STUDIES IN THE METABOLISM OF CRASSULACEAN PLANTS: DIURNAL VARIATION OF ORGANIC ACIDS AND STARCH IN EXCISED LEAVES OF *BRYOPHYLLUM CALYCINUM*

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(WITH TWO FIGURES)

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It has been shown in a previous paper (**7**) that the diurnal variation of the organic acid content of excised leaves of *Bryophyllum calycinum* cultured in water under normal light conditions in the greenhouse is accompanied by a diurnal variation of the starch content in the opposite sense. The acidity decreased during the day while the starch increased, but the acidity increased during the night while the starch decreased. In terms of the quantity of organic substances concerned, these two changes in composition were by far the largest observed, and their relative magnitude was such as to lend color to the view that the metabolism of organic acids in the leaves is closely connected with that of starch. The observations thus supported the hypothesis of BENNET-CLARK (**1, 2**) that certain of the polysaccharides and the malic acid in succulent plants of this type are the respective beginning and end products of a series of chemical equilibria, the relative concentration of these components at any point of time being a function of the conditions of illumination to which the leaves had previously been exposed. The observations of WOLF (**12, 13**) have laid particular emphasis upon starch as the polysaccharide concerned in the case of *Bryophyllum calycinum*.

The previous experiment carried out in this laboratory was limited to a study of the changes in composition of samples of *Bryophyllum calycinum* leaves cultured in water over a period of 24 hours. It seemed desirable, therefore, to repeat the experiment and study the changes for a longer period in order to see to what extent the chemical transformations would continue to respond to repeated alternations of light and darkness. Furthermore, a moderately accurate analytical method for isocitric acid has since become available, and direct examination of the behavior of this component is now possible. In the previous work, isocitric acid had been estimated by difference, a procedure that might well introduce error into the conclusions reached.

The present paper is, therefore, a report upon the composition of ex-

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cised *Bryophyllum calycinum* leaves cultured in water in the greenhouse for three days with special attention to the diurnal changes in composition with respect to organic acids and starch.

Experimental

The samples were collected from plants derived from the same clone as those used in the earlier work and were grown under the same conditions. Small plants had been transplanted to sand in crocks on February 2, 1947. The collection was made at 4 A.M. (standard time) on July 7 (sunrise, 4.26 A.M.; sunset, 7.28 P.M.), the leaflets being cut from the upper 8 to 10 petioles from 28 plants. The individual samples were selected by the leaf size method (**10**), 11 samples of mean weight 262.2 ± 2.2 grams and including from 49 to 53 leaflets each being taken. Two of

TABLE I

FUNDAMENTAL DATA ON SAMPLES OF EXCISED *Bryophyllum calycinum* LEAFLETS SUBJECTED TO CULTURE IN WATER IN ALTERNATE LIGHT AND DARKNESS

	PER SAMPLE			PER KILOGRAM INITIAL FRESH WEIGHT				
CULTURE PERIOD	FRESH WEIGHT AT START	FRESH WEIGHT AT END	EQUILIBRATED CRUDE DRY WEIGHT	CRUDE DRY WEIGHT	NITROGEN	PROTEIN NITROGEN	ORGANIC SOLIDS	ISOCITRIC ACID
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*m.eq.*
0	266.7	266.7	29.8	111.7	2.00	1.62	88.6	168
0	260.5	260.5	28.5	109.4	2.00	1.73	88.7	170
10	261.0	253.6	29.3	112.3	2.04	1.67	90.4	173
16	260.0	250.4	28.2	108.5	1.97	1.66	87.6	178
24	260.6	258.3	28.3	108.6	2.13	1.74	87.4	173
34	261.8	256.9	28.6	109.2	2.07	1.67	87.7	174
40	261.1	256.2	30.1	115.3	2.02	1.64	93.0	172
48	265.3	263.8	29.7	111.9	2.04	1.61	90.2	171
58	261.1	259.0	28.3	108.4	2.08	1.69	86.9	160
64	262.1	260.0	29.6	112.9	2.05	1.63	93.1	166
72	264.1	263.2	31.5	119.3	2.10	1.72	93.3	171
Mean	262.2				2.045	1.67	89.7	170.5
Standard deviation	±2.2				±0.047	±0.045	±2.5	±4.7
Coefficient of variation (%)	0.83				2.3	2.7	2.7	2.8

these samples were at once dried for use as controls and the remainder were arranged in troughs in the greenhouse, the bases being immersed in distilled water. Single samples were removed and dried for analysis at 2 P.M., 8 P.M., and 4 A.M., (standard time) during the following 72 hours. The first day was clear and bright, the second was cloudy until 8 A.M. and then intermittently bright and cloudy for the rest of the day. The third day was moderately fine although there was a brief thunderstorm at noon.

As will become evident in the discussion of the starch and organic acid composition of the samples, the variations in light conditions from day to day appear to have had an influence upon the extent and rate of the changes that occurred.

The first five columns of table I show the fundamental data from which the factor is derived to convert the composition of each sample, as obtained by chemical analysis in terms of percentage of the crude dry weight, to grams per kilogram of initial fresh weight. This correction of the data is essential in order that the comparisons of the separate samples shall be valid, for it was only at the time of collection that they were all in the same metabolic condition.

The samples were selected with special care to secure uniformity in initial fresh weight. The coefficient of variation of the fresh weight was only 0.83 per cent. That a moderately satisfactory degree of uniformity in composition was also achieved is evident from the results for the total nitrogen (table I, column 6); the coefficient of variation was 2.3 per cent. This value could be improved upon only by the application of the statistical method of collection of the leaflets (**10**), a method which was not devised until after the present culture experiment was completed.

Results

Protein nitrogen

In view of the unfortunate error that was made in computing the protein nitrogen (**9**) in three of the earlier papers of this series (**6, 7, 8**), special attention was devoted to this component in the present experiment. The data (table I, column 7) show that no significant change in the quantity of protein took place during culture in alternate light and darkness for three days. The coefficient of variation was 2.7 per cent., which is negligibly greater than that for total nitrogen. No significant change in total nitrogen could occur under the present conditions of culture.

Organic solids

The organic solids of the tissues did not change significantly throughout the period studied. The coefficient of variation (table I, column 8) was 2.7 per cent., identical with that for protein nitrogen. One might anticipate that photosynthesis during a period of daylight would lead to a detectable increase in organic solids, for such an increase is easily demonstrable in tobacco leaves cultured under similar conditions (**11**). The present data show that the net changes in organic solids in Bryophyllum leaves from photosynthesis and respiration were smaller than the variation among samples.

Organic acids

The changes in the acidity of the leaves are shown in the top curve of figure 1 in which the pH is plotted. Starting at the strongly acid reac-

tion of pH 3.95 at dawn, the acidity diminished to pH 5.5 by 2 P.M. and remained essentially unchanged in the sample collected shortly after sunset. During the night, the acidity increased to pH 4.1, but the cloudy weather of the second day appears to have interfered to some extent with

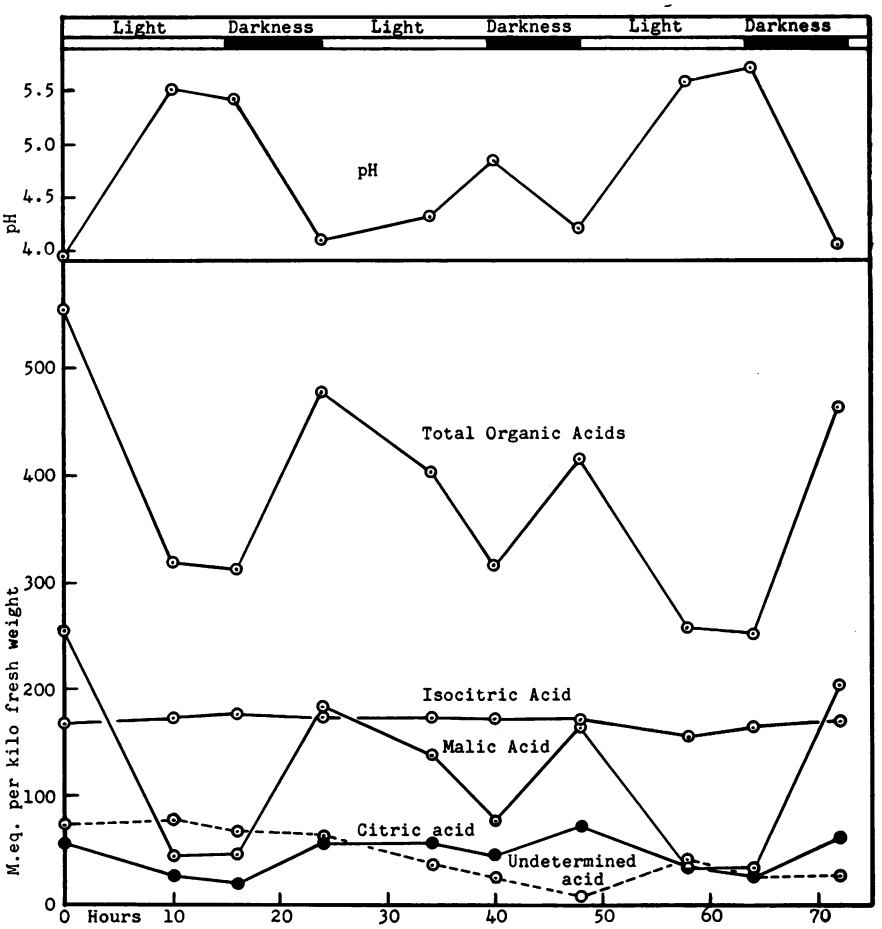

FIG. 1. Diurnal variation in pH (upper part of figure), total organic acids and individual organic acids of excised leaves of *Bryophyllum calycinum* cultured in water. Alternations of light and darkness indicated at top of figure. Data are milliequivalents of acid per kilogram of initial fresh weight.

the subsequent rise in pH; the maximum of 4.8 was not attained until the evening sample was taken. During the third day, however, the changes were quite similar to those of the first day.

The curve for total organic acids shows corresponding fluctuations; starting at 557 m.eq. per kilo of initial fresh weight at dawn, the acids had decreased by 244 m.eq. at sunset. The greater part of the loss arose from the disappearance of 213 m.eq. (14.3 grams) of malic acid, the remainder being citric acid which diminished by 37 m.eq. (2.37 grams).

During the night, the acids increased by 166 m.eq. most of which was malic acid although citric acid rose to its initial level. The decrease in acids during the second day was less precipitous than on the first, possibly because of the cloudy weather. Furthermore, citric acid diminished by only 14 m.eq. and malic acid by only 107 m.eq.

During the third period of daylight, the total organic acids dropped by 164 m.eq. and again the change was nearly complete by 2 P.M. Of this quantity, 135 m.eq. consisted of malic acid and 43 m.eq. of citric acid; together, these amount to 13.7 grams of organic substance. During the last night of the experiment, the acids rose by 212 m.eq. of which 173 m.eq. (11.6 grams) were malic acid.

It is clear, therefore, that excised Bryophyllum leaves retain the capacity to undergo diurnal variation in acidity for at least three days, and that the greater part of the change arises from alterations in the quantity of malic acid in the tissues. Citric acid shares in the variation but to only a moderate extent in relation to malic acid.

Isocitric acid

Especial significance attaches to the results for isocitric acid plotted in figure 1. The analytical method used depends upon the fact that isocitric acid is converted to citric acid by the enzyme aconitase present in mammalian heart muscle. Krebs and Eggleston (**5**) have shown that the equilibrium mixture of acids contained in a properly buffered system incubated with heart muscle aconitase contains 89.5 per cent. of citric acid, 6.2 per cent. of isocitric acid, and 4.3 per cent. of aconitic acid. Accordingly, as these authors have shown, isocitric acid can be determined from the difference in citric acid content of a sample of tissue before and after treatment with an extract or suspension of heart muscle. This method was applied by Krebs and Eggleston to several succulent species and gave results which indicated that isocitric acid shares to a small extent in diurnal changes in acidity, although malic acid was found to be the main acid component that altered in concentration. Bonner and Bonner (**3**) have employed a similar method in their studies of the effect of various partial pressures of carbon dioxide in the air upon the changes in acid content of several succulent species.

The curve for isocitric acid (figure 1) shows that this component of the tissues did not change significantly during the period of the experiment; the results[3] given in detail in table I (last column) yield a coefficient of variation of 2.8 per cent., only slightly greater than the variation in total nitrogen. Within the limits of accuracy of the sampling method used,

[3] In calculating the results of the analyses of these samples, the possible presence of traces of aconitic acid has been neglected. Although the precision is excellent, the accuracy is open to slight question; that is to say, the correct curve may occupy a position a little different from that in the diagram. These points are at present under investigation.

isocitric acid does not share in the diurnal variation of acidity in *Bryophyllum calycinum* leaves.

Undetermined organic acid

The difference between the sum of the malic, citric, isocitric acids and the total organic acids is plotted in figure 1 (dotted line) as undetermined organic acid. Inasmuch as this difference contains all of the analytical errors of the separate determinations, too much weight cannot be placed upon the accuracy of the results. However, it is evident that the samples contained a small proportion of organic acids in addition to those that were directly determined. One of the components of this fraction is oxalic acid, for the presence of traces of oxalic acid in Bryophyllum leaves has been established in earlier papers from this laboratory (**6, 7**). Determinations were not made in the present series of samples, but, from previous experience, the quantity would be expected to be 20 m.eq. per kilo or less; oxalic acid therefore presumably makes up only a fraction of the undetermined organic acid shown in the figure.[4]

The trend of the curve suggests that a part of the undetermined acid is irreversibly decomposed during the culture period but there is no indication of a diurnal variation in quantity. However, further study by more sensitive analytical methods will be needed before the part played by these substances becomes clear.

Starch

Figure 2 shows the behavior of the starch. At dawn, the leaves contained 9.6 grams per kilo of starch but this had increased to 25.3 grams by early afternoon of the sunny first day and to 25.9 grams (*i.e.* by 16.3 grams per kilo) by evening. Starch formation practically ceased by 2 P.M. of this day; the additional four or five hours of bright sunlight gave rise to only a small further increase. During the night, starch dropped to 12.7 grams but increased again, although slowly, throughout the cloudy second day of the experiment reaching the level of the previous evening by sunset. The starch content changed less strikingly during the second night, but, the following day, reached the level of 26 grams per kilo by sunset and again underwent a sharp fall during the third night. It is important to note that starch attained essentially the same maximum on three successive days.

The curve in general resembles that for the total acidity, although the fluctuations are in the reverse direction. In order to illustrate this point more vividly, the total organic acids have been plotted on a suitable scale

[4] Preliminary chromatographic tests by Mr. C. W. H. Partridge of this laboratory suggest that there are nine components in the organic acid fraction of *B. calycinum* leaves of which four have been identified as oxalic, malic, citric, and isocitric acids. The other components are present in extremely small proportions; one of them is possibly succinic acid.

downward from the top of the figure (dotted line, ordinate at right of figure 2) so that the vertical mirror image of the actual organic acid curve can be directly compared with the curve for starch. The close relationship between them is obvious. As further evidence, the correlation coefficient of the total organic acid vs. starch was calculated; this gave the negative value -0.916 for the 11 cases. The probability that this result

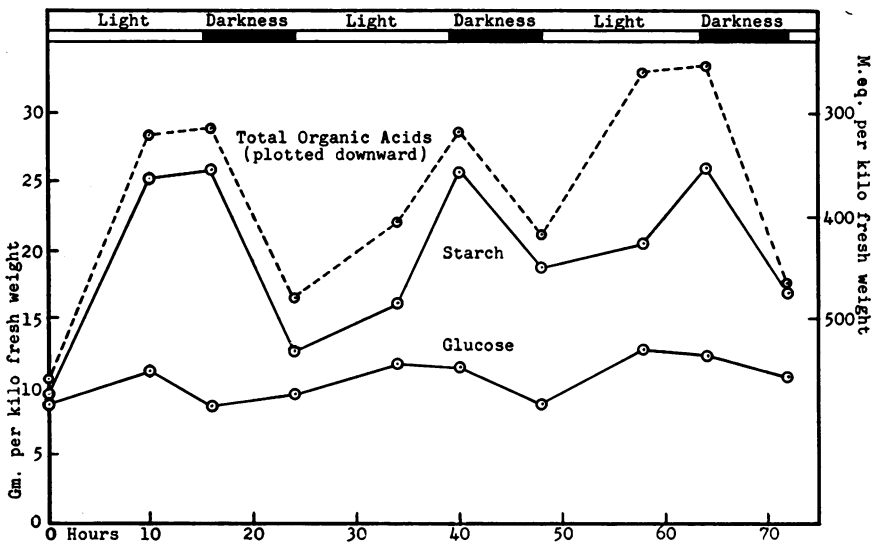

FIG. 2. Diurnal variation of starch and glucose of excised leaves of *Bryophyllum calycinum* cultured in water. Alternations of light and darkness indicated at top of figure. Data are grams per kilogram of initial fresh weight. Total organic acids plotted downward (ordinate at right) as dotted line to show close relationship between variation of acidity and that of starch.

is one of chance is less than one in a thousand ($r = 0.847$ for $P = 0.001$; 9 degrees of freedom). An even closer correlation was found during the initial 48 hours; the first 8 observations gave $r = -0.977$ ($r = 0.925$ for $P = 0.001$; 6 degrees of freedom).

A calculation of the relationship between the observations for pH and the quantities of starch gave the smaller but still highly significant correlation coefficient 0.854 for the 11 samples. Inasmuch as pH is a function that is inverse to the acidity, the correlation coefficient of pH vs. starch is positive.

The behavior of glucose (i.e. copper reducing material after hydrolysis calculated as glucose) is also shown in figure 2; the curve indicates that only small fluctuations took place. Inasmuch as hydrolysis of starch to glucose is the probable first step in the decomposition of starch, while condensation of glucose is the final step in its formation, a certain degree of regularity in the relative proportions of starch and glucose might be anticipated. However, it seems clear that mechanisms exist which maintain

the glucose concentration in the tissues at a fairly constant level in spite of the wide variations in the quantity of starch.

Discussion

The present experiment confirms and extends the result of the earlier one (**7**) and again emphasizes the close connection between the metabolism of organic acids and of starch. The evidence for an equilibrium relationship between organic acids on the one hand and starch on the other suggested that a calculation of the relative molar quantities of the reactants would be of interest. The results of such a calculation are shown in table II. The figures are offered for consideration with some reserve and

TABLE II

RELATIONSHIP OF CHANGES IN STARCH AND GLUCOSE TO CHANGES IN MALIC AND CITRIC ACIDS

	CHANGE IN GLUCOSE*	CHANGE IN MALIC AND CITRIC ACIDS†	RATIO Δ ACIDS: Δ GLUCOSE	RATIO Δ MALIC: Δ CITRIC ACID
	millimoles	*millimoles*		
First day	+101.0	−117.6	1.16	8.9
First night	−76.6	+82.1	1.07	5.5
Second day	+91.6	−58.2	0.64	11.0
Second night	−58.3	+53.4	0.92	4.7
Third day	+65.5	−81.8	1.25	4.8
Third night	−64.9	+98.2	1.51	7.5
Mean			1.09 ± 0.29	

* The change in glucose is computed from the change in starch content between the samples taken at sunrise and at sunset by multiplying by the factor 1.11; this is corrected for the change in glucose which, save during the first night, was in the same direction in all cases. The figures are millimoles per kilo of initial fresh weight.

† The change in malic and citric acid is the sum of the changes in each acid between the samples taken at sunrise and sunset expressed as millimoles per kilo of initial fresh weight.

should be regarded as merely preliminary in nature, for only analytical determinations of unusual accuracy made upon samples that duplicate each other in initial composition even more closely than the present ones could be expected to yield reliable ratios. However, the figures show, with an overall uncertainty of the order of 27 per cent., that the relative quantities are such that one mole of organic acid most of which was malic acid was converted to one mole of glucose the greater part of which accumulated as starch during each of three successive periods of daylight while the reverse change took place during the alternate periods of darkness. The energy necessary to provide for carbohydrate synthesis was obviously derived from sunlight and it would appear that a fairly high level of illumination is required if the change is to be rapid and extensive. It should further be pointed out that, although these interconversions involved quantities of the order of 15 grams of organic solids per kilo of

leaf tissue, that is to say, about 17 per cent. of the organic solids of the samples, no significant net change in the total quantity of organic solids present in the system was detected.

The relative molar quantities of malic and citric acids concerned in the successive interconversions are shown in the last column (table II); during the first day, malic and citric acids entered into reaction in the proportion of 8.9 moles of malic acid to 1 mole of citric acid; during the following night, 5.5 moles of malic acid were produced for each mole of citric acid formed, and so forth. There is little regularity in these figures and probably none could be expected unless the experiment were carried out under rigidly controlled conditions with respect to light supply, temperature, and humidity, and with samples of closely similar initial composition.

With regard to the enzymatic mechanisms whereby these interconversions were brought about, there is little to add to the views that have previously been expressed (**7**). The evidence suggests that the organic acids concerned are members of a chain of reversible enzymatic reactions which presumably represent a series of oxidation, decarboxylation and other steps whereby glucose derived from the hydrolysis of starch is alternately consumed and reproduced. A systematic series of reactions such as those which comprise the well-known Krebs tricarboxylic cycle is a possible expression of the sequence of chemical events. However, there are two observations which make it difficult to conclude that exactly this system is the one that operates in the case of diurnal variation of acidity in Bryophyllum leaves. The first is the fairly prominent part taken by citric acid in the reactions. In the tricarboxylic acid cycle as at present proposed to account for the metabolism of carbohydrate in animal tissues (**4**), citric acid is represented as being a product of a side reaction whereby *cis*-aconitic acid is in equilibrium with citric acid through the mediation of, presumably, the enzyme aconitase. *Cis*-aconitic acid occurs in the main sequence of reactions. This is not an insuperable objection in the present case but it is, nevertheless, difficult to understand.

The other objection is the fact that isocitric acid does not appear to share at all in the diurnal variation of acidity in Bryophyllum. This substance is present in large relative proportions in Bryophyllum leaves and it occurs in the main sequence of reactions of the Krebs cycle. It is difficult to see how so extensive a chemical change as that observed in the present experiment could take place, by means of reactions in which isocitric acid plays an active part, without any detectable influence upon the quantity of isocitric acid in the system.

This observation further raises the important question of the function of isocitric acid in Bryophyllum leaves. It is a curious fact that, so far

as is now known, this rare substance is present in substantial quantities only in those plant species which are characterized by the phenomenon of a broad diurnal variation in acidity. It seems, however, that isocitric acid takes no part in these changes in the particular species which provides the classical example of diurnal variation. There is an anomaly here which requires explanation.

Summary

Excised leaves of *Bryophyllum calycinum* cultured in water under greenhouse conditions for three days undergo successive alterations in organic acid and starch content in mutually opposite directions in a manner which suggests a close relationship between the metabolism of these substances. The observations thus accord with the views of Bennet-Clark. The extent and speed of the reactions appear to be influenced by the weather conditions, being appreciably greater on a bright sunny day than upon a partially cloudy day.

Malic acid is the organic acid which undergoes the greatest fluctuations in concentration, but citric acid shares in them to a moderate extent; isocitric acid, however, does not appear to vary at all.

In spite of the fact that the interconversions of starch to organic acids and the reverse involve quantities of the order of 17 per cent. of the organic solids of the tissues, no significant alteration in organic solids from sample to sample was observed. It may be inferred that the reactions which involve exchanges of gas with the atmosphere were such that there was no detectable net change in weight.

Preliminary calculations of the relative molar quantities of the substances concerned suggest that approximately one molar proportion of glucose (mostly derived from starch) is transformed to one molar proportion of malic and citric acids (malic acid the main component) during the night and that the reverse reaction occurs during the day. It is possible that the enzymatic mechanisms involved are analogous to those described by the Krebs tricarboxylic acid cycle, but the direct part taken by citric acid and the failure of isocitric acid to share at all cannot easily be accounted for by the hypothesis that the Krebs cycle, in the form widely accepted as the explanation of carbohydrate oxidation in animal tissues, exactly defines the mechanism of diurnal variation of acidity in *Bryophyllum calycinum* leaves.

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