STUDIES IN THE METABOLISM OF CRASSULACEAN PLANTS: THE BEHAVIOR OF EXCISED LEAVES OF *BRYOPHYLLUM CALYCINUM* DURING CULTURE IN WATER

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(WITH TWELVE FIGURES)

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A fundamental problem in experimental work with living tissues is the selection of samples in such a way that differences in composition which result from the treatment to which the material is exposed shall represent the effects of the treatment alone. There should be no opportunity for other processes to intervene and obscure the results. Complete isolation of the system under study can be effected, in the case of leaf tissue, only by excision from the plant. This has the disadvantage that the leaves are then merely "surviving organs" and soon begin to undergo changes that are ultimately lethal. Nevertheless, for a reasonable length of time, such leaves can be assumed to behave in a manner that reflects the normal course of metabolic events.

The technique of excised leaf culture as applied in this laboratory (**4, 5**) consists in the selection at random of a group of approximately equal samples from a quantity of leaves picked from the plants at the same time, and therefore, presumably, in the same biological and chemical condition. One or more samples are at once prepared for analysis, in order to provide a point of departure for the series, and the others are subjected to treatment. Samples are subsequently withdrawn from time to time for analysis, the data obtained being computed in terms of equal quantities of fresh tissue as weighed at the start of the experiment. Biological variation is minimized by the use of moderately large numbers of leaves per sample. Although the results are expressed in concentration units, they refer to originally equal and similar lots of tissue. Accordingly, the differences from sample to sample furnish, within the limitations of biological and statistical variation, a measure of the actual magnitudes of the changes that have occurred. Errors arising from translocation are eliminated and, presumably, the only phenomena that affect the data are those associated with photosynthesis, respiration, absorption of culture solution, transpiration, and chemical transformations occurring within the cells.

The diurnal variation of acidity characteristic of crassulacean plants is a phenomenon admirably suited for study by this general method. The entire cycle of changes is normally completed within 24 hours, a period sufficiently short to justify the assumption that irreversible catabolic changes which may be initiated are of minor significance in comparison with the

relatively much larger chemical changes associated with the normal metabolism of organic acids and carbohydrates. Accordingly, in the present paper, three experiments are described in which excised leaves of *Bryophyllum calycinum* were subjected to culture in water. In the first, leaves collected in the afternoon, at the time of low acidity, were cultured for 24 hours in the greenhouse exposed to the normal variation in illumination; in the second, similar leaves were cultured in complete darkness for 2 days and, in the third, leaves collected in the early morning, at the time of high acidity, were cultured in complete darkness also for 2 days.

Methods

Preparation of samples

On March 12, 1940, 9 Bryophyllum plants from the lot described in a previous paper (**3**) were selected, and the compound leaves, beginning at the second from the top, were removed from 5 of them at 4 P.M. The individual leaflets were cut from the petioles and rapidly sorted into a number of piles according to size. The samples for the experiment were then chosen so that the same number of leaflets from each pile was included in each, there being three samples of 33 leaflets taken for one experiment and four samples of 34 leaflets for the second. One sample from each group was immediately placed in the drying oven at 80° C.

The samples to be subjected to culture were arranged in long V-shaped troughs with the bases of the leaflets immersed in water, the blades being supported without overlap by the walls of the troughs. The troughs containing the 33-leaflet samples were placed in the greenhouse, the others in a completely dark room. All samples were in position within half an hour. The temperature of the greenhouse ranged from 20 to 27° C. during the 24-hour period of the experiment, that of the dark room from 21 to 23°.

The following morning at 7, shortly after sunrise, the other 4 plants were treated in the same way, four 39-leaflet samples being taken of which three were set up in the dark room by 7:15, the fourth being at once dried. The experiment carried out in the greenhouse was continued for 24 hours; the two experiments in darkness were extended for a second day in order to obtain information on the initiation of catabolic changes.

The process of selection of samples provided for randomization within each sample with respect to the plant of origin. The plants chosen for the afternoon samples corresponded in size with the larger plants described in a previous paper (**3**), those chosen for the morning samples corresponded to the smaller plants. Fundamental data on all of the samples are shown in table I. Analytical results obtained as percentages of the crude dry weight were calculated, with the use of factors derived from these figures, so as to give the quantity of the component that would have been obtained if each sample of fresh leaf had weighed exactly 1000 grams at the start.

The limits within which the samples duplicated each other with respect to composition may best be illustrated by the data for total nitrogen and ash.

Regardless of metabolic transformations within the cells during the period of culture, there should be no significant loss of nitrogen nor of inorganic components from the samples and no gain from the water used as culture medium. The mean value for the total nitrogen of the 11 samples was 1.747 ± 0.072 gms. per kilo of original fresh weight. The standard deviation is ± 4.1% of the mean. The mean value for the ash was 8.94 ± 0.020 gm. per kilo, the deviation being ± 2.3% of the mean. Accordingly, it may be assumed that differences substantially greater than ± 4% observed for other components represent significant alterations in composition as a result of the treatment.

TABLE I

FUNDAMENTAL DATA ON SAMPLES OF EXCISED *Bryophyllum calycinum* LEAFLETS SUBJECTED TO CULTURE IN WATER. FIGURES NOT OTHERWISE DESIGNATED ARE GRAMS. THE TIMES ARE STANDARD TIME

TIME OF SAMPLING	ELAPSED TIME	NUMBER OF LEAFLETS	FRESH WEIGHT: START	FRESH WEIGHT: END	CRUDE DRY WEIGHT	CRUDE DRY WEIGHT PER KILO	FRESH WEIGHT PER KILO: END
	hrs.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
ALTERNATE DARKNESS AND LIGHT							
4 P.M.	0	33	193.1	.....	18.2	94.26	.....
6 A.M.	14.0	33	182.5	185.6	19.0	104.1	1017
4 P.M.	24.0	33	180.0	177.5	18.9	105.0	986
DARKNESS: STARTING IN AFTERNOON							
4:30 P.M.	0	34	231.3	.....	21.3	92.08	.....
6:30 A.M.	14.0	34	217.6	219.9	21.2	97.4	1010
4:30 P.M.	24.0	34	219.8	221.9	21.2	96.45	1010
4 P.M.	47.5	34	202.3	201.5	18.7	92.43	996
DARKNESS: STARTING AT DAYBREAK							
7 A.M.	0	39	249.4	.....	23.7	95.01	.....
4:15 P.M.	9.25	39	243.5	252.3	23.1	94.86	1036
8 A.M.	25.0	39	250.5	257.3	21.7	86.64	1027
8 A.M.	49.0	39	251.1	247.0	21.5	85.61	983.5

Results

CULTURE IN ALTERNATE DARKNESS AND LIGHT

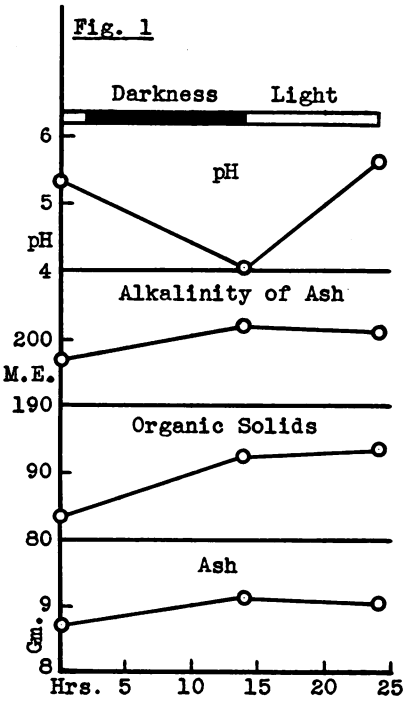
That diurnal variation in acidity occurs in detached leaves of Bryophyllum subjected to culture in water is evident from the curve for pH in figure 1. During the night, the pH dropped by 1.3 units, owing to synthesis of acids, and rose again during the day to a value even higher than that observed initially. The change was about twice as great (in pH units) as that observed in the leaves of intact plants described in a previous paper (**3**). These observations alone are sufficient to justify the fundamental assumption on which the present experiments are based, namely, that excision of the leaves does not seriously interfere with the normal metabolic functions for at least 24 hours.

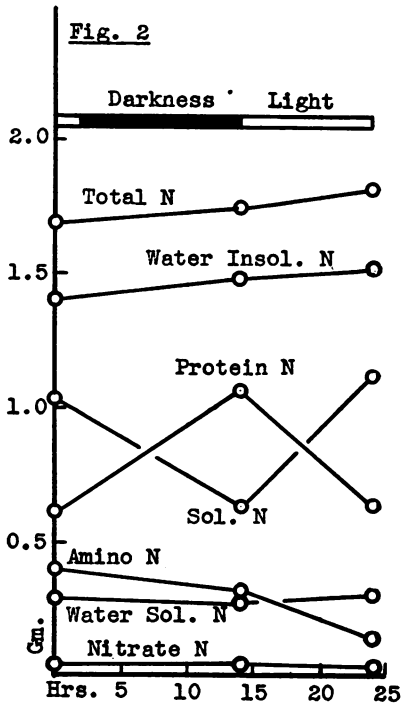
Notwithstanding the large change in total acid reflected in this change in pH, the alkalinity of the ash (fig. 1) remained constant. The mean value for the alkalinity of the ash of the entire set of 11 samples was 195.1 ± 5.8 milliequivalents per kilo, the deviation being ± 3% of the mean. This is the same order of magnitude as the deviation in the nitrogen and ash content and is clearly a measure of the reproducibility of the samples rather than of any effect of treatment. The alkalinity of the ash is a determination of the difference between the number of equivalents of inorganic acidic and of inorganic basic components present after ignition of the tissue; inasmuch as no opportunity was afforded for translocation of inorganic acid or base into or out of the leaves, there was no way in which this difference could be altered by the treatment to which the leaves were subjected.

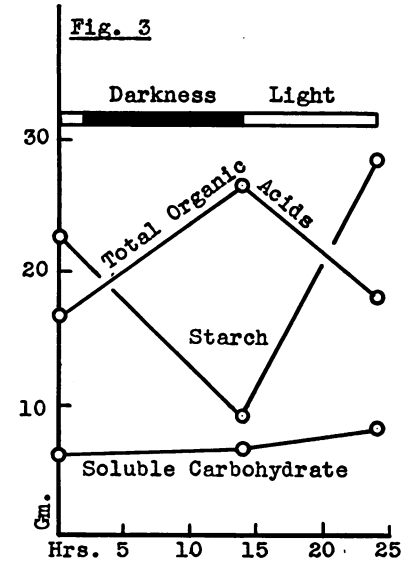
Organic solids apparently increased during the period of darkness and remained essentially constant during the day (fig. 1). However, the analytical determination of organic solids is especially difficult and subject to error and it is by no means certain, in view of the small number of samples, that the apparent increase, although it amounted to about 10%, was real. The similar samples cultured in the dark room (fig. 5) showed no comparable effect. The ash (fig. 1) and the total nitrogen (fig. 2), as has already been pointed out, showed no significant change in the course of the experiment.

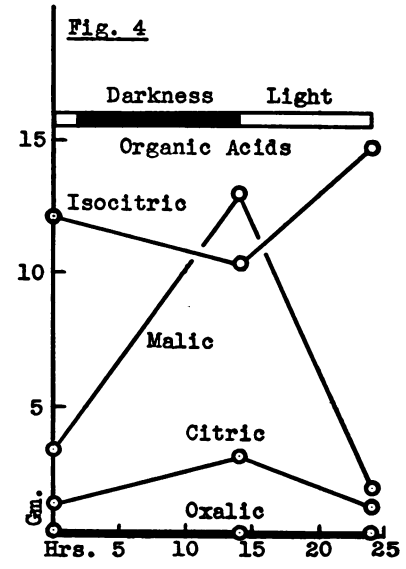
The data presented so far are thus in agreement with the view that the three samples used for this experiment were initially sufficiently alike in composition to warrant the conclusion that changes in excess of 10% with respect to any analytical component represent alterations in composition as a result of the treatment to which they were exposed. The change in protein nitrogen (fig. 2) furnishes an illustration of this; during the night, the protein nitrogen increased from 0.61 to 1.06 gm. per kilo and then diminished during the day to 0.65 gm. The change was similar both in magnitude and in its relationship to light to that observed in leaves of intact plants subjected to analogous treatment (**3**). Furthermore, as in the previous experiment, there were changes in soluble organic nitrogen symmetrical with those of protein nitrogen, the one increasing when the other diminished and *vice versa.*

That the change in organic soluble nitrogen should be roughly equal and opposite to the change in protein nitrogen is to be expected. The quantity denoted protein nitrogen represents the nitrogen of the dried leaf tissue that remains insoluble after a sample is exhaustively extracted with hot 70% alcohol, a procedure designed to remove chlorophyll and other pigments together with the soluble carbohydrates and simpler nitrogenous components, and subsequently further extracted with boiling water to remove a small additional quantity of water-soluble nitrogenous material. It is assumed that nitrogen that remains insoluble under these conditions belongs exclusively to denatured protein. Wide application of the method has hitherto revealed no case that throws doubt on this conclusion. The soluble organic









FIGS. 1 to 4. Composition of excised leaflets of *Bryophyllum calycinum* cultured in water for 24 hours in alternate darkness and light. Data are expressed in grams per kilo of fresh weight.

nitrogen is obtained by subtracting the protein nitrogen from the total nitrogen and correcting the difference for nitrate and for the trace of free ammonia nitrogen present. If these are constant, as was very nearly the case in the present samples, the curve for soluble nitrogen must be nearly symmetrical with that for protein nitrogen.

Determinations were also made of the quantity of nitrogen that remained insoluble when samples of the dry tissue were extracted with hot water. With leaf tissues such as tobacco, results so obtained are practically the same as those after extraction successively with hot alcohol and hot water. With Bryophyllum leaves, the quantity of water-soluble nitrogen (fig. 2) is much smaller than that of soluble organic nitrogen as usually determined, and the difference between the two curves (fig. 2) furnishes a measure of the additional nitrogen extracted by hot alcohol. Correspondingly, the quantity of water-insoluble nitrogen (fig. 2) is much greater than the protein nitrogen. Neither curve shows any clear effect of the treatment to which the leaves were subjected. Bryophyllum leaves obviously contain nitrogenous substances that possess solubility relationships quite unlike those of the proteins of such more thoroughly studied leaves as tobacco, and the interpretation of the present data is correspondingly uncertain. If the method usually employed in this laboratory for the determination of protein nitrogen is in fact applicable to Bryophyllum, the data imply that a substantial part of the protein present in the early morning is converted, under the influence of light, into products that are soluble in hot alcohol. During the night, these products are reconverted into material, presumably protein, that has the same solubility as before. That the alteration in solubility is not merely an effect of the changed pH of the leaves was established by suitable separate experiments.

The question of the nature of the products that are soluble in hot alcohol remains for more detailed study. That they are not normal products of proteolytic action such as amino acids or simple peptides is evident from the curve for amino nitrogen (fig. 2) for, at the time the protein nitrogen was increasing by substantially 0.4 gm., the amino nitrogen decreased by less than 0.1 gm.; correspondingly, while the protein nitrogen was diminishing by 0.35 gm. the amino nitrogen also diminished by 0.17 gm. instead of increasing. The failure of these analytical quantities to correspond with each other either in magnitude or in the anticipated direction of change is evidence that the diurnal alteration in the apparent solubility of the protein is something other than protein digestion such as is observed in tobacco leaves cultured under similar conditions.

Diurnal variation of the total organic acids is shown in figure 3. During the night, the acids increased from 16.9 to 26.7 gm. per kilo, an increase of 58% of the evening value; during the following day, they decreased to 18.1 gm. As has already been shown by the curve for pH, the normal diurnal process of organic acid metabolism characteristic of this species was in no detectable way interfered with by excision of the leaves. Corresponding in

point of time with the change in the acids, there was an even more extensive change, although in the opposite sense, in the quantity of starch. Starch decreased during the night from 22.6 to 9.2 gm. per kilo (calculated as glucose) and increased during the day to 28.5 gm. Nevertheless, the soluble carbohydrates scarcely changed significantly; there was at most a slight increase during the day.

The chemical possibilities presented by these changes in composition are far too complex for detailed interpretation, and all that can be attempted is to point out certain inferences that may justifiably be drawn. The quantities of starch and organic acids involved are such that a roughly quantitative relationship of the nature of an equilibrium between starch and acid might be assumed to exist, that is to say, a reaction analogous to that offered as a speculation by BENNET-CLARK (1) to account for the chief chemical reactions of crassulacean metabolism. During the night, 13.4 gm. of starch disappeared while 9.8 gm. of organic acids were formed. There was no change in the soluble carbohydrates so that the net change represents a loss of 3.6 gm. of organic substance. No such loss appears on the curve for organic solids; rather, there was an apparent increase of about 9 gm. which, from other considerations, it seems more conservative at present to interpret as being possibly due to biological variation between samples. Thus the evidence, as far as it goes, suggests that the respective alterations in the quantities of acids and of starch were the result of intracellular chemical reactions and it is quite possible that the acids arose in the course of a series of oxidation reactions which consumed starch.

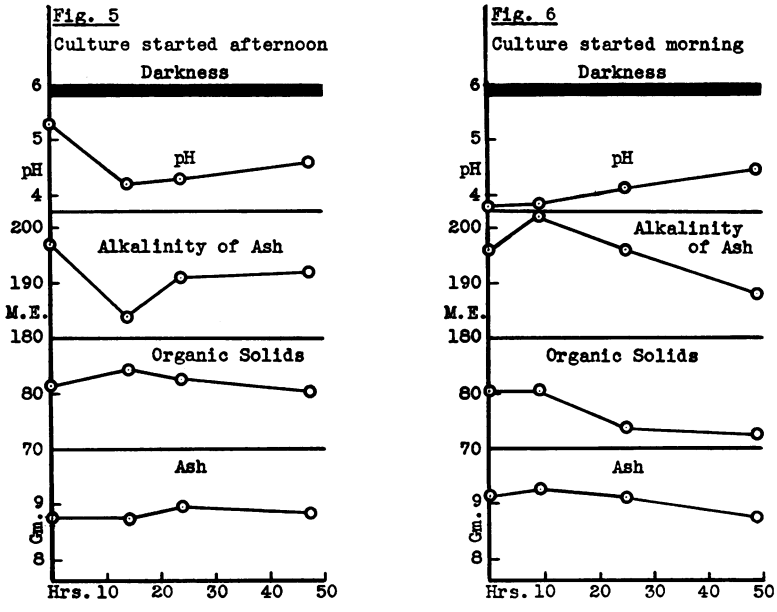
During the day, however, 19.3 gm. of starch appeared and 8.6 gm. of acids disappeared. The net increase in organic solids from these two reactions is about 12 gm. since there was also an increase of about 1 gm. of soluble carbohydrate. However, no increase of this magnitude is shown by the curve for the organic solids (fig. 1); these in fact remained constant and it seems reasonable to assume that, if about 12 gm. of starch were newly formed by photosynthesis from carbon dioxide acquired from the air, there would have been a clearly evident rise of the curve in figure 1. Thus the formation of starch in light must, in this case, have also been largely the result of intracellular reactions and the quantity is considerably greater than could possibly have arisen from the organic acids even on the most favorable assumptions as to the efficiency of the reversal of the equilibrium which may have operated during the night.

By far the greater part of the change in total organic acids arose from the change in malic acid (fig. 4). This substance increased from 3.3 to 13.0 gm. per kilo, or by a factor of 4 during the night and decreased to less than the original value during the day. Citric acid underwent a parallel series of changes increasing from 1.3 to 3.1 gm. per kilo during the night and falling again during the day to 1.2 gm. Isocitric acid, however, behaved quite differently; it decreased from 12.1 to 10.4 gm. per kilo during the night and increased to 14.8 gm. during the day. These changes are in the opposite

sense to those of the malic and citric acids and they are proportionally much smaller ones; isocitric acid does not respond to illumination as extensively as the other two acids. The small quantity of oxalic acid present showed no significant change as a result of the treatment of the samples.

Culture in complete darkness

The data for the experiments carried out for 2 days in complete darkness are plotted in separate figures placed side by side for more convenient comparison of the effects upon leaves picked in the one case in the afternoon at the time of low acidity and, in the other, in the morning at the time of high acidity, these leaves, of course, having been in darkness during the night.

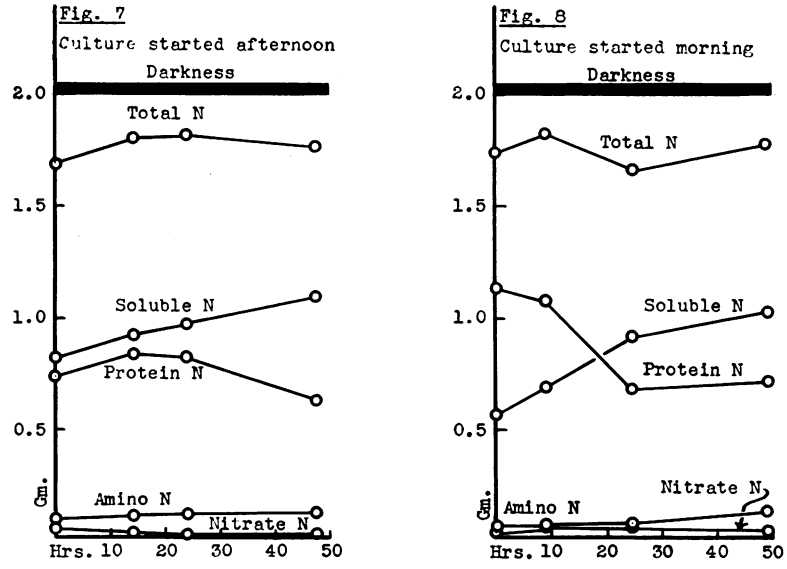


Figs. 5, 6. Composition of excised leaflets of *Bryophyllum calycinum* cultured in water for 2 days in complete darkness. Data are expressed in grams per kilo of fresh weight. Figure 5 refers to leaves started in the afternoon at a time of low acidity; figure 6 to leaves started in the morning at a time of high acidity.

The pH of the samples placed on experiment in the afternoon was high (fig. 5) and dropped for the first few hours in the same way as it did in the first experiment. It then began slowly to rise and had increased by 0.4 units at the end of 2 days. The pH of the samples started in the morning was low (fig. 6) and there was a slow and steady rise over the 2-day period that totaled about 0.6 units. The early parts of these curves reflect metabolic changes that were probably not greatly different from those that occur in normal leaves; the events that took place during the second day, however, doubtless reflect the behavior of cells placed under gradually mounting metabolic stress. One of the first demonstrable effects of this stress is clearly a diminution in the acidity of the leaves.

The curves for alkalinity of ash, organic solids, and ash (figs. 5 and 6) and for total nitrogen (figs. 7 and 8) show, as did the similar data for the first experiment, no significant effect of the treatment of the samples. It is quite likely that the small drop in organic solids at the end of these two experiments represents a loss of organic material from respiration but the samples were too few in number and the experiment was not sufficiently prolonged to make this certain. Analogous experiments with tobacco leaves (**4**) and rhubarb leaves (**5**), however, have shown that such a result is to be anticipated.

The behavior of protein nitrogen in the two experiments was entirely different (figs. 7 and 8); in the samples started in the afternoon at a low



FIGS. 7, 8. Composition of excised leaflets of *Bryophyllum calycinum* cultured in water for 2 days in complete darkness. Data are expressed in grams per kilo of fresh weight. Figure 7 refers to leaves started in the afternoon at a time of low acidity; figure 8 to leaves started in the morning at a time of high acidity.

level, protein at first increased. After passing through a flat maximum, the quantity of protein nitrogen then decreased during the second day of the experiment. However, neither change was as striking as those encountered in the first experiment (fig. 2); the increase was about 13% of the afternoon value and was thus probably significant, the decrease was 25% of the maximum value and of the validity of this there can be little doubt.

The samples started in the morning at a high level of protein decreased only slightly in protein content during the first 9 hours but had dropped by 39% at the end of 24 hours. There was no significant change during the second day. The effect of culture in darkness on the protein of Bryophyllum leaves is thus dependent upon the level of the protein present in the

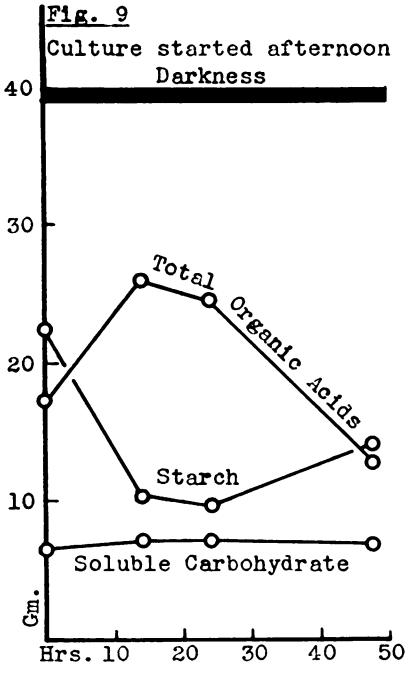
leaves at the beginning of the experiment. If the culture is started at a low level at the end of the day, there appears to be a small increase, but if it is started in the morning at a high level, there is little if any significant change for about 9 hours; then, however, a change analogous to that observed in light supervenes and there is a marked fall. Interpretation of these changes must await more detailed analytical studies; for the present, all that can be stated is that the fall in protein is not accompanied by a marked rise in amino nitrogen (fig. 8) as would be expected if the reaction were one of protein hydrolysis. Rather, it would appear to be one of change in solubility of a substantial part of the protein such as might be supposed to result from some form of disaggregation into units that are not rendered insoluble by hot alcohol.

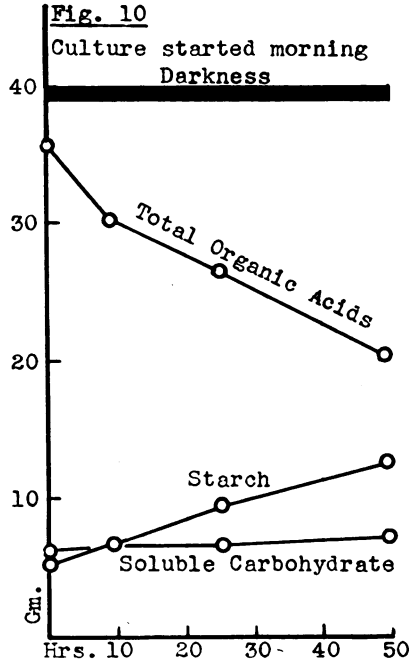
The failure of the curves for soluble nitrogen to follow courses exactly symmetrical with those for the protein arises in part from the minor irregularities in the curves for total nitrogen. The changes that occurred in the early phases of the culture started in the afternoon were small and were about at the limit of certain detection by the analytical techniques.

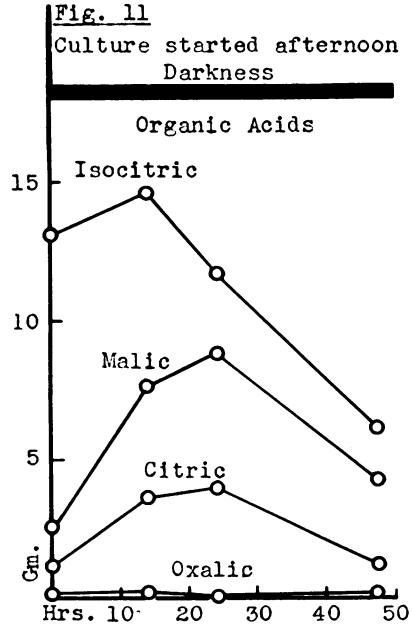
The leaves placed on experiment in the afternoon at low acidity promptly increased in total organic acids (fig. 9) just as in the first experiment. The acids remained at a high concentration for the whole of the first day but then rapidly diminished to a level lower than that at the start. Comparison of the curve with that for pH (fig. 5) shows that the drop in total acids was not accompanied by a correspondingly large increase in the pH and it is evident that pH by itself is an unreliable index of the magnitude of such changes. On the other hand, the leaves started in the morning at high acidity (fig. 10) did not maintain the level of organic acids then present; a continuous loss occurred although this did not become immediately evident on the curve for pH (fig. 6).

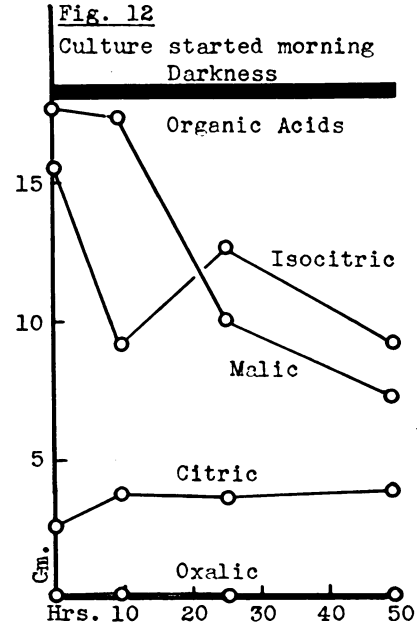
The behavior of the starch was surprising; in the leaves started in the afternoon (fig. 9), starch decreased during the period that the acids were increasing, remained constant while the acids were maintained at a high level but then *increased* during the period that the acids were falling in concentration. This represents synthesis of starch in leaves in total darkness, an unusual observation at best, and one that might well be attributed to error had it not been confirmed by an even more striking synthesis of starch in the leaves started in the morning (fig. 10). In these, starch increased from 6.2 to 12.6 gm. over the entire 2-day period at a rate which followed a smooth curve essentially symmetrical with that for the total acids.

It will be useful to consider the relative quantities of starch and organic acids involved in these changes. Figure 9 shows that, during the first 14 hours in culture in darkness, 13.4 gm. of starch disappeared while 8.9 gm. of organic acids were formed. The change in soluble carbohydrate was insignificant being only 0.5 gm.; thus it is possible that the organic acids that were formed arose from oxidation reactions in which starch was consumed. There was a net decrease of 4.5 gm. of organic material, too little









FIGS. 9 to 12. Composition of excised leaflets of *Bryophyllum calycinum* cultured in water for 2 days in complete darkness. Data are expressed in grams per kilo of fresh weight. Figures 9 and 11 refer to leaves started in the afternoon at a time of low acidity; figures 10 and 12 to leaves started in the morning at a time of high acidity.

to be revealed with certainty on the curve for organic solids (fig. 5) in any case even if these were the only chemical reactions that occurred. Actually, the organic solids in the 14-hour sample were slightly greater than those in the initial sample although the difference was well within the limits of uncertainty in the determination.

During the second day of the culture period, organic acids diminished by 11.7 gm. and starch increased by 4.3 gm. Compared with the experiment carried out in daylight, this is a small increase in starch. It was inferred from the data from the experiment in daylight that the organic acids could have contributed at most only a part of the material from which the starch was synthesized during illumination even on the assumption of complete reversibility of the reactions whereby the acids may have been produced in darkness. In the present case, however, judging from the quantities alone, there is a possibility that all of the starch formed in darkness could have arisen from such a reversal. However, this is not proof that such reactions occurred; all that has been established is that a significant quantity of starch was formed in darkness during a period when a substantially larger quantity of organic acids disappeared.

In the experiment in which the leaves were high in organic acids at the start (i.e., leaves which had remained attached to the plants during the night and in which the normal reactions of darkness had taken place), 9.4 gm. of acids disappeared during the first day and 6.1 during the second. The quantities of starch formed in these intervals were, respectively, 3.1 and 3.2 gm. Thus the behavior throughout was analogous to that during the second day of the other experiment in darkness.

The details of the transformations of the organic acids are shown in figures 11 and 12. In the leaves started in the afternoon, there was a large increase of malic acid and a substantial one of citric acid during the first 24 hours. During this interval, isocitric acid first increased and then diminished. Throughout the second day, all three acids diminished at almost equal rates. In the leaves started in the morning, malic acid remained nearly constant for 9 hours and then diminished rapidly, citric acid increased slightly at first, while isocitric acid underwent a sharp drop followed by a rise. During the second day, both malic and isocitric acids diminished while citric acid remained essentially constant. The relationships among the three acids strongly suggest that interconversions may have occurred; the temporary maintenance of malic acid at the initial high level and the small production of citric acid may both be results of reactions that consumed isocitric acid. If malic acid was produced as rapidly from isocitric acid as it was decomposed in the course of other reactions, the net quantity present would remain unchanged. The increase in citric acid may be assumed to result from transformation of isocitric acid *via* aconitic acid, a reaction well known to occur in muscle tissue (**2**). In the interval between the ninth and twenty-fifth hours of the culture period, the sharp fall in malic acid and the corresponding increase in isocitric acid may have been the effect of a reversal of the reaction that occurred during the first 9 hours.

Discussion

The chemical changes that occur during the first 24 hours of culture of excised leaves of Bryophyllum are, qualitatively, closely similar to those observed in normal leaves (**3**). Marked fluctuations occur in the form of nitrogen that is insoluble in hot alcohol and hot water and which has been interpreted as representing protein nitrogen. Leaves that have recently been illuminated are low in this component but protein is rapidly formed in such leaves when they are placed in the dark. Corresponding changes in the opposite direction take place in the soluble nitrogen. The acidity, whether measured as pH or by the titration of the organic acids extracted from the tissue, likewise undergoes wide variations, acids being synthesized in darkness and decomposed in light. Starch is formed during illumination but, unless the organic acids are undergoing decomposition, disappears in darkness so that the fluctuations are in the opposite sense to those of the organic acids. The largest share in the changes in the organic acids is taken by malic acid; citric acid varies in the same direction as malic acid but through a smaller range. Isocitric acid, however, varies in a less regular manner and the changes that it undergoes are frequently in the opposite sense to those of malic acid, sometimes being large, sometimes small, in relation to it.

The chief technical advantage of the leaf culture method is that, because of the complete isolation of the biological system, inferences may be drawn from the quantities of substances involved in the reactions provided the changes are large enough to be outside the range of probable biological variation among samples. Within certain limits, therefore, specific interpretations of some of the reactions that occur may be attempted.

It will be useful to point out the contrasts between the behavior of Bryophyllum leaves and those of tobacco when subjected to culture. Excised tobacco leaves cultured in water in light (**4**) exhibit a prompt and surprisingly large increase in organic solids, for the most part because of the synthesis of soluble carbohydrates and starch. In darkness, they undergo equally prompt losses owing to the uncompensated effect of respiration, and these losses fall heavily upon the carbohydrates and malic acid. The protein nitrogen diminishes rapidly under both conditions of culture; evidence was secured that implied the complete destruction of that part of the protein that disappeared, and indicated that the simple nitrogenous products formed underwent a succession of complex transformations. The experiments with tobacco leaves were extended for several days so that there is little doubt that the greater part of the changes observed, especially in the later stages, were catabolic in nature. They showed, however, that leaf cells are the seat of intense metabolic activity, and many of the changes were initiated within the first 24 hours.

Excised Bryophyllum leaves likewise undergo profound metabolic changes in composition during culture but, in this species, some of the more conspicuous changes are a part of the normal cyclic behavior of the tissue

under the influence of diurnal variation in illumination. Certain of the reactions so clearly observed in tobacco leaves were, on the other hand, scarcely if at all demonstrable in this species in part because of the brevity of the experiment. This statement applies in particular to the reactions between the tissues and the oxygen and carbon dioxide of the air; no conclusive evidence was secured of photosynthesis in terms of an increase of organic solids in light, nor could loss of solids by respiration in darkness be demonstrated although it is possible that experiments more prolonged than the present ones might be successful in this. The evidence, as far as it goes, suggests what may be termed a more closed metabolic economy than that of the tobacco leaf, and observations that bear on this aspect of the problem are to be found in the literature. The work of BENNET-CLARK (**1**) and of WOLF (**6**) on the respiratory quotient, for example, shows that the liberation of carbon dioxide drops to a very low level shortly after the leaves are placed in darkness at the time that acid production increases in intensity. It is reasonable to infer that carbon dioxide is being utilized within the tissues for the synthesis of organic acids, possibly, for example, of oxalacetic acid from pyruvic acid by the Wood and Werkman reaction. Later, during continued culture in darkness (i.e., after about 12 hours), carbon dioxide liberation by the leaves increases and this occurs at the time when the organic acids pass their maximum and begin to drop in concentration. Its liberation is an evidence of decarboxylation reactions.

BENNET-CLARK likewise observed that oxygen uptake is high during the early phase of acid production but later diminishes. This is in agreement with the assumption that the formation of organic acids is a result of oxidation reactions involving carbohydrates, particularly starch. It is to be noted that both oxidation and carbon dioxide fixation are essential if the organic acid metabolism of Bryophyllum leaves is to be accounted for in terms of the tricarboxylic acid cycle of KREBS. Pyruvic acid may be assumed to arise from the decomposition of hexoses but an increase in length of the three carbon chain of pyruvic acid to four carbon atoms (i.e., conversion to oxalacetic acid) is essential if the subsequent production of malic acid is to be accounted for. Furthermore, oxalacetic acid is also an essential intermediate for the formation of isocitric and citric acids. Regardless, therefore, of the absence of direct information on the presence of the specific enzyme systems necessary for these reactions, one is justified in advancing, as an hypothesis, the view that the transformations of organic acids observed in Bryophyllum leaves are an expression of the activity of such enzyme systems. On this view, the several organic acids are intimately linked with each other by chemical equilibria and transformation of one acid into another, of carbohydrates into organic acids and even the reverse transformation of organic acids to carbohydrates receive a rational explanation. The synthesis of starch in darkness when the concentration of organic acids is high becomes a phenomenon that is to be anticipated. It should be pointed out that all of the analytical data of the present experiments are in agreement with the requirements of this hypothesis.

During the second 24-hour period of the present experiments, however, other phenomena began to make their appearance. The early culture experiments of BENNET-CLARK showed that the organic acids of *Sedum praealtum* leaves passed through a maximum in concentration after about 12 hours in darkness and subsequently declined. At the same time, carbon dioxide production increased. WOLF has made somewhat similar observations on *Bryophyllum calycinum* leaves, and the phenomena are very likely general for the Crassulaceae. In the present experiments, certain reactions were initiated which were the reverse of those noted during the first day. It is probably correct to assume that such reactions represent the beginning of the catabolic processes that inevitably follow excision of the leaf. One of the first to be demonstrable is, as a rule, the transformation of the nitrogen of protein into forms that are soluble and, in particular, an increase in amino nitrogen presumably produced by proteolytic action. These effects are clearly evident in tobacco and rhubarb leaves subjected to culture under analogous conditions (**4, 5**). They are not especially marked in the present case, however, although the culture started in the afternoon does show them to a possibly significant extent (fig. 7).

Another effect well shown by tobacco and rhubarb leaves is the loss of organic solids from respiration but, again, the present experiments were not sufficiently prolonged for this to become unequivocally clear (figs. 5 and 6). However, the disappearance of organic acids, especially malic acid, with a corresponding increase in pH is invariably observed and the present experiments show this especially well. All three of the chief organic acid components decreased sharply during the second day of the experiment started in the afternoon, two of them followed the same course in the experiment started in the morning. This effect may be regarded as an evidence of the increasing metabolic stress to which the excised leaves were subjected. Even in this case, however, the analogy with tobacco and rhubarb leaves is not close. The Bryophyllum leaves became enriched in starch and there was no effect upon the soluble carbohydrates. In tobacco and rhubarb leaves, on the other hand, carbohydrates are early involved in the general picture of catabolism.

Taken together, then, the chemical evidence of the present experiments suggests a general metabolism adapted for survival under unfavorable conditions. The response, rather early in the period of stress, is to lay up a store of starch. It is quite possible that the well-known capacity of excised leaves of Bryophyllum to survive and reproduce by buds which develop at the margins may be the ultimate expression of such a provision.

Summary

Equal samples of leaflets excised from *Bryophyllum calycinum* plants were subjected to culture with the base of each leaflet immersed in water, one set of samples taken in the late afternoon being treated in the greenhouse and a second set in a completely dark room. A third set collected

early in the morning was likewise placed in the dark room. Analytical data were computed in terms of 1 kilogram of fresh tissue weighed at the time of sampling.

There were no significant effects in any case upon the quantity of organic solids nor upon the ash, the alkalinity of the ash, or the total nitrogen; even the soluble carbohydrates failed to change. The samples cultured in the greenhouse increased sharply in organic acids during the night and decreased during the day, the greater part of the change being due to malic acid with citric acid playing a smaller but similar role. Isocitric acid decreased at night and increased during the day.

Starch varied in a manner the converse of organic acids, the fluctuations being in each case through a wider range than those of the acids. Thus it is possible that organic acids synthesized at night arose from oxidation of carbohydrates. However, some source of starch other than the organic acids must be invoked to account for the starch synthesized during the day, yet the failure of the organic solids to increase significantly indicates that photosynthesis played only a small part.

Protein increased during the night and decreased during the day, soluble nitrogen undergoing similar changes in the reverse direction.

The leaves cultured in darkness starting in the afternoon at low acidity increased in malic, citric, and isocitric acids for 14 hours. The increase in malic and citric acids continued for the rest of the first day but isocitric acid then began to decrease. All three acids decreased during the second day.

Starch decreased during the first day in darkness but increased significantly during the second. Protein increased slightly for 14 hours and then decreased slowly.

The leaves cultured in darkness starting in the morning at high acidity began to lose isocitric acid at once. Malic acid dropped slightly but citric acid increased; later malic acid dropped sharply, isocitric acid increased and citric acid remained constant. During the second day, isocitric acid and malic acid decreased, and citric acid remained unchanged.

Starch increased slowly but continuously throughout the 2-day period in darkness. Protein decreased during the first day and remained unchanged during the second.

The changes in organic acids and starch can be largely accounted for if it is assumed that the several substances are linked by enzymatic reactions in a system analogous to the Krebs tricarboxylic acid cycle.

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LITERATURE CITED

1. BENNET-CLARK, T. A. The role of organic acids in plant metabolism. Part II. New Phytol. **32**: 128–161. 1933.
2. KREBS, H. A., and EGGLESTON, L. V. Micro-determination of *iso*citric and *cis*-aconitic acids in biological material. Biochem, Jour. **38**: 426–437. 1945.

3. 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. in press. 1947.
4. VICKERY, H. B., PUCHER, G. W., WAKEMAN, A. J., and LEAVENWORTH, C. S. Chemical investigations of the tobacco plant. VI. Chemical changes that occur in leaves during culture in light and in darkness. Connecticut Agr. Exp. Sta. Bull. **399.** 1937.
5. ————, ————, ————, and ————. Chemical investigations of the rhubarb plant. Connecticut Agr. Exp. Sta. Bull. **424.** 1939.
6. WOLF, J. Beiträge zur Kenntnis des Säurestoffwechsels sukkulenter Crassulaceen. III. Stoffliche Zusammenhänge zwischen gärfähigen Kohlehydraten und organischen Säuren. Planta **28**: 60–86. 1938.