THE BEHAVIOR OF ISOCITRIC ACID IN EXCISED LEAVES OF *BRYOPHYLLUM CALYCINUM* DURING CULTURE IN ALTERNATING LIGHT AND DARKNESS

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(WITH TWO FIGURES)

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In an earlier study of the diurnal variation of organic acids and starch in excised leaves of *Bryophyllum calycinum* (**3**), it was observed that the quantity of isocitric acid present did not change significantly during a three-day period of culture in water under greenhouse conditions of illumination. Because of the contrast between the behavior of this substance and that of malic and citric acids, it seemed essential to repeat the experiment under even more carefully controlled conditions and to employ improvements in the method for the determination of isocitric acid which have now become available (**1**). The outcome has been a confirmation of the earlier experiment. The changes in the quantity of isocitric acid in Bryophyllum leaves exposed to normal diurnal alternations of light and darkness, if significant at all, are scarcely in excess of the combined errors involved in the sampling and in the method of determination.

Experimental procedure

The plants were propagated from the same clone used in earlier work. They had been transplanted to sand in pots February 15, 1949, and were grown with the culture solution previously described (**2**). The samples of leaflets were collected July 28 by the statistical method (**8**), the upper three pairs of fully developed opposite leaves (five leaflets each) from 20 plants being taken to provide 10 samples each of 60 leaflets. Collection was begun at 4 A.M. (standard time) and all samples were in position in the culture troughs with the petioles in water by 5:30. Sunrise occurred at 4:43 A.M., sunset at 7:14 P.M. Zero time was taken at 5:00 A.M. when the control sample was placed in the drying oven.

The troughs were set up in a greenhouse under white parachute cloth shade. During the first day, there was a slight haze in the morning and light cloud was present in the early afternoon; the temperature reached 33° C and the humidity was high. The second day was clear save for a little cloudiness near noon and the maximum temperature was 36° C. The effect of the cloth shade was to diffuse the light but the intensity was of the order of 1600 to 2000 foot-candles throughout most of both days.

Samples were removed at times selected to give information on the rates of change of the organic acids and starch during the day and night periods. They were dried at 80° C, broken up and equilibrated at 24° C and 50% relative humidity before being finally ground in a Wiley mill for analysis, and were then stored in closed bottles in the air-conditioned room.

Table I gives the fundamental data on the samples. The precision with which they duplicated each other in initial composition is shown by the results for total nitrogen which have a coefficient of variation of 0.9%. The protein nitrogen also remained constant at 1.74 ± 0.017 gm. per kilo. The coefficient of variation of the initial fresh weight was 1.5% while that of the equilibrated dry weight was 1.8%. This last figure, however, is affected by an increase of from 5 to 6 gm. of organic solids per kilo of fresh weight which occurred during the 48-hour period. This result is in contrast to the

TABLE I

FUNDAMENTAL DATA ON SAMPLES OF EXCISED *Bryophyllum calycinum* LEAFLETS COLLECTED BY THE STATISTICAL METHOD AND SUBJECTED TO CULTURE IN WATER IN ALTERNATING LIGHT AND DARKNESS.

	Per sample			Per kilo initial fresh weight				
Culture period	Fresh weight at start	Fresh weight at end	Equilibrated dry weight	Equilibrated dry weight	Nitrogen	Ash	Organic solids	Isocitric acid
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*m.eq.*
0	225.5	225.5	23.00	102.0	2.16	11.4	83.3	164
5	230.8	234.3	23.45	101.6	2.17	11.3	83.7	169
10	228.7	225.2	23.45	102.5	2.19	11.0	84.1	169
15	232.0	228.6	23.75	102.4	2.19	10.7	84.9	176
19	231.7	228.8	23.60	101.9	2.18	10.6	85.3	174
24	228.2	226.3	23.95	104.9	2.16	10.8	87.0	164
29	226.4	225.8	23.70	104.7	2.21	10.9	87.7	166
34	226.9	224.4	23.55	103.8	2.20	10.7	86.2	171
39	220.9	215.2	23.00	104.1	2.22	11.2	86.3	163
48	230.0	219.7	24.45	106.3	2.20	10.6	89.6	176
Mean	228.1		23.59		2.19	10.9		169
Standard deviation	±3.37		±0.43		±0.02	±0.3		±4.8
Coefficient of variation %	1.5		1.8		0.9	2.8*		2.9*

*These figures are affected by a larger analytical error than that in the results for total nitrogen.

previous experiment in which no increase in organic solids was detected and is evidence of the improvement in the accuracy of such experiments to be obtained with use of the statistical method of sampling.

Figure 1 shows the behavior of the organic acids and the changes in the pH of the tissue. As in the previous experiment, malic acid reached a minimal value at 3:00 P.M. on each of the two successive sunny days and this is also true of citric acid. Nevertheless, as is evident from figure 2, starch formation continued, although at a diminished rate, until sunset. It may be inferred that, although the formation of starch in light is obviously correlated with the decrease in organic acids and this relationship accounts for the greater part of the increase in starch, there is, nevertheless, a moderate and probably continuous increase during the day as the result of photosynthetic reactions. This conclusion is in conformity with the observations of VARNER and BURRELL (**6**) who demonstrated the presence of radioactive carbon in the starch of Bryophyllum leaves exposed to radioactive carbon dioxide in light for a short period.

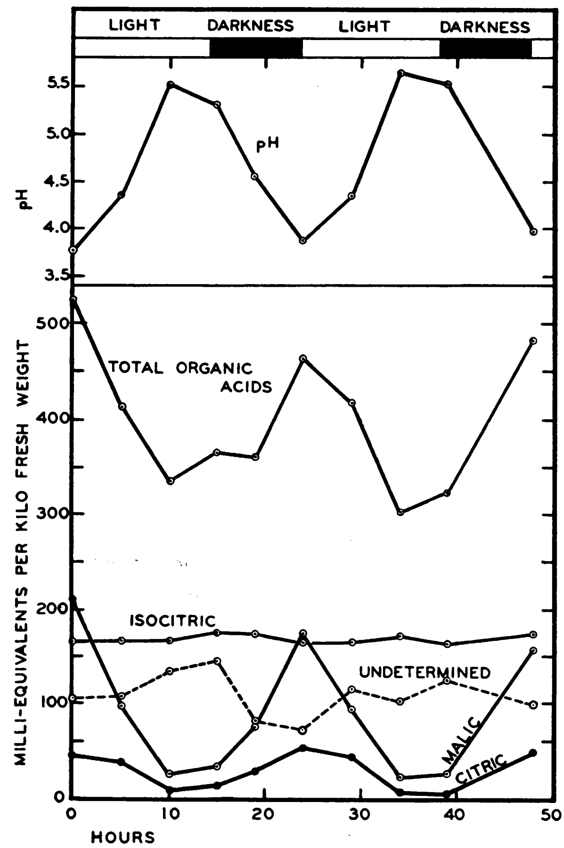

FIG. 1. Changes in the organic acid composition and pH of excised leaves of *Bryophyllum calycinum* cultured in water in alternating light and darkness. Data are milliequivalents of acid per kilo of initial fresh weight.

The curve for isocitric acid is not a horizontal straight line as it would be if there were no effect of light conditions and the methods of sampling and of analysis were completely accurate. There is a suggestion of an increase in isocitric acid during both periods of illumination and of a drop during the first night. However, the maximum range is small, being of the order of 6% of the quantity measured, or about 0.8% of the organic solids of the leaves, and the coefficient of variation is 2.9%. The change, if real, is negligible in comparison with the behavior of malic and citric acids which altered in quantity in response to the changes in illumination by factors of from 5 to 10, and involved chemical transformation of about 17% of the organic solids of the tissues in the first 10 hours.

In view of the accumulation of analytical error in the data for undetermined organic acids (fig. 1), no interpretation can be attempted of the fluctuations in the total quantity of these substances present save to point out that an increase in light and a decrease in darkness is suggested. Preliminary observations by the method of paper chromatography have indicated that there are at least eight and possibly as many as 10 organic acid components present in dried Bryophyllum leaves,[1] only oxalic and succinic acid among the minor ones being as yet provisionally identified. Until all of these components have been recognized and analytical methods developed for their determination, no comment can be made upon the share the individual substances may take in the phenomenon of diurnal variation.

The curve for starch (fig. 2) shows variations in quantity that are correlated with those for both total organic acids and pH in a highly significant manner. The correlation coefficient of starch *vs.* total organic acids is -0.91, that against pH is $+0.91$. The probability that this is the result of chance is less than one in a thousand ($r = 0.872$ for $P = 0.001$ with eight degrees of freedom). An even higher degree of correlation is found for starch *vs.* malic acid, the figure being -0.97; that for starch *vs.* citric acid is -0.88. As in the previous work, the maximum starch content attained was the same on both days.

The behavior of the soluble carbohydrates is shown in the lower part of figure 2, the scale of ordinates being five times greater than that of the curve for starch. Synthesis of glucose (free reducing sugar computed as glucose) began promptly after sunrise, but the curves suggest that transformation of glucose to starch soon assumed dominance so that, from mid-morning until sunset, the level of glucose dropped slightly. There was no significant change during the night; but in the early morning, the conditions were such that glucose soon rose to an even higher level than that attained the first day. However, it diminished rapidly during the afternoon, presumably owing to formation of starch; and there was again no significant change at night, a point of considerable importance in view of the continued increase in organic solids (fig. 2).

Unfermentable carbohydrate (free reducing sugar after treatment of the extract with yeast) likewise fluctuated in response to light but the maxima were attained only in mid-afternoon and the evidence suggests that a small proportion of this component was utilized during the dark periods. The total amount of material involved was small inasmuch as the range in quantity was less than 1 gm. per kilo of leaves and, consequently, no attempt was made to determine sedoheptulose in these samples.

Sucrose (computed from increase in reducing power after treatment with

[1] The author is indebted to Dr. C. A. Hargreaves of this laboratory for these observations.

invertase) behaved in a manner closely similar to that of the unfermentable carbohydrate with respect to the time of day the maximum was attained; but, again, the quantities involved were small, being only approximately 0.8 gm. Furthermore, little more than traces of sucrose were present in the leaves at midnight of the first day and at dawn of the second. The increase during the day and decrease during the late afternoon and evening are thus strikingly large proportional fluctuations of this component. It is doubtless

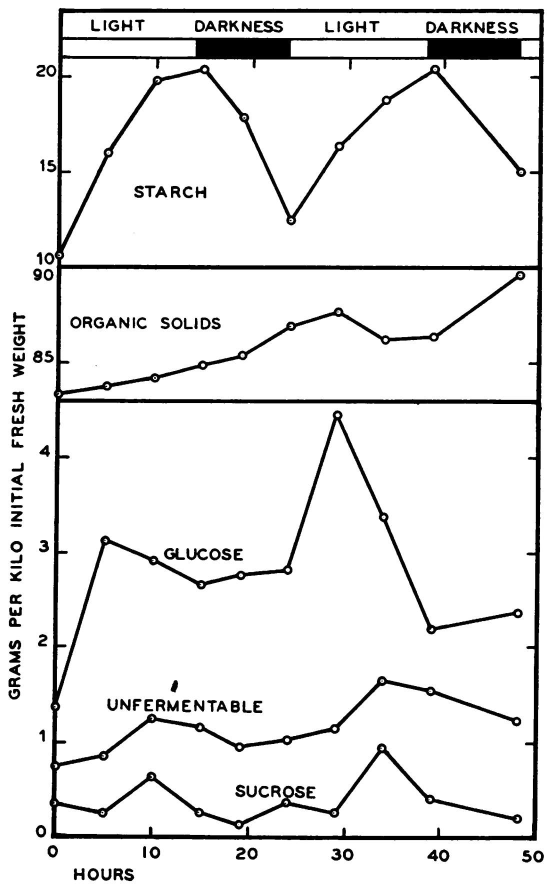

FIG. 2. Changes in the carbohydrates and organic solids of excised leaves of *Bryophyllum calycinum* cultured in water in alternating light and darkness. Data are grams per kilo of initial fresh weight.

significant that both unfermentable carbohydrate and sucrose increased moderately during the middle of the day when synthesis of starch was proceeding at so high an intensity that glucose did not accumulate.

The curve for organic solids (fig. 2; ordinates on the same scale as those for starch) shows a net increase for the first 29 hours along a nearly straight line. Thus, carbon fixation took place not only in light but also in darkness. There was a moderate net loss of organic solids during the second day, but carbon assimilation was apparently reestablished at nightfall and continued throughout the dark period. The observations are thus in conformity with those of VARNER and BURRELL (**6**) who have found, in short time experiments, that radioactive carbon dioxide is assimilated by Bryophyllum leaves both in light and in darkness.

Discussion

The ratios between the quantities of malic acid and of starch which underwent change in the successive periods of the experiment are shown in table II, starch being expressed in terms of the molar equivalent of glucose. During the morning and early afternoon of the first day and during the periods from 12 midnight to dawn and from dawn to 10 A.M. of the second day, the relationship between these quantities was essentially constant in spite of the fact that the overall reaction was proceeding in the direction of

TABLE II

RATIO OF CHANGES OF MALIC ACID TO CHANGES OF STARCH CALCULATED AS GLUCOSE.

Time interval (standard time)	Change in quantity		Ratio
	Δ Starch as glucose	Δ Malic acid	Δ Malic acid / Δ Glucose
	millimoles	*millimoles*	
5 A.M. to 10 A.M.	+33.2	−56.0	−1.7
10 A.M. to 3 P.M.	+23.4	−36.7	−1.6
3 P.M. to 8 P.M.	+ 3.8	+ 3.9	+1.0
8 P.M. to 12 Mid.	−16.6	+21.2	−1.3
12 Mid. to 5 A.M.	−32.0	+49.7	−1.6
5 A.M. to 10 A.M.	+23.9	−39.7	−1.7
10 A.M. to 3 P.M.	+14.9	−37.2	−2.5
3 P.M. to 8 P.M.	+ 9.7	+ 2.7	+0.3
8 P.M. to 5 A.M.	−33.2	+65.3	−2.0

acid formation during one of these intervals and of starch formation during the other three. These were periods in which the rate of chemical change attained maximal intensity with respect to the quantities of starch that became involved. However, during experimental periods when smaller quantities of starch underwent change, the ratio departed more or less widely from this constant value, indicating that reactions other than the main one were influencing the overall results. In the late afternoon of both days, malic acid and starch both increased, suggesting that the main interconversion reaction had either ceased or had diminished in intensity to negligible proportions in relationship to the other chemical changes that were taking place.

The situation is clearly one that is far from simple from the chemical point of view; and, in spite of the occasional constancy of the relationship between the main reactants and of the highly significant negative statistical correlation between the sets of data for malic acid and starch, it is obvious that a number of complicating factors which form a part of the general metabolic scheme are being overlooked. Some of these factors may be noted. Citric acid, as well as some at least of the acids of the undetermined group, responds to the alternation of illumination, and the highly significant correlation between the data for citric acid and starch suggests that this relationship is of great importance. Manifestly, it must receive the closest attention when attempts are made to arrive at an interpretation of the phenomena in terms of specific chemical reactions. Furthermore, glucose, together with other soluble carbohydrates, is also involved. Superimposed upon the chemical changes of these components are the reactions whereby the organic solids increased in quantity not only during the day, presumably as a result of the assimilation of carbon through photosynthesis, but also at night. With such species as tobacco, loss of organic solids takes place owing to uncompensated respiration when light is withdrawn. With Bryophyllum, as has long been known, little or no carbon dioxide is evolved by the leaves in darkness under normal conditions. On the contrary, the increase in organic solids observed suggests that carbon assimilation reactions continue, a point that has been emphasized by THOMAS and BEEVERS (**5**), who maintain that oxygen is also taken up. The mechanisms whereby many of these reactions occur doubtless involve organic acid components even if only as transition products. The general organic acid metabolism is thus a highly complex process and its predominating feature, the reversible transformation of malic acid to starch, is easily demonstrated only because of its relative magnitude.

It is against this background of rapid and extensive changes of organic acid components in Bryophyllum leaves that the apparent inactivity of isocitric acid stands out in contrast. The observation suggests that this substance may, in great part, be physically remote within the leaf cells from the centers at which the major reactions are taking place or that it is not one of the intermediates in the chain of enzymatically controlled equilibria concerned with the normal diurnal variation of acidity. Either view is in conformity with the observations with radioactive carbon dioxide by STUTZ and BURRIS (**4**) and by VARNER and BURRELL (**6**). These workers have shown that isocitric acid acquires only a small proportion of radioactive carbon in comparison with the quantities found in malic and citric acids after exposure of the leaves to light.

Nevertheless, it would be inaccurate to assume, merely on the present evidence, that isocitric acid is a chemically indifferent component of Bryophyllum leaves. As is shown in another paper (**7**), this substance soon becomes involved in the chemical transformations of the organic acids when the period of darkness to which the leaves are exposed is abnormally prolonged; it then promptly attains a new and lower level of concentration which is subsequently maintained relatively constant. Much remains to be learned, however, of the stimuli which can bring about such responses.

Summary

The behavior of isocitric acid in excised leaves of *Bryophyllum calycinum* subjected to culture in water under greenhouse conditions of illumination has been reexamined with improved technical methods. The observation that this substance does not change significantly in quantity in the normal course of the phenomena of diurnal variation of acidity has been confirmed.

Although the relative quantities of starch and of malic acid which undergo diurnal fluctuations in response to light conditions are such as to indicate that mutual interconversion occurs, these substances being respective end-products of what is doubtless a complex series of enzymatically controlled chemical reactions, the simple hypothesis that diurnal variation of acidity is to be accounted for in terms of a reversible equilibrium between starch and malic acid is inadequate to account for all of the observations. Although this may be an expression of the most important reaction from the point of view of quantity, it furnishes an incomplete picture of the metabolic events. Notable among the complicating factors are the evidences of assimilation of carbon both in light and in darkness, and the behavior of citric acid and of the soluble carbohydrates. The chemical details of all of these reactions remain to be explained.

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