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THE CAPACITY OF LEAVES OF BRYOPHYLLUM CALYCINUM TO RECOVER FROM PROLONGED EXPOSURE TO DARKNESS OR TO LIGHT[1]

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Emphasis was placed in a recent paper (1) on the observation that the normal diurnal rhythm of the changes in the starch and organic acid content of leaves of Bryophyllum calycinum appears to be closely adapted to the average length of a day or night. If the period of darkness or of light is artificially prolonged, the changes in composition, which had been initiated at their usual speed, come more or less to a standstill after the elapse of about 12 hours. The effects of reactions other than those that are expressed by the major changes in composition can then in certain instances be perceived. Such reactions may not be abnormal, but in the usual course of events their effects are concealed by the larger and more rapid changes of composition, and, if they are allowed to proceed for an unusual length of time, there is a possibility that some degree of damage to the system may occur. It was, accordingly, of interest to expose Bryophyllum leaves to a prolonged period of darkness or of light, and to examine the extent to which the normal variations in composition are resumed after transfer to the other condition of illumination.

In discussing the results, it will be convenient to employ the concept of physiological stress. Disregarding the fact that excision of a Bryophyllum leaf from the plant immediately places it under certain not clearly defined but real stresses, the effects of which eventually become evident from the formation of rootlets at the margins of the leaf, observation shows that such leaves, if picked at sunrise and placed

in light, behave in the normal fashion, for malic acid diminishes and starch is formed. Furthermore, it has been demonstrated that excised leaves, when exposed to the usual diurnal alternation of light and dark in a greenhouse, undergo profound changes in composition in response to this alternation for at least 3 days (2). Excision from the plant thus by itself places no serious restriction upon the speed or amplitude of the normal chemical changes.

However, if leaves are exposed to artificially maintained illumination for many hours, increasing evidences of abnormality become apparent (3). They become flaccid, and, after the main chemical changes have been completed, irregular although small oscillations of both starch and organic acid content occur. In the converse case, when leaves are picked at nightfall and placed in darkness at ordinary temperatures, starch diminishes in amount and malic acid is formed for about 12 hours; subsequently the starch remains approximately constant while malic acid slowly decreases. Accordingly, under either condition, the leaves may be assumed gradually to have passed into a stage characterized by an increasing degree of physiological stress.

In the present experiments, individual samples of leaves have been removed from such presumably stressed conditions and have been either illuminated or placed in darkness, as the case may be, for 11 to 14 hours to see to what extent recovery could occur. The main criteria employed were the capacity to resume the normal changes in composition with respect to starch and organic acids.

Experimental Procedure

The ten samples used for the culture experiment in darkness were collected by the statistical method (4) in the early evening of June 20, 1955, a hot, humid and sunny day. The plants had been grown in soil in crocks in the greenhouse, and the top three pairs of fully developed but still young five-leaflet leaves were used. The leaf-culture troughs were set up in a room maintained at 24° C and 50 % relative humidity, and the room was kept in complete darkness after the leaflets had been arranged with their bases in water, 8:00 P.M. being taken as the zero hour of the experiment. After the expiration of 36, 60 and 84 hours (i.e., at 8:00 A.M. on three successive days), an individual sample was transferred to the greenhouse and placed in a trough exposed to full daylight for 11 hours. The sample was then dried for analysis. Meanwhile, samples had been taken from the troughs in the dark room at intervals chosen so as to afford maximal information regarding the rates of the reactions which affect the main components of the leaves. The precise times at which samples were so withdrawn are indicated in the figures.

The ten samples used for the culture experiment in light were collected in the same way, starting at 5:00 A.M. July 21, 1953 (sunrise 5:36 A.M. daylight saving time), also from plants grown in soil in the greenhouse. The culture troughs were arranged in an insulated room under a bank of fluorescent lights which gave a light intensity of approximately 1100 ft-c at the surfaces of the leaves. The temperature varied between 20 and 22° C and the humidity between 58 and 66 % with the operation of the control equipment which automatically came into play at intervals of from 1 to 2 hours. Zero hour was taken at 6:00 A.M. when the initial control sample was placed in the drying oven. After the expiration of 27, 50 and 74 hours, individual samples were transferred to troughs in the dark room where they were held, respectively, for 11, 12 and 14 hours at 24° C and 50 % relative humidity. Samples were also removed from the culture troughs in light and dried for analysis at times indicated in the figures. Fuller details of the technique and of the treatment of the

samples in preparation for chemical analysis have been given in previous papers (1, 5). The analytical methods are described in recent bulletins from this station (6, 7).

Experimental Results

Table I shows the evidence for the uniformity of composition within each set of samples. The fresh weights of the individual samples had a coefficient of variation of 1.2 % in one set and 1.15 % in the other, and the nitrogen and ash contents of both sets were satisfactorily constant. Alkalinity of ash was determined in only a few of the samples of the set cultured in darkness, and only the average value is given since the variability of this quantity in other sets of samples has always been found to be less than that of the total nitrogen. The coefficient of variation of the protein nitrogen in both sets of samples was notably higher than that of the components which would be assumed to remain constant, and plots of the data indicated that a slow but continuous loss occurred. The significance of this observation will be enlarged upon later.

The main analytical results are plotted in figures 1 and 2. The solid lines show the changes in composition in continuous darkness at the left, and in continuous light at the right of the figures. The curves which represent the changes in the amounts of each component are placed side by side, and the data confirm in remarkably close detail the results of previous studies of this species (3, 5).

The effect of the transfer of samples from darkness to light or from light to darkness at successive intervals during the course of the experiment is shown by the broken lines, and it is obvious that with few exceptions the change in composition took place in the direction which would be predicted from earlier results. Whether or not the changes actually followed straight line functions, as they are represented to have done, must be left for subsequent study. The mean rates of the reactions which occurred are indicated by the slopes of these broken lines, and close examination shows that these slopes changed progressively with time. Accordingly, the rates at which the sev-

TABLE I

ANALYTICAL DATA ON TWO SETS OF TEN SAMPLES EACH OF LEAFLETS OF BRYOPHYLLUM CALYCINUM COLLECTED BY THE STATISTICAL METHOD

ANALYTICAL DETERMINATION	CULTURED IN LIGHT WITH TRANSFER TO DARKNESS (SAMPLES 1953-B)		CULTURED IN DARKNESS WITH TRANSFER TO LIGHT (SAMPLES 1955-C)	
	MEAN ± S.D.*	C.V., % *	MEAN ± S.D.*	C.V., % *
Initial fresh wt., gm/sample	311.6 ± 3.86	1.2	176.2 ± 2.03	1.15
Total N, gm **	1.95 ± 0.033	1.7	2.51 ± 0.031	1.3
Protein N, gm **	1.51 ± 0.040	2.6	1.93 ± 0.050	2.8
Ash, gm **	11.86 ± 0.12	1.02	9.78 ± 0.15	1.6
Alkalinity of ash, meq **	286.2 ± 1.7	0.6	245.3	...

* S.D., standard deviation; C.V., coefficient of variation as percentage.
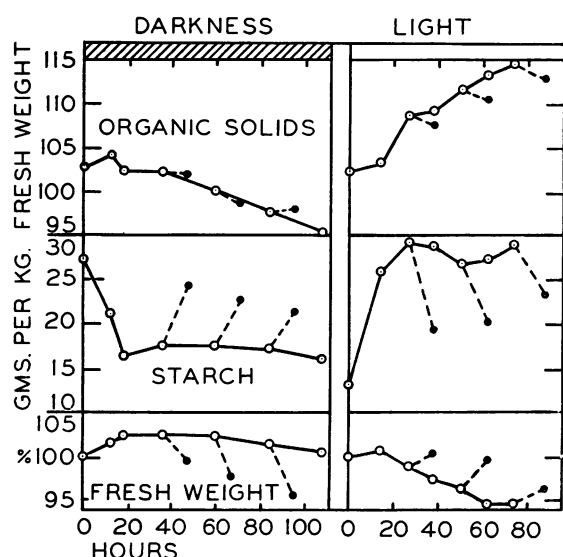** Data expressed in terms of 1 kilo of initial fresh wt.

FIG. 1. Changes in organic solids, starch and in fresh weight of leaves of *Bryophyllum calycinum* cultured in water. The solid lines show at the left the effect of culture in darkness (samples 1955-C), those at the right the effect of culture in light (samples 1953-B). The broken lines show the effect of transfer of samples from culture in darkness to light at the left, and of transfer from light to darkness at the right.

eral reactions occurred after transfer are dependent upon the time that had elapsed before the transfer was made.

Before considering this aspect of the data, it is necessary to draw attention to certain details. The curve for organic solids in figure 1 shows that a small increase occurred during the first 12 hours in darkness as would be expected from previous work (1, 5), but that this was followed by continuous loss. Transfer to light, however, gave rise to no marked increase in organic solids; during 11 hours, the sample transferred at the 36-hour point maintained its weight, that at 60 hours lost 1.5 gm per kilo, and that at 84 hours gained only 0.25 gm per kilo. Nevertheless, these 3 samples increased in starch content by 6.5, 4.9 and 4.0 gm per kilo in the 11 hours each of them was exposed to light. Photosynthesis (i.e., net fixation of carbon dioxide) is thus entirely inadequate to account for the formation of the starch. On the other hand, the samples cultured in light increased continuously in organic solids as would be expected. Transfer to darkness brought about losses of organic solids within the next 11 to 14 hours of 0.9, 1.3 and 1.7 gm after, respectively, 27, 50 and 74 hours of culture in light. It would seem that, under the conditions of stress occasioned by long exposure to light, the capacity of the leaves to continue to accumulate organic solids for a short time in darkness is seriously interfered with, although more detailed examination will be required to discover whether or not it is eliminated.

Attention should also be directed to the changes in fresh weight in figure 1. The leaves placed in

darkness quickly took up about 2 % of water and became unusually turgid; even the sample held in darkness for 108 hours was still crisp although the weight had dropped almost to its original value and evidence of the initiation of rootlets at the margins of the leaves was noted. The samples transferred to light, however, soon lost their turgidity and were definitely limp at the end of the period of exposure.

The samples cultured in light increased slightly in fresh weight for the first 14 hours, but subsequently lost weight continuously and became markedly flaccid; nevertheless, on transfer to darkness, they retained a moderate capacity to take up water again although only the sample exposed to light for 27 hours recovered all of its initial fresh weight.

The behavior of the starch is also shown in figure 1. The two curves are obviously symmetrical with each other in general pattern, and it is unfortunate that the samples were not removed at precisely the
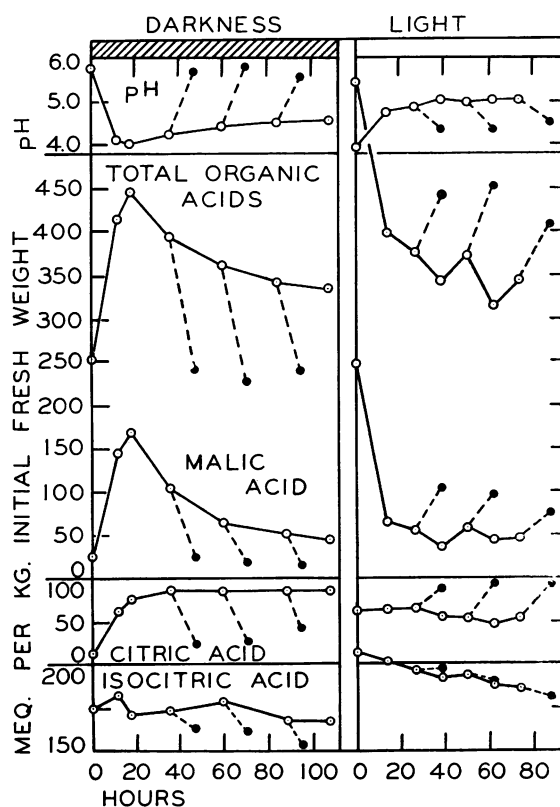

FIG. 2. Changes in pH and organic acids in leaves of *Bryophyllum calycinum* cultured in water. The solid lines show at the left the effect of culture in darkness (samples 1955-C), those at the right the effect of culture in light (samples 1953-B). The broken lines show the effect of transfer of samples from culture in darkness to light at the left, and of transfer from light to darkness at the right. The total organic acids of the sample collected at 0 hours in the set exposed to light was 572 meq per kilo of initial fresh weight, the isocitric acid content of the same sample was 207 meq.

same time intervals so that a calculation of the coefficient of correlation would be justified. The symmetry suggests, however, that the behavior of the two sets of samples was reasonably comparable even though the leaves were subjected to experiment two years apart in point of time.

Data on the rates at which starch disappears in darkness or is synthesized in light in a number of experiments with Bryophyllum leaves are assembled in table II. The sets of samples labeled 1955-C and 1953-B are those for which the full information is shown in figures 1 and 2; more complete data for 1952-A, 1951-C and 1951-B are to be found in references (1, 5, 3), but the data for 1953-A have not been published. The rate of change of starch is expressed in millimoles per hour per kilo of initial fresh weight ($C_6H_{10}O_5$ is taken as 1 mole), and the data give the mean rates during the total indicated periods of exposure. Thus the leaves of set 1955-C in the unstressed condition, that is, immediately after collection in the evening, lost starch at the mean rate of 3.2 millimoles per hour during the first 12 hours and at 3.7 millimoles per hour during the first 18 hours in darkness. It will be noted that the curve in figure 1 slopes downward a little more steeply in the interval between 12 and 18 hours, and thus the mean rate for the whole 18-hour period is slightly increased. Consideration of the figures in column 4 of table II shows that the rate at which starch disappears from Bryophyllum leaves in darkness, provided that they are

unstressed or have been stressed for not more than 27 hours of exposure to light, is moderately constant. The figures range from -3.2 to -5.8 millimoles per hour. Within these limits, therefore, different sets of samples of leaves from the clone grown in this laboratory are comparable with each other.

The rates at which starch is synthesized in Bryophyllum leaves collected at sunrise and placed in light are shown in the last column of table II. The range observed for the different sets of unstressed samples is from $+5.6$ to $+12$ millimoles per hour for periods of from 10 to 18 hours. Samples 1952-A appear to have been unusually vigorous in this respect. The rates observed in samples that had been stressed by exposure to darkness for 24 or more hours are, however, all smaller and suggest that such exposure interferes to a greater or less extent with the subsequent capacity of the starch-synthesizing mechanism.

The behavior of the organic acids of samples 1955-C and 1953-B is shown in figure 2, and it is obvious that the transformations which led to the formation or loss of malic and citric acids played the largest part in the observed changes in pH and in total organic acids. The behavior of malic acid during prolonged culture of the leaves in darkness is complex since two main effects are superposed; there was a rapid synthesis during the first 18 hours, but malic acid subsequently diminished at a rate such that, after 108 hours in darkness, the level had dropped almost to that in the freshly picked leaves.

TABLE II

RATE OF CHANGE OF THE STARCH CONTENT OF LEAVES OF BRYOPHYLLUM CALYCINUM
DURING EXPOSURE TO DARKNESS OR TO LIGHT

SAMPLES	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO DARKNESS	RATE	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO LIGHT	RATE
		hrs	*milli-moles/hr* *		*hrs*	*milli-moles/hr* *
1955-C	Unstressed **	12	-3.2	Stressed in darkness** 36 hrs	11	$+ 3.6$
	"	18	-3.7	" " ". 60 "	11	$+ 2.8$
	....	..	...	" " " 84 "	11	$+ 2.3$
1953-B	Stressed in light 27 hrs	11	-5.5	Unstressed	14	$+ 5.6$
	" " " 50 "	12	-3.5	....	..	...
	" " " 74 "	14	-2.5	....	..	...
1953-A	Stressed in light 24 hrs	6	-5.1	Unstressed	10	$+ 8.8$
	" " " 24 "	16	-5.3	"	18	$+ 6.4$
	....	..	...	Stressed in darkness 24 hrs	7	$+ 4.9$
	....	..	...	" " " 24 "	16	$+ 3.0$
1952-A	Stressed in light 24 hrs	8	-4.1	Unstressed	8	$+13.3$
	" " " 24 "	12	-5.4	"	12	$+12.2$
	" . " " 24 "	17	-4.1	"	18	$+ 8.6$
1951-C	Unstressed	16	-5.8	....	..	...
1951-B	....	..	...	Unstressed	10	$+ 7.2$
	....	..	...	"	15	$+ 6.5$
	....	..	...	"	20	$+ 5.2$

* Data expressed in terms of 1 kilo of initial fresh wt.
** "Unstressed" leaves had been cultured in darkness or in light for the indicated period after being picked. "Stressed" leaves were cultured in light or in darkness for the indicated period and were then transferred to the other situation for the periods indicated under "Total exposure." The change in composition took place during this subsequent exposure at the rate shown.

TABLE III

RATE OF CHANGE OF MALIC ACID CONTENT OF LEAVES OF BRYOPHYLLUM CALYCINUM DURING EXPOSURE TO DARKNESS OR TO LIGHT

SAMPLES	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO DARKNESS	RATE	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO LIGHT	RATE
		hrs	milli-moles/hr *		hrs	milli-moles/hr *
1955-C	Unstressed **	12	+ 5.0	Stressed in darkness ** 36 hrs	11	− 3.6
	"	18	+ 4.0	" " " 60 "	11	− 2.2
	....	..	...	" . " " 84 "	11	− 1.6
1953-B	Stressed in light 27 hrs	11	+ 2.1	Unstressed	14	− 6.5
	" " " 50 "	12	+ 1.6	....	..	...
	" " " 74 "	14	+ 1.1	....	..	...
1952-A	Stressed in light 24 hrs	8	+ 8.1	Unstressed	8	− 11.0
	" " " 24 "	12	+ 7.2	"	12	− 9.9
	....	..	...	"	18	− 7.1
1951-C	Unstressed	16	+ 3.2	....	..	...
1951-B	....	..	...	Unstressed	10	− 8.7
	....	..	...	"	15	− 7.5
	....	..	...	"	20	− 5.5

* Data expressed in terms of 1 kilo of initial fresh wt.
** See footnote to table II.

Notwithstanding the continuous loss of malic acid which began after 18 hours in darkness, the samples when later transferred to light lost malic acid at a greatly accelerated rate so that, after 11 hours in light, the quantity had decreased considerably below that present when the leaves were picked. In these samples, indeed, malic acid had become almost a minor acid component.

The leaves exposed to continuous light lost malic acid rapidly for 14 hours and slowly thereafter although in no case did the level to which it diminished drop as low as that seen in the transferred leaves of the parallel experiment. The irregular fluctuations which occurred after 24 hours are almost certainly greater than the experimental error, and may reflect minor irregularities in the conditions (e.g., temperature) in the culture room. The outstanding result is, however, that after transfer to darkness, the enzyme systems of these leaves were unable to synthesize malic acid in sufficient amounts to restore even one-half of the quantity present when the leaves were picked. Different lots of Bryophyllum leaves may be expected to contain between 200 and 300 meq of malic acid per kilo of fresh weight when collected at daybreak, so that the present samples, which contained 247 meq per kilo, were of about average composition. Inasmuch as the malic acid content of leaves that have been excised at daybreak and exposed to the normal diurnal variation in illumination is restored nearly to its initial level during the subsequent night period (8), it is clear that exposure to an artificially prolonged period of illumination adversely affects the mechanisms concerned in the synthesis of malic acid during a subsequent period of darkness.

In table III are callected data on the rates at which malic acid is synthesized in darkness or at which it disappears in light in a number of sets of samples of Bryophyllum leaves. In normal leaves picked at nightfall (i.e., unstressed and held in darkness), the rates of synthesis range from 3 to 5 millimoles per hour per kilo, and the unusually vigorous set marked 1952-A, which had contained 299 meq of malic acid at daybreak, increased in malic acid content at the rate of 8 millimoles per hour even after the leaves had been exposed to light for 24 hours. Nevertheless, stress brought about by long exposure to light (samples 1953-B) diminishes the rate at which malic acid is synthesized after transfer of leaves to darkness, and the effect is more pronounced the longer the period of stress.

On the other hand, leaves picked at daybreak (unstressed and exposed to light) lose malic acid at the rate of from about 6 to 11 millimoles per hour. The effect of the stress occasioned by long exposure to darkness is clearly evident in the much diminished rates at which malic acid disappeared in a subsequent light period, as is shown by the successively lower rates of loss of malic acid in the stressed leaves of samples 1955-C. However, inasmuch as the level of malic acid in these samples had diminished materially during prolonged culture in darkness (fig 2), the lower rates of loss after transfer to light do not necessarily imply impairment of the metabolic system concerned.

The response of citric acid to change of the conditions of illumination of Bryophyllum leaves as a rule is less spectacular than is that of malic acid. In general, however, the responses are parallel and, although they may be of smaller absolute magnitude in terms of the number of milliequivalents of citric acid formed or decomposed, the changes are often substantial in terms of relative amounts. The curve for citric acid at the left of figure 2 illustrates this point un-

usually well. The citric acid in the leaves cultured in darkness increased 6-fold from 13 meq per kilo in the leaves picked in the afternoon to 76 meq after 18 hours in darkness. Malic acid increased nearly 7-fold in the same time. When the leaves were transferred to light after 36 hours in darkness, citric acid dropped from 87 meq per kilo to 25 meq in 11 hours, or to less than one-third of the largest quantity present. The changes that occurred are closely correlated with the changes in starch. The coefficient of correlation between the data for citric acid and for starch in this set of samples was -0.956 (r = 0.872 for P = 0.001 with 8 degrees of freedom) which is highly significant. The close correlation between the data for malic acid and for starch, which is so striking in experiments on normal diurnal variation (8), is masked in the present instance because of the slow and continuous loss of malic acid which supervened after the leaves had been in darkness for 18 hours.

The leaves exposed to continuous light, the data for which are plotted at the right of figure 2, show the converse of these last relationships. The data for malic acid and for starch are closely related, the coefficient of correlation being -0.921 which is highly significant at the 0.1 % level, whereas the data for citric acid and starch, on the other hand, have a coefficient of correlation of only -0.499 which is not significant even at the 10 % level (r = 0.549 for P = 0.1 with 8 degrees of freedom). However, the relationship of the behavior of citric acid to that of starch in this instance is confused by an uncertainty regarding experi-

mental conditions to be discussed in a subsequent paragraph.

In previous papers of this series on the metabolism of the organic acids in Bryophyllum leaves, the behavior of citric acid has received only passing attention since the chief concern has been with the malic acid and starch. Sufficient data are now at hand to warrant discussion of the somewhat discordant results that have been obtained. In table IV are data from 7 separate experiments in which the behavior of citric acid has been examined in samples collected by the statistical method and subjected to culture in water under various conditions.

When leaves are sampled in the late afternoon and cultured in water in darkness, citric acid increases for many hours at a remarkably constant rate which averages about 3.8 ± 0.7 millimoles per hour per kilo of initial fresh weight. Even if collected at sunrise and subsequently held in darkness (samples 1949-C), accumulation of citric acid at about this same rate continues for at least 7 hours. The data for samples 1949-B represent two successive cycles of 9 hours of darkness, each of which was preceded by a full day of exposure of detached leaves to sunlight; the conditions are reckoned to be "unstressed" since the exposure to light was normal in length. If, however, the leaves are subjected to stress by prolonged exposure to light (samples 1953-B), the rate at which citric acid is formed is somewhat diminished although it is still substantial.

The behavior of citric acid in leaves picked at day-

TABLE IV

RATE OF CHANGE OF CITRIC ACID CONTENT OF LEAVES OF BRYOPHYLLUM CALYCINUM DURING EXPOSURE TO DARKNESS OR TO LIGHT

SAMPLES	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO DARKNESS	RATE	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO LIGHT	RATE
		hrs	milli-moles/hr *		hrs	milli-moles/hr *
1955-C	Unstressed **	12	+4.0	Stressed in darkness ** 36 hrs	11	−5.7
	"	18	+3.5	" " " 60 "	11	−5.4
	....	..	...	" " " 84 "	11	−4.1
1953-B	Stressed in light 27 hrs	11	+2.2	Unstressed	14 †	+0.09
	" " " 50 "	12	+3.3	"	27 †	+0.07
	" " " 74 "	14	+2.8	....	..	...
1952-A	Stressed in light 24 hrs	8	+3.3	Unstressed	8 †	−1.3
	" " " 24 "	12	+3.0	"	12 †	−1.7
1951-C	Unstressed	16	+3.8	....	..	...
	"	24	+3.1	....	..	...
1951-B	....	..	...	Unstressed	10 †	−0.5
	....	..	...	"	15 †	−0.4
1948-B	Unstressed	9	+4.5	Unstressed	10	−3.6
	"	9	+4.7	"	15	−3.1
1949-C	Unstressed	7 ††	+3.8	....	..	...
	"	14 ††	+2.7	....	..	...

* Data expressed in terms of 1 kilo of initial fresh wt.
** See footnote to table II.
† In artificial light.
†† Leaves collected at sunrise and cultured in darkness.

break and exposed to light is, however, by no means always predictable. The stressed leaves of samples 1955-C and the unstressed leaves of samples 1949-B (table IV), which were exposed in the greenhouse, lost citric acid at rates comparable with those at which it is synthesized in darkness. The leaves of samples 1953-B, 1952-A and 1951-B which were exposed to artificial light at controlled temperature and humidity immediately after being picked at daybreak lost citric acid much more slowly; indeed, those of samples 1953-B apparently gained slightly (fig 2). A question is at once raised whether the situation under the conditions of artificial illumination was truly comparable with that which obtained in the greenhouse where samples 1955-C and 1949-B were treated. The conditions with respect to temperature were certainly different. The leaves under the artificial lights were maintained near 22° C as observed on thermometers placed close to the surfaces of the leaves, although this was at best only an approximate indication of the temperature within the tissues. The temperature of the greenhouse, on the other hand, varied widely and, since the experiments were done on warm sunny days, rose to the vicinity of 38° C or even higher in the early afternoon. The mean temperature over the whole experimental period was doubtless considerably higher than that in the temperature-controlled room.

That either the intensity or the quality of the artificial light was responsible for the difference in behavior seems less probable. The leaves of samples 1953-B increased rapidly and extensively in both organic solids and starch as is shown in figure 1, and the same is true of samples 1951-B (3). Under the same conditions of illumination, the starch content of samples 1953-A (table II) showed the most extensive and rapid increase that has been observed in this laboratory although the increase of organic solids was not striking (1). Accordingly, the light intensity in the room used was adequate for photosynthetic reactions and for the reactions concerned in the formation of starch, and there is no indication that there was an insufficiency with respect to quality. It is obvious that the discrepancies can only be cleared up by suitably planned additional experiments.

The behavior of isocitric acid in the present samples (fig 2) is of special interest. It has been pointed out previously that isocitric acid remains constant within the limits of the error of measurement in Bryophyllum leaves exposed to the normal diurnal variation of illumination (8), although a possibly significant loss occurs on long exposure to light (3). The curves for the behavior of isocitric acid in figure 2 show that, during culture in darkness, this substance followed a somewhat irregular course. However, the mean content of the 7 samples was 173 ± 5.7 meq per kilo. The variation was thus only 3.3 % of the quantity measured, and accordingly, there is no convincing evidence of a change in isocitric acid during the treatment of the leaves which was significantly greater than the variability of the observations. On transfer to light, however, there was a significant decrease in

the amount of isocitric acid in these samples. The 3 observations are consistent and are supported by the data of the curve at the right of the figure which show the behavior of isocitric acid in the leaves cultured in continuous light. In these samples, isocitric acid diminished continuously although slowly. A regression line calculated for the 7 samples cultured in light shows that approximately 9 % of the isocitric acid present at the start disappeared during the 74 hours of the culture period. This is greater than the analytical error, and the conclusion may be drawn that isocitric acid is to some extent utilized under these conditions. The tentative conclusion in an earlier paper (3) is thus confirmed. However, in these leaves, transfer to darkness brought about only negligibly small changes which were well within the error of the observations.

DISCUSSION

EFFECTS OF STRESS: The effect of prolonged exposure to light or to darkness upon the rate at which starch disappears or is synthesized after transfer of the leaves from one situation to the other is shown by the data for samples 1955-C and 1953-B in table II. These rates furnish measures of the slopes of the broken lines in figure 1. The progressive change in the rates represents the effect of the increase in the stress to which the physiological systems had been exposed. The analogous data for the rates at which malic acid underwent change are given for the same sets of samples in table III, and those for citric acid in table IV. These rates are indicated by the slopes of the broken lines in figure 2. In all instances, with the exception of the data for the rate of change of citric acid in the leaves that had been stressed in light and subsequently transferred to darkness, the rates diminished with the increase in the period of culture before the transfer was made. The decrease in the rates of the reactions furnishes evidence of progressive impairment of the ability of the enzymatic systems involved to recover from the effects of the stress.

The close inverse correlation that has repeatedly been observed between the data for decrease of malic acid and increase of starch in Bryophyllum leaves exposed to light, as well as for the apparent reversal of this relationship in leaves exposed to darkness, has been advanced as evidence in favor of the view that a sequence of chemical reactions occurs whereby actual transformation of the one substance into the other takes place. The present data reflect disturbances in the efficiency or speed of these reactions brought about by the onset of what has been designated "stress." In general, the speed of chemical reactions is influenced by temperature, and by the relative proportions of the reactants involved. With enzymatically catalyzed reactions, it is also a function of the affinity of the substrate for the enzyme, a quantity which is measured by the Michaelis constant. The speed of reactions is likewise affected by the presence of inhibitors and, in addition, if the concentration of one or more of the essential enzymes or co-

factors is changed, a corresponding change in reaction speed would occur. In the present experiments, there was no reason to suppose that the temperature, at which the three samples in each set subjected to transfer were treated, diminished in a systematic way so as to retard progessively the rates at which the chemical reactions proceeded. On the contrary, the physical conditions after each transfer were duplicated as closely as possible. With respect to the possibility that the speed of the reactions diminished because of progressive failure of the supply of one of the reactants, it may be pointed out that malic acid did indeed slowly drop to a low level during prolonged culture of the leaves in darkness (fig 2), and this may have affected the rate at which starch was synthesized in these samples after transfer to light. On the other hand, in the samples cultured in light, malic acid was at approximately the same low level in all three samples which were transferred to darkness, and starch was at a high level in all, yet a progressive change in the rates of reaction occurred. Accordingly, it is difficult to account for the decrease in reaction rate after transfer in terms of a deficiency of one of the reactants.

That change occurred in the Michaelis constants of individual enzymes as the period of culture increased is highly improbable; the affinity of an enzyme for its substrate is a property characteristic of the protein itself and, within the limits of present knowledge, is fixed in magnitude. One is left, therefore, with two hypotheses to account for the observed progressive change in the rates of the reactions; the possibility of the generation of inhibitory substances, or the possibility of the progressive destruction of one or more of the enzymes or co-factors essential for the catalysis of the reactions. Whether or not inhibitory substances are gradually formed must be left for future investigation, and at present little information regarding co-factors is available. In any case, however, the most likely hypothesis is that a slow destruction of the enzymes occurred. Information that may bear on this point was obtained by an examination of the protein nitrogen. It has been mentioned that a plot of the data suggested that a progressive loss of protein took place throughout the period of culture. The regression line calculated for the 7 points which represent the protein nitrogen of the leaves cultured in darkness indicated that about 6.5 % of the protein had disappeared at the 84-hour point when the third sample was transferred to light. The regression line for the protein nitrogen of the samples cultured in light suggested that about 3.8 % of the protein had disappeared at the 74-hour point when the third transfer to darkness was made. That these losses of protein reflect the gradual decomposition of enzymes essential for the chemical reactions which occurred is indeed speculative, but it can be concluded that such an event is in conformity with the observations on the changes in protein content.

QUANTITATIVE RELATIONSHIPS BETWEEN STARCH AND ACIDS: The hypothesis that starch is converted into organic acids in darkness and acids are converted into starch in light can be tested by comparison of the quantities of the presumed reactants which underwent change subsequent to transfer from the one to the other condition of illumination. The essential information is collected in table V. In order to avoid all considerations of chemical mechanisms, the data are expressed in terms of millimoles of carbon, and the figures are arranged so as to answer the question: Was sufficient carbon supplied, by the component that diminished in quantity, to account for the increase in the component to which it was presumably converted? The upper part of table V refers to the leaves which were cultured in darkness and transferred to light, the lower to the leaves subjected to the converse treatment. The data for the changes in starch are given in gm per kilo in column 3 in order to furnish an easily appreciated measure of the direction and magnitude of the phenomena. Column 7 gives the ratio between the sum of the carbon involved in the change in the quantity of malic and citric acids to the carbon involved in the change in the amount of starch. For the unstressed leaves cultured in darkness, the ratio of 1.46 indicates that appreciably more of these acids were formed than could have arisen from the starch. Carbon from some other source must have entered the system, and such observations as those of Thurlow and Bonner (9) and of Varner and Burrell (10) with radioactive carbon dioxide indicate that fixation of carbon can account for a part at least of the extra carbon found in these acids. The rather precise agreement between the loss of carbon from starch after 12 hours and the corresponding gain of carbon of malic acid is, however, worthy of attention.

At the end of 18 hours, although the rate of loss of starch had been maintained, the rate of gain of the acids had diminished, with the result that the ratio of the quantities of carbon approached unity. Thus the quantities of carbon involved in the overall reactions which had occurred during 18 hours are in conformity with the hypothesis.

After transfer of the leaves to light, starch was synthesized and malic and citric acids diminished. The ratios between the quantities of carbon involved in these reactions in each of the 3 samples remained essentially constant notwithstanding the wide differences in the amounts of the substances concerned, and thus also conform with the hypothesis of actual transformation. That the ratios are slightly greater than unity suggests that the transformation was somewhat short of being quantitative, that is to say, a little of the malic or citric acid or possibly of both was converted into products other than starch.

The data in the lower part of table V show that considerably more carbon appeared in the starch formed during the first 14 hours of culture in light than can be accounted for by the carbon of the acids that disappeared. If a correction is applied to the amount of starch synthesized by subtracting the weight of the organic solids that were formed

(see data in parentheses), these solids being assumed to represent photosynthesized starch, the ratio is somewhat increased and, when this calculation is carried out for the leaves that had been cultured for 27 hours in continuous light, reaches 1.07. A previous observation (3) is thus confirmed. It may be concluded, therefore, that unstressed leaves cultured in light behave in a manner which conforms with the hypothesis that much of the starch formed may arise from chemical transformation of the organic acids.

The leaves that had been stressed by prolonged exposure to light before transfer to darkness were, however, no longer able to make efficient use of the starch for the synthesis of acids. The ratio between the quantities of carbon is much depressed. Attention has already been drawn to the point that the increase in malic acid was far less striking (fig 2 and table III) than that in unstressed leaves both in amount and in the rate at which the reaction occurred. The damage appears to have been especially severe to the systems involved in the synthesis of this substance, although the systems concerned with the formation of citric acid do not seem to have been so gravely affected. No information was obtained regarding the fate of that part of the starch which did not appear to contribute to the acid metabolism.

The present investigation has provided further evidence on the interrelationships of starch and organic acids in Bryophyllum leaves in light and darkness inasmuch as it is shown that almost quantitative transformation of the one into the other is within the bounds of possibility. The crux of the problem is, however, the elucidation of the mechanisms whereby these chemical events are brought about. Upon this matter, there is nothing to offer at the present time save speculation. It seems certain, however, that a careful study of the enzyme systems of this species will ultimately lead to at least a partial understanding of the extraordinary chemical behavior of crassulacean plants.

SUMMARY

Initially identical samples of excised leaves of Bryophyllum calycinum have been subjected to prolonged culture in water in darkness, individual samples being transferred to light at intervals. A second set of samples was subjected to the converse conditions, the object being to test the capacity of the leaves to resume the normal rhythm of variation in composition with respect to starch and organic acids.

The effect of prolonged culture either in darkness or in light was to set up a condition which is interpreted as representing a steadily increasing degree of physiological stress. This condition was characterized by the diminishing speed at which the major chemical changes in composition subsequent to transfer were

TABLE V

MOLAR RELATIONSHIP BETWEEN THE CARBON OF STARCH AND OF MALIC AND CITRIC ACIDS
INVOLVED IN CHANGES IN COMPOSITION WHICH OCCUR IN BRYOPHYLLUM
LEAVES SUBJECTED TO CULTURE IN WATER

CONDITION BEFORE TREATMENT	HRS OF PRELIMINARY CULTURE	Δ STARCH, GM *	A Δ STARCH CARBON, MILLIMOLES *	B Δ MALIC ACID CARBON, MILLIMOLES *	C Δ CITRIC ACID CARBON, MILLIMOLES *	RATIO $\frac{(B+C)}{A}$
Unstressed: cultured in darkness (Samples 1955-C)	12	− 6.3	− 234	+ 240	+ 98	1.46
	18	− 10.8	− 401	+ 290	+ 127	1.04
Stressed by culture in darkness for period shown: Change after transfer to light for 11 hrs	36	+ 6.5	+ 239	− 160	− 125	1.19
	60	+ 4.9	+ 182	− 95	− 119	1.17
	84	+ 4.0	+ 149	− 68	− 90	1.06
Unstressed: cultured in light (Samples 1953-B)	14	+ 12.7	+ 469 (+ 440) **	− 363	+ 2.6	0.77 (0.82) **
	27	+ 15.9	+ 590 (+ 353) **	− 385	+ 5.8	0.64 (1.07) **
Stressed by culture in light for period shown: Change after transfer to darkness for 11, 12 and 14 hrs, respectively	27	− 9.8	− 362	+ 93	+ 53	0.40
	50	− 6.8	− 250	+ 77	+ 79	0.62
	74	− 5.7	− 212	+ 59	+ 77	0.64

* Data expressed in terms of 1 kilo of initial fresh wt.
** Corrected for increase of organic solids assumed to represent starch.

observed to take place as the culture period was prolonged. The data refer particularly to the synthesis and decomposition of starch, and the correlated reciprocal changes in malic and citric acids.

During the culture period, the total protein of the leaf slowly diminished in amount. It is tentatively suggested that the changes in the rates at which the leaves are able to recover from the stressed condition are correlated with the loss of protein and specifically with the gradual, although only partial, destruction of the enzymes essential for the chemical transformations to occur. The enzymes concerned with the metabolism of malic acid appear to have been especially sensitive to prolonged culture in light.

An examination of the data for the reciprocal changes in starch and organic acids showed that, in general, approximately the correct quantity of carbon was supplied by the component which diminished in amount to account for the carbon of the component which was synthesized. However, this relationship did not hold for the synthesis of organic acids in leaves which had been stressed by prolonged culture in light and were then placed in darkness. In these leaves, there was a marked deficiency in the synthesis of organic acids.

In contrast to its behavior in Bryophyllum leaves exposed to light under greenhouse conditions, citric acid was observed to diminish slowly, if at all, when the leaves were exposed to artificial light at a controlled temperature in the vicinity of 20° C.

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THE DARK FIXATION OF CO₂ BY SUCCULENT LEAVES: THE FIRST PRODUCTS [1,2]

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It has long been realized that green leaves have the ability to fix CO₂ in the dark to form organic acids with the concomitant loss of carbohydrate stores. A group of plants known as the succulents is characterized by a particularly active dark fixation of CO₂. In the case of the succulents, the organic acids synthesized in the dark are transformed to carbohydrates during a subsequent light period (4).

Despite the considerable amount of work which has been done on succulent metabolism (11), the exact metabolic pathways by which the dark fixation of CO₂ proceeds have not, hitherto, been elucidated.

The techniques of paper chromatography and radioautography developed by Calvin, Benson, and their co-workers (3), to determine the pathway of C¹⁴O₂ in photosynthesis are particularly applicable, with modifications, to the study of the dark fixation of C¹⁴O₂ by succulents. That C¹⁴O₂ is incorporated into the organic acids of succulents has been demonstrated by Thurlow and Bonner (13) who exposed Bryophyllum crenatum leaves to C¹⁴O₂ for 60 hours. By means of a gross chemical separation of the prod-