observed to take place as the culture period was prolonged. The data refer particularly to the synthesis and decomposition of starch, and the correlated reciprocal changes in malic and citric acids.

During the culture period, the total protein of the leaf slowly diminished in amount. It is tentatively suggested that the changes in the rates at which the leaves are able to recover from the stressed condition are correlated with the loss of protein and specifically with the gradual, although only partial, destruction of the enzymes essential for the chemical transformations to occur. The enzymes concerned with the metabolism of malic acid appear to have been especially sensitive to prolonged culture in light.

An examination of the data for the reciprocal changes in starch and organic acids showed that, in general, approximately the correct quantity of carbon was supplied by the component which diminished in amount to account for the carbon of the component which was synthesized. However, this relationship did not hold for the synthesis of organic acids in leaves which had been stressed by prolonged culture in light and were then placed in darkness. In these leaves, there was a marked deficiency in the synthesis of organic acids.

In contrast to its behavior in Bryophyllum leaves exposed to light under greenhouse conditions, citric acid was observed to diminish slowly, if at all, when the leaves were exposed to artificial light at a controlled temperature in the vicinity of 20° C.

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THE DARK FIXATION OF CO_2 BY SUCCULENT LEAVES: THE FIRST PRODUCTS[1,2]

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It has long been realized that green leaves have the ability to fix CO_2 in the dark to form organic acids with the concomitant loss of carbohydrate stores. A group of plants known as the succulents is characterized by a particularly active dark fixation of CO_2. In the case of the succulents, the organic acids synthesized in the dark are transformed to carbohydrates during a subsequent light period (4).

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Despite the considerable amount of work which has been done on succulent metabolism (11), the exact metabolic pathways by which the dark fixation of CO_2 proceeds have not, hitherto, been elucidated.

The techniques of paper chromatography and radioautography developed by Calvin, Benson, and their co-workers (3), to determine the pathway of $C^{14}O_2$ in photosynthesis are particularly applicable, with modifications, to the study of the dark fixation of $C^{14}O_2$ by succulents. That $C^{14}O_2$ is incorporated into the organic acids of succulents has been demonstrated by Thurlow and Bonner (13) who exposed *Bryophyllum crenatum* leaves to $C^{14}O_2$ for 60 hours. By means of a gross chemical separation of the prod-

ucts, they found that about 50 % of the activity was associated with the organic acid fraction, the remainder with amino acids, sugars, and insoluble materials. More refined techniques for the separation of organic acids on silica gel columns were employed by Varner and Burrell (16) in their study of $C^{14}O_2$ fixation by excised *B. calycinum* leaves. After two hours of dark fixation, radioactivity was found in malic, oxalic, succinic, citric, and isocitric acids. The malate contained 76 % of the activity fixed in the organic acid fraction, confirming that this compound is the principal acid concerned with acidification in the succulents. Ranson (12) who used paper chromatography, $C^{14}O_2$, and radioautography identified aspartate and glutamate as well as the above-named organic acids in 14-hour experiments with excised Kalanchoë leaves.

In all of the experiments outlined above, relatively long dark CO_2 fixation periods were used and mixtures of products were obtained. It should be possible to identify the product or products of the initial carboxylation reaction by shortening the reaction period. This paper will describe experiments in which we have been able to identify the products of the 6-second dark fixation of $C^{14}O_2$ by excised *B. calycinum* leaves as malate and aspartate. It is suggested that the reaction mechanism for the process involves oxaloacetate as the common, but transient precursor. Evidence for the occurrence of oxaloacetate as well as the presence of the enzyme mediating the synthesis of this compound from PEP[3] and CO_2 in the leaves of *B. calycinum* supports the postulated mechanism.

Materials and Methods

CO_2 Dark Fixation Apparatus: An apparatus was devised in which succulent leaves were exposed to $C^{14}O_2$ in complete darkness, and which permits the leaves to be homogenized in situ after very short exposure intervals. The apparatus is shown in figure 1. A stainless steel micro-Waring blendor was fitted with a large two-holed rubber stopper. The blades were modified, as indicated, to insure total disruption of the leaves. All glass connections were coated with an opaque wax to exclude light. Approximately 1 gm of intact excised leaves are placed around the blade, the stopper securely clamped in place, a vacuum created in the chamber by means of an aspirator, and $C^{14}O_2$, previously generated from $BaCO_3$ and collected in the trap, drawn into the chamber. To terminate the reaction, a vacuum is again created in the chamber and 30 ml of boiling 80 % ethanol drawn into the chamber with simultaneous homogenization. The homogenate was filtered, extracted with Skelly A solvent to remove chlorophyll and carotenoids, and concentrated to a final volume of 2.0 ml under reduced pressure.

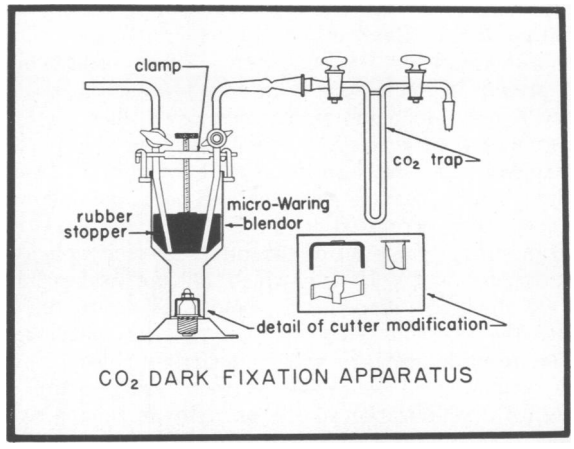

Fig. 1. Apparatus designed for the exposure of intact leaves to $C^{14}O_2$ in complete darkness.

B. calycinum from the clone maintained by the Division of Biology, California Institute of Technology, were obtained through the generosity of Dr. James Bonner and Mr. Charles Newman. The plants were 10 to 15 cm tall when used. Only the young leaves, 1 to 2 cm in length, near the apex of the stem were used. No special care was exercised in maintaining environmental conditions of light or temperature during growth. Prior to use, the whole plants were exposed to sunlight for at least four hours to insure that they possessed an appreciable carbohydrate store.

Chromatographic and Radioautographic Methods: The procedures of Benson et al (3) were used with slight modification. Aliquots (0.1 to 0.2 ml) of the concentrated ethanolic extract were chromatographed on Whatman No. 1 (18″ × 22″) by descending chromatography. The first solvent was 80 % phenol : 20 % water (w/w), the second solvent *n*-butanol (79) : acetic acid (19) : water (50) (v/v). Compounds were located by radioautography made with "no-screen" x-ray film. Activity of each area was determined using an end window Geiger tube directly on the paper. Authentic samples of suspected compounds were cochromatographed with the extract and identification made by superposition. Amino acids were located with the ninydrin in collidine spray of Levy and Chung (8); organic acids with a mixed indicator spray of 3 gm brom-phenol blue–1 gm methyl red per 1000 ml of 95 % ethanol.

Preparation of Enzyme: Homogenates from leaf tissue were prepared by the method of Waygood (18). The leaves are infiltrated with 1 % NH_4OH to neutralize the acidic compounds present in the leaf which will otherwise inactivate the enzyme during grinding. Ten gm of infiltrated leaves were ground with sand in a cold mortar with 10 ml of 0.1 M TRIS buffer, pH 7.5. The homogenate was centrifuged at 500 × g for 5 minutes to remove cell debris and the cloudy

[3] The following abbreviations are employed: PEP, phosphoenolpyruvate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; DPN^+, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TRIS, tris (hydroxymethyl) aminomethane; OA, oxaloacetate; ITP, inosine triphosphate; IDP, inosine diphosphate.

supernatant assayed for PEP-carboxylase activity by the method of Bandurski and Greiner (2).

ISOLATION OF OXALOACETATE: Since oxaloacetate is present in the leaf tissue in very low concentrations and is very labile and readily destroyed when the leaf tissue is homogenized, a satisfactory procedure for trapping the oxaloacetate with carrier and 2,4-dinitrophenylhydrazine was carried out as follows: leaves were permitted to fix $C^{14}O_2$ in the dark for 20 minutes. Fifty ml of saturated 2,4-dinitrophenylhydrazine in 2 *N* HCl in which 100 mg oxaloacetate were dissolved immediately before use were drawn into the reaction vessel and the tissue homogenized. The reaction mixture was immediately filtered, and the supernatant set aside at room temperature to permit crystallization of the phenylhydrazone derivative. The phenylhydrazone was isolated and recrystallized to constant specific activity following the procedures outlined by Bandurski and Greiner (2).

RESULTS AND DISCUSSION

Radioautography of a chromatogram of a leaf extract after a fixation period of 6 seconds is shown in figure 2. Only two products have incorporated $C^{14}O_2$ in this time. These are malate and aspartate. The radioactivity in each of these two compounds was determined for successively longer periods of exposure to $C^{14}O_2$. These activities expressed as percent of total activity in the extract for each exposure time are plotted in figure 3. Extrapolation of these values to zero time shows, however, that there might be some common precursor to the malate and aspartate that is too labile to appear on the chromatograms. A possible hypothesis is that oxaloacetate may be such a precursor. It is also possible that aspartate and malate would incorporate label via independent pathways.

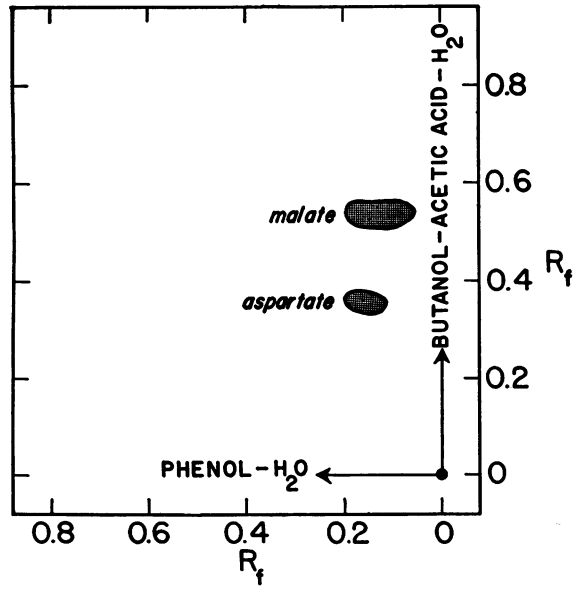

FIG. 2. Tracing of radioautogram obtained from extract of *Bryophyllum calycinum* leaves after 6-sec exposure to $C^{14}O_2$.

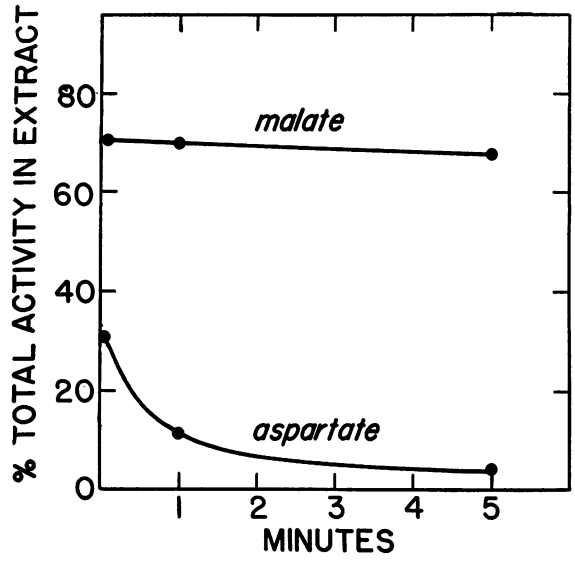

FIG. 3. Radioactivity of malate and aspartate expressed as percent total activity in extract as a function of time.

Much speculation has appeared in the literature concerning the mechanism for the initial fixation of CO_2 in the succulents. Several workers have proposed that the responsible system is the "malic enzyme" (9).

(*1*) CO_2 + pyruvate + TPNH + H^+ → malate + TPN^+

Others have favored the Wood-Werkman reaction (19).

(*2*) CO_2 + pyruvate → oxaloacetate

It should be pointed out that this reaction is a purely speculative one and has not, as yet, been experimentally verified. Utter and Wood (15) demonstrated that in the presence of ATP and Mg^{++}, pyruvate could be carboxylated to yield oxaloacetate

(*3*) $$\text{pyruvate} + CO_2 \xrightarrow[Mg^{++}]{ATP} \text{oxaloacetate}$$

The experiments of Bandurski and Greiner (2) with spinach leaves and of Utter and Kurahashi (14) with liver have clarified the mechanism for the carboxylation of PEP. The enzyme phosphoenolpyruvylcarboxylase from spinach leaves catalyzes the reaction

(*4*) PEP + CO_2 → oxaloacetate + orthophosphate

It is an essentially irreversible reaction and is not dependent upon any nucleotide coenzyme (1). The liver system, on the other hand, is readily reversible and requires the participation of nucleotide coenzymes

(*5*) PEP + CO_2 + IDP ⇆ ITP + oxaloacetate

Thus, the direct phosphorylation of pyruvate

(*6*) pyruvate + ATP → PEP + ADP

TABLE I

ACTIVITY OF PEP-CARBOXYLASE IN BRYOPHYLLUM CALYCINUM EXTRACT

CONDITION	CPM FIXED IN REACTION MIXTURE
Enzyme + PEP	5,430
Enzyme − PEP	30
Boiled enzyme + PEP	0

Each tube contained 60 micromoles TRIS hydrochloride, pH 7.5, 20 micromoles $MgSO_4$, 100,000 cpm $NaHC^{14}O_3$, 0.2 ml enzyme, 6 micromoles PEP. Total volume 1.5 ml. Incubated 60 min at 37° C. Reaction stopped with 0.1 ml 1 *N* HCl and unreacted $C^{14}O_2$ removed by bubbling N_2 through the mixture. An 0.2-ml aliquot of the reaction mixture was counted with an end-window Geiger tube.

linked to either reaction *4* or reaction *5* constitutes a likely reaction mechanism for reaction *3*.

Both the reversible and irreversible carboxylation reactions have been found together in wheat germ (10). That the enzyme of Bandurski's experiment is present in succulent tissue is shown by the data of table I which concerns results from a typical experiment with a homogenate, prepared as indicated above. An estimate of the in vivo activity of PEP carboxylase in *B. calycinum* leaves is impossible because of the losses in activity incurred during preparation.

The presence of oxaloacetate in plant tissues in rather low concentrations has been noted by Virtanen (17). Virtanen also made the observation that the level of oxaloacetate rises in the light and falls in the dark. Bradbeer (5) has obtained evidence for the presence of oxaloacetate in Kalanchoë leaves. He was further able to show that oxaloacetate incorporates $C^{14}O_2$ during a 6-hour dark fixation. We have confirmed Bradbeer's results by permitting leaves of *B. calycinum* to fix $C^{14}O_2$ and by subsequently killing the tissue with carrier oxaloacetate and isolating the 2,4-dinitrophenylhydrazone as described above. Table II presents data from such an experiment. Characterization of the 2,4-dinitrophenylhydrazone of oxaloacetate was made by elemental analysis and cochromatography with an authentic sample confirmed its identity.

Thus it is clear, from the evidence of figure 2, that a single compound gives rise to both malate and aspartate, and the identification of both oxaloacetate and the enzyme, PEP carboxylase, which mediates the synthesis of oxaloacetate, suggests the following scheme for the initial reactions of dark CO_2 fixation by succulent leaves:

TABLE II

CRYSTALLIZATION OF THE 2,4-DINITROPHENYLHYDRAZONE OF OXALOACETATE TO CONSTANT SPECIFIC ACTIVITY

NO. OF CRYSTALLIZATIONS	WT (MG)	CPM/MG
1	95.0	30.1
2	67.1	27.2
3	40.6	27.0

Conditions of the experiment are described in the text. The phenylhydrazone was recrystallized from ethyl acetate by the addition of hexane. Radioactivity was determined on an aliquot with an end-window Geiger tube.

(*7*)

$$\text{Carbohydrate} \xrightleftharpoons{Glyc} \text{PEP}$$

$$\text{PEP} \xrightarrow[PC]{+CO_2} \text{oxaloacetate}$$

$$\text{Oxaloacetate} \begin{cases} \xrightleftharpoons[MD]{} \text{malate} \\ \xrightarrow[T]{+NH_3} \text{aspartate} \end{cases}$$

Glyc = glycolysis *MD* = malic dehydrogenase
PC = PEP carboxylase *T* = transaminase

Such a mechanism has been proposed by Tchen and Vennesland (10). The alternative scheme involving malic enzyme in the initial reaction appears unlikely despite the fact that net dark fixation of small amounts of malate is possible (7) by this system. If malate were the first product, it would be difficult to conceive of its oxidation to oxaloacetate and subsequent transamination to aspartate. The equilibrium for the reaction

(*8*) $$\text{malate} + DPN^+ \rightarrow OA + DPNH + H^+$$

greatly favors the reduction of OA (6). The transaminase reaction is essentially a freely reversible chemical equilibrium. Since succulents have the ability to accomplish a great deal of net CO_2 fixation, it seems more probable that the reaction scheme (*7*) is operative.

SUMMARY

1. The major products of the dark fixation of $C^{14}O_2$ by *Bryophyllum calycinum*, a typical succulent, have been identified as malate and aspartate during a 6-second fixation period.

2. The kinetics of the labeling from these two compounds during longer periods of dark fixation indicate that they must arise from a common precursor. It is suggested that this precursor may be oxaloacetate.

3. Radioactive oxaloacetate has been identified in leaves exposed to $C^{14}O_2$. The enzyme catalyzing the carboxylation of PEP to oxaloacetate has been identified in *B. calycinum* homogenates.

4. A scheme to account for the initial fixation of CO_2 during the dark acid metabolism of succulents is proposed.

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PERMEABILITY OF VICIA FABA ROOT SEGMENTS TO WATER AS MEASURED BY DIFFUSION OF DEUTERIUM HYDROXIDE[1,2]

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In experiments with Avena coleoptiles reported previously (7) the time required for internal concentrations of deuterium hydroxide (DHO) to attain half of the external concentrations was found to be 8 or 9 minutes. It was found in those experiments that segments of coleoptiles killed by treatment with 2,4-dinitrophenol (DNP) exhibited only a slight increase in permeability to water, the half time being decreased to about 6.5 minutes in dead coleoptile segments. This contrasts with results obtained by Brouwer (1) and Kramer (4) with roots, and results of Wartiovaara (12) with Tolypellopsis, all of these workers finding a large increase in permeability to water after killing. These workers concluded that most of the resistance to water movement in roots resides in the cytoplasm but the results of the experiments with Avena coleoptiles suggest that in the latter most of the resistance to water movement resides in the cell walls. A series of experiments similar to those on Avena coleoptiles was, therefore, performed on roots in order to learn if roots behave differently from coleoptiles. It will be shown below that the half time of diffusion of water in *Vicia Faba* root segments is less than one-tenth that in Avena coleoptile segments and that killing either by DNP treatment or by heat treatment results in a considerable decrease in the half time, i.e., permeability increases.

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Materials and Methods

The material used in this work consisted of 10-mm segments cut 3 to 4 mm above the apices of secondary roots of *Vicia Faba*. The diameter of the segments was about 0.5 mm. The *Vicia Faba* (var. Windsor Long Pod) seedlings were grown as follows. About 120 seeds were soaked overnight in an enameled tray containing a shallow layer of distilled water. The seed coats were removed and seeds which appeared healthy, i.e., not attacked by parasites, were planted on water-soaked and drained vermiculite and covered by wet vermiculite in a stainless steel tray. Four