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Crystal Morphology and ^{13}Carbon/^{12}Carbon Composition of Solid Oxalate in Cacti[1]

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ABSTRACT

Morphology, crystal structure, and carbon isotopic composition of calcium oxalate from representative species from the family Cactaceae were determined using scanning electron microscopy, x-ray diffraction, and isotope ratio mass spectrometry. Crystals from one species in the Opuntieae tribe of the Cactaceae were druses with acute points composed of the monohydrate form of calcium oxalate (whewellite). Crystals from three species in the Cereeae tribe were the dihydrate form of calcium oxalate (weddellite) forming druses made up of tetragonal and isodiametric crystallites. The oxalate was relatively enriched in ^{13}C isotope (−7.3 to − 8.7 ‰) compared with woody fibers (−13.3 to −14.1 ‰) from the same plants.

Most cacti have large amounts of crystalline calcium oxalate distributed throughout their tissues. These deposits are prevalent in crystal idioblasts and the quantity of these cells in a single plant may be related to the amount of Ca available in the soil (7). In the United States large populations of cacti are primarily found in calcareous soils, and thus Ca is not expected to be a limiting factor for crystal idioblast differentiation and subsequent crystal deposition. As oxalate crystallizes, it assumes various morphological aspects. Star-like conglomerates of small crystallites (druses), large single needles (styloids), and bundles of long thin crystals (raphides) are all commonly found in higher plants. In a survey of different plant species, Al-Rais et al. (1) found that crystalline calcium oxalate dihydrates occurred as druses whereas the monohydrates were found as raphides or styloids. In this study oxalate druses were found as both monohydrates (whewellite) and dihydrates (weddellite).

Calcium oxalate has been analyzed for its ^{13}C/^{12}C composition in only a few studies. Hoefs (9) determined the ^{13}C/^{12}C ratio of crystalline oxalate from rhubarb. Of all other plant crystalline deposits, only aragonite crystals from hackberry have also been analyzed for their ^{13}C/^{12}C ratios (13). Since it has been demonstrated that the isotope ratio of the metabolites reflects fractionation effects with respect to biosynthetic pathway (12), it is necessary to compare isotope ratios between plant components to establish their metabolic position (14). Oxalate from idioblasts found in both green and nongreen parts of the stem has been analyzed and compared to other components of the stem to establish such a position. The relatively high ^{13}C content of the oxalite crystals implies that an unusual mechanism is responsible for oxalate synthesis.

MATERIALS AND METHODS

Plant Material and Atmospheric CO_2. *Opuntia imbricata* Haw. and *O. englemannii* Parry from the tribe Opuntieae were collected from the campus of the University of Texas. Other cacti from the tribe Cereeae were collected from West Texas. *Echinomastus intertextus* Engelm. was collected in Paradise Canyon; *Escobaria tuberculosa* Brit. and Rose, in the Marathon Basin; and *Echinocactus horizonthalonius* Lemaire, near Packsaddle Mountain. Spinach (*Spinacia oleracea* L.) was purchased at a local market and used for comparison with the cacti. Oxalate was removed mechanically from the plants as they were brought into the laboratory. Air samples were collected in evacuated 5-liter bottles at the sites of cacti collection in Paradise Canyon, Marathon Basin, and Packsaddle Mountain.

Separation of Oxalate Crystals. Crystalliferous regions of *Echinomastus*, *Echinocactus*, *Escobaria*, and *Opuntia* stems were removed from the plants and allowed to dry in air. The dried material from each species was placed in 12-cm Petri dishes and covered with absolute ethanol. The material was macerated with dissection knives which freed the crystals from their respective cells. Spinach leaves were placed in a glass bowl and covered with water and then macerated with razor blades. The macerates were filtered through four layers of cheesecloth allowing the crystals through, but excluding the plant tissue. About 500 mg of purified oxalate was collected in this manner. These crystals were washed repeatedly with distilled H_2O and ethanol until plant debris was no longer evident under a 30× dissecting microscope.

Microscopy. Representative crystals from each species were manually placed on a piece of mica (1 × 1 cm) with mounting medium. All specimens were coated with gold-palladium from a distance of 5 cm at a 45° angle. Scanning electron microscopy was carried out with a JSM-2 scanning electron microscope (JEOL Co., Inc., Tokyo) at an acceleration voltage of 20 and 25 kv.

Oxalate Identification. The chemical composition of the crystals was identified from x-ray diffractometer scanning patterns. About 200 mg of crystalline material was ground to a fine powder (about 300 mesh) in an agate mortar and pestle. The powder was put in a glass slide to fit a diffractometer on a Norelco x-ray unit. Ni-filtered Cu Kα radiation (λ = 1.54 Å) was used in all analyses. The Cu tube was operated at 35 kv and 20 mamp. The results were compared to the published analyses in the American Society for Testing and Materials (ASTM) file. The reflections in the diffraction patterns are indicated by the plane minimum distance periodicities (d Å) with corresponding relative intensity (I/I_o).

^{13}C/^{12}C Analyses. Oxalate and other organic matter were combusted to CO_2 at 800 C. The resulting samples of CO_2 and the collected atmospheric CO_2 samples were submitted to mass spectrographic analysis in a Nier-type isotope-ratio mass spectrometer. Results are expressed relative to the PDB carbonate standard: δ-

$$^{13}C\ ‰ = \frac{R_{sample}}{R_{standard}} - 1 \times 1000$$ where R = the mass 45 to mass 44 ratio of sample of standard. Precision of measurement was ± 0.2 ‰ for combustion and analysis.

RESULTS

Morphology of Oxalate Crystals. Crystals in species of cacti from Opuntieae and Cereeae tribes observed in these studies were found as druses. Figure 1 shows the general aspect of these structures after they were removed from the plants. In general, these druses are aggregates of hundreds of small crystals. The differences among the various druses depends on the gross shape of the individual crystallites that make up the conglomerate. In each species all of the crystallites that make up the druse have the same general appearance. In *Opuntia*, the individual crystallites have acute points and all appear to grow out from the center of the druse (Fig. 1). On the other hand, druses from *Echinomastus* appear to be made up of symmetrical tetragonal crystallites piled up on one another (Fig. 2). Forms of druse crystallites intermediate between those in *Opuntia* and *Echinomastus* druses were also found. For example, the druses in *Echinocactus* (Fig. 3) and *Escobaria* (Fig. 4) are made up of more isodiametrically shaped crystallites than those of *Opuntia*, but they do not have the more definite tetragonal symmetry of the crystallites from *Echinomastus*.

Identification of Calcium Oxalate. X-ray diffractometer scans of pulverized crystalline material from all cacti show that these crystals are various types of calcium oxalate. Standards for various oxalate forms are not available, therefore, the results were compared to published x-ray analyses in the ASTM file. Scan data of the cacti crystals fall into two categories. Crystal data from the *Opuntia* crystals best fit data in the ASTM file that corresponds to calcium oxalate monohydrate. Data in Table I indicate that there were some additional diffraction lines obtained from the *Opuntia* crystals that are not present in the published data. The most important difference is the presence of a moderately intense line at a d spacing of 5.81 Å ($I/I_o = 25$) and another at 3.78 Å ($I/I_o = 15$). The ASTM data cards do not include these lines. There were additional reflections obtained from these crystals, but these
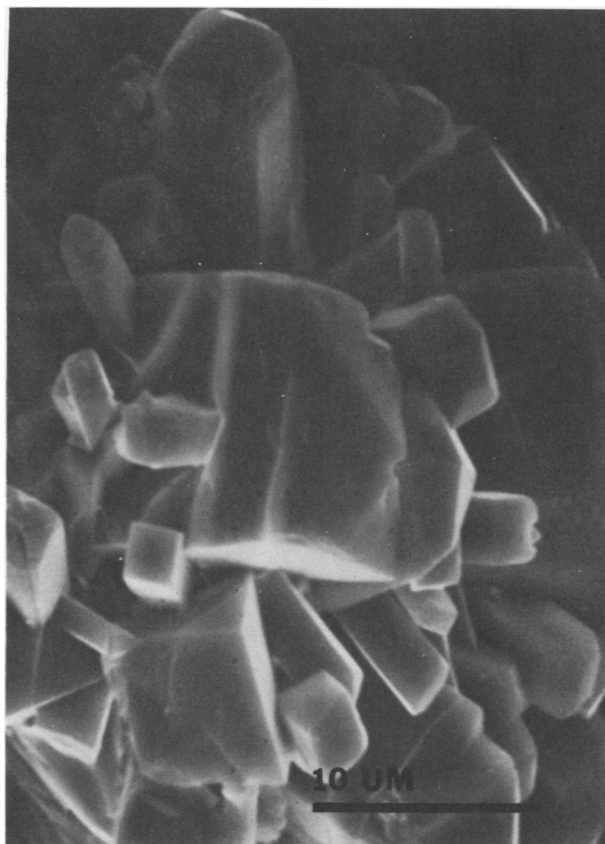

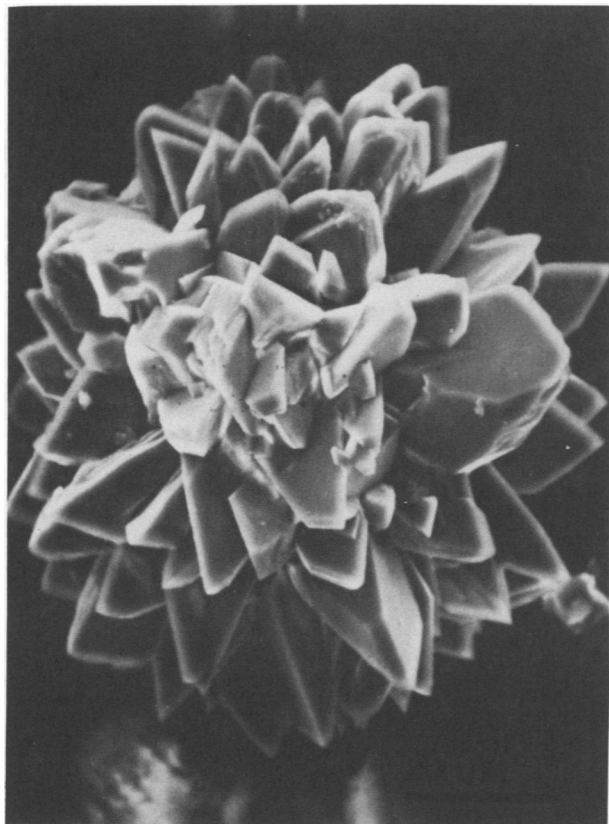FIG. 2. Surface view of a druse from *Echinomastus*.

FIG. 1. Crystal druses of *Opuntia* demonstrating the crystallites with acute points.
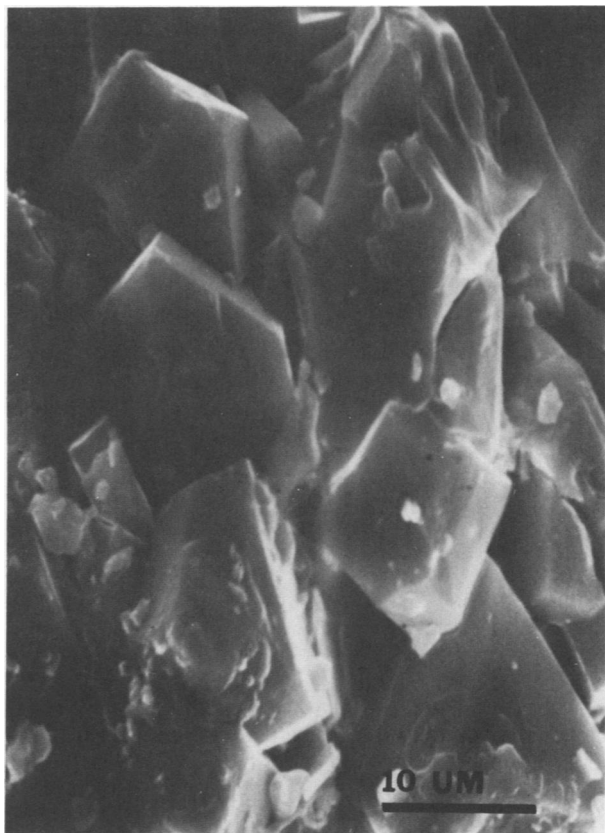

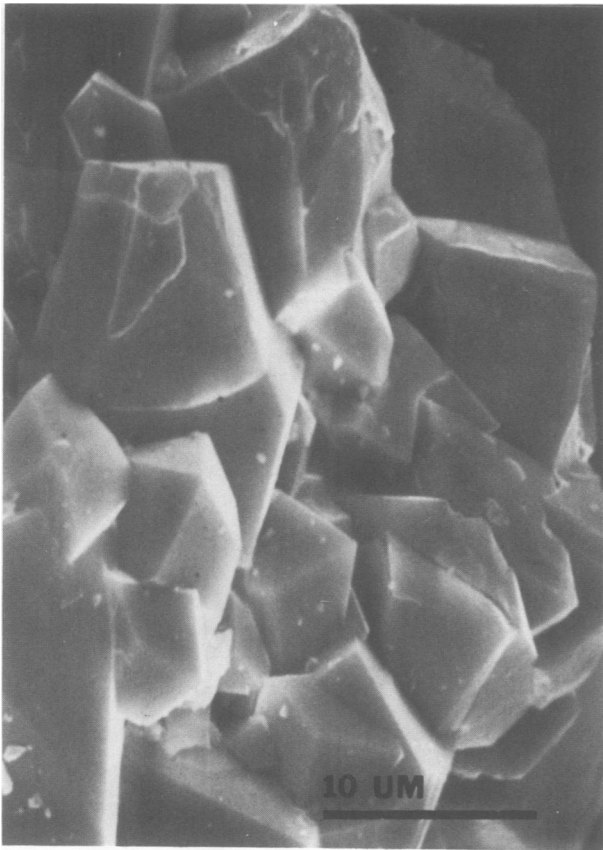FIG. 3. Crystallites from a druse from *Echinocactus*.

FIG . 4. View of druse from *Escobaria*.

are of very low intensity and are often difficult to distinguish from detector background. The three main reflections at $d = 5.95$ Å, 3.65 Å and 2.98 Å correspond to the ASTM calcium oxalate monohydrate data, however, and it is evident that this material is some form of monohydrate calcium oxalate.

Crystals obtained from different species in the Cereeae tribe were found to be calcium oxalate dihydrate. In this case, the x-ray scanning data from the crystals conforms well to the ASTM file data (Table II). The data which have been indexed in the ASTM file show that these patterns are due to a tetragonal form of calcium oxalate. Some of the low intensity reflections reported in the ASTM file were not present in the scans of *Echinomastus* crystals, but all of the reflections of intensities above $I/I_o = 8$ were observed. Of the samples from three Cereean species only *Escobaria* crystals show an additional diffraction line at $d = 3.03$ Å that was not recorded on the ASTM file data, but this was not an important reflection ($I/I_o = 8$). The crystal powder scan pattern of *Echinocactus* fits the ASTM data particularly well.

$^{13}C/^{12}C$ **Analysis of Oxalate and Other Plant Components.** Table III summarizes δ-^{13}C values obtained for various components of the plants as well as values for atmospheric CO_2 at some of the collection sites. The δ-^{13}C values are the same at Paradise Canyon (collection site of *Echinomastus*) and Packsaddle Mountain (site of *Echinocactus*). There is a slight difference at Marathon Basin (site of *Escobaria*).

The various cacti all were found to have similar δ-^{13}C values of oxalate. These values varied from -8.7 ‰ for *Opuntia* to -7.3 ‰ for *Echinomastus*. *Echinomastus* oxalate is 1 ‰ heavier in ^{13}C than the atmospheric CO_2 of its environment. By comparison oxalate

Table I. *Comparison of ASTM Data of Calcium Oxalate Monohydrate and Diffractometer Scan Data of Crystal Druses in* Opuntia imbricata

Whewellite Ca₂O₄·H₂Oᵃ		O. imbricataᵇ	
dÅᶜ	I/Iₒᵈ	dÅ	I/Iₒ
*5.9ᵉ	100	*5.95	100
		5.81	25
		3.78	15
*3.65	83	*3.65	80
		3.00	10
*2.98	58	*2.97	50
		2.90	10
2.85	6	2.83	15
2.50	33	2.49	30
2.35	58	2.35	43
2.26	20	2.26	10
2.21	3	2.21	5
2.13	3	2.12	2
2.07	20	2.08	10
		1.97	5
1.95	13	1.95	5
		1.89	5
		1.86	3
1.82	7	1.82	5
1.73	10		
Others—low intensity			

ᵃ ASTM data were obtained with Zr-filtered CuKα (1.54 Å) radiation.
ᵇ Cactus data were obtained with Ni-filtered CuKα radiation.
ᶜ dÅ is minimum distance of periodicity of crystallographic planes given in angstrom units (Å).
ᵈ I/Iₒ is relative intensity of diffraction response for each analysis.
ᵉ The three major peaks are indicated by * in each analysis.

Table II. *Comparison of ASTM x-Ray Data of Calcium Oxalate Dihydrate to Diffractometer Data from Crystal Druses of Three Species of Cacti from the Tribe Cereeae*

For abbreviations see footnotes for Table I.

Weddellite CaC₂O₄·2H₂O		Echinomastus intertextus		Escobaria tuberculosa		Echinocactus horizonthalonius	
dÅ	I/Iₒ	dÅ	I/Iₒ	dÅ	I/Iₒ	dÅ	I/Iₒ
8.73	4	8.66	5	8.75	6	8.58	5
6.32	6					6.32	8
*6.18	100	*6.14	100	*6.18	100	*6.14	100
*4.42	30	*4.39	40	*4.43	40	*4.39	40
4.37	2					4.30	3
3.91	8	3.89	10	3.91	6	3.89	10
3.68	12	3.65	15	3.68	12	3.65	10
3.59	2						
3.39	4			3.34	15	3.37	4
3.16	4			3.24	10	3.15	5
3.09	10	3.07	15	3.09	15	3.07	30
2.82	14	2.80	15	2.81	20	2.79	30
*2.78	65	*2.77	80	*2.77	80	*2.77	80
2.76	4						
2.68	2					2.66	3
2.42	8			2.42	10	2.42	8
2.41	16	2.40	20	2.40	10	2.39	20
2.37	2						
2.34	4	2.33	6	2.34	6	2.33	3
2.28	2					2.27	3
2.24	25	2.24	35	2.24	35	2.23	25
2.21	6	2.20	8	2.21	6	2.20	10
2.12	8	2.11	10	2.12	20	2.11	18
2.02	6	2.02	10	2.02	6	2.02	8
1.96	10	1.94	15	1.96	20	1.95	10
1.90	10	1.94	15	1.96	20	1.95	10
1.83	10	1.83	15	1.84	10	1.83	10
1.74	6	1.74	3	1.74	3	1.74	3
Others—low intensity							

Table III. δ-^{13}C Values (o/oo) for Various Components of Cacti

Species	Atmospheric CO_2	Calcium Oxalate	Spines	Fibers
Echinomastus intertextus	−8.3[a]	−7.3	−9.2	−13.4
Echinocactus horizonthalonius	−8.3[b]	−7.8	−11.1	−13.0
Escobaria tuberculosa	−7.8[c]	−8.3	−10.4	−12.3
Opuntia englemannii		−8.5	−10.1	−13.3
Opuntia imbricata		−8.7	−12.8	−14.1
Spinacia oleracea		−19.5		−25.7

[a] Paradise Canyon.
[b] Packsaddle Mountain.
[c] Marathon Basin.

from spinach, a C_3 plant, was much lighter (−19.5 ‰). Plant parts rich in carbohydrates like the wood fibers of *Opuntia imbricata* (−14.1 ‰) and the epidermis of *Echinomastus* (−13.4 ‰) were much lighter than the oxalate. Spines from the cacti in both the Cereeae and Opuntieae tribes have intermediate δ-^{13}C values between their oxalate and fiber components. This heavily sclerified tissue was found to have δ-^{13}C values between −9.2 ‰ (*Echinomastus*) and −12.8 ‰ (*Opuntia imbricata*).

DISCUSSION

Crystalline oxalates are known from a wide spectrum of plant sources and in some plant species the amounts of oxalates produced may account for more than 8% of dry weight (2). In spite of their ubiquity in plants the presence of these deposits has yet to be correlated with specific physiological functions. It has been suggested that oxalates are produced for the purpose of retaining Ca which is important for over-all cell function (2). Because cacti live in calcareous soils, it seems that oxalate should serve some additional purpose in these plants. Recently, it has been suggested that both mono- and divalent ion supply control in plants and soil is the result of the chelating qualities of oxalate (8). Thus, it would be important for plants growing under certain soil conditions to produce such a chelating agent to insure adequate amounts of ions needed for growth. In *Echinomastus* and *Echinocactus* the cortex in the older regions of the stem contained as much as 50% dry weight of oxalate salt. Since the cortex in these species accounts for more than half of the entire plant the total carbon shuttled into oxalate production appears remarkably high. In the present study, it was found that cacti produce both mono- and dihydrate oxalate and that the method of production may have some unique qualities.

Oxalates often have slightly different powder patterns or diffractometer reflections as evidenced by the large number of oxalate data cards in the ASTM file. Recently, Al-Rais et al. (1) found extra reflections in an x-ray analysis of calcium oxalate monohydrate (whewellite) crystals from *Monstera* and *Coleus*. Similarly, the same type of oxalate in *Opuntia* has several extra reflections, although only two are of moderate intensity and the rest are low intensity. The reflections at d spacings of 5.80 Å (I/I_o = 25) and 3.78 Å (I/I_o = 15) cannot be explained at this time. Sometimes oxalates have natural contaminants which may give rise to the extra d spacings (2). Besides the crystals from *Opuntia imbricata* which are indicated in these analyses, other *Opuntia* species also have the monohydrate form of calcium oxalate (Rivera, unpublished). All druses observed here from cacti of the tribe Cereeae are the dihydrate form of calcium oxalate (weddellite). Whatever phylogenetic schemes one might derive for the cacti on the basis of these results should be regarded with caution because other members of the Cereeae are known to have styloid crystals which may be the monohydrate form of oxalate (1, 3). Similarly, one should be wary of assigning amount of hydration to a crystal on the basis of its gross habit without actual x-ray or chemical analysis. Al-Rais et al. (1) found that in a group of plants which

they had selected, the monohydrate form of calcium oxalate was associated with raphides, but the dihydrate form was found as both druses and "solitary forms" except raphides. In our analyses the druses of *Opuntia* are clearly the monohydrate form of calcium oxalate and therefore the association of crystal habit to the amount of hydration associated with calcium oxalate without actual analysis does not hold. Perhaps in a large enough sampling of different plant species one might find raphides which are dihydrate oxalates. Although raphides are known in cacti, none were analyzed in these experiments, nor were the styloids which were found in Cereeaen cacti.

The $^{13}C/^{12}C$ study of oxalate was undertaken to determine the metabolic position of this abundant material in plants such as cacti. Although Hoefs (9) determined the δ-^{13}C value of rhubarb oxalate to be −28.6 ‰, he did not compare it to other parts of the plant making it impossible to determine its relative δ-^{13}C. In these studies oxalate was found to have relatively more ^{13}C than other plant components. For comparison, spinach was chosen as a representative C_3 plant against which the cacti (CAM plants) could be compared (6, 15). In both spinach and cactus species, the fibers were depleted in ^{13}C relative to oxalates from the same plant (Table III). Spinach oxalate had a δ-^{13}C value (−19.5 ‰) that was remarkably close to published values for the National Bureau of Standards oxalate standard of −19.3 ‰ (II). The oxalate δ-^{13}C value of −7.3 ‰ in *Echinomastus* is the highest ever found for oxalate.

In C_3 plants glycolate is an early product of photosynthesis and may subsequently be oxidized to glyoxylate and then to oxalate (10). In ^{13}C content, the glycolate and oxalate will be similar to the sugar fraction of the plant (12). In plants such as the cacti which exhibit Crassulacean acid metabolism, the first product of dark fixation of CO_2 by PEP carboxylase is oxaloacetic acid which may be only 3 ‰ depleted in ^{13}C relative to the source CO_2 (16). Enzymic cleavage of some oxaloacetic acid into oxalate and acetate (4, 5) could account for the observed isotopic values (Table III), especially if the acetate moiety were somewhat depleted in ^{13}C and the oxalate molecule enriched. Oxaloacetate formed by dark CO_2 fixation in cacti could be enzymically cleaved into oxalate and acetate before reduction to malate and subsequent sequestration of the 4-carbon acid in the central vacuole. This possibility is now under investigation.

The δ-^{13}C values of the spines are unusual in that they differ so much from other carbohydrate components. At this time we can only suggest that perhaps some of the lignin and tanin components in this highly sclerified tissue contain relatively larger amounts of ^{13}C.

LITERATURE CITED

1. AL-RAIS AH, A MYERS, L WATSON 1971 The isolation and properties of oxalate crystals from plants. Ann Bot 35: 1213–1218
2. ARNOTT HJ, FGE PAUTARD 1970 Calcification in plants. In H Schraer, ed, Biological Calcification: Cellular and Molecular Aspects. Appleton-Century-Crofts, New York, pp 375–446
3. ARNOTT HJ, FGE PAUTARD, H STEINFINK 1965 Structure of calcium oxalate monohydrate. Nature 208: 1197–1198
4. BREIDENBACH RW, H BEEVERS 1967 Association of the glyoxylate cycle enzymes in a novel subcellular particle from castor bean endosperm. Biochem Biophys Res Commun 27: 462–469
5. CHANG CC, H BEEVERS 1968 Biogenesis of oxalate in plant tissues. Plant Physiol 43: 1821–1828
6. DINGER BE, DT PATTEN 1972 Carbon dioxide exchange in selected species of *Echinocereus* (Cactaceae). Photosynthetica 6: 345–353
7. FRANK E 1972 The formation of crystal idioblasts in *Canavalla ensiformis* D.C. at different levels of calcium supply. Z Pflanzenphysiol 67: 350–358
8. GRAUSTEIN WC, K CROMACK JR, P SOLLINS 1977 Calcium oxalate: occurrence in soils and effect on nutrient and geochemical cycles. Science 198: 1252–1254
9. HOEFS J 1969 Natural calcium oxalate with heavy carbon. Nature 223: 396

10. RICHARDSON KE, NE TOLBERT 1961 Oxidation of glyoxylic acid to oxalic acid by glycolic acid oxidase. J Biol Chem 236: 1280-1284

11. SCHWARCZ HP 1969 The stable isotopes of carbon. In KH Wedepohl, ed, Handbook of Geochemistry, Springer-Verlag, New York, Vol 11/1 6-B-1 to 6-B-1b

12. SMITH BN 1972 Natural abundance of the stable isotopes of carbon in biological systems. BioScience 22: 226-231

13. SMITH BN, GK HOOPS, LS LAND, RL FOLK 1971 $^{13}C/^{12}C$ ratios of aragonite from fruit of Celtis laevigata Wield. Naturwissenschaften 58: 365-366

14. SMITH BN, BS JACOBSON 1976 $^2H/^1H$ and $^{13}C/^{12}C$ ratios for classes of compounds isolated from potato tuber. Plant Cell Physiol 17: 1089-1092

15. TING IP, WM DUGGER 1968 Non-autotrophic carbon dioxide metabolism in cacti. Bot Gaz 129: 9-15

16. WHELAN T, WM SACKETT, CR BENEDICT 1973 Enzymatic fractionation of carbon isotopes by phosphoenolpyruvate carboxylase from C_4 plants. Plant Physiol 51: 1050-1054