Phytochemistry, 1970, Vol. 9, pp. 2097 to 2104. Pergamon Press. Printed in England.

DIURNAL VARIATIONS IN ENZYMIC ACTIVITIES IN SUBCELLULAR FRACTIONS OF CACTUS PHYLLOCLADES

A. A. KHAN, C. P. TEWARI, P. S. KRISHNAN and G. G. SANWAL

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

(*Received* 28 *January* 1970)

Abstract—The activities of phosphopyruvate carboxylase (E.C. 4.1.1.31), malic enzyme (malate dehydrogenase (decarboxylating) E.C. 1.1.1.40), selected enzymes of TCA cycle and electron transport chain were determined in mitochondrial and supernatant fractions isolated from phylloclades of the cactus *Nopalea dejecta*, collected at noon and midnight. There were significant and consistent increases in the activities of mitochondrial and supernatant phosphopyruvate carboxylase, mitochondrial aconitate hydratase (E.C. 4.2.1.3) and soluble malate dehydrogenase (E.C. 1.1.1.37) and decreases in mitochondrial malate dehydrogenase and fumarate hydratase (E.C. 4.2.1.2) and in both mitochondrial and soluble malic enzyme in the midnight samples. Of the respiratory chain enzyme complexes tested, cytochrome *c* oxidase (E.C. 1.9.3.1) and succinate cytochrome *c* reductase had higher activities at noon, while reduced NAD-cytochrome *c* reductase and reduced NAD dehydrogenase (E.C. 1.6.99.3) activities were higher at midnight. The rate of succinate oxidation and coupled phosphorylation also varied, with a maximum at noon. The diurnal pattern of alteration in enzymic activities has a bearing on crassulacean acid fluctuation.

INTRODUCTION

MEMBERS of *Cactaceae* exhibit the peculiar metabolic behaviour of accumulating organic acids in their tissues in the dark.[1-3] The dark carbon dioxide fixation by the stem and root of cactus leads to the synthesis of malic acid as the primary product, together with smaller amounts of citric, succinic, aspartic and glutamic acids.[4] Whereas a considerable amount of information is available regarding the enzymatic mechanism of carbon dioxide fixation,[5-6] very little seems to be known about the roles of enzymes in bringing about the diurnal variations in acids in succulent plants.

In an earlier communication, Khan and Sanwal[3] reported diurnal variations in the activities of various tricarboxylic acid (TCA) cycle enzymes and glucose-6-phosphate dehydrogenase in homogenates of cactus phylloclades collected at various hours of the day and night and pointed out that these were in keeping with the acid "accumulation" in the night and deacidification in the day. Mukerji,[7] from this laboratory, studied the 4-hourly variations in the activities of malic enzyme and phosphopyruvate carboxylase in homogenates of cactus. In view of the plural or multiple localization of some enzymes, a study of diurnal pattern of enzymic activity should take into account the changes occurring in the individual subcellular fractions. The present communication deals with diurnal changes in the enzymic activity of the mitochondrial and supernatant fractions prepared from phylloclades of the cactus *Nopalea dejecta*. The results are interpreted in relation to crassulacean acid metabolism.

[1] T. A. BENNET-CLARK, *New Phytologist* **32**, 37 (1933).
[2] G. G. SANWAL and P. S. KRISHNAN, *Nature* **187**, 918 (1960).
[3] A. A. KHAN and G. G. SANWAL, *Indian J. Biochem.* **2**, 207 (1965).
[4] I. P. TING and W. M. DUGGER, *Botan. Gaz.* **129**, 9 (1968).
[5] D. A. WALKER and J. M. A. BROWN, *Biochem. J.* **67**, 79 (1957).
[6] D. A. WALKER, *Biochem. J.* **74**, 216 (1960).
[7] S. K. MUKERJI, *Indian J. Biochem.* **5**, 62 (1968).

RESULTS

Localization of Enzymes

The results obtained in typical experiments for the distribution of the TCA enzymes, cytochrome *c* oxidase and phosphopyruvate carboxylase are recorded in Table 1.

About 80 per cent of the recovered activity of the marker enzyme, cytochrome *c* oxidase, was contained in the mitochondrial fraction, the supernatant fraction being devoid of the activity. About 65 per cent of the total activity of succinate dehydrogenase recovered was concentrated in the mitochondrial fraction. All the other enzymes of TCA cycle were recovered in a higher proportion from the supernatant than from the mitochondrial fraction, the recovery of malate dehydrogenase and NADP-linked isocitrate dehydrogenase being the highest. Eighty-four per cent of phosphopyruvate carboxylase activity of the homogenate was also recovered from the supernatant fraction; the mitochondrial fraction contained only 8 per cent of the activity in homogenate.

Diurnal Variations in Mitochondrial Activity

Tender phylloclades collected at noon and midnight were immediately used in the isolation of mitochondria. Although there occurred a 3-fold increase in the total acidity in the midnight samples of phylloclades,[2] the buffering capacity of the medium was adequate to give neutral homogenates.

The mitochondrial preparations were analysed for the oxidation of succinate with coupled phosphorylation. The results obtained in three experiments are recorded in Table 2. No variation was observed in protein recovered in the mitochondrial fractions. The mitochondria isolated at midnight showed a lowered rate of oxidation of succinate and of phosphorylation. The oxidation was lowered by 36–40 per cent and phosphate esterification by 30–38 per cent. The P/O ratio was essentially unaltered.

Cytochrome *c* oxidase and succinate cytochrome *c* reductase activities were respectively 25–31 per cent and 30–31 per cent lower at midnight than at noon (Table 3). In contrast, the activities of reduced NAD cytochrome *c* reductase and reduced NAD dehydrogenase were respectively 27–36 per cent and 102–113 per cent higher at midnight than at noon.

The activities of selected enzymes of TCA cycle, malic enzyme and phosphopyruvate carboxylase in mitochondrial preparations isolated at noon and midnight are recorded in Table 4. Also included are the data for malate dehydrogenase, malic enzyme and phosphopyruvate carboxylase present in the supernatant fraction obtained on sedimentation of mitochondria (15,000 *g*, 30 min).

Aconitate hydratase activity was 56–100 per cent higher at midnight. In contrast, fumarate hydratase activity decreased by 41–51 per cent in the midnight samples of mitochondria. Whereas mitochondrial malate dehydrogenase activity was reduced by 50–86 per cent, malate dehydrogenase activity in the 15,000 *g* supernatant fraction of the homogenate increased by 123–138 per cent at midnight as compared to noon. Malic enzyme activity was higher at noon; the activity at midnight was lowered 20–22 per cent in the mitochondrial fraction and 44 per cent in the supernatant fraction. Phosphopyruvate carboxylase activity was at least 2-fold higher at midnight, both in the mitochondrial and supernatant fractions, as compared with those at noon.

DISCUSSION

During recent years, three groups of workers have attempted to explain crassulacean acid fluctuation in terms of enzymatic activity. Ting and Duggar[4] studied the non-autotrophic metabolism of $^{14}CO_2$ and associated acid accumulation in *Opuntia ramosissima*. Malic acid

TABLE 1. INTRACELLULAR DISTRIBUTION OF TCA CYCLE ENZYMES, CYTOCHROME OXIDASE AND PHOSPHOPYRUVATE CARBOXYLASE IN CACTUS PHYLLOCLADES

Subcellular fraction	Enzyme activity* (proportion of whole homogenate (%))								
	AH	IDH-P	IDH	OGD	SDH	FH	MDH	CO	PC
Debris fraction and starch	6·1 (0·032)†	8·4 (0·044)	6·0 (0·001)	8·4 (0·019)	9·2 (0·020)	10·1 (0·105)	7·4 (0·067)	10·3 (0·008)	6·0 (0·018)
Chloroplast	10·3 (0·089)	7·6 (0·053)	7·6 (0·026)	12·2 (0·039)	6·1 (0·018)	6·7 (0·115)	8·1 (0·088)	11·0 (0·021)	1·7 (0·006)
Mitochondria	27·4 (0·118)	19·4 (0·066)	33·6 (0·058)	27·9 (0·050)	65·3 (0·110)	20·4 (0·178)	19·6 (0·090)	83·5 (0·80)	8·3 (0·016)
Supernatant	57·1 (0·083)	72·0 (0·076)	36·1 (0·019)	50·0 (0·033)	28·9 (0·018)	63·3 (0·174)	72·0 (0·099)	0 (0)	84·2 (0·054)

* AH—Aconitate hydratase; IDH-P—isocitrate dehydrogenase (NADP-linked); IDH—isocitrate dehydrogenase (NAD-linked); OGD—oxoglutarate dehydrogenase; SDH—succinate dehydrogenase; FH—fumarate hydratase; MDH—malate dehydrogenase; CO—cytochrome oxidase; and PC—phosphopyruvate carboxylase.

† The figures in parentheses indicate the specific activities of the enzymes.

TABLE 2. DIURNAL ACTIVITY OF CACTUS MITOCHONDRIA FOR SUCCINATE OXIDATION AND COUPLED PHOSPHORYLATION

Hr of sample	Temp. inside phylloclades (deg)	Protein (mg/g fr. wt.)	μ-atom/mg Protein		P/O	Decrease at midnight (%)	
			O_2 Uptake	P_i Esterified		O_2 Uptake	P_i Esterified
Noon	48	0·97	2·15	3·03	1·41		
Midnight	28	0·97	1·29	1·90	1·47	40·0	37·6
Noon	47	1·03	1·48	2·04	1·38		
Midnight	30	1·03	0·94	1·42	1·50	37·5	30·4
Noon	47	1·16	1·74	2·57	1·48		
Midnight	31	1·16	1·12	1·78	1·59	35·6	30·7

TABLE 3. DIURNAL VARIATIONS IN THE ACTIVITIES OF RESPIRATORY CHAIN ENZYMES

Enzyme	Spec. act. (units/mg protein) Noon	Midnight	Increase (+) or decrease (−) at midnight (%)
Cytochrome *c* oxidase	0·065	0·046	(−) 30
	0·048	0·033	(−) 31
	0·052	0·039	(−) 25
	0·039	0·027	(−) 31
Succinate-cytochrome *c* reductase	0·033	0·023	(−) 30
	0·026	0·018	(−) 31
	0·031	0·022	(−) 30
	0·027	0·019	(−) 30
Reduced NAD-cytochrome *c* reductase	0·028	0·037	(+) 32
	0·026	0·033	(+) 27
	0·043	0·057	(+) 33
	0·031	0·042	(+) 36
Reduced NAD dehydrogenase	0·060	0·128	(+) 113
	0·093	0·193	(+) 108
	0·063	0·127	(+) 102

TABLE 4. DIURNAL VARIATIONS IN THE ACTIVITIES OF TCA CYCLE ENZYMES, MALIC ENZYME AND PHOSPHOPYRUVATE CARBOXYLASE IN MITOCHONDRIA AND SUPERNATANT FRACTIONS

	Spec. act. (units/mg protein) Noon	Midnight	Increase (+) or decrease (−) at midnight (%)
Mitochondrial enzymes			
Aconitate hydratase	0·071	0·142	(+) 100
	0·160	0·250	(+) 56
Fumarate hydratase	0·094	0·048	(−) 41
	0·168	0·082	(−) 51
Malate dehydrogenase	0·042	0·006	(−) 86
	0·104	0·050	(−) 52
	0·110	0·055	(−) 50
Malic enzyme	0·036	0·028	(−) 22
	0·055	0·042	(−) 20
Phosphopyruvate carboxylase	0·013	0·028	(+) 115
	0·017	0·044	(+) 159
Supernatant enzymes			
Malate dehydrogenase	0·160	0·381	(+) 138
	0·161	0·364	(+) 123
Malic enzyme	0·216	0·120	(−) 44
Phosphopyruvate carboxylase	0·055	0·110	(+) 100
	0·057	0·130	(+) 128

not only accumulated, but turned over at a relatively rapid rate. The steady-state decarboxylation proceeded at a much greater rate in the dark than in the light. The authors concluded that acid accumulated in the dark since carboxylation exceeded decarboxylation; in

the light much of the available CO_2 was assimilated photosynthetically so that non-autotrophic carboxylation was slower than steady-state decarboxylation.

Wilkins[8] concluded that the rhythm of dark fixation of CO_2 in the leaves of *Bryophyllum fedtschenkoi* was due to the *in vivo* periodic fluctuation in activity of phosphopyruvate carboxylase. The activity of this enzyme, when extracted periodically, was high at all times and showed no rhythmic variation. It was postulated that the enzyme activity was regulated *in vivo* by an inhibitor such as citric acid, whose concentration was altered by metabolism or transport to a new intracellular location such as the vacuole.

Brandon[9] claimed to have demonstrated the occurrence in mitochondria of *B. tubiflorum* the enzymes and systems needed for the synthesis of malic acid from glucose-6-phosphate in a series of reactions involving double carboxylation. He then studied the temperature effect on the activities of enzymes singly and in mixture and concluded that acid accumulated as a result of dominance of acid-synthesizing enzymes at low temperature and decreased due to dominance of acid-utilizing enzymes at high temperature. However, the response of mitochondrial enzyme activities to temperature changes *in vitro* fitted in with the diurnal pattern of acid fluctuation only if varying amounts of malate were present in the assay system at the commencement. Possibly, *in vivo*, this variation is brought about by transport of malate from mitochondria to storage pools and vice versa.

The data obtained by the present authors for the activities of mitochondrial and supernatant fractions isolated at noon and at midnight permit a rational explanation of the crassulacean metabolism of the plant. A basic assumption has been made that the enzymes involved are present in limiting amounts and are rate limiting.

Considerable amounts of oxaloacetate become available in the night by the β-carboxylation reaction in the cytoplasm, since phosphopyruvate carboxylase occurs predominantly in the soluble fraction of cactus phylloclades. Phosphopyruvate itself is synthesized in cytoplasm from carbohydrates by the glycolytic series of reactions.[10] The doubling of soluble phosphopyruvate carboxylase activity at midnight could result in large amounts of oxaloacetate because the carbon dioxide fixation by way of phosphopyruvate carboxylase is essentially an irreversible reaction.[11] Since there is a similar increase in soluble malate dehydrogenase activity and there would be a high potential concentration of NADH owing to the continued formation of 3-phosphoglycerate by glycolytic reactions,[2] malate would be formed reductively from oxaloacetate in the cytoplasm. Malate would be stored in the vacuoles,[12] inaccessible to phosphopyruvate carboxylase so that feedback inhibition of the enzyme would not occur. Oxaloacetate does not exert any inhibitory effect on soluble malate dehydrogenase.[13] The mitochondria would not be a suitable site for malate synthesis in cactus phylloclades, since at night mitochondrial phosphopyruvate carboxylase (representing only a small proportion of the total activity) and malate dehydrogenase activities were considerably reduced; also the doubling in the activity of reduced NAD dehydrogenase might render NADH a rate-limiting factor for the reaction.

Malic enzyme was reduced in activity both in the soluble and mitochondrial fractions at night. This will limit the utilization of malate formed in cytoplasm or any formed in mitochondria.

[8] M. B. WILKINS, *Planta* **72**, 66 (1967).
[9] P. C. BRANDON. *Plant Physiol.* **42**, 977 (1967).
[10] J. BONNER and J. E. VARNER, *Plant Biochemistry*, p. 213, Academic Press, New York (1965).
[11] R. S. BANDURSKI, *J. Biol. Chem.* **217**, 137 (1955).
[12] S. L. RANSON and M. THOMAS, *Ann. Rev. Plant Physiol.* **11**, 81 (1960).
[13] A. DELBRUCK, H. SCHIMASSEK, K. BARTSCH and T. BUCHER, *Biochem. Z.* **331**, 297 (1959).

The increased activity of mitochondrial aconitate hydratase in the night time would promote the turnover of increased amounts of citrate and isocitrate. Isocitrate may be expected to be rapidly transformed along the cycle in view of the increased availability of NAD (owing to oxidation of NADH by reduced NAD dehydrogenase) and NADP (owing to reduced utilization by malic enzyme). Since succinic dehydrogenase activity is apparently lowered (which is consistent with the lowering noticed in succinate oxidase and succinate cytochrome *c* reductase), an accumulation of succinic acid would occur. It may be pointed out that Ting and Dugger[4] observed the accumulation of citric and succinic in addition to malic acid during the dark carbon dioxide assimilation in cactus.

Oxaloacetate formation by β-carboxylation was diminished in the day time both in the cytoplasm and mitochondria, since phosphopyruvate carboxylase activity was decreased. Malate formation was also considerably reduced in cytoplasm in the day-time because of decreased soluble malate dehydrogenase activity. However, increased mitochondrial malate dehydrogenase, fumarate hydratase and succinate dehydrogenase (owing to increased succinate oxidase and succinate cytochrome *c* reductase) would lead to rapid rate of malate oxidation in mitochondria in spite of decreased aconitate hydratase activity. Malate would be brought to the mitochondria from the storage pool.

A contributing factor to malate utilization is the increased malic enzyme activity in the cytoplasm and mitochondria. The increased cytoplasmic malic enzyme would promote malate transformation to pyruvate at a rapid rate at noon. The NADPH formed during malate oxidation could transfer its H to NAD and the NADH could penetrate mitochondria, or be made available to the mitochondria by the operation of a malate dehydrogenase-mediated shuttle mechanism.[14,15]

Pyruvate formed either in the mitochondria or outside would be oxidized via the regular Krebs cycle and respiratory chain.

In essence, the scheme postulated pictures malate formation at night as taking place largely in the supernatant fraction by malate dehydrogenase action on oxaloacetate. Malate utilization occurs in the day-time in the mitochondria through the Krebs cycle, aided in part by the action of malic enzyme. The utilization may also be initiated in the cytoplasm through the activity of malic enzyme, the pyruvate being transferred to mitochondria for total oxidation. The hypothesis makes use of the concepts of enzyme activity, enzyme localization, enzyme activity, activity of inhibitors and compartmentation of substrate within the cytoplasm itself.

EXPERIMENTAL

Sampling of tissues. Tender phylloclades of *Nopalea dejecta* Salm Dyck, the same age physiologically and growing under similar conditions of moisture, temperature, light and darkness in the University campus, were marked off. Twelve to fifteen phylloclades were collected for each sample at noon and midnight. The samples were thoroughly washed with H_2O, sliced and pooled. A 25-g sample was used for the isolation of mitochondria.

Isolation of cell fractions. The dispersion medium was after Wiskich and Bonner.[16] The procedure for the isolation of cell fractions was the same as described earlier.[17]

Enzyme activity determination. Enzyme assays were carried out as described earlier.[17] In studies on the intracellular distribution of enzymes, spectrophotometric measurements were made after deproteinization of

[14] N. O. Kaplan, *Bacteriol. Rev.* **27**, 155 (1963).
[15] T. Ragland and D. P. Hackett, *Arch. Biochem. Biopyhs.* **108**, 479 (1964).
[16] J. T. Wiskich and W. D. Bonner, *Plant Physiol.* **38**, 594 (1963).
[17] A. A. Khan, C. P. Tewari, P. S. Krishnan and G. G. Sanwal, *Phytochem.* **9**, 1423 (1970).

the homogenates and subcellular fractions with EtOH–Na_2SO_4.[18] Units of enzyme activities are μmoles substrates transformed per min under the assay conditions at 30°. The specific activity of an enzyme is unit of activity per mg protein.

Oxidative phosphorylation was measured as described in the previous communication.[17]

Other estimations. Orthophosphate estimation was according to Lowry and Lopez[19] and protein according to Lowry *et al.*[20] All biochemicals used were from Sigma Chemical Co., U.S.A.

Acknowledgement—The authors are grateful to the Council of Scientific and Industrial Research, New Delhi, for financing the Unit on Plant Metabolism.

[18] G. E. GLOCK and P. MCLEAN, *Biochem. J.* **55**, 400 (1953).
[19] O. H. LOWRY and J. A. LOPEZ, *J. Biol. Chem.* **162**, 421 (1946).
[20] O. H. LOWRY, N. J. ROSEBROUGH, A. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).