DIURNAL CHANGES AND GROWTH RATES AS ASSOCIATED WITH ASCORBIC ACID, TITRATABLE ACIDITY, CARBOHYDRATE AND NITROGENOUS FRACTIONS IN THE LEAVES OF *ANANAS COMOSUS* (L.) MERR.[1]

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(WITH FIVE FIGURES)

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Introduction

Tissues from the leaves of *A. comosus* collected at different diurnal intervals may contain different amounts of various products of metabolism. As examples, the acidity of the chlorophyllose tissues of leaves collected in the afternoon may be very small (**61**) in comparison with that of similar tissues from leaves collected early in the morning (**63**). It has also been observed that plants with greater plant weights and, presumably, with a high rate of growth may contain more acid in the chlorophyllous tissues of the leaves than similar plants growing at lower rates (**66, 67, 68, 69**).

The information contained herein reports and discusses certain relationships between plant growth, on the one hand, and titratable acidity and sugars at different diurnal intervals on the other. Morever, it shows the effects of different amounts of acidity on the degree of dispersion of proteins, presumably related to particle size as affected by hydration at different pH, at various diurnal intervals.

Literature

The literature on organic acid metabolism in plants was ably summarized in 1932 by BENNET-CLARK (**10, 11, 12**). Since then investigations by VICKERY *et al.* (**75, 76**), ALLSOPP (**1**), WOLF (**83**), PUCHER *et al.* (**52**), and KREBS and EGGLESTON (**40**) have greatly contributed towards our general knowledge of the chemistry and metabolism of these acids in plants and animals.

Considerable controversy, in the past, beclouded the identity of the so-called crassulacean malic acid; some (**44, 45**) believing it to be isomalic acid and others something different but closely related to the latter. PUCHER and VICKERY (**52**) showed that the crassulacean malic acid was identical with isocitric acid, which has also been confirmed by KREBS and EGGLESTON (**43**). However, establishment of the identity of isocitric with crassulacean malic acid affords better means of identifying the other organic acids in the tissues and of measuring, also, the extent of changes which any or all such acids may undergo at different diurnal intervals.

As to the nature of the substances which contribute to the formation of the various organic acids in the tissues at various diurnal intervals, dif-

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ferent investigators hold dissimilar views. KRAUS (**37, 38**) observed that fluctuations in acidity were associated with fluctuations in the carbohydrate content of the same tissues in the opposite direction, and that changes in titratable acidity were not connected with neutralization of the acid by base. WOLF (**83**) claims that during organic acid accumulation, at night, the sugar content of the tissues of *Bryophyllum calycinum* decreased; also, there was interdependence between fermentable hexose sugars and starch, on the one hand, and organic acids, on the other.

In contrast with the above, RUHLAND and WETZEL (**53, 54**) attribute the diurnal variations in organic acids to the breakdown of amino acids, stored in the rhizome of *Rheum hybridum,* which yield l-malic acid and an equivalent amount of ammonia. The same investigators (**57**) claim that, in Begonia, where oxalic acid predominates as ammonium oxalate, the rise in titratable acidity during the night is followed by a decrease in ammonia; but fail to state the fluctuations in ammonia at other diurnal intervals. The possibility of nitrogen metabolism contributing to the formation of organic acids has been considered by KOSTYTCHEV (**36**) who asserts that the common plant acids, malic, tartaric, citric, etc., are not at all normal intermediate products of oxygen respiration but that they arise from amino acids or from intermediate products of the synthesis of proteins and that their oxidation is more like "protein respiration" than "sugar respiration."

But STEINMANN (**72**) is in disagreement with both the above findings, claiming that in *Rheum sativum* a slight gain in titratable acidity was observed during the day and this was increased if the transport was hindered by cutting the main veins of the leaf; thus attributing the formation of acid directly to photosynthesis.

Organic acids accumulate mostly in the chlorophyllous regions of the leaves, according to SIDERIS *et al.* (**63**). ASTRUC (**3**) found that the green regions in variegated leaves of Pelargonium and Acer contained about five times as much titratable acidity as the adjoining white parts.

According to GUSTAFSON (**26**), the total acid of the plant juice is not responsible for the H-ion concentration gradient found in plants. BENDRAT (**8**) claims that the titratable acidity yielded no information of value regarding the acid metabolism in succulents since the sign of the change of total acidity was opposite to that of the change in titratable acidity in many cases.

The disappearance of organic acids from tissues during the day is attributed to various factors. SPOEHR (**71**) showed that the acidity of the expressed sap of Opuntia decreased when the sap was exposed to light, but this was not due to an enzyme because addition of the ash of the plant to pure malic acid solutions enabled this photodeacidification to take place at about the same rate as in the sap. Citric acid, being readily oxidized *in vitro* to acetone-dicarboxylic acid, is believed by some investigators (**14**) to undergo similar changes in the tissues.

Methods

Greenhouse and field grown plants were employed separately for the various studies. The light averaged in the outdoors 5800 foot candles from January to August 1944, but under greenhouse conditions was approximately 30% lower. Temperature, depending on wind movement, was from 3 to 5 degrees higher in the greenhouse than outdoors. The average temperature from January to August 1944 was 77.5° F. in the day, and 71.3° F. at night; while average maximal temperature was 83.6 and minimal 66.5° F. for the same period.

The greenhouse plants were grown in solution cultures, composed as follows: Water, 1000 ml.; KNO_3, 0.184 gm.; $Ca(NO_3)_2 \cdot 4H_2O$, 0.236 gm.; $MgSO_4 \cdot 7H_2O$, 0.246 gm.; $NaH_2PO_4 \cdot H_2O$, 0.138 gm.; $FeSO_4 \cdot 7H_2O$, 0.028 gm.; $MnSO_4 \cdot 4H_2O$, 0.011 gm.; $ZnSO_4 \cdot 7H_2O$, 0.015 gm.; and $K_2B_4O_7 \cdot 5H_2O$, 0.016 gm.

The plants, tops or crowns, were suspended, in January 1944, in porcelain crocks of 17 liter capacity containing the nutrient solution which was aerated continuously and changed at three-week intervals. These plants were sampled on June 5, August 2, 8, and 9, 1944.

The field-grown plants, approximately 5000, set in twelve different treatments, each replicated four times, occupied an area about one-third of an acre.

The various treatments consisted mainly of different amounts of soil fumigants per acre injected before planting. There were three different controls or checks: X_1, for the fumigants applied to the 1943 planting, and X_2 and X_3. The soil in the X_2 was never treated before with any fumigant but in the X_3 it was treated with 225 lbs. of chloropicrin per acre in 1939, but not in 1943. Both X_2 and X_3 served to demonstrate possible residual effects of the 1939 chloropicrin treatment on the second cycle plants planted in December 1943. The other treatments were as reported in table VI.

The field-plant samples were collected in September 1944 when the plants were nine months old. Plant weights are reported in table VI.

Ten leaves or more of the active (D) group (**62**), one leaf per plant, were collected at each diurnal interval. These leaves occupying a position between the young (E) and mature (C) are the longest in the plant and their position with reference to the main axis of the stem is not truly perpendicular but slightly inclined. These leaves after washing were weighed, sectioned, and the medial cross section (No. 4) of the chlorophyllous region was selected for chemical analysis. The tissues of these sections were cut into 2-mm. widths with a rotatory stainless knife slicer; some were dried while others were used in the fresh state for analyses.

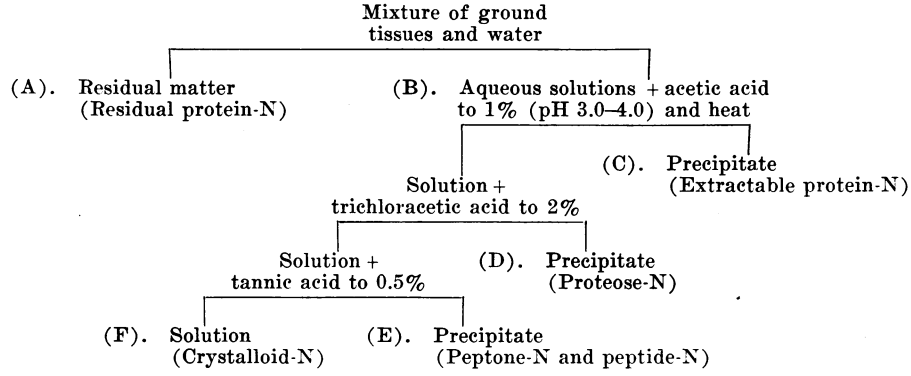
Where the substances sought for chemical analysis were water soluble, the tissues were mixed with water and macerated in a Waring Blendor, and the water-soluble substances extracted and strained through Canton flannel by the application of manual pressure at four successive intervals, mixing

the residue with 50 ml. of water at each interval until a volume of 400 ml. was obtained. Aqueous extracts were employed for the determination of titrimetric acidity, pH, sugars, and nitrogenous fractions.

Titrimetric acidity was determined as soon as possible after extraction on a 10-ml. aliquot with 0.1 N NaOH, using phenolphthalein as indicator; pH was determined with a Beckman pH glass electrode apparatus. Determination of malic and citric acids was made by the method of PUCHER *et al.* (**51**).

For the determination of total sugars the method of QUISUMBING and THOMAS (**55**) was employed after precipitation of the suspended matter with lead acetate, removal of the excess lead with potassium phosphate and inversion of sucrose by invertase.

The nitrogenous fractions were segregated following the aqueous extraction into water soluble-N and residual-N, the former comprising all the nitrogen in the solution and the latter that in the residual tissue matter. The different fractions were obtained and classified on the basis of the scheme shown below:



The nitrogen in all fractions was determined by Kjeldahl's method.

For the determination of ascorbic acid the tissues were mixed with a 5% aqueous solution of oxalic acid and ground in a Waring Blendor, the aqueous fraction strained as before, filtered, and ascorbic acid was titrated in the filtrate with sodium 2,6-dichlorobenzeneoneindophenol.

Chlorophyll was determined in all cases colorimetrically with a Klett-Summerson photoelectric colorimeter using No. 60 light filter. It was also found necessary to record optically changes in color ranging from green to brown presumably resulting from chlorophyll decomposition by chlorophyllase in tissues with low pH values.

Results

GREENHOUSE PLANTS

ASCORBIC ACID.—The leaves were collected at two-hour intervals for twenty-four hours on June 5 and 6, 1944.

TABLE I

ASCORBIC ACID, TITRATABLE ACIDITY, pH, TOTAL-N, RESIDUAL-N, EXTRACTABLE-N PER GRAM OF FRESH TISSUE, ALSO CHLOROPHYLL, AND DECOMPOSITION OF THE LATTER AS INDICATED BY THE COLOR OF THE EXTRACTED SAP, IN THE CHLOROPHYLLOSE NO. 4 SECTIONS OF THE ACTIVE (D) LEAVES OF *A. comosus* COLLECTED AT TWO-HOUR INTERVALS FROM JUNE 5 TO 6, 1944

TIME OF HARVEST	ASCORBIC ACID	ACIDITY			NITROGEN			CHLOROPHYLL		
		T.A. 0.1 N NaOH	CITRIC-MALIC ACID*	pH	TOTAL	RESIDUAL	EXTRACTABLE	FRESH TISSUE	OPTICAL ESTIMATE	
									COLOR	BROWNING
	mg.	*ml.*	*mg.*		*mg.*	*mg.*	*mg.*	*mg./gm.*		
6–5–44										
6: 00 A.M.	4.23	2.80	17.9	3.4	2.80	2.02	0.78	0.455	Brown	++++
8: 00 A.M.	3.90	2.66	17.0	3.6	2.74	2.08	0.66	0.400	Brown	++++
10: 00 A.M.	4.08	1.60	10.2	4.6	2.74	1.94	0.80	0.414	Brown	+++
12: 00 P.M.	3.92	0.62	4.0	5.6	2.75	1.89	0.86	0.546	Br–Gr	+
2: 00 P.M.	3.81	0.30	1.9	6.3	2.55	0.75	1.80	0.695	Green	0
4: 00 P.M.	3.79	0.38	2.4	6.2	2.53	0.71	1.82	0.675	Green	0
6: 00 P.M.	3.79	0.38	2.4	6.2	2.63	1.23	1.40	0.644	Green	0
8: 00 P.M.	3.29	0.64	4.1	5.6	2.84	1.80	1.04	0.618	Green	+
10: 00 P.M.	3.66	0.98	6.3	5.2	2.84	2.14	0.70	0.480	Gr–Br	++
6–6–44										
12: 00 A.M.	3.39	1.40	9.0	4.7	2.59	1.83	0.76	0.372	Brown	++++
2: 00 A.M.	3.26	1.70	10.9	4.3	2.61	2.01	0.60	0.371	Brown	++++
4: 00 A.M.	3.11	2.20	14.1	3.8	2.49	1.85	0.64	0.278	Brown	++++
Average	3.69	.....	.....	.....	2.68	.....	.....	.....	.....	.....

* Titratable acidity calculated as citric acid but is expressed as citric-malic acid because of the small difference in the conversion factors (malic acid = 67 and citric acid = 64 for 1.0 N NaOH).

Changes in ascorbic acid at different diurnal intervals, in table I, were very small and insignificant. There were fluctuations which cannot be explained satisfactorily, but they were probably associated more with carbohydrate changes in the tissues than diurnal effects.

RAY (**56**) has found that certain sugars (mannose) increased to a greater extent the ascorbic acid content of tissues than others (arabinose or xylose) but their effects on plant growth were opposed. Ascorbic acid, according to PURR (**54**), inhibited the activity of the Beta-type of amylase, "the sac-

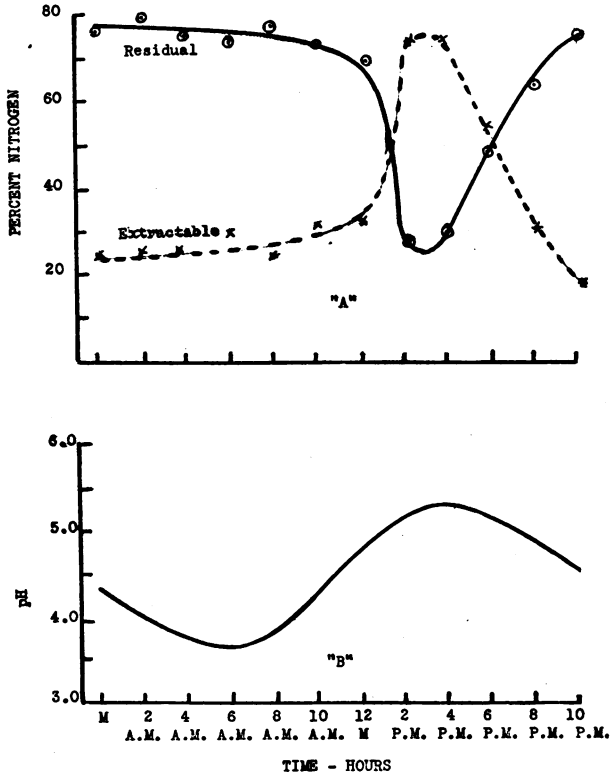


FIG. 1. "A," residual and extractable nitrogen as percentage of total and "B," pH of the chlorophyllous sections (4) of the active (D) leaves of *A. comosus* grown in the greenhouse and collected at different hours of June 5–6, 1944.

charifying enzyme," but had no effect on the Alpha-type, "the dextrinizing enzyme," while dehydroascorbic acid had directly opposite effects on both types of amylases, suggesting that the predominance of either form of ascorbic acid may change the concentrations of certain carbohydrate fractions in the tissues.

ACIDITY.—The titratable acidity and pH of the leaves collected on June 5–6, 1944, reported in figure 1 and table 1, underwent great changes between day and night intervals. Maximal values of titratable acidity and minimal of pH were attained at 6 A.M. and the opposite at 6 P.M. Also, the leaves

collected, at four-hour intervals, on August 2 and 9, 1944, reported in tables II and III, respectively, showed maximal titratable acidity at 6 A.M. and minimal at 6 P.M., while pH values for the same intervals were in reverse order to acidity values. However, titratable acidity was higher, for similar diurnal intervals, in the leaves collected on June 5–6 and August 2 than on August 9, 1944. Examination of sunlight records, indicating the amount of light by the area formed by the temperature differential curves of two blackened thermometers, one of which was kept in the shade and the other exposed to light, shows that the amount of light, determined by measuring the area by planimeter, was approximately 50% greater on June

TABLE II

EFFECTS OF DIURNAL CHANGES ON TITRATABLE ACIDITY (CITRIC AND MALIC ACIDS), pH, TOTAL SUGARS, REDUCING SUGARS, SUCROSE, STARCH, AND CHLOROPHYLL IN THE CHLOROPHYLLOUS NO. 4 SECTIONS OF THE ACTIVE (D) LEAVES OF EIGHT-MONTH-OLD *A. comosus* GROWN IN SOLUTION CULTURES UNDER GREENHOUSE CONDITIONS AND HARVESTED AUGUST 2, 1944

Time	Acidity			Carbohydrate fractions				Chlorophyll	
	T.A.* 0.1 N NaOH	Citric-malic acids	pH	Total sugars	Reducing sugars	Sucrose	Starch	Mg. per Gm.	Color
	ml.	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg./ gm.*	
6: 00 A.M.	2.20	14.1	4.2	30.2	21.9	8.3	0.1	0.210	Brown
10: 00 A.M.	1.50	9.6	4.5	33.1	23.4	9.7	0.2	0.240	Brown
2: 00 P.M.	0.57	3.6	5.8	38.4	28.0	10.4	0.4	0.589	Green
6: 00 P.M.	0.35	2.2	6.2	39.5	28.1	11.4	0.3	0.661	Green
10: 00 P.M.	0.60	3.8	5.5	36.3	27.0	9.3	0.5	0.625	Green
2: 00 A.M.	1.10	7.0	4.8	36.0	27.4	8.9	0.2	0.494	Br–Gr

* T.A. = Titratable acidity as ml. of 0.1 N NaOH.

5–6 and August 2 than on August 9, 1944. The differences in titratable acidity between the samples of June 5–6 or August 2 and 9, probably resulting from differences in the carbohydrate content of the tissues, were presumably related to differences in sunlight and photosynthetic activity for the respective periods. BENNET-CLARK (**10**) has reported somewhat comparable results for the diurnal titratable acidity of *Sedum praealtum* at different seasons of the year, the acidity being greater from May to September when sunlight was more abundant than at other seasons.

Definite identification and determination of the organic acids in the leaf tissues of *A. comosus* was made by the method of PUCHER *et al.* (**51**). The data, in table IV, show that in the leaves collected at 6 A.M. the recovered acids were 90% of total titratable acidity of which 54.7% was malic and 45.3% citric acids. In the leaves collected at 6 P.M. the recovered acids constituted 85% of total titratable acidity of which 30.3% was malic and 69.7% citric acid. The total acidity of the leaves collected at 6 A.M. and

TABLE III

TITRATABLE ACIDITY, pH, CITRIC AND MALIC ACIDS, TOTAL-N, RESIDUAL-N AND EXTRACTABLE-N, ALSO FRACTIONS OF EXTRACTABLE-N AS PROTEIN-N, PROTEOSE-N, PEPTONE-N, AND CRYSTALLOID-N PER GRAM OF FRESH TISSUE IN THE CHLOROPHYLLOSE NO. 4 SECTIONS OF THE ACTIVE (D) LEAVES OF *A. comosus* COLLECTED AT FOUR-HOUR INTERVALS ON AUGUST 9, 1944

TIME OF HARVEST	ACIDITY			NITROGEN						
	0.1 N NaOH	CITRIC-MALIC ACIDS	pH	TOTAL	RESIDUAL	EXTRACTABLE	EXTRACTABLE-N FRACTIONS			
							PROTEIN	PROTEOSE	PEPTONE	CRYSTALLOID
	ml.	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
2: 00 A.M.										
6: 00 A.M.	1.30	8.3	4.8	3.58	0.918	2.66	0.342	0.088	0.085	2.145
10: 00 A.M.	1.90	12.2	4.4	3.57	1.090	2.48	0.386	0.057	0.083	1.954
2: 00 P.M.	1.63	10.4	4.6	3.67	0.935	2.74	0.445	0.066	0.089	2.140
6: 00 P.M.	0.57	3.7	6.0	3.98	0.475	3.51	0.570	0.086	0.113	2.710
10: 00 P.M.	0.44	2.8	6.2	4.09	0.429	3.66	0.975	0.107	0.118	2.460
Average	0.70	4.5	5.7	4.10	0.593	3.51	0.880	0.088	0.109	2.421
8–9–44	.....	.....	.....	3.84	.....	.....	.....	.....	.....	.....

6 P.M. differed considerably, being in the morning 2.23 times greater than in the evening. Similar differences, also, existed in the malic and citric content of the tissues for the same periods. The concentration of malic acid was reduced approximately 75%, from 6 A.M. to 6 P.M., and of citric acid 37.0%; the reduction in malic acid being almost twice as great as in citric acid.

The breaking down of organic acids in tissues, presumably because of enzymatic activity (**83**), is not the result of diurnal periodicity but of light effects. It is known that malic acid gives oxalacetic acid after gentle oxidation with hydrogen peroxide and citric acid may be oxidized to acetonedicarboxylic acid (**14**) or to pyruvic and oxalacetic acids, suggesting that

TABLE IV

MALIC AND CITRIC ACID AS MG. PER GRAM OF FRESH TISSUE IN THE CHLOROPHYLLOUS NO. 4 SECTIONS OF THE ACTIVE (D) LEAVES OF EIGHT-MONTH-OLD *A. comosus* COLLECTED AT 6: 00 A.M. AND 6: 00 P.M. ON AUGUST 8, 1944; ALSO PERCENTAGE DIFFERENCES OF THE SAME

TIME	TOTAL ACIDITY		CITRIC ACID	MALIC ACID	CITRIC AND MALIC	UNDETERMINED ACIDS (DIFFERENCE)	
	0.1 N NaOH	CITRIC* AND MALIC				0.1 N NaOH	CALCULATED AS CITRIC-MALIC*
	ml.	*mg.*	*mg.*	*mg.*	*mg.*	*ml.*	*mg.*
6: 00 A.M.	2.48	15.85	6.54	7.90	14.44	0.28	1.80
6: 00 P.M.	1.11	7.10	4.10	1.94	6.04	0.18	1.15
% acid decomposed from 7: 00 A.M. to 7: 00 P.M.	.....	55.2	37.3	75.5	58.2	.....	.....

* Ml. of 0.1 N NaOH multiplied by 6.4, the factor for citric acid.

under ordinary conditions, both acids are subject to oxidation. Great light intensities and durations were found to accelerate more the rate of disappearance of such acids from the tissues than small ones. Some idea of the effects of diurnal periodicity in relation to the acidity of the tissues of leaves exposed to light or darkness may be obtained from the data in table V.

The data are from two lots of plants, grown simultaneously and with identical nutrition in the greenhouse, one of which was covered with a black cloth from 6 A.M. to 2 P.M. and the other was exposed to the daylight intensity of the greenhouse. They show that the acidity of the exposed plants decreased 17.4% of the initial value from 6 A.M. to 10 A.M. and 78.0% from 6 A.M. to 2 P.M., but that of the covered plants increased 23.6% from 6 A.M. to 10 A.M. and 4.4% from 6 A.M. to 2 P.M. The covered plants gained, instead of losing, in acidity and the reduction in gain of 19.2%, i.e., 23.6 – 4.4 from 10 A.M. to 2 P.M. might have resulted from deficiencies in the supplies of readily available carbohydrates due to cessation of photosynthetic activity by exclusion of sunlight.

Somewhat similar results were obtained by PURJEWITSCH (**53**) in the crassulacean Aeonium where the acid content reached its maximal value eight hours after the leaves had been placed in darkness, and twenty-four hours in Robinia. The longer period in the latter plant was attributed to the supposedly greater stability of citric acid which is more predominant in this plant than malic acid.

These results indicate that light is necessary for the processes responsible in the breaking down of organic acids in the tissues and that, in the absence of light, plants continue to form organic acids as long as carbohydrate supplies are available.

Generation of organic acids from amino acids, via "protein respiration," suggested by KOSTYCHEV (**36**), is highly questionable because in *A. comosus* (**64, 65**) grown in darkness for 17, 56, or 105 days with great reductions in protein-N and approximately proportional increases in amino-N, the

TABLE V

TITRATABLE ACIDITY, AS MG. OF CITRIC ACID PER GRAM OF FRESH TISSUE, IN THE CHLOROPHYLLOUS NO. 4 SECTIONS OF THE ACTIVE (D) LEAVES OF TWO LOTS OF *A. comosus*, ONE OF WHICH WAS COVERED WITH A BLACK CLOTH FROM 6: 00 A.M. TO 2: 00 P.M. AND OTHER EXPOSED TO SEASONAL DAYLIGHT

TIME	TITRATABLE ACIDITY AS CITRIC ACID		DIFFERENCE IN CITRIC ACID BETWEEN 6: 00 A.M. OR 10: 00 A.M. AND 2: 00 P.M.	
	EXPOSED	COVERED	EXPOSED	COVERED
	mg.	*mg.*	%	%
6: 00 A.M.	11.01	11.40	0.0	0.0
10: 00 A.M.	9.09	14.09	- 17.4	23.6
2: 00 P.M.	2.43	11.90	- 78.0	4.4

acidity of the sap was very low in comparison with that of plants exposed to light which generated presumably organic acids via "carbohydrate respiration."

SUGARS.—Total sugars in table II, showing maximal values at 6 P.M. and minimal at 6 A.M., were in converse order with titratable acidity (malic-citric acid) in the same table. The results suggest that during respiration sugars in the tissues were oxidized, in the absence of light, to malic and citric and possibly other organic acids. The combined values of malic-citric acid, estimated from titratable acidity by multiplying the values of NaOH by 6.41 and contrasted with sugars, in figure 4, show that the former increased as the latter decreased and vice versa. This is, also, in agreement with the findings of KRAUS (**37, 38**) and of BENNET-CLARK (**11**) with *Sedum praealtum.*

CHLOROPHYLL.—Ten grams of the 2-mm.-thick slices of fresh leaf tissues were placed in flasks with 100 ml. of 95% ethyl alcohol and left standing overnight. They were then macerated in a Waring Blendor, the chlorophyll extracted and determined with a Klett-Summerson photoelectric colorimeter, using a No. 60 light filter.

Tables I and II show that chlorophyll from leaves collected at different diurnal intervals was subject to different degrees of self-destruction either by the inherent acidity of the tissues or by chlorophyllase presumably operating more favorably at high than low acidities. The products of chlorophyll decomposition, supposedly phaeophytins, changing the extract from from green to varions intensities of brown, were directly related to the acidity of the tissues; the extracts of leaves collected at 2 to 6 P.M. were green but at all other times showed different intensities of admixed brown and green colors. The amounts of chlorophyll in the extracted sap of macerated leaf tissues correlated negatively with the amounts of organic acids, shown in figure 3-A, indicating that a higher acidity accelerated the destruction of chlorophyll more than a lower acidity.

Neutralization of the tissues with alkali to pH 7.0, also, inhibited the development of brown color, suggesting that the high acidity of the tissues, presumably in association with chlorophyllase, was mostly responsible for the breaking down of chlorophyll in dying or dead tissues.

NITROGENOUS FRACTIONS.—Residual nitrogen, mostly or all protein-N, consists of the insoluble fraction left in the tissues after extraction of the water soluble fractions. The effects of diurnal intervals on residual nitrogen, in tables I and III, were presumably related to the acidity of the tissues prevalent at such intervals. As for example, leaves collected at 2 A.M. to 10 A.M. with higher acidity, had more residual-N than leaves at other intervals with lower acidity. And, conversely, extractable-N (water soluble-N) was greater in leaves collected at 2 P.M. to 10 P.M. with lower acidity than at other intervals with higher acidity.

The relationship of acidity to the extractable-N fraction, in table I, where the rise or fall was mainly due to protein-N, is indicated by the correlation coefficient, $r = -0.72$ and $t = 3.280$ requiring $t = 3.169$ for statistical significance at the P 0.01 level.

The association of acidity with changes in residual-N or in extractable-N suggests reversible changes in the relative degree of dispersion of proteins resulting from hydration at different pH which had affected the ease of extraction from the cell. At the high acidities, prevailing late at night and early in the morning, protein particles, because of lower hydration, become presumably less mobile and their dispersal decreases than at the low acidities during the day intervals at which considerable protein from the residual-N fraction enters the aqueous phase in suspension due to the greater hydration.

Examination of the fractions of extractable-N, in tables I and III, shows that it increased from 2 P.M. to 10 P.M. but decreased from 2 A.M. to 10 A.M. Also, the data in figure 1 show that there was an inverse relationship in the amounts of extractable-N and residual-N, that is, as the former decreased the latter increased, and vice versa.

The data in table III, containing the various fractions of extractable-N, indicate that the protein-N fraction changed in reverse order with the

residual-N fraction, suggesting that the gains in the protein-N fraction of the extractable-N group had resulted from losses in the residual-N group. Some changes were, also, observed in the crystalloid-N fraction of the extractable-N group which were in reverse order with those in residual-N.

A much better idea of the relationship of the various nitrogenous fractions is obtainable from figure 2 where such fractions are plotted as percentage of total extractable-N. It will be noted that protein-N was low

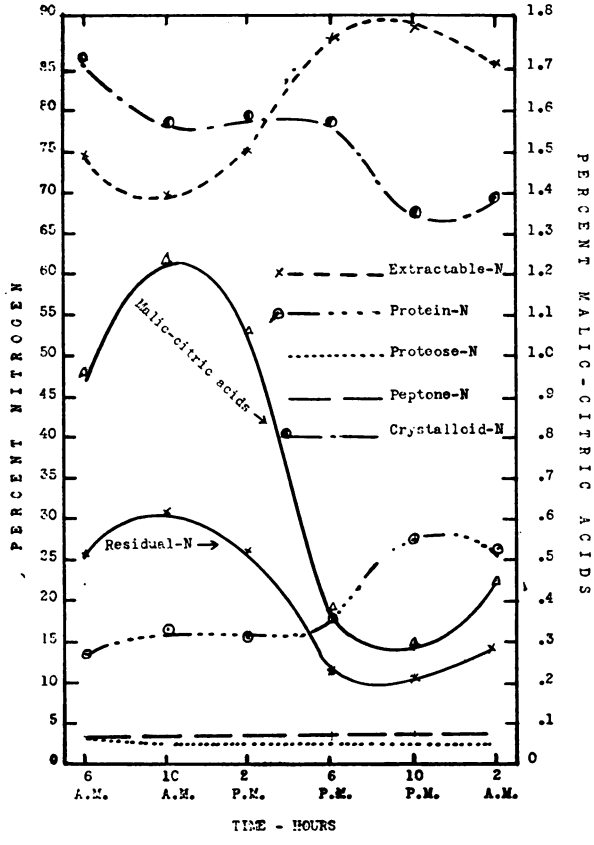


FIG. 2. Residual-N and extractable-N as percentage of total-N, also, protein-N, peptone-N, proteose-N and crystalloid-N as percentage of extractable-N in the chlorophyllous No. 4 sections of the active (D) leaves of *A. comosus*, grown in the greenhouse, collected August 9, 1944, and malic-citric acid values of the same tissues.

from 6 A.M. to approximately 2 P.M. when it began to increase until 10 P.M., then it decreased until 2 A.M. and it remained almost at the same level from 2 to 10 A.M. Crystalloid-N began to increase from 6 A.M. to 2 P.M., but decreased from the latter time to 6 P.M. and remained relatively at the same level from 6 P.M. to 6 A.M. Comparison of the curves of crystalloid-N and malic-citric acid, in the same figure, shows that as the former increased, from 6 A.M. to 2 P.M. the latter decreased, possibly suggesting conversion of malic

or citric acids, presumably in the presence of diphosphopyridine nucleotide [$(Py(PO_4)_2)$] to amino forms (**16, 73, 78**) as:

$$\text{malic acid} + Py(PO_4)_2 \longrightarrow H_2Py(PO_4)_2 + \text{oxalacetic acid (I)}$$
$$\text{oxalacetic acid} + NH_3 \longrightarrow \text{aspartic acid (II)}$$
$$\text{aspartic acid} + NH_3 \longrightarrow \text{asparagine}$$

and aspartic acid, in the presence of pyruvic acid which is present in plant tissues, via transamination to alanine.

$$\text{Aspartic acid} + \text{pyruvic acid} \rightleftharpoons \text{oxalacetic} + \text{alanine}$$

Similar comparison of the curves of crystalloid-N and protein-N shows that the former began to rise from 10 A.M. to 2 P.M. but the latter from 2 P.M. to 10 P.M. suggesting possible conversion of crystalloid-N to protein-N. But light is not essential for the above reaction according to PRIANISCHNIKOW (**50**), and more studies are necessary to establish the relationship of titratable acidity changes to crystalloid-N.

The curves of peptone-N and proteose-N remained practically at the same level at all times suggesting that they were affected the least by diurnal changes. These fractions, presumably fragments of various cell proteins resulting from cellular metabolic activity, in accordance with the theory of BORSOOK and HUFFMAN (**16**), possibly undergo breakdown and recombination at rates somewhat proportional with the nitrogenous constituents of the cell (**70**).

FIELD PLANTS

These plants, all from a single clone, were grown under identical conditions of sunlight, water and amounts of applied fertilizer salts excepting the soil fumigation treatments prior to planting which are reported in table VI.

LEAF WEIGHTS, ACIDITY, SUGARS.—The data, in table VI, show that leaf weights, which were directly related to plant weights, differed in the various treatments. All chloropicrin and D-D and the 400 pounds of ethide per acre treatments produced greater leaf weights than all others.

Titratable acidity in the chlorophyllous No. 4 sections of the active (D) leaves, reported as citric-malic acids, was greater at 7 A.M. than at 3 P.M. which is in agreement with findings in tables I, II and III. It was directly related to leaf weights collected at 7 A.M., depicted in figure 3-B. With a coefficient of correlation, $r = 0.92$, and $t = 7.60$ and requiring for significance $t = 3.169$ at the P 0.01 level, the relationship between titratable acidity and leaf weights was very great. Also, in figure 3-C, sugars and leaf weights were inversely related, that is, the former decreased as the latter increased and vice versa. This relationship, presumably associated with the utilization of sugars at a greater rate by large than small plants and depicted in figure 4, shows a negative correlation coefficient, $r = -0.95$, which is statistically highly significant.

TABLE VI

PLANT WEIGHTS, WEIGHT PER LEAF OF THE ACTIVE D GROUP, AND TITRATABLE ACIDITY AS CITRIC-MALIC ACIDS, SUGARS, AND MINERAL NUTRIENTS PER GRAM OF FRESH TISSUE OF THE CHLOROPHYLLOUS NO. 4 SECTIONS OF ACTIVE (D) LEAVES FROM *A. comosus* GROWN UNDER FIELD CONDITIONS WITH DIFFERENT KINDS AND AMOUNTS OF SOIL FUMIGANTS, AND HARVESTED AUGUST 15, 1944

TREATMENTS		PLANT WTS.	LEAF WEIGHTS		TITRATABLE ACIDITY ML. 0.1 N NaOH		SUGARS MG. PER GM.		NUTRIENT ELEMENTS MG. PER GM.				
FUMIGANT	PER ACRE		7:00 A.M.	3:00 P.M.	7:00 A.M.	3:00 P.M.	7:00 A.M.	3:00 P.M.	N	K	Ca	Mn	P
	lbs.	*gm.*	*gm.*	*gm.*	*ml.*	*ml.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Check, X–1	0	2000	27.9	27.2	18.3	2.2	34.8	42.9	2.72	3.75	0.52	0.09	0.10
Check, X–2	0	.....	22.9	22.6	14.2	1.9	35.6	42.3	.....	.....	.....	.....	.....
Check, X–3	0	.....	32.4	32.8	20.3	2.6	35.4	41.5	.....	.....	.....	.....	.....
Chloropicrin	90	2400	51.2	52.0	28.0	3.5	27.0	35.1	3.45	3.90	0.78	0.14	0.12
Chloropicrin	180	4300	57.2	57.4	30.8	3.6	24.6	30.8	3.69	4.46	1.89	0.27	0.15
Chloropicrin	360	5800	64.3	66.6	29.8	3.7	19.5	27.8	3.86	6.40	1.36	0.42	0.16
D-D mixture	150	4800	50.4	51.6	29.5	3.5	23.2	31.6	3.32	6.47	1.30	0.18	0.15
D-D mixture	300	4620	50.5	52.0	29.7	3.7	24.6	31.2	3.14	7.15	1.12	0.20	0.14
D-D mixture	450	4580	47.0	49.0	29.2	3.6	26.2	32.2	3.90	6.56	1.49	0.19	0.15
Ethide	100	2040	37.2	36.9	22.2	3.0	34.1	42.0	3.00	4.50	0.76	0.11	0.12
Ethide	200	3170	45.9	44.0	26.7	3.3	28.6	37.8	3.80	3.88	1.55	0.18	0.12
Ethide	400	4890	54.9	46.0	28.8	3.7	23.6	35.4	3.58	4.00	2.12	0.19	0.15

The fact that leaf weights correlated positively with titratable acidity and negatively with sugars indicated a sort of interrelationship between titratable acidity and sugars and suggests possible conversion of the former to the latter. The correlation of titratable acidity to sugars, depicted in

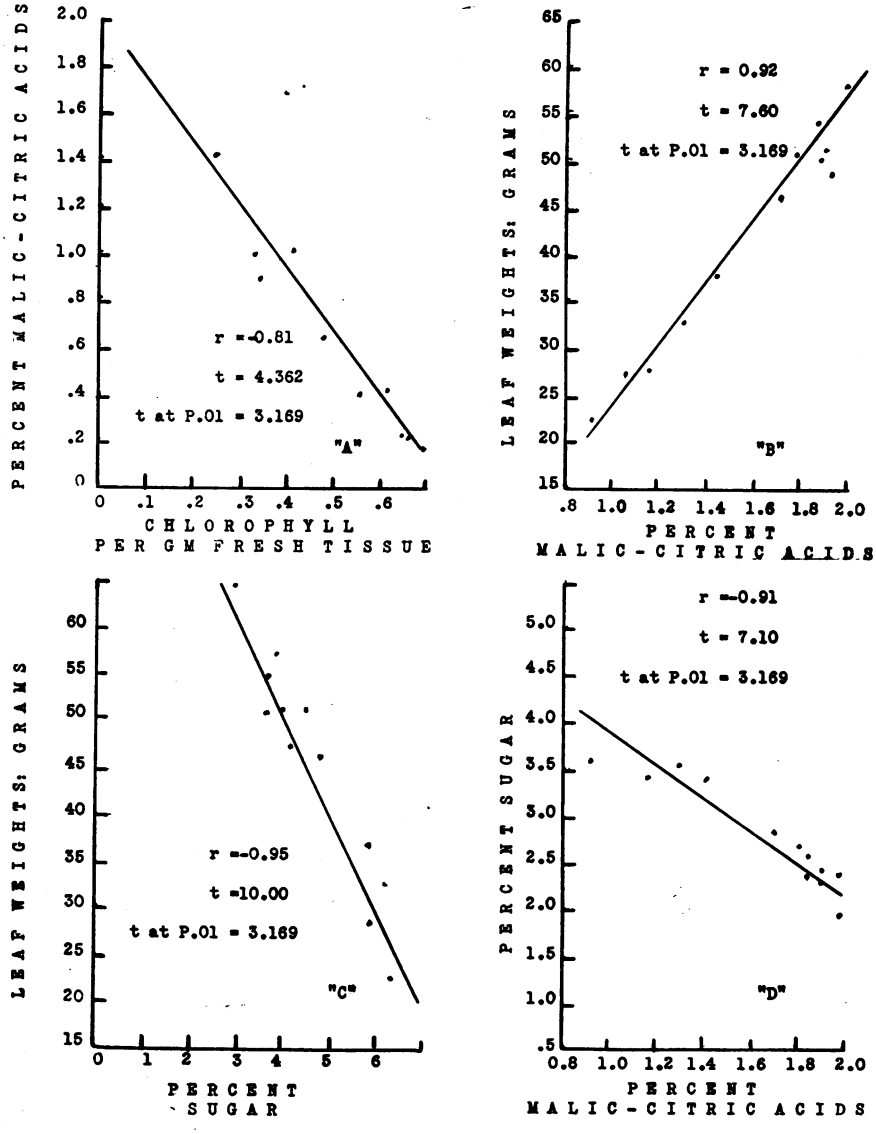


FIG. 3. Curves of correlation: "A," titratable acidity (malic-citric acids) and chlorophyll showing the effects of acidity on chlorophyll destruction in the sap of macerated tissues collected at different diurnal intervals; "B," leaf weights and titratable acidity; "C," leaf weights and sugar; and "D," sugar and titratable acidity in the chlorophyllous No. 4 sections of the active (D) leaves of *A. comosus*, collected at 7:00 A.M. on September 6, 1944, grown in the field with equal amounts of fertilizer salts but with different kinds and amounts of fumigants applied to the soil before planting.

figure 3-D, shows a coefficient, r = – 0.91, which is statistically highly significant at the P 0.01 level.

These data, although susceptible to other interpretations, strongly indicate that sugars are the raw substances for the formation of organic acids by chlorophyllous tissues in darkness and that such acids are produced and sugar utilized at much greater rates by plants growing more than less rapidly.

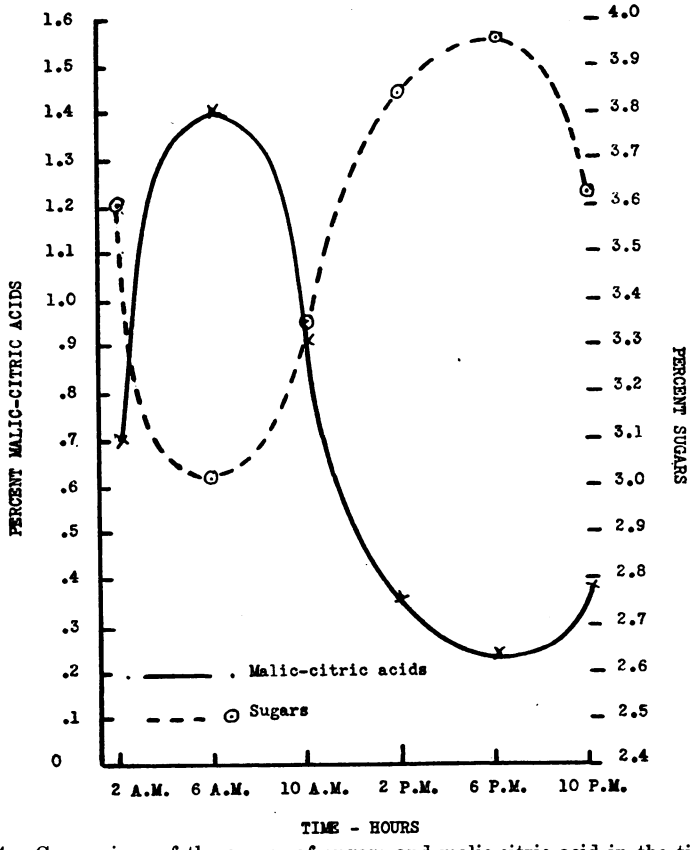


FIG. 4. Comparison of the curves of sugars and malic-citric acid in the tissue of the chlorophyllous No. 4 sections of the active (D) leaves of *A. comosus*, grown in solution cultures in the greenhouse, collected August 2, 1944.

NITROGENOUS FRACTIONS.—Nitrogen, potassium, or calcium per gram of fresh tissue, in table VI, although fluctuating at various intervals, were not related to leaf weights, titratable acidity or sugars. But total amounts of these elements per plant, obtainable by multiplying plant weights by the concentrations of the various ions in table VI, were positively correlated with leaf weights, as follows:

Required t = 2.306 for significance at P 0.05.

ITEMS		r	t
Leaf wt.	: N per plant	0.76	3.313
" "	: K " "	0.71	2.840
" "	: Ca " "	0.62	2.220

TABLE VII

TOTAL NITROGEN, AS MILLIGRAMS PER GRAM OF FRESH TISSUE, RESIDUAL-N AND EXTRACTABLE-N, AS PERCENTAGE OF TOTAL-N, AND PROTEIN-N, PROTEOSE-N, AND CRYSTALLOID-N AS PERCENTAGE OF EXTRACTABLE-N, IN THE CHLOROPHYLLOUS NO. 4 SECTIONS OF THE ACTIVE (D) LEAVES OF NINE-MONTH-OLD *A. comosus*, COLLECTED AT 8: 00 A.M. AND 4: 00 P.M. ON SEPTEMBER 6, 1944, FROM PLOTS TREATED BEFORE PLANTING WITH DIFFERENT KINDS AND AMOUNTS OF SOIL FUMIGANTS

TREATMENTS		7: 00 A.M.						3: 00 P.M.					
FUMIGANT	LBS. PER ACRE	TOTAL-N MG./GM. FRESH WT.	RESIDUAL N	EXTRACTABLE N	EXTRACT-N FRACTIONS			TOTAL-N MG./GM. FRESH WT.	RESIDUAL N	EXTRACTABLE N	EXTRACT-N FRACTIONS		
					PROTEIN N	PROTEOSE N	CRYSTALLOID N				PROTEIN N	PROTEOSE N	CRYSTALLOID N
	lbs.	*mg.*	%	%	%	%	%	*mg.*	%	%	%	%	%
Check, X–1	0	2.010	46.0	54.0	51.8	2.1	46.1	2.130	38.7	61.3	64.2	12.8	23.0
Check, X–2	0	1.760	51.8	48.2	56.9	9.8	33.3	1.780	40.9	59.1	62.0	7.9	30.1
Check, X–3	0	2.230	44.7	55.3	51.8	3.6	44.6	2.270	35.8	64.2	67.7	10.7	21.6
Chloropicrin	90	2.500	39.8	60.2	51.8	2.7	45.5	2.330	35.9	64.1	69.2	7.4	23.4
Chloropicrin	180	2.610	35.8	64.2	54.5	3.7	41.8	2.660	32.3	67.7	62.1	5.5	32.4
Chloropicrin	360	2.460	35.3	68.7	56.2	1.8	42.0	2.600	38.0	62.0	73.1	3.1	23.8
D-D mixture	150	2.540	39.8	60.2	53.1	4.3	42.6	2.460	34.7	65.3	65.7	11.4	22.9
D-D mixture	300	2.670	36.3	63.7	49.7	4.2	46.1	2.700	37.6	62.4	61.8	10.5	27.7
D-D mixture	450	2.620	34.7	65.3	47.0	4.5	48.5	2.760	30.8	69.2	59.9	9.6	30.5
Ethide	100	2.220	36.7	63.3	46.3	3.9	49.8	2.090	38.0	62.0	60.1	7.7	32.2
Ethide	200	2.620	39.1	60.9	61.1	4.5	34.4	2.670	35.3	64.7	59.8	12.2	28.0
Ethide	400	2.700	46.0	54.0	47.9	4.2	47.9	2.780	40.2	59.8	57.2	11.6	31.2
Mean	.....	.....	40.5	59.5	52.3	4.1	43.6	.....	36.5	63.5	63.6	9.2	27.2

The above may suggest that leaf weights increased in direct proportion with the total amounts of nitrogen and potassium per plant. As the large plants were grown in treatments that had been more efficiently sterilized by certain fumigants and presumably had healthier and better root systems, the amounts of nitrogen and potassium per plant removed from the soil was greater by the large than small plants.

The nitrogenous fractions from the chlorophyllous tissues, reported in table VII, differed considerably in the different treatments between the 7 A.M. and 3 P.M. collections.

Residual-N was greater and extractable-N smaller in the leaves of the 7 A.M. than 3 P.M. collections, which is in agreement with the data in tables I and III. Protein-N and proteose-N were smaller but crystalloid-N was greater in the 7 A.M. than 3 P.M. leaves, which, also, agree with the data in table III. The lack of samples between 10 A.M. and 2 P.M. in the field plants

TABLE VIII

MEAN PROTEIN-N, PROTEOSE-N, AND CRYSTALLOID-N AS PERCENTAGE OF EXTRACTABLE-N (FROM THE DATA IN TABLE VII) AND DIFFERENCES OF THE SAME BETWEEN LEAVES COLLECTED AT 7: 00 A.M. AND 3: 00 P.M.; ALSO, STATISTICAL SIGNIFICANCE OF THE DIFFERENCES

EXTRACTABLE-N FRACTIONS	TIME OF COLLECTION		DIFF.	T
	7: 00 A.M.	3: 00 P.M.		
	%	%	%	%
Protein-N	52.5	63.6	−11.1	6.90
Proteose-N	4.1	9.2	− 5.1	5.32
Crystalloid-N	43.7	27.2	16.5	8.65
Required for significance at P 0.01				3.106

as in figure 2 for the greenhouse plants failed to disclose possible changes in protein-N and crystalloid-N.

Crystalloid-N was higher in the 7 A.M. leaves of the field than in the 6 A.M. leaves of the greenhouse plants, presumably because of longer exposure to sunlight of the former than latter plants. Moreover, the field plants grown at an elevation of approximately 1000 feet at Wahiawa and not shaded by surrounding mountains were exposed to light at sunrise, whereas the greenhouse plants grown in Manoa Valley which is surrounded by mountains lost approximately two hours of sunlight in the morning.

Differences of protein-N, or crystalloid-N between leaves collected at 7 A.M. and 3 P.M. were statistically significant as shown in table VIII.

The greater extractable protein values in the leaves collected at 3 P.M. than 7 A.M. presumably resulted, on the one hand, from solubilized residual-N which entered in suspension the aqueous phase due to the decreased acidity of the sap, and on the other, from conversion of crystalloid-N to protein-N.

Discussion

The above data, briefly stated, indicate that the chlorophyllous regions of the leaves of *A. comosus* are subject, at different diurnal intervals, to changes in titratable acidity and sugars, according to figure 4, the former increasing and the latter decreasing in darkness or the opposite in light. Also, comparable changes were observed in certain nitrogenous fractions.

These results are in general agreement with the findings of others but the present interpretation is at variance with that of a few investigators.

MAYER (**44, 45**), KRAUS (**37, 38**), DE VRIES (**79, 80**), PURJEWITSCH (**53**), NATHANSON (**47**) and BENNET-CLARK (**10, 11**) support the view that malic acid is formed in succulents by the partial oxidation of sugars. However, RUHLAND and WETZEL (**58, 59**) and KOSTYCHEV (**36**) attribute the formation of organic acids to the oxidative deamination of amino acids resulting from "protein respiration." This view has been severely criticized by BENNET-CLARK and WOODRUFF (**10**) who consider malic acid a product of "sugar respiration." VICKERY *et al.* (**76**) in the interpretation of their results claim to have obtained a much closer association of malic acid with carbohydrates than proteins although the participation of the latter was not excluded, and state the case as follows: "Although the interpretation assigned to the various changes is speculative, there is no doubt that respiration in the leaf blades involved substances other than carbohydrates very early in the culture period and the conclusion is drawn that a part of the protein of the blade was included in the reaction. It is suggested that the residues of the amino acids, after these had been oxidatively deaminized, were subsequently to an appreciable extent completely oxidized, presumably by mechanisms allied to those provided for the oxidation of fatty acids."

The investigations of SIDERIS *et al.* (**64, 65**) with *A. comosus* grown in light or darkness for different periods under field and greenhouse conditions show that titratable acidity was reduced more in the leaves and fruits of the plants in darkness than in those exposed to light, although protein hydrolysis in the plants grown in darkness was evident by the low values of protein-N and the accumulations of soluble organic-N.

The reduction of titratable acidity, in light, has been explained by BENNET-CLARK (**11**) on the principle of Le Chatelier:

$$\text{Carbohydrate} \rightleftharpoons \text{malic acid} + CO_2 + \text{energy}$$

where rises in temperature displace the equilibrium in the direction in which energy is absorbed. Although high temperatures accelerate the rate of organic acid disappearance from tissues more than low, light is considerably more effective, in accordance with the data in table V.

Intelligent interpretation of the results herein reported may be best made in the light of the findings of others in related investigations with modern methods of experimental enzymology.

The data in tables II and VI and in figures 3-D and 4 indicate that the

formation of organic acids, at night, was at the expense of sugars. The oxidation of carbohydrates has been explained by SZENT-GYORGYI (**73**) by a series of reactions involving dicarboxylic acids with 4-carbon atoms. According to this scheme, these acids, because of the great reactivity of H of the α and β C-atoms, serve, after activation by hydrogenase, as catalytic H-carriers, the H-atoms oxidized by cytochrome while the dehydrogenated acid is ready to take up two new H-atoms from the H-donator.

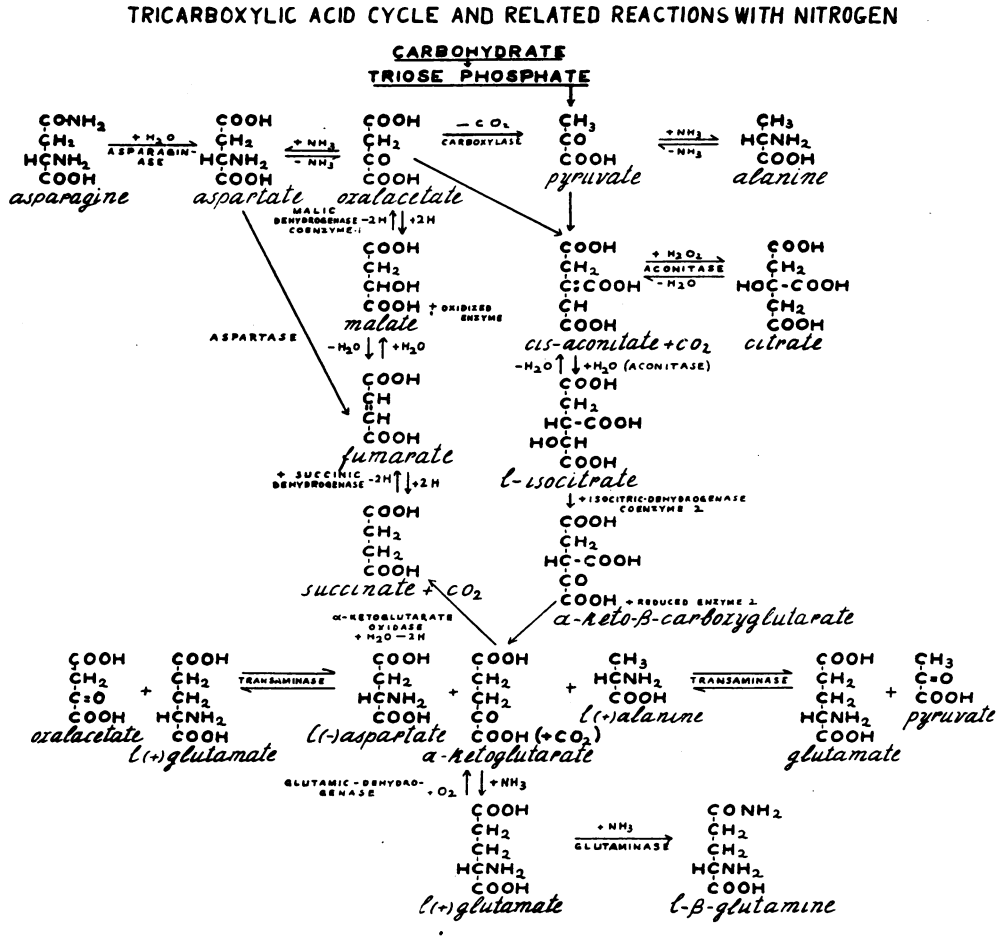


FIG. 5. Tricarboxylic acid cycle according to KREBS (42).

However, before sugars are oxidized to four carbon atom dicarboxylic acids they undergo a series of reactions, according to BARRON (**6**), involving glycolysis, phosphorylation, dismutation, and dephosphorylation, converting them to pyruvic acid. All such reactions are reversible except the dephosphorylation of phosphopyruvate, the latter possibly involving uptake of CO_2 leading to the formation of four carbon dicarboxylic acids which upon oxida-

tion yield phosphopyruvate. Pyruvic acid may be converted after CO_2 fixation to oxalacetic acid, a four carbon atom dicarboxylic acid.

KREBS (**39**) has introduced a modified form of the four-carbon dicarboxylic acid scheme of reactions of SZENT-GYORGYI (**73**), known as the tricarboxylic acid cycle, whereby the oxidation of carbohydrates follows a course via citric and other related tricarboxylic acids, as indicated in figure 5.

The mechanism of oxidation of carbohydrates depends on oxalacetate which, acting as a catalyst, bears no stoichiometrical relationship to the amount of carbohydrate oxidized. Oxalacetate may be derived from substances mostly always present in plant tissues, such as aspartate, glutamate, citrate, malate, fumarate, succinate and from pyruvate by CO_2 fixation. Also, certain amino acids yield oxalacetate by transamination.

The presence, in plant tissues, of the various dicarboxylic acids, mentioned above, and the corresponding dehydrogenases coupled with the ability of plant tissues to utilize these acids as metabolic substrata, according to certain investigators (**18, 21, 52, 57, 58, 59, 69, 74, 75, 76, 77**), suggests that the series of reactions involved in carbohydrate oxidation follow a course similar to that outlined by KREBS (**42**) for the tricarboxylic acid cycle in figure 5.

Although the various reactions, as outlined by BARRON (**6**) have not been studied as extensively in plant tissues as in yeasts and animal tissues, there is evidence indicating that plant tissues constitute no exception. JAMES (**58, 59, 60**) observed that hexosediphosphate, phosphoglycerate and pyruvic acid were formed by barley sap from glucose in the presence of adenylic acid, thus demonstrating the necessity of phosphorylation for carbohydrate breakdown, and the ratio of CO_2 formation to inorganic P disappearance constitutes additional evidence in favor of phosphorylation. Also, the results of BONNER and WILDMAN (**15**) show that *brei* from spinach leaves was capable of producing fructose diphosphate and glycerophosphate in the presence of glucose, and the amount of phosphorylated compounds produced was augmented in the presence of adenosine triphosphate, suggesting that hexose breakdown possibly follows a phosphorylytic pattern similar to that in yeast and muscle.

The data in table IV show that malic acid disappearance in the tissues from 6 A.M. to 6 P.M. was 75.5% of the total while citric acid was 37.3%. Citric acid was presumably more stable than malic acid in *A. comosus* as well as in Robinia, according to PURJEWITSCH (**35**).

The disappearance of organic acids from leaves in light, at day, is attributable by WOLF (**83**) to carbohydrate resynthesis which may be effected by a reversal of the series of reactions involved in carbohydrate oxidation. All such reactions are reversible, according to BARRON (**6**) except the dephosphorylation of phosphopyruvate. Reversal of the reaction from glucose to starch, in plants, was effected by HANES (**27, 28**) and from glucose to glycogen in animals by COLOWICK and SUTHERLAND (**18**). Reversibility of the series of reactions from glucose-6-phosphate to lactate was effected by

GREEN *et al.* (**25**) and from lactate to pyruvate by ELLIOT *et al.* (**22**) and BARRON and LYMAN (**7**).

Certain of the reactions for the oxidation of carbohydrates, suggested by SZENT-GYORGYI (**73**) and KREBS (**39**), may not apply to all plants. For instance, in animals the oxidation of H-atoms in the systems

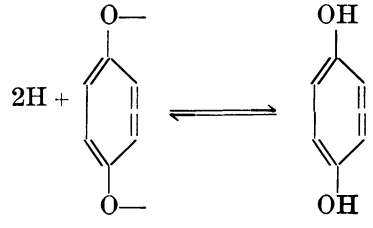
$$\text{(I)}\quad \text{Fumarate} \xrightarrow{+2H} \text{succinate}$$

$$\text{(II)}\quad \text{Succinate} + \text{cytochrome B} \xrightarrow[\text{succinic dehydrogenase}]{} \text{Fumarate} + \text{2H-ions} + \text{cytochrome-C}$$

$$\text{(III)}\quad \text{Cytochrome-C} + \text{2H-ions} \xrightarrow[\text{cytochrome oxidase}]{} \text{cytochrome A} + H_2O$$

may be accounted for by the presence of cytochrome. But in *A. comosus*, where the presence of cytochrome is questionable because of the negative results obtained by chemical analysis of the tissues by the method of KEILIN and HARTREE (**35**), the oxidation of H atoms may follow a different course. Also, negative results for cytochrome-C were obtained in spinach leaves by BONNER and WILDMAN (**15**) and those obtained spectroscopically by GODDARD (**24**) in wheat germ were very low.

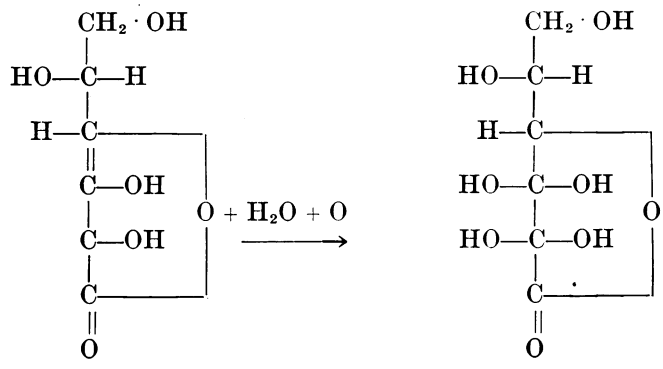
In order to explain the discrepancy BONNER and WILDMAN (**15**) assumed that succinic dehydrogenase in spinach may be different from that in animal tissues and its possible oxidation in the absence of cytochrome is effected by a quinone carrier, in accordance with MICHAELIS' (**46**) concept



Hydroquinone ion Hydroquinone

where the 2H from succinic dehydrogenase reduce the hydroquinone ion to hydroquinone.

However, the leaves of *A. comosus* either lack completely or contain exceedingly small amounts of phenolic compounds related to hydroquinone to take up H from dehydrogenases, but they contain great amounts of ascorbic acid. According to SZENT-GYORGYI (**73**) ascorbic acid undergoes reversible oxidation by ascorbic oxidase, in the presence of oxygen to dehydroascorbic acid, the H presumably transferred to oxygen forming, respectively OH or hydrogen peroxide, and the latter reaction with peroxidase, which according to unpublished data of the authors, is plentiful in the chlorophyllous tissues of the leaves of *A. comosus* to oxidize more H atoms.



Ascorbic acid Dehydroascorbic acid

The system ascorbic acid-dehydroascorbic acid has been investigated by BARRON (**5**) who claims that the reaction of ascorbic acid with cytochrome (electromotively active iron-porphyrins) provides a mechanism for the generation of peroxides in the cell:

(1) Ascorbic acid + 2 Fe^{+++} ⟶ dehydroascorbic acid + 2 Fe^{++} + 2H

(2) 2 Fe^{++} + O_2 ⟶ 2 Fe^{+++} + H_2O_2

HUSZAK (**30**) found that ascorbic acid may be oxidized by H_2O_2 in the presence of flavones acting as catalysts.

Peroxides generated in the tissues in the manner suggested by BARRON (**5**) may be decomposed by peroxidase which is plentiful in the chlorophyllous tissues of the leaves of *A. comosus*. GAFRON (**23**) has suggested a type of peroxides, not hydrogen peroxide but of the type $Y(OH)_4$, may be generated in tissues which play a very important role in the respiration of the cells and, also, in the oxidation of malic acid. The $Y(OH)_4$ type of peroxide may be decomposed by photocatalase during photosynthesis.

The changes in residual-N and extractable-N were attributed presumably to changes in the degree of hydration and dispersion of the proteins in the cell at different H-ion. This is in agreement with the observations of KRAUSS (**39**) which indicate that the physical state of the chloroplasts in the leaves of Bromeliaceae undergoes changes in the morning, noon and afternoon. The chloroplasts are shown in the morning to be ellipsoidal in shape and

TABLE IX

PLANT WEIGHTS AND TOTAL ACIDITY IN TOMATO PLANTS GROWN IN NITRATE AND AMMONIUM CULTURES FROM CLARK (**17**)

CULTURES	FRESH WT.		TOTAL ACIDS M.E. IN 100 GM. DRY TISSUE	
	LEAVES	STEM	LEAVES	STEM
	gm.	*gm.*	*m.e.*	*m.e.*
NO_3	94.4	175.6	153.0	147.0
NH_4 (concentr.)	38.7	53.7	71.0	65.4
NH_4 (dilute)	37.6	69.1	.....	73.8

their constituting material to have a definite structural configuration which conforms with the concept of SHARP (**60**), that the chlorophyll is confined to numerous small platelets, or grana, embedded in the cytoplasmic stroma. During the progress of the day, starch begins to form within the chloroplast until the latter appears to be made up almost entirely of a conglomerate of starch grains which gradually break away from the chloroplast and become free in the cell. When no more starch grains remain within the chloroplast the latter appears as a pale green, sometimes hardly visible, body, the contents of which appear wholly homogeneous, that is, not segregated into distinct ellipsoid bodies.

These observations on the physical state of the chloroplasts, that, in the

TABLE X

COMPOSITION OF THE SOLUTIONS OF NUTRIENT CULTURES IN N, K, CA AND CL, PLANT WEIGHTS AND TITRATABLE ACIDITY, AS PERCENTAGE OF CITRIC-MALIC ACIDS, OF THE CHLOROPHYLLOUS NO. 4 SECTIONS OF THE ACTIVE (D) LEAVES AND COMPARISONS OF PLANT WEIGHTS AND ACIDITIES AND REMARKS ON POSSIBLE CAUSES FOR THE DIFFERENCES OBSERVED; ALSO, CORRELATION OF PLANT WEIGHTS WITH LEAF ACIDITY

Culture no.	Elements in cultures mg. per liter				Plant		Comparisons			
								Ratios		
	N*	K	Ca	Cl	Weights	Acidity	Cultures no.	Weights	Acidity	Remarks
	mg.	*mg.*	*mg.*	*mg.*	*gm.*	*mg.*				
5	140	39	40	70	3226	19.5				
6	140	39	4	7	2361	16.0	5–6	1.37	1.22	.....
11	28	195	40	72	4672	20.8				
12	28	195	4	7	4550	20.1	11–12	1.03	1.03	.....
13	28	39	200	280	3492	12.5				High Cl.
14	28	39	40	0	3588	19.3	14–13	1.03	1.55	
16	28	4	200	280	1646	6.4				High Cl.
17	28	4	40	0	2059	18.5	17–16	1.25	2.89	.....
20	3.5	195	40	0	1840	11.5				Low N
23	3.5	39	200	360	1486	10.2	20–23	1.23	1.13	Low N and high Cl.

Correlation coefficient, r = 0.86; t = 4.870; required for significance at P 0.01 t = 3.355.
* N = nitrate-N.

morning, are organized into distinctly visible particles, which, with the progress of the day, lose gradually their original structure and finally become a homogeneous mass of matter conform with assumptions advanced to explain the differences in non-extractable protein-N, designated as residual-N, at different diurnal intervals. The greater values of residual-N at the high H-ion concentrations of the sap, in the night and early morning suggest that the chloroplastic proteins were in a state of contraction and low degree of dispersal in the sap of the cell by the loss of water of hydration thus forming the distinct ellipsoid particles observed by KRAUSS (**39**). But

with the advance of the day, increased light and the lowering of the concentration of H-ions the chloroplastic proteins presumably expanded by increased hydration and dispersal of their particles in the cell, which, according to KRAUSS (**39**), appeared as a wholly homogeneous pale green mass without the distinct ellipsoid bodies.

The assumption of chloroplastic protein particles being subject to expansion or contraction by differential hydration is supported by the investigations of OSTERHOUT (**48, 49**) who observed in Nitella that chloroplasts contract under natural conditions or under the influence of certain reagents or when a sufficient amount of water enters any part of the cell. Such contractions may be reversed by removal of the agents causing contraction.

Protein particles may change size by differential hydration and subsequent expansion or contraction following neutralization of the electric charge by H–, OH–, or other ions at pH above or below the isoelectric point. According to HITCHCOCK (**29**), the permeability of collodion membranes coated with protein, as measured by the flow of water or dilute solutions, was greater near the isoelectric point of the protein and smaller with increasing concentration of acid or alkali, suggesting dehydration with subsequent contraction at the isoelectric point and hydration with subsequent expansion at pH above or below. IRWIN (**31**) observed that the rate of exit of the dye was increased when the pH value of the sap of Nitella was raised by penetration of NH_3, but when the pH was lowered by an entrance of acetic acid the rate of penetration of dye was increased or decreased depending on the condition of the protoplasm in the cell.

The studies of BANGA and SZENT-GYORGYI (**4**) on the structure of the proteins of chloroplasts reveal that they are highly viscous, thixotropic and exhibit streaming double refraction; the last property placing them in the fibrous group. This group, according to ASTBURY (**2**) and EDSALL and MEHL (**21**), have the ability to unfold and refold, become stretched or contracted, and being exceedingly sensitive to the effects of denaturing agents cease to exhibit streaming double refraction.

The relationship of environmental factors to tissue acidity at different diurnal intervals is not clear. RUHLAND and WETZEL (**57**) are of the opinion that, in Begonia, rich nitrogen manuring causes a considerable rise in acidity. BENNET-CLARK (**10**) found that two plants of *Crassula laectea* of the same age, which were cuttings from the same parent plant but grown in two different soils, had titratable acidities of 8.3 and 2.2 mg. per 100 gm. of fresh weight at the diurnal maximal acidity in June. BENECKE (**9**) and CLARK (**18**), finding that plants supplied with nitrate-ions produced more acidity (total acidity not titratable or free acidity) in the tissues than similar plants with ammonium-ions, attributed the increased acidity in the NO_3^- cultures to the neutralizing effects of the bases with which NO_3^- was associated which were absorbed more from the NO_3^- than NH_4^+ cultures. BENNET-CLARK (**13**), attempting to interpret the results of CLARK (**18**) that plants cultured on ammonium salts produced almost no oxalic, malic, or

citric acids, but that asparagine and glutamine were increased, comments, as follows: "The significance of this is hard to interpret: it suggests that ammonia traps acids of the malic-oxalic group, or some precursor of them, with resultant amide formation, but it is not clear why the same equilibrium concentration of these acids should not be established in both nitrate and ammonium cultures, etc." However, recapitulation of CLARK's (**18**) data, in table IX, shows that plant weights were related directly to tissue acidity in both nitrate and ammonium cultures, which is in agreement with the results in tables VI and X. Also, the data of VICKERY *et al.* (**77**) show relationship between organic acids and tissue weight in *Narcissus poeticus*. Moreover, other studies of the authors (**68, 69**) have shown that the amounts of free organic acids in the tissues of *A. comosus*, grown in solution cultures and supplied with nitrate or ammonium ions, were related directly to the rate of growth as indicated by plant weights rather than to the kind of nitrogen supplied to the roots.

Data from unpublished studies by the authors reproduced in table X, show that tissue acidity at comparable diurnal intervals is directly related to plant weights and presumably to the rate of metabolic activity. This relationship, indicated by a correlation coefficient of $r = 0.86$ and $t = 4.87$, is statistically significant at the P 0.01 level. Reduction of plant weights, in table X, was caused either by insufficient amounts (3.5 mg. per liter) of nitrogen or by high concentrations of chlorides in the culture solution.

The omission of plant weights in the studies of BENNET-CLARK, RUHLAND and WETZEL and other investigators makes impossible any comparison between plant growth and tissue acidity.

The generation of greater acidity in the green tissues of plants with high rather than low growth rates is presumably related to the rate of sugar oxidation by respiration with malic acid and citric acids as end products. BENNET-CLARK (**11**) claims, from data on respiration, that during the phase of acid accumulation, RQ values much lower than unity were obtained and these were succeeded during the phase of acid disappearance, by values higher than 2.0, indicating that more than half of the acid reduced was possibly converted into carbohydrate.

It is suggested that in *A. comosus* the index of respiration in relation to malic acid synthesis or breaking down should be studied simultaneously in chlorophyllous and nonchlorophyllous leaf tissues to yield satisfactory comparative results, since the chlorophyllous tissues are better adapted to acid accumulations, in darkness, than the nonchlorphyllous tissues.

Summary

Diurnal changes affected greatly the chemical composition of the chlorophyllous tissues of the leaves of *Ananas comosus*.

The concentrations of ascorbic acid in the chlorophyllous tissues at different diurnal intervals varied slightly. Such variations were related pos-

sibly more to the carbohydrate supplies in the leaves than to diurnal intervals.

Titratable acidity of the tissues, composed mostly of malic and citric acids, increased in darkness, at night, but decreased in light, at day; being highest at 6 A.M. and lowest at 6 P.M. About 75% of malic acid and 37% of citric acid disappeared in the tissues from 6 A.M. to 6 P.M.

Total sugars decreased in darkness, at night, but increased in light, at day, being lowest at 6 A.M. and highest at 6 P.M. Also, more sugars were found in the chlorophyllous tissues of the leaves of plants with low than high growth rates, indicating less utilization by the former than latter plants.

Titratable acidity was inversely related to total sugars, suggesting possible generation of acidity after oxidation of sugars by processes allied to respiration.

Plant growth, as measured by leaf or plant weights, correlated positively with titratable acidity and negatively with total sugars, suggesting that the rate of metabolic activity and, in turn, of respiration, being greater in plants growing more than less rapidly, caused the generation of more acidity at the expense of sugars in the large than small plants.

Residual nitrogen, i.e., the insoluble fraction in macerated tissues after extraction of the water soluble fraction, was greater from tissues collected at intervals in darkness, at night, than in light, at day, but extractable nitrogen, in the water soluble fraction, was reversed. The increase in extractable nitrogen was attributed to a greater hydration and dispersion in the cells of the proteinaceous residual-N fraction in light than in darkness by changes in the acidity of the tissues which presumably modified the sizes of protein particles by swelling or contraction and either facilitated or retarded the extraction of residual nitrogen from the cells at different diurnal intervals.

The various data are discussed and interpreted as well as possible in the light of recent advances in enzymology.

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