamate, glutamine, alanine, glycine, and serine. The activities of these compounds expressed as percent of total activity fixed at the time intervals tested are presented in table I. After 60 minutes no new labeled compounds appear, although total activity in these compounds continues to increase. It should be noted that neither phosphorylated intermediates of glycolysis nor carbohydrates appear during dark CO_2 fixation. Table II presents the activities of the various α-keto acids isolated as the hydrazone derivatives.

Probable metabolic pathways by which the labeled compounds may be formed after the initial fixation of $C^{14}O_2$ as oxaloacetate are suggested in figure 2.

The classical Krebs tricarboxylic cycle would appear to play a key role in succulent metabolism. The preparation of biochemically active mitochondrial particles from green pea leaves by Smillie (16) indicates the functioning of this metabolic system in leaf tissue. The previous inability of many workers (5) to isolate such preparations seems entirely due to procedural difficulties and not to the absence of mitochondria in green tissue. Stutz and Burris (18) found that the specific activities of some of the acids of the cycle failed to reach equilibrium in *B. calycinum* leaves exposed to $C^{14}O_2$. They interpreted their results to indicate that the cycle, if operative at all, must be quite sluggish. However, they fail to consider the rates at which some of the acids, particularly malic, citric, and iso-citric are removed from the metabolic activities of the cycle into the vacuole or into some other more static area of the cell. Evidence from our laboratory, to be presented elsewhere, indicates that all organic and amino acids are not equally available for metabolism. The experiments of Tolbert

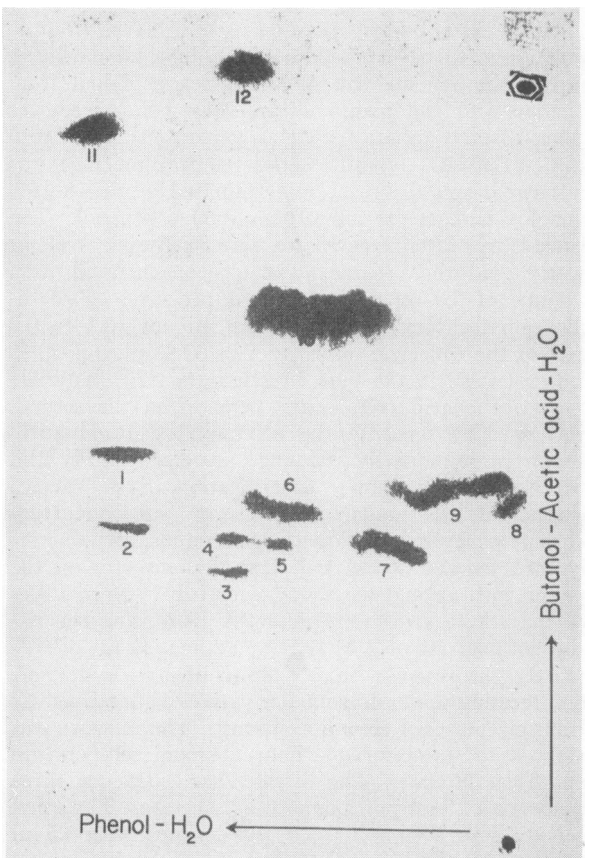

Fig. 1. Photographic reproduction of radioautogram from two dimensional paper chromatogram of extract from 60-min dark fixation of $C^{14}O_2$ by *Bryophyllum calycinum*. Compounds identified are: 1. alanine; 2. glutamine; 3. asparagine; 4. glycine; 5. serine; 6. glutamate; 7. aspartate; 8. citrate; 9. isocitrate; 10. malate 11. fumarate; 12. succinate.

Table I

Products from the Dark Fixation of $C^{14}O_2$ by Bryophyllum calycinum Leaves *

Compound	1 Min	5 Min	10 Min	30 Min	60 Min	120 Min
Malate	60	65	57	71	61	75
Aspartate	20	10	8.7	7.6	11	4.1
Isocitrate	11	10	14	7.5	15	8.2
Citrate	4.1	4.3	3.4	4.6	6.1	5.3
Glutamate	4.0	2.1	3.9	2.6	2.1	2.0
Glutamine	0.3	0.5	3.8	0.7	0.2	0.5
Alanine	0.0	1.3	3.4	0.4	0.2	0.3
Succinate	0.0	1.9	1.0	0.5	0.9	0.7
Fumarate	0.0	0.8	1.8	0.7	0.4	0.5
Serine	0.0	0.0	0.0	0.2	0.3	0.1
Glycine	0.0	0.0	0.0	0.0	0.2	0.2
Asparagine	0.0	0.0	0.0	0.0	0.3	0.1
Totals	99.4	95.9	97.0	95.8	97.7	97.0

* All values as percentage of total C^{14} counted on the chromatogram. Small amounts of unidentified compounds and the origin account for the percentages not listed.

These are the results of a single experimental run, but are typical of 3 expts.

Table II

Activities of 2,4-Dinitrophenylhydrazone Derivatives after a 10-Minute Dark Fixation of $C^{14}O_2$ by Bryophyllum calycinum Leaves *

Keto acid	% Activity
Oxaloacetate	38
α-Ketoglutarate	28
Pyruvate	33
Total	99

* Values are given as percentage of total C^{14} counted on the chromatogram. Small amounts of unidentified hydrazone derivatives and the origin account for the percentages not listed.

These are the results of a single experimental run, but are typical of 4 expts.

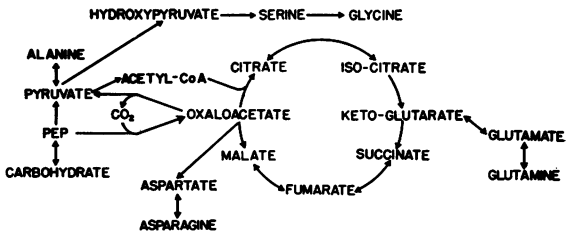

FIG. 2. Proposed metabolic pathways involved in the dark fixation of CO_2 by *Bryophyllum calycinum* leaves. The pathway for the biosynthesis of serine from pyruvate via hydroxypyruvate is hypothetical.

(21) with large uni-cellular algae also indicate that differential transport of organic compounds is involved in plant metabolism. Ramstad and Lieberman (12) have expressed the view that the Krebs cycle is not functioning in succulent metabolism. Their fundamental assumptions with respect to the concentrations of acids and the equilibrium of citrate, iso-citrate, and cis-aconitate could be explained by differential transport mechanisms.

Further evidence supporting an active cycle in succulents is found in the experiments of Thomas (1) who demonstrated that dark CO_2 fixation is markedly inhibited at low oxygen levels. Not only is the cycle sensitively dependent upon oxygen for optimal activity, but also, anaerobiosis increases the amount of adenylate acceptor (i.e., ADP[4]) available. Since PEP can either be carboxylated to yield oxaloacetate, or transfer its phosphate group to ADP to yield ATP, anaerobiosis would favor the later reaction, and, as a direct consequence, a decrease in the carboxylation reaction would be observed. It is difficult to conceive of metabolic sequences other than the Krebs cycle that would account for the pattern of compounds identified during the dark fixation of CO_2 by succulent leaves.

The rapid formation of glutamate could occur from α-ketoglutarate by either transamination or by the direct amination through the action of glutamic acid dehydrogenase. The activity found in alanine and aspartate would appear to be dependent upon synthesis via the activity of the transaminases (26). That the oxaloacetate must "shuttle" via malate to the symmetrical molecule of fumarate and back to oxaloacetate is apparent from the activity found in alanine. Were this "shuttle" not operative, the decarboxylation of oxaloacetate would yield non-radioactive pyruvate and consequently, inactive alanine. The presence of α-ketoglutarate, oxaloacetate, and pyruvate in plant tissue has been demonstrated by Virtanen (23) and by Barker and Mapson (1). We have confirmed and extended their observations by isolating 2,4-dinitrophenylhydrazone derivatives of these keto acids which contained radioactivity after a 10-minute exposure to $C^{14}O_2$ in the dark. Thus the key α-keto acids of the Krebs cycle seem to be directly involved in the dark metabolism of the succulents. That glutamine incorporates label coincidentally with the glutamate is evidence for a very active glutamine synthetase in Bryophyllum leaves similar to the enzyme system studied by Elliot (7) and Webster (24) in a variety of plant tissues. The asparagine synthease (25) enzyme is much slower than the glutamine enzyme as demonstrated by the relatively late appearance of radioactive asparagine despite the fact that aspartate is one of the first compounds to incorporate label.

The proposed pathways for the synthesis of serine and glycine are based only on the observation that serine incorporates radioactivity before glycine. Much evidence is now available to indicate that serine is the precursor to glycine in animal tissue. Sallach (14) has administered carboxyl-C^{14} glyceric acid to rats and has observed its direct conversion to carboxyl labeled serine. He has also shown the presence of an active transaminase which transfers the amino group of alanine to hydroxypyruvate. Kitt (10) has incubated glucose-1-C^{14} with tumor tissue for short periods of time and has demonstrated that serine is labeled prior to glycine. To explain these results, Kitt postulates a pathway leading from phosphoglycerate to phosphohydroxypyruvate to phosphoserine. Confirmation for this metabolic scheme for serine biosynthesis is given by the study by Greenberg and Ichihara (8) who with the use of preparations from rat liver, were able to isolate and identify the phosphorylated intermediates in the metabolic pathway. Stafford et al (17) have investigated an enzyme from parsley which catalyzes the reaction

$$\text{DPNH} + \text{H}^+ + \text{hydroxypyruvate} \rightleftharpoons \text{D-glycerate} + \text{DPN}^+.$$

Once labeled carbon has been incorporated into pyruvate or phosphopyruvate, as is indicated by our experiments, a reversal of glycolysis would lead to phosphoglycerate. We are not able at this time to ascertain whether a phosphorylated or non-phosphorylated pathway is operative in the synthesis of serine by higher plants.

We have been unable to demonstrate the incorporation of label into oxalate by cochromatography of our extracts with carrier oxalate. This finding is in contrast to the studies of Varner and Burrell (22) and Stutz and Burris (18) who have isolated the oxalate by column chromatography and have shown appreciable incorporation of $C^{14}O_2$. There was no detectable activity associated with lactate, glyoxylate, and glycolate, compounds which are associated with the organic acid metabolism of higher plants (6).

SUMMARY

1. Compounds which incorporate $C^{14}O_2$ during dark fixation by *Bryophyllum calycinum* leaves have been identified by two dimensional paper chromatog-

[4] The following abbreviations are employed: PEP, phosphoenolpyruvate; DPN^+, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

raphy. The compounds detected are: malate, citrate, iso-citrate, succinate, fumarate, aspartate, asparagine, glutamate, glutamine, alanine, glycine, and serine.

2. The kinetics of the incorporation has been studied by exposing the excised leaves to $C^{14}O_2$ for periods of time from one minute to 2 hours.

3. The pattern of incorporation observed in the organic and amino acids suggests the operation of the Krebs tricarboxylic acid cycle in its entirety. Amino acids are synthesized from their respective α-keto acid analogues by transamination or by reductive amination.

4. Labelling is observed in serine before it can be detected in glycine during the dark fixation of $C^{14}O_2$ by succulents.

We wish to thank Drs. J. Bonner and G. Laties of the California Institute of Technology for their valuable advice and suggestions as well as for the plants used in these experiments.

Literature Cited

1. Barker, J. and Mapson, L. W. Studies in the respiratory and carbohydrate metabolism of plant tissues. V. Experimental studies of the formation of carbon dioxide and of the changes in lactic acid, sucrose and in certain fractions of keto-acids in potato tubers in air following anaerobic conditions. Proc. Roy. Soc. (London) B 141: 321–337. 1953.
2. Benson, A. A., Bassham, L. A., Calvin, M., Goodale, T. C., Haas, V. A. and Stepka, W. The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. Jour. Amer. Chem. Soc. 72: 1710–1718. 1950.
3. Bonner, J. Plant Biochemistry. Pp. 154–159. Academic Press, New York 1950.
4. Bradbeer, W. Ph.D. thesis, Durham University. November, 1954.
5. Brummond, D. O. and Burris, R. H. Reactions of the tricarboxylic acid cycle in green leaves. Jour. Biol. Chem. 209: 755–765. 1954.
6. Burris, R. H. Organic acids in plant metabolism. Ann. Rev. Plant Physiol. 4: 91–114. 1953.
7. Elliot, W. H. Isolation of glutamine synthetase and glutamotransferase from green peas. Jour. Biol. Chem. 201: 661–672. 1953.
8. Greenberg, D. M. and Ichihara, A. Pathway of serine formation from carbohydrate. Report Pacific Slope Biochem. Conf., Seattle, Washington June 11–12, 1956.
9. Isherwood, F. A. and Cruickshank, D. H. Chromatographic separation and analysis of mixtures of pyruvic, oxaloacetic and alpha-ketoglutaric acids. Nature 173: 121–122. 1954.
10. Kitt, S. The biosynthesis of free glycine and serine by tumors. Cancer Research 15: 715–718. 1955.
11. Pucher, G. W. The organic acids of the leaves of *Bryophyllum calycinum*. Jour. Biol. Chem. 145: 511–523. 1942.
12. Ramstad, E. and Lieberman, H. Studies on the acid metabolism of Crassulaceae. Medd. Norsk Farm. Selskap 17: 214–225. 1955.
13. Ranson, S. L. The use of isotopically labelled carbon dioxide in experiments bearing on two matters raised in the main body of this paper. New Phytologist 53: 28–30. 1954.
14. Sallach, H. J. Evidence for a specific alanine-hydroxypyruvic transaminase. In: Amino Acid Metabolism, W. D. McElroy and H. B. Glass, eds. Pp. 782–787. John Hopkins Press, Baltimore, Maryland 1955.
15. Saltman, P., Kunitake, G., Spolter, H. and Stitts, C. The dark fixation of CO_2 by succulent leaves: The first product. In press.
16. Smillie, R. M. Enzymic activities of sub-cellular particles from leaves. I. The occurrence of mitochondria in green leaves of the pea plant. Australian Jour. Biol. Sci. 9: 81–91. 1956.
17. Stafford, H. A., Magaldi, A. and Vennesland, B. The enzymatic reduction of hydroxypyruvic acid to D-glyceric acid in higher plants. Jour. Biol. Chem. 207: 621–629. 1954.
18. Stutz, R. F. and Burris, R. H. Photosynthesis and metabolism of organic acids in higher plants. Plant Physiol. 26: 226–243. 1951.
19. Thomas, M. and Ranson, S. L. Physiological studies on acid metabolism in green plants. III. Further evidence of CO_2-fixation during dark acidification of plants showing crassulacean acid metabolism. New Phytologist 53: 1–30. 1954.
20. Thurlow, J. and Bonner, J. Fixation of atmospheric CO_2 in the dark by leaves of Bryophyllum. Arch. Biochem. 19: 509–511. 1948.
21. Tolbert, H. E. and Zill, L. P. Photosynthesis by cut cells and cell free sap from Chara and Nitella. Report, A.I.B.S. Meeting, Madison, Wisconsin 1953.
22. Varner, J. E. and Burrell, R. C. Use of C^{14} in the study of the acid metabolism of *Bryophyllum calycinum*. Arch. Biochem. 25: 280–287. 1950.
23. Virtanen, A. I., Miettinen, J. K. and Kunttu, H. Alpha-keto acids in green plants. Acta Chem. Scand. 7: 38–44. 1953.
24. Webster, G. C. Enzymatic synthesis of glutamine in higher plants. Plant Physiol. 28: 724–727. 1953.
25. Webster, G. C. and Varner, J. E. Aspartate metabolism and asparagine synthesis in plant systems. Jour. Biol. Chem. 215: 91–99. 1955.
26. Wilson, D. G., King, K. W. and Burris, R. H. Transamination reactions in plants. Jour. Biol. Chem. 208: 863–874. 1954.