THE FORMATION OF STARCH IN LEAVES OF *BRYOPHYLLUM CALYCINUM* CULTURED IN DARKNESS

HUBERT BRADFORD VICKERY

(WITH TWO FIGURES)

Received June 28, 1951

In experiments carried out in this laboratory several years ago, it was noted that excised leaves of *Bryophyllum calycinum* collected at daybreak and cultured in water in darkness may increase appreciably in their content of starch (**3**). Such leaves contain a high proportion of organic acids when picked but undergo a marked loss of acidity when kept in darkness for two or three days. The suggestion was advanced that the newly formed starch may have arisen from organic acids as a result of reversal of the glycolytic reactions. Experiments on the normal diurnal variation respectively of organic acids and starch in Bryophyllum leaves (**4**, **6**) have lent considerable support to the view that such a course of events is possible, and an unpublished experiment carried out by the late Dr. G. W. Pucher shortly before his death in 1947 showed that starch accumulation in leaves cultured in darkness may at times reach considerable proportions. Accordingly, renewed study has been given to this problem.

Bryophyllum calycinum leaves collected at daybreak were maintained in a closed dark cabinet under a continuous fine spray of water for several days, individual samples being removed for analysis at appropriate times. The leaves, which contained 3.7 gm. per kilo of initial fresh weight of starch at dawn, contained only 1 gm. after a further seven hours in darkness but then began to accumulate starch so that, after 26 hours, they contained 3 gm. and, after 75 hours, 3.6 gm. An even larger relative increase had been observed in the unpublished experiment of Pucher (see also **3**) so that there appears to be no doubt of the capacity of Bryophyllum leaves to behave in this manner, notwithstanding a report to the contrary of VARNER and BURRELL (**5**).

Experimental procedure

Ten samples of leaflets were collected June 29, 1949 by the statistical method (**7**) from plants of the same lot as those described in another paper (**6**) and in the same manner. Collection was begun at 4:00 A.M. (standard time, sunrise at 4:22 A.M.). Zero time was taken as 5:00 A.M. when the control sample was placed in the drying oven, and all samples were in place in the closed sterilized metal cabinet by 5:07 A.M. The samples were held in wire baskets, previously sterilized with steam, supported on two shelves, and water at a constant temperature of 23° C was supplied continuously from two centrally located spray nozzles immediately above each layer of baskets. Sampling times were chosen so as to obtain frequent observations in the early part of the experiment. As each successive sample was removed, the leaflets were individually blotted as dry as possible, and the sample was weighed and dried at 80° C. The first evidence of rootlet formation at the indentations in the margins of the leaves was seen at 75 hours. At 99 hours, most of the leaflets had developed rootlets some of which were 1 cm. long and, at the end of the experiment at 196 hours, in spite of the fact that these leaflets were relatively young, all save the smallest had rootlets many of which were 3 cm. long.

TABLE I

FUNDAMENTAL DATA ON SAMPLES OF EXCISED *Bryophyllum Calycinum* LEAVES COLLECTED BY THE STATISTICAL METHOD AND SUBJECTED TO CULTURE IN WATER IN DARKNESS.

Culture period	Per sample			Per kilo initial fresh weight				
	Fresh weight at start	Fresh weight at end	Equilibrated dry weight	Equilibrated dry weight	Nitrogen	Organic solids	Ash	Starch
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0	128.5	128.5	14.50	112.8	2.83	93.2	11.4	3.74
7	131.8	135.1	14.70	111.5	2.78	91.4	11.1	0.93
14	126.0	132.8	13.65	108.3	2.83	89.1	11.0	1.59
26	129.3	140.0	13.40	103.6	2.81	85.6	11.1	3.05
34	129.6	141.6	13.05	100.7	2.78	83.7	11.0	2.90
50	126.7	137.2	12.50	98.66	2.79	81.8	10.9	3.10
75	127.2	137.7	12.40	97.48	2.75	81.1	10.8	3.63
99	130.7	141.1	12.60	96.40	2.81	79.9	10.9	3.41
147	129.6	143.8	11.90	91.82	2.72	76.1	10.7	3.13
195	131.7	148.6	12.15	92.25	2.79	75.9	10.9	3.20
Mean	129.1				2.79		11.0	
Standard deviation	±2.01				±0.03		±0.20	
Coefficient of variation, %	1.6				1.2		1.8	

The techniques for drying, equilibrating, grinding and storing the samples are described in the previous paper (6). They were removed from the air-conditioned room only when subsamples were being weighed for analysis.

Table I gives the fundamental data on the weights and composition of the samples. The accuracy with which they duplicated each other is evidenced by the results for total nitrogen which had a coefficient of variation of 1.2%. Notwithstanding the apparent slight loss of ash constituents to be seen in the observations at zero and at seven hours, the ash weights had the unusually low coefficient of variation of 1.8%. The alkalinity of the ash averaged 256 ± 5 m.eq. per kilo of initial fresh weight (fig. 1), and the data did not reflect the slight loss to be seen in the initial figures for ash. It is possible, therefore, that the lost material consisted merely of dirt on the surface of the leaves which was washed away by the water. The organic solids show continuous loss owing to the effect of respiration, the data for the first 34 hours (fig. 2) falling upon an approximately straight line; the rate of loss of organic solids then diminished.

The initial behavior of the starch (fig. 2) suggests that, for the first few hours of the experiment, starch was being used in metabolism in a manner

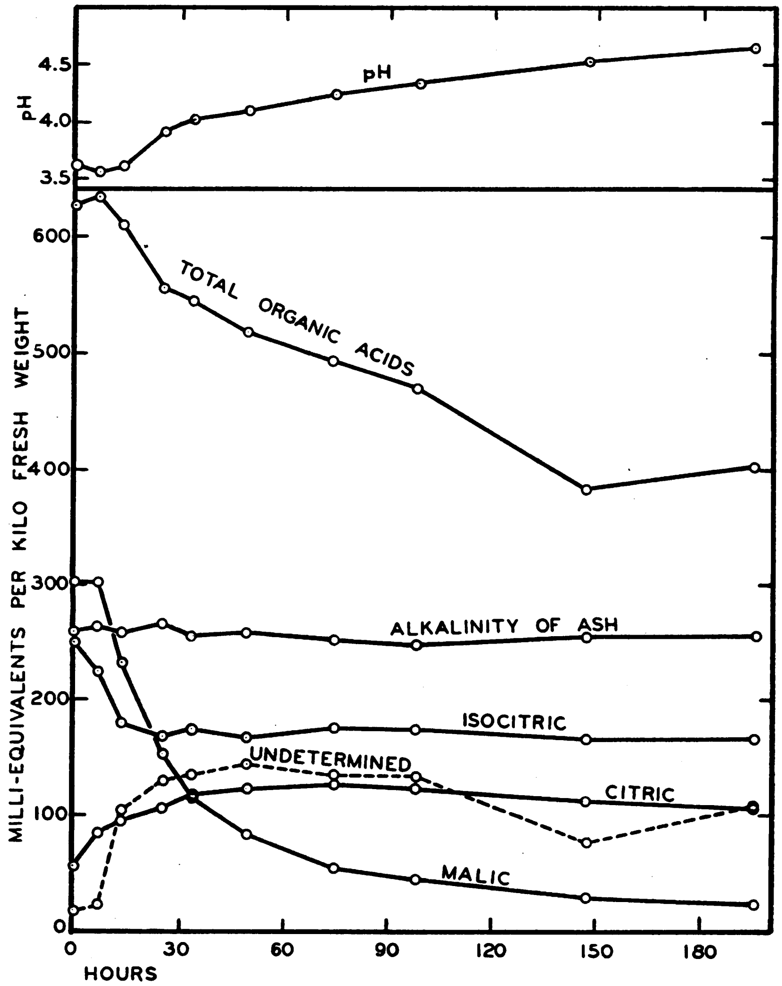

FIG. 1. Changes in the organic acid composition and pH of excised leaves of *Bryophyllum calycinum* cultured in water in darkness. Data are milliequivalents per kilo of initial fresh weight.

that was, in effect, a continuation of its behavior during the night. Leaves closely similar to these had been found to contain approximately 20 gm. of starch per kilo at sunset when exposed to light throughout the day, and it is to be presumed that the 3.7 gm. per kilo found in the present samples at dawn represent the part of this quantity which still remained when the samples were collected.

A significant change in the organic acid composition was initiated shortly after the start of the experiment, for at the end of seven hours citric acid had increased and isocitric acid had decreased (fig. 1). Under the stress set up by the prolonged period of darkness, more of the components of the tissue then became involved in the chemical changes that occurred. Respiration, as evidenced by the loss of organic solids (table I), continued at a high rate, 1 gm. of organic matter (about 1% of the organic solids) being

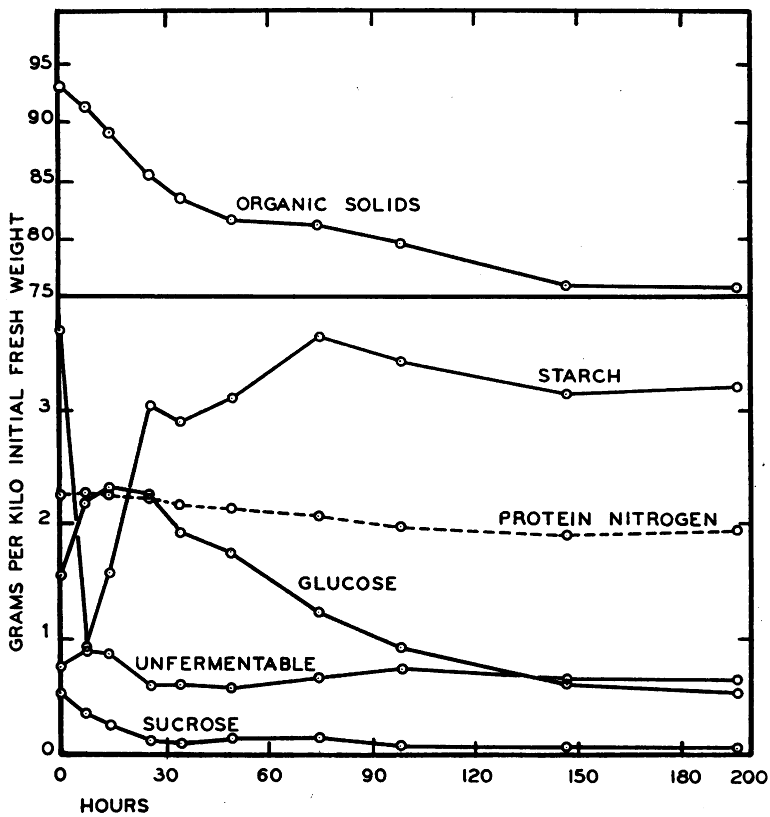

FIG. 2. Changes in soluble carbohydrates, starch, and organic solids of excised leaves of *Bryophyllum calycinum* cultured in water in darkness. Data are grams per kilo of initial fresh weight.

completely oxidized every 3.6 hours for the first 34 hours. Beginning after seven hours, malic acid (fig. 1) diminished rapidly in quantity and there was also a sudden increase in the organic acids of the so-called undetermined fraction. Nevertheless, the total quantity of organic acids present in the system diminished continuously and the pH began to rise. Most striking of all was the formation of an appreciable quantity of starch.

During the second day, the protein (fig. 2) began to be used in the metabolism and, in the interval between 26 and 99 hours, protein nitrogen diminished by about 10% of the quantity initially present; thus about 1.7

gm. of protein disappeared as such during this interval. Subsequently, however, the loss of protein was only slight.

Probably associated with the behavior of the starch is that of the soluble reducing sugar calculated as glucose (fig. 2). This increased by about 0.7 gm. in the first seven hours (the starch diminished by nearly 3 gm.) but then passed through a maximum and subsequently decreased until 99 hours had elapsed. Between 14 and 75 hours, about 1 gm. of glucose disappeared while about 2 gm. of starch were formed. The unfermentable carbohydrate likewise had increased slightly at seven hours and subsequently decreased, but it soon reached a level that remained substantially constant for the rest of the period studied. The quantities involved in the changes of this component were, however, small, being only from 0.2 to 0.3 gm. Sucrose dropped rapidly from its initial level of 0.5 gm. per kilo and only negligible amounts remained after the first day.

The more rapid phase of the general metabolism continued for about 75 hours. By that time the rate of respiration had decreased materially and starch began to diminish slowly. The rate at which malic acid was diminishing also had become slow and citric acid had passed through a flat maximum. Isocitric acid, however, appeared to have reached a new equilibrium level within 26 hours and thereafter changed not at all.

Discussion

To visualize the relative magnitude of the major chemical transformations that occurred, the behavior of malic acid may be considered. Starting at 302 m.eq. per kilo of fresh weight, malic acid diminished to 22 m.eq. after 195 hours. Thus, 280 m.eq. or 18.7 gm. of malic acid per kilo were ultimately converted into other substances. This amount is 20% of the organic solids present in the leaves at the start.

The confirmation of earlier observations on the formation of starch during culture of Bryophyllum leaves in darkness for a prolonged period is of special interest. Synthesis of starch took place contemporaneously with the extensive decrease in malic acid and, because of the obvious close relationship of these two phenomena noted in studies of the normal diurnal variation of acids and starch (**4, 6**), it is natural to associate them in the present case as well. Although the data for starch and for malic acid over the period from 7 hours to 195 hours inclusive are closely correlated mathematically ($r = -0.93$; $r = 0.898$ for $P = 0.001$ with seven degrees of freedom), an inquiry into the relative quantities of starch and of malic acid that underwent change during the successive periods of study suggests that the metabolic relationship is not a simple one. During the first seven hours, starch equivalent to 17 millimoles of glucose (3% of the organic solids) disappeared but there was no change in malic acid. In the next interval of seven hours, starch equivalent to four millimoles of glucose was formed while malic acid dropped by 35 millimoles. During the next 12 hours, starch equivalent to nine millimoles of glucose was formed and 39 millimoles of malic acid disappeared. The molar relationships in the subsequent intervals are equally irregular. Although the transformation of malic acid into other substances continued at a rate which follows a smooth curve, the changes in starch fluctuated irregularly and by quantities that were in all cases small in comparison with those of the acid. In the absence of studies with tracer elements, no definite conclusion as to the chemical fate of the malic acid can be drawn and none regarding the origin of the starch. The purely analytical method of searching for metabolic relationships breaks down when the changes observed are small in comparison with the total mass of metabolizing material present.

Nevertheless, there are two relationships among the organic acid components of the tissue that merit particular attention. Isocitric acid remains unchanged in quantity during the course of the normal diurnal variation in acidity of Bryophyllum leaves (**4**, **6**), but it enters into the system of metabolic changes almost at once as a result of the unusual stresses imposed under the conditions of the present experiment. During the first 26 hours, 82 m.eq. per kilo (5.2 gm.) of isocitric acid or one third of the total quantity present disappeared; this amount is 6% of the organic solids of the tissues. During the remainder of the culture period, the quantity of isocitric acid was constant within the limits of accuracy of the measurement (coefficient of variation 2.3%). The effect is therefore an important one in relation to the total quantity of metabolizing substances in the system and clearly indicates that isocitric acid may under certain circumstances become an active participant in the reactions of the leaf in spite of its apparent inactivity in the course of normal diurnal variation in acidity. The observations suggest that, in the initial phases of culture in darkness for a period beyond the normal length of a night, the position of equilibrium of isocitric acid changes in order to meet the new conditions. This is the first demonstration based on direct analytical determinations that isocitric acid may play an active part in the organic acid metabolism of the Bryophyllum plant.

That the decrease in isocitric acid may be correlated chemically, via an aconitase type of reaction, with the simultaneous increase in citric acid is possible but furnishes at best an incomplete picture of the relationships. The increase in citric acid was only 50 m.eq. (3.25 gm.) in the first 26 hours and, more significantly, continued along a smooth curve until about 70 m.eq. (4.5 gm.) had accumulated after 75 hours. A part, or possibly all, of the newly formed citric acid may have been derived from the relatively much larger quantity of malic acid that disappeared during this period. The possibility that some of the citric acid may have arisen from the carbohydrate components is not excluded.

The other notable organic acid transformation was the formation of a substantial quantity of acids in the undetermined group. Although the data for undetermined acids (fig. 1) are obtained by difference and are subject to the combined analytical errors of the separate determinations, uncompensated error alone could hardly account for more than 10 to 15% of the undetermined acid apparently formed. It is necessary to assume, therefore, that unknown acid components increased from a level of approximately 20 m.eq. per kilo to about 140 m.eq. at the expiration of 75 hours of culture. This quantity is 8 to 9% of the organic solids if calculated as its equivalent of malic or citric acid. Some of this newly formed acid may have arisen from transformations of malic acid, and some may have been derived from the isocitric acid; other possibilities are also open. Decision on these points must await the identification of the components of this group of reactive substances and also the accumulation of knowledge of the enzyme systems in the leaves.

The more rapid metabolic changes seem to have been approximately completed at the expiration of 75 hours at which time a different sequence of events began. The rate of respiration had dropped, starch began to diminish slowly and both citric acid and the group of undetermined acids passed beyond their flat maxima and began to decrease slowly; malic acid continued to decrease. It is not impossible that these subtle changes in the behavior of the components may be associated with the initiation of the reproductive phase in these leaves, for it was at this point that the first evidence of the formation of rootlets at the indentations of the margins of the leaves was seen. During the remainder of the experimental period, growth of rootlets became increasingly notable. However, it is doubtful that the initiation of this biological event is to be directly attributed to the change in the organic acid composition. Growth of plantlets at the margins of old basal leaves in *Bryophyllum calycinum* is a common observation in plants maintained under greenhouse conditions, and in such leaves normal diurnal variation in acid and starch content is still continuing although probably at a lower intensity than in young upper leaves such as those used in the present study (**2**). Accordingly, it does not seem likely that the unusual organic acid composition of the present leaves at the time the reproductive phase began was, *per se*, the condition which elicited this response.

The present experiment, then, lends further emphasis to the possibilities of rapid and extensive alterations in the organic acid composition of plant leaves in response to artificially applied stimuli. These acids are universally regarded as intermediates in the processes of carbohydrate metabolism, not only in connection with the phenomena of photosynthesis (**1**), but also with those associated with respiration in the more thoroughly investigated animal tissues. Pending the identification of the specific enzyme systems active in Bryophyllum leaves, it is speculative in the extreme to attempt to organize the reactions that are observed into systems analogous to those operative in better known cases. Nevertheless, the behavior of the acids in Bryophyllum leaves is consistent with the view that among the enzymatically controlled reactions are some which bear a close analogy to certain of the changes recognized as components of the well-known Krebs tricarboxylic acid cycle.

The possible substrates of the high respiration observed in these leaves in the early phase of the culture experiment may be indicated by some of the data. The loss of organic solids in the first 26 hours was 7.6 gm. In this period, glucose increased by 0.7 gm., sucrose decreased by 0.4 gm., and starch at first decreased by 2.8 gm. and then increased by 2.1 gm. It seems unlikely, therefore, that simple sugars or starch played a direct and important role in the respiration at this stage. Malic acid decreased by 150 m.eq. (10.0 gm.) but the total organic acids decreased by only 69 m.eq., a quantity equivalent to only about 4.6 gm. of solids. This may indeed represent respiration loss but there remain 3 gm. of lost organic matter still to be accounted for. The protein nitrogen was substantially unchanged during this period so that significant respiration of protein components did not take place. Therefore nearly one half of the organic matter that underwent complete oxidation during the first day consisted of substances of as yet undetected chemical nature.

Summary

An earlier observation that moderate quantities of starch may be formed in excised leaves of *Bryophyllum calycinum* collected at dawn and cultured for several days in water in darkness has been confirmed. The increase in starch is accompanied by an extensive and continuous loss of malic acid, and by a slow increase of both citric acid and the group of undetermined organic acids to flat maxima. In addition, about one third of the isocitric acid present disappeared within the first 26 hours but, subsequently, no change in the quantity of this substance was observed. There was a net continuous loss of total organic acids.

The loss of organic solids through respiration was rapid for the first 50 hours but subsequently was slow although continuous. The responses of the simple carbohydrates were small in relation to the much larger quantities of organic acids which underwent change. The loss of protein from the tissues was also relatively small.

Because of the complexity of the metabolic changes, it is impossible to conclude that the newly formed starch was derived through chemical transformations from the malic acid which disappeared. Nevertheless this remains a possibility.

Certain changes in the curves after 75 hours of culture, especially that for starch, are tentatively associated with the initiation of rootlet formation at the indentations of the leaf margins.

The observations as a whole indicate the presence in this species of a carbohydrate metabolism which is independent of photosynthesis but which can lead to the synthesis of starch.

Grateful acknowledgment is made to Marjorie D. Abrahams, Laurence S. Nolan, and Katherine A. Clark for technical assistance.

DEPARTMENT OF BIOCHEMISTRY
THE CONNECTICUT AGRICULTURAL EXPERIMENT STATION
NEW HAVEN, CONNECTICUT

LITERATURE CITED

1. BENSON, A. A., BASSHAM, J. 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.
2. PUCHER, G. W., LEAVENWORTH, C. S., GINTER, W. D., and VICKERY, H. B. Studies in the metabolism of crassulacean plants: the diurnal variation in organic acid and starch content of *Bryophyllum calycinum*. Plant Physiol. **22**: 360–376. 1947.
3. PUCHER, G. W., LEAVENWORTH, C. S., GINTER, W. D., and VICKERY, H. B. Studies in the metabolism of crassulacean plants: the behavior of excised leaves of *Bryophyllum calycinum* during culture in water. Plant Physiol. **22**: 477–493. 1947.
4. PUCHER, G. W., VICKERY, H. H., ABRAHAMS, M. D., and LEAVENWORTH, C. S. Studies in the metabolism of crassulacean plants: diurnal variation of organic acids and starch in excised leaves of *Bryophyllum calycinum*. Plant Physiol. **24**: 610–620. 1949.
5. 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.
6. VICKERY, H. B. The behavior of isocitric acid in excised leaves of *Bryophyllum calycinum* during culture in alternating light and darkness. Plant Physiol. **27**: 9–17. 1952.
7. VICKERY, H. B., LEAVENWORTH, C. S., and BLISS, C. I. The problem of selecting uniform samples of leaves. Plant Physiol. **24**: 335–344. 1949.