Diurnal Variations in Some Enzymes of Carbohydrate Metabolism in Tapioca Leaves [1, 2]

P. N. Viswanathan, L. M. Srivastava, & P. S. Krishnan

Division of Biochemistry, Lucknow University, Lucknow, U.P., India

Although diurnal rhythmicity in the overall metabolic activity of plants and lower organisms has been recognized (2, 8), there have been only a few studies of any possible associated rhythmicity at the enzymatic level. Diurnal variations in oxidase and catalase in the tobacco plant (23), in amylase and phosphatase in *Phaseolus multiflorus* and *Kalanchoe blossfeldiana* (4, 5), and in amylase in *Spinacea oleracia* and *Perilla ocymoides* (25) have been reported. One of the most convincing evidences showing that physiological rhythm is a reflection of biochemical alterations relates to the luminescence in Gonyaulax, where luciferin and luciferase are found in greater amounts in cell-free extracts prepared during the dark period (9, 21).

Members of this laboratory have been engaged in investigations aimed at elucidating rhythmicity, if any, in the level of plant enzymes and their substrates. Sanwal and Krishnan (15) observed that aldolase and phosphatase of cactus showed distinct diurnal variations and the authors put forward a hypothesis to explain the significance of the alterations in the activities of these two enzymes. This was followed by a report by Mukerji et al. (12) that transaminase displayed a marked diurnal variation in cactus. The activity in the evening was several-fold that of samples collected at night and early morning, when the titratable acidity was maximum. The conclusions of these authors are subject to the criticism that homogenates were prepared in water and, as such, the marked changes in the acidity of the different homogenates may have resulted in inactivation of transaminase to a different extent, yielding values for activity which are artifacts. Sufficiently sensitive methods are not available to determine the influence, if any, of vacuolar acids on the pH of protoplasm in vivo in the succulent plants. It was also observed in this laboratory that cactus tissue showed a significant diurnal variation in protein and in nonprotein nitrogen content; the former increased and the latter decreased in the day time; the levels were restored at night. It was of interest to examine whether or not such diurnal variations are exhibited also by nonsucculent tissues, which do not possess the crassulacean type of organic acid metabolism. In the bougainvillaea plant (*Bougainvillaea spectabilis*) Srivastava and Krishnan (unpublished results) found that oxalic acid oxidase showed a marked diurnal fluctuation with a minimum at 4 PM and maximum at 12 PM. Oxalic acid also fluctuated, but in a reciprocal manner. The present report deals with the diurnal activities in the leaves of the tapioca plant (*Manihot utilissima* Pohl) of some of the enzymes involved in the synthesis and breakdown of carbohydrates and protein, as well as the diurnal contents of protein and of nonprotein and total nitrogen. The enzymes studied were fructosediphosphate aldolase, fructosediphosphatase (acid, neutral, & alkaline), phosphoglucomutase, starch phosphorylase, and glutamic-oxaloacetic transaminase.

Materials & Methods

The tapioca plants used in these experiments were raised in the University campus on garden soil under natural conditions of light and darkness and temperature. Most of the analyses were conducted during February and March, when nights were comparatively cool and days hot. The sun rose at 6 to 6:15 AM and set at about 6:15 to 6:25 PM. The plants selected were 6 to 8 months old, so that tuber formation had set in. A total of about 40 leaves, containing 6 to 7 leaflets each and receiving full sunlight, was marked off on different plants. These leaves were fully green and more or less of the same physiological age, being situated about 5 to 7 places before the tendermost leaf. From each leaf a leaflet was cut out at 4 hourly intervals over a 24-hour period, commencing at 8 AM. Care was taken not to injure the tissue during this process. The ambient temperature was taken to be the same as that of the leaf. The leaflets collected at any time were pooled, freed of midribs, and analysed either fresh or after storage in frozen condition at −18 C in a deep freeze.

► Preparing Homogenates: The homogenizations were effected in a chilled Waring blendor using water as the grinding fluid. In a typical run, 20 g leaf material were ground with 60 ml water. The period of homogenization was kept constant at 3 minutes. After filtering through muslin, the homogenates were diluted so as to make them 20 % with respect to the fresh weight of leaf material. Aliquots of the homogenates were removed immediately after

[1] Received Sept. 6, 1961.

[2] This investigation was financed by the Council of Scientific and Industrial Research, New Delhi.

preparation for determining enzyme activity, dry weight, protein, and total nitrogen.

► Determining Enzyme Activities: All the following enzyme activities were determined under conditions where the enzymes were present in limiting amounts.

Aldolase activity was determined by the method of Sibley and Lehninger (16), but at 30 C, instead of at 37 C. One unit of activity was the splitting of 1 μmole of fructosediphosphate in 1 hour at 30 C and pH 8.5.

Phosphoglucomutase activity was determined by the method of Cardini (3), but instead of the pure coenzyme, glucose-1,6-diphosphate, the filtrate from a 25 % homogenate of liver heated in boiling water for 30 minutes was used. One unit of the enzyme corresponded to the formation of 1 μmole of stable phosphorus from glucose-1-phosphate in 1 hour at 30 C at pH 7.5.

Glutamic-oxaloacetic transaminase activity was determined according to Tonhazy et al. (24). One unit of enzyme activity corresponded to the formation of 1 μmole oxaloacetic acid at 30 C in 1 hour at pH 7.4.

The assay system for fructosediphosphatase activity consisted of 5 μmoles of neutralized tetra sodium salt of fructosediphosphate, 50 μmoles of tris buffer pH 8.5, or borate buffer pH 6.5, or citrate buffer pH 5.5, depending on whether the alkaline, neutral, or acidic enzyme was being estimated, 0.2 ml of 20 % leaf homogenate, any supplements and water in a final volume of 1.0 ml. In case of the alkaline enzyme, 5 μmoles magnesium sulphate and 1.6 μmoles ethylenediaminetetraacetate adjusted to pH 8.5 were added. One unit of enzyme represented the splitting of 1 μmole of orthophosphate in 1 hour at 30 C at the particular pH value.

Starch phosphorylase activity was determined both in the presence and absence of fluoride, as reported by Srivastava and Krishnan (18). One unit was the splitting of 1 μmole of orthophosphate in 1 hour at 30 C.

The various enzyme activities have been reported as specific activity, calculated per mg protein.

► Other Estimations: Protein was estimated by the method of Lowry et al. (10) and total nitrogen by the micro-Kjeldahl method. In some experiments total nonprotein nitrogen was determined in the supernatants after trichloroacetic acid precipitation of protein. Phosphorus was estimated by the method of Fiske and Subbarow (6). The pH of the homogenates was measured with a Pye pH meter.

Results

► I. Diurnal Values for Total Nitrogen, Protein, & Dry Solid Content of Tapioca Leaves. Results obtained in a typical experiment are reported in table I.

There was no significant change in total nitrogen and protein content. Also, in spite of the large variation in the atmospheric temperature during the 24-hour period of observation there was not much difference in the dry weight content of the leaf material. The pH of the leaf contents, as determined in the fresh homogenates, remained constant at 6.2.

Table I

Diurnal Content of Total Nitrogen, Protein, & Dry Solids of Tapioca Leaves*

Hr of collection of samples	Ambient temp C	Dry wt	Protein	Total Nitrogen
		mg/100 mg	fr wt	
8 AM	20.0	11.3	2.61	0.57
12 AM	34.0	11.6	2.65	0.55
4 PM	28.0	12.1	2.63	0.58
8 PM	19.5	11.3	2.63	0.55
12 PM	16.0	11.9	2.61	0.55
4 AM	15.0	11.8	2.68	0.56

* March 29, 1961.

► II. Aldolase & Phosphorylase Activities in Tapioca Leaves: The activities of aldolase and phosphorylase are reported in table II.

Aldolase and phosphorylase activities varied diurnally with a maximum at 12 AM and minimum at 12 PM. There was almost a threefold difference between the maximum and minimum activities in the case of aldolase and a twofold difference in phosphorylase (as determined in the presence of fluoride). The same degree of variation persisted also when the activities were calculated on the basis of fresh weight, dry weight, and total nitrogen of leaf material. As the activity of a mixture of equal volumes of the most active (12 AM) and the least active (12 PM) homogenates was about the same as the arithmetic mean of the individual activities, the low activity observed in the night cannot be due to the presence of an inhibitor.

If the difference in the activities of phosphorylase in the absence and presence of added fluoride may be

Table II

Diurnal Activities of Aldolase & Phosphorylase in Tapioca Leaves*

Hr of collection of samples	Ambient temp C	Aldolase	Phosphorylase	
			Without fluoride	With fluoride
		Units/mg	Protein	
8 AM	15.5	0.62	0.42	0.21
12 AM	29.0	0.84	0.49	0.26
4 PM	27.5	0.66	0.42	0.21
8 PM	23.5	0.54	0.44	0.16
12 PM	16.5	0.30	0.34	0.14
4 AM	13.5	0.38	0.33	0.16

* Feb. 26, 1961.

Table III

Distribution of Phosphorylase Activity Between Plastids & Supernatant in Tapioca Leaves Collected at Noon & Midnight*

	Phosphorylase activity at noon		Phosphorylase activity at midnight	
	Without fluoride %	With fluoride %	Without fluoride %	With fluoride %
Whole homogenate	(100)	(100)	(100)	(100)
Supernatant	65	47	69	50
Particulates	40	56	32	54

* March 29, 1961.

taken as an estimate of phosphatase activity toward glucose-1-phosphate it will be seen that phosphatase activity was fairly constant, indicating that the diurnal change was confined to phosphorylase.

► III. Localization of Phosphorylase Under Conditions of Maximum & Minimum Activity. The phosphorylase of tapioca leaves was reported by Srivastava and Krishnan (19) to be distributed between the supernatant and the particulates sedimenting at 1,600 × *g* from aqueous homogenates. It was of interest to determine whether the increased phosphorylase activity in the day time and its decrease in the night were accompanied by any change in its distribution between plastids and soluble fraction, especially in view of Madison's observation (11) that the location of the enzyme in the leaves of the tobacco plant is dependent on the metabolic status of the plant. Samples collected at noon and midnight were immediately homogenized in 0.5 M sucrose in 0.05 M citrate buffer, pH 7.1 and the resulting homogenates were centrifuged and the activity determined in the separated fractions.

There was no marked change in the distribution of the enzyme accompanying the diurnal increase and decrease of activity (table III).

► IV. Fructose Diphosphatases in Tapioca Leaves: The activities of alkaline, neutral, and acidic fructosediphosphatases in the six samples collected over a 24-hour period are reported in table IV.

Table IV

Diurnal Activity of Acid, Neutral, & Alkaline Fructosediphosphatases in Tapioca Leaves*

Hr of collection of samples	Ambient temp C	Alkaline	Neutral	Acid
		Units/mg Protein		
8 AM	19.5	0.84	0.46	1.08
12 AM	34.0	1.04	0.48	1.04
4 PM	33.0	0.82	0.48	1.06
8 PM	25.5	0.58	0.46	1.06
12 PM	22.5	0.36	0.46	1.06
4 AM	17.5	0.44	0.44	1.04

* March 13, 1961.

There was practically no change in the acidic and neutral enzymes, but the alkaline enzyme showed a marked diurnal change. The activity remained low at the night time, but the sample collected at 8 AM showed a marked increase. There was a further, but small, rise leading to maximum activity at 12 AM. This activity was almost thrice that at 12 PM. The same degree of variation existed when activities were expressed also in terms of unit fresh weight, mg nitrogen, and dry weight. As in the case of aldolase and phosphorylase, the alkaline fructosediphosphatase activity of homogenates with maximum and minimum activities remained unaltered on mixing in equal volumes.

► V. Phosphoglucomutase & Transaminase in Tapioca Leaves: The diurnal activities of phosphoglucomutase and transaminase are reported in table V.

Transaminase activity did not follow a diurnal rhythm. Phosphoglucomutase activity tended to be maximum at 4 AM and minimum at 12 AM, but the magnitude of alteration was not as pronounced as in the case of aldolase, alkaline fructosediphosphatase, or phosphorylase.

Table V

Diurnal Activity of Phosphoglucomutase & Transaminase in Tapioca Leaves*

Hr of collection of samples	Ambient temp C	Phospho-glucomutase	Transaminase
		Units/mg	Protein
8 AM	20.0	1.38	4.12
12 AM	34.0	1.18	3.62
4 PM	28.0	1.44	3.82
8 PM	19.5	1.58	3.82
12 PM	16.0	1.58	4.04
4 AM	15.0	1.40	3.68

* March 29, 1961.

Discussion

Since metabolic processes are for the most part mediated through enzymes, it should be possible to regulate metabolism by modifying enzymic activities. Such a control of enzyme action is possible either by regulating the number of catalytically active sites by the process of synthesis and degradation, or by modifying the activity of these sites by the action of an inhibitory or stimulatory agent. The present investigation has revealed a diurnal rhythm in some, but not all, enzyme activities. Since the plants were grown under natural conditions of light and darkness and temperature alterations, the changes recorded are likely to be of physiological significance. However, the very nature of the experiments precluded the quantitative assessment of the relationship to light intensity. Yet another uncertainty in interpretation centered around the contributing influence of alterations in temperature.

Of the three types of fructosediphosphatase activities in the leaves of the tapioca plant, only the alkaline enzyme shows a diurnal variation. The increase in this enzyme in the day time may be interpreted as showing that the alkaline activity is more likely to be related to photosynthetic activity than are the acidic and neutral enzymes. Such a hypothesis is in harmony with the recent observation by Smillie (17) that the alkaline fructosediphosphatase is localized in the chloroplasts, contradicting the earlier conclusion by Racker and Schroeder (13). The rise of temperature occurring in the day may be expected to accelerate the hydrolytic activity of the alkaline enzyme, along with that of the other enzymes.

The diurnal variation in the activities of fructosediphosphate aldolase and phosphorylase is in the same direction as that of alkaline fructosediphosphatase: the activities at noon are three- and twofold, respectively, those at midnight. The equilibrium of the aldolase reaction is known to be shifted in vitro in the direction of triosephosphate formation at higher temperature (20). Assuming that the in vitro observations find a parallel in vivo, the increased activity of aldolase in the day should actually result in an increased split of fructosediphosphate. However, since the fructosediphosphatase activity is increased in the day, fructosediphosphate existing in equilibrium with triosephosphates would be irreversibly hydrolysed to fructose-6-phosphate, thereby tending to force the aldolase reaction in the direction of synthesis. Since phosphofructokinase activity was not determined, the contributing effect of this enzyme cannot be assessed at present. The pathway of synthesis of starch in plants is not quite clear (14). The rate of starch synthesis by potato phosphorylase in vitro is increased when the temperature is raised (1). Assuming that starch synthesis is catalysed by phosphorylase, the increased enzymic acivity in the day may be expected to cause increased synthesis of the polysaccharide in the tapioca plant.

From assays by the mixed homogenate technique it has been shown that the alterations in enzymic activities are not due to formation of an inhibitor or stimulator. In the absence of clearcut evidence for the presence in plant tissue of inactive precursors and a mechanism to activate them, it may be supposed that the diurnal fluctuation in enzymes in tapioca leaves is due to increase and decrease in the amount of the enzyme proteins. That fresh protein is not synthesized to any significant extent would be clear from the observation that there was no detectable diurnal change in protein, nonprotein nitrogen, and total nitrogen, in marked contrast to the diurnal rhythm in the protein content of the cactus plant (12, 15).

The mechanism whereby the level of an enzyme is diurnally controlled is not quite clear. The levels of glycolic acid oxidase in barley (22) and indole acetic acid oxidase in pea seedlings (7) have been reported to be under the regulating influence of the respective substrates. Galston and Dalberg (7) believed that the adaptive formation and deadaptive disappearance of an enzyme, together with the existence of a mechanism of substrate formation, provide the essential features of a self-contained mechanism of diurnal rhythmicity.

In conclusion, it may be emphasized that the interpretations offered above have been based mainly on results from activity determinations in vitro of individual enzymic reactions and that the assumption has been made that some of the enzymes occur in limiting amounts, so that changes in their concentration affect the overall metabolic processes.

Summary

1. A diurnal variation in fructose-1,6-diphosphate aldolase and starch phosphorylase was observed in the leaves of the tapioca plant; the activities were higher in the day time than at night.

2. The fluctuation in phosphorylase activity was not associated with a change in its intracellular distribution.

3. Of the three fructosediphosphatase activities, alkaline, neutral, and acidic, only the alkaline enzyme showed a diurnal variation; the activity was maximum at noon and minimum at midnight. These results lend support to the hypothesis that it is the alkaline enzyme which is directly involved in photosynthesis.

4. Phosphoglucomutase activity did not show a prominent fluctuation, but there seemed to be a distinct tendency for higher activities at night.

5. Glutamic-oxaloacetic transaminase activity seemed to be constant in all the samples collected during a 24-hour period.

6. Assays by the mixed homogenate technique eliminated the presence of activator or inhibitor as the cause of alterations in activity. The data can be interpreted in terms of a de novo formation of enzymes.

7. There was no significant change in the protein content or total and nonprotein nitrogen. It is likely that enzyme protein was being formed and removed by transformation occurring among the various proteins and not by fresh synthesis from nonprotein nitrogen.

8. The significance of the observed diurnal activity in enzymes is discussed from the point of view of carbohydrate synthesis in the day time and breakdown in the night.

Acknowledgment

Two of the authors (P.N.V. & L.M.S.) desire to express their gratitude to the Council of Scientific and Industrial Research, New Delhi, for the award of research fellowships.

Literature Cited

1. Arreguin-Lozano, B. & J. Bonner. 1949. Experiments on sucrose formation by potato tubers as influenced by temperature. Plant Physiol. 24: 720–738.

2. BUNNING, E. 1956. Endogenous rhythms in plants. Ann. Rev. Plant Physiol. 7: 71–90.
3. CARDINI, C. E. 1951. Activation of plant phosphoglucomutase by glucose-1,6-diphosphate. Enzymologia 15: 44–48.
4. EHRENBERG, M. 1950. Beziehungen zwischen fermenttätigkeit und blattbewegung bei *Phaseolus multiflorus* unter verschiednen photoperiodischen bedingungen. Planta 38: 244–279.
5. EHRENBERG, M. 1954. Einflus verschiedenen lichtdunkelwechsels auf die rhythmik der phosphataseaktivität in den blättern von *Kalanchoë blossfeldiana*. Planta 43: 528–536.
6. FISKE, C. H. & Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375–400.
7. GALSTON, A. W. & L. Y. DALBERG. 1954. The adaptive formation & physiological significance of indole acetic acid oxidase. Am. J. Botany 41: 373–380.
8. HASTINGS, J. W. 1959. Unicellular clocks. Ann. Rev. Microbiol. 13: 297–312.
9. HASTINGS, J. W. & B. M. SWEENEY. 1957. The luminescent reaction in extracts of the marine dinoflagellate *Gonyaulax polyedra*. J. Cell. Comp. Physiol. 49: 209–226.
10. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, & R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
11. MADISON, J. H. 1956. The intracellular location of phosphorylase in tobacco. Plant Physiol. 31: 387–391.
12. MUKERJI, S. K., G. G. SANWAL, & P. S. KRISHNAN. 1961. Diurnal variation in transaminase activity in the cactus plant. Nature 191: 516–517.
13. RACKER, E. & E.A.R. SCHROEDER. 1958. The reductive pentose phosphate cycle. II. Specific C-1 phosphatases for fructose-1,6-diphosphate & sedoheptulase-1,7-diphosphate. Arch. Biochem. Biophys. 74: 326–344.
14. RONGINE DE FEKETE, M. A., L. F. LELOIR, & C. E. CARDINI. 1960. Mechanism of starch biosynthesis. Nature 187: 918–920.
15. SANWAL, G. G. & P. S. KRISHNAN. 1960. Diurnal variation in aldolase & phosphatase in the cactus plant. Nature 188: 664–665.
16. SIBLEY, J. A. & A. L. LEHNINGER. 1949. Determination of aldolase in animal tissues. J. Biol. Chem. 176: 233–234.
17. SMILLIE, R. M. 1960. Role of alkaline fructosediphosphatase in photosynthesis. Nature 187: 1024–1025.
18. SRIVASTAVA, L. M. & P. S. KRISHNAN. 1961. Distribution of starch phosphorylase in the tapioca plant (*Manihot utilissima*). Enzymologia 23: 270–279.
19. SRIVASTAVA, L. M. & P. S. KRISHNAN. 1961. Localization of starch phosphorylase in the leaves of the tapioca plant (*Manihot utilissima*). J. Sci. Ind. Res. India. 20 C: (In press)
20. STUMPF, P. K. 1948. Carbohydrate metabolism in higher plants. (i) Pea aldolase. J. Biol. Chem. 176: 233–241.
21. SWEENEY, B. M. & J. W. HASTINGS. 1957. Characteristics of the diurnal rhythm of luminescence in *Gonyaulax polyedra*. J. Cell. & Comp. Physiol. 49: 115–128.
22. TOLBERT, N. E. & M. S. COHAN. 1953. Activation of glycolic acid oxidase in plants. J. Biol. Chem. 204: 639–648.
23. TOMBESI, L. 1951. Glutathione & ascorbic acid content & oxidase & catalase activity on the leaves of *Nicotina tabaccum* var. Virginia bright leaf during vegetative cycle & subsequent curing, as functions of soil moisture. II. Tabacco 55: 15–25.
24. TONHAZY, N. E., N. G. WHITE, & W. W. UMBREIT. 1950. Estimation of glutamic aspartic transaminase in tissues & its application to radiation sickness. Arch. Biochem. 28: 36–42.
25. VENTER, J. 1956. Die periodizität der variierung in amylaseakitivität. Z. Botan. 44: 59–76.