Phytochemistry, 1974, Vol. 13, pp. 1695 to 1702. Pergamon Press. Printed in England.

MALIC ENZYME IN THE LEAVES OF *BRYOPHYLLUM DAIGREMONTIANUM*

JEANNINE GARNIER-DARDART and ORLANDO QUEIROZ*
Laboratoire de Physiologie Cellulaire Végétale, associé au C.N.R.S.,
Université de Paris XI, Centre d'Orsay Bât. 430, 91405–Orsay, France

(*Received* 4 *September* 1973)

Key Word Index *Bryophyllum daigremontianum*; Crassulaceae; acid metabolism; malic enzyme; regulation by substrate.

Abstract—Malic enzyme (E.C. 1.1.1.40) was purified 125-fold from the leaves of *Bryophyllum daigremontianum*, and shown to be activated allosterically by its substrate.

INTRODUCTION

MALIC enzyme (L-malate: NADP oxidoreductase decarboxylating E.C. 1.1.1.40) is thought to play an important role in Crassulacean acid metabolism and in C_4-photosynthesis. The presence of the enzyme has been reported in mitochondria of *Bryophyllum tubiflorum* leaves,[1] in chloroplasts of *B. daigremontianum*[2] and in bundle-sheath chloroplasts of *Zea Mays*.[3] In *Opuntia*, malic enzyme activity was shown in chloroplasts of the phylloclades[4] and three isoenzymes, identified as mitochondrial, chloroplastic and cytoplasmic forms, were isolated from the stem.[5] To our knowledge, no allosteric effects have been reported on plant malic enzyme; studies with *Pennisetum purpureum* have shown non-allosteric properties for the enzyme.[6] On the contrary, allosteric control of malic enzyme was reported in *Escherichia coli*.[7,8]

This paper reports allosteric activation of malic enzyme from the leaves of *Bryophyllum daigremontianum*. The regulatory role of malate on the activity of the enzyme in Crassulacean acid metabolism is discussed.

RESULTS

Malic enzyme has been separated from malate dehydrogenases and 6-phosphate gluconate dehydrogenase (see experimental). Specific activities after elution from the Biogel column were about 2 μmol NADP reduced/min/mg protein for malic enzyme, 35 μmol NADH oxidized/min/mg protein for NAD-malic dehydrogenase, and 2 μmol NADPH oxidized/min/mg protein for NADP-malic dehydrogenase. 6P-gluconate dehydrogenase was almost completely eliminated by precipitation with ammonium sulfate.

* Laboratoire du Phytotron, C.N.R.S., 91190—Gif-sur-Yvette, France.

[1] BRANDON, P. C. (1963) *Kld. Nederl. Akad. van Wetensch., Amsterdam, Proc. Serv. C* **66,** 406.
[2] GARNIER-DARDART, J. (1965) *Physiol. Vég.* **3,** 215.
[3] SLACK, C. R., HATCH, M. D. and GOODCHILD, D. J. (1969) *Biochem. J.* **114,** 489.
[4] MUKERJI, S. K. and TING, I. P. (1968) *Phytochemistry* **7,** 903.
[5] MUKERJI, S. K. and TING, I. P. (1968) *Biochim. Biophys. Acta* **167,** 239.
[6] COOMBS, J., BALDRY, W. and BUCKE, C. (1973) *Planta* **110,** 109.
[7] SANWAL, B. D., WRIGHT, J. A. and SMANDO, R. (1968) *Biophys. Biochem. Res. Commun.* **31,** 623.
[8] SANWAL, B. D. and SMANDO, R. (1969) *J. Biol. Chem.* **244,** 1824.

The highest activity observed for purified malic enzyme was 5 μmol NADP reduced/min/mg protein, and the enzyme was purified about 125 times. Activity was free of malate dehydrogenase activity and no reaction occurred if $MgCl_2$ was omitted. NADP reduction was followed for at least 20 min: there was a linear relationship between increase in absorbance at 340 nm and time. No oxidation was found and there was no evidence that reaction products have any effect on the enzyme.

Effect of pH on malic enzyme activity

Figure 1(a) shows the effect of different pH values on the activity of the enzyme measured at 26° in presence of 0·33 mM NADP and different concentrations of malate. For malate concentrations between 0·33 and 3·33 mM, pH optimum was between 6·85 and 7·25; for higher values, maximum activity is attained at pH 7·25 and broadens up to pH 8·25. The activity was not modified by the composition of the buffer. Changes in V_{max} and in apparent $1/K_m$ for malate are shown in Fig. 1(b). Affinity increases with pH up to 6·45, then stays constant for a range of pH between 6·45 and 7·25 (K_m = 0·59 mM) and decreases over 7·25 (K_m = 2·1 mM at pH 7·9 and 6·66 mM at pH 8·65). V_{max} increases with the pH up to 6·85 and then stabilizes. Optimum pH for the enzyme ranges from 6·85 to 7·25 for all the malate concentrations.

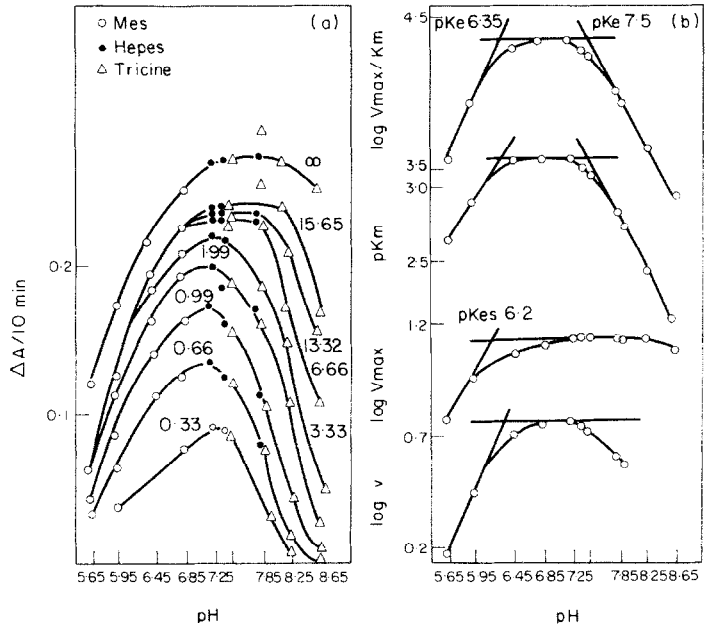

FIG. 1. (a) EFFECT OF pH ON ENZYME ACTIVITY AT DIFFERENT MALATE CONCENTRATIONS RANGING FROM 0·33 TO 15·65 mM.
(b) VARIATION OF V_{max}, v, K_m AND V_{max}/K_m AS A FUNCTION OF pH.

Variations expressed as log $1/K_m$, log V_{max}, log V_{max}/K_m and log v (activity at low substrate concentration) as a function of pH afford an evaluation of pK values for the enzyme-substrate complex and for free enzyme (Fig. 1b). It is suggested that the imidazole group could play a role in the enzyme-substrate complex (pK for the imidazole group: 5·6–7 at 26°) and that the optimum pH is around 6·9 (mean value between 6·3 and 7·45). The fact

that a single pK value is found for the free enzyme would suggest that there is no formation of intermediary complexes.

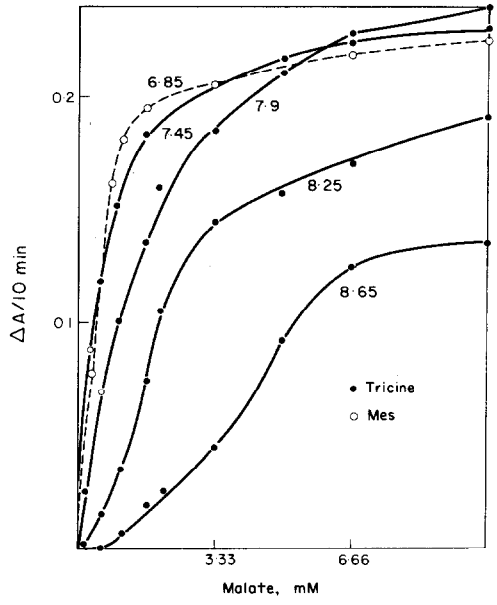

FIG. 2. EFFECT OF pH ON THE ACTIVITY OF MALIC ENZYME.
Rate, measured as change in absorption, vs malate concentration. pH ranges from 6·85 to 8·65; NADP 0·33 mM; $MgCl_2$ 6·66 mM.

At pH values higher than 8 (Fig. 2), sigmoidal kinetics in enzyme activity are apparent for malate concentrations ranging from 0·33 to 6·66 mM with a corresponding Hill number of about 2 (Fig. 3). At lower pH, this was not observed (n from 0·94 to 1·1). But a more detailed study at lower malate concentrations, below 3·3 mM (Fig. 4), shows that under these conditions kinetics are also sigmoid at optimum pH (pH 6·9). These results suggest the presence of two binding sites for malate and also that eventual cooperativity effects could be masked at optimum pH, when concentration in malate is too high. A clear S-shape appears at pH 6·9 for low malate and coenzyme concentrations (Fig. 4).

Effect of substrate, coenzyme and cofactor on the activity of malic enzyme

Effect of $MgCl_2$ and NADP on the activity of malic enzyme as a function of malate concentration. This study was carried out at pH optimum (pH 6·9) and at 21°. Activity as a function of malate concentration follows sigmoid kinetics (Fig. 4). The threshold concentration for measurable enzyme activity depends on the concentration of NADP and is lower than 66 and 166 μM for 330 and 8 μM NADP respectively. These curves show a higher cooperativity for malate at lower NADP and malate concentrations.

Double reciprocal plots show clear non-linearity, suggesting cooperativity for malate. Variation in NADP or in $MgCl_2$, in the ranges utilized, did not modify the K_m for malate: K_m = 0·82 mM at pH 6·9, 21°. At 26°, the K_m is 0·59 mM at pH 6·9 and 2·1 mM at pH 7·9.

For $MgCl_2$ concentration of 6·6 mM, n changes slightly according to the concentration in NADP: n is 1·2 for 0·33 mM NADP and 1·4 for 0·033 mM. With 0·33 mM NADP and 1·66 mM $MgCl_2$, n is 1. At the same NADP concentration (0·33 mM), the effect of two concentrations of $MgCl_2$ suggests that cooperativity for malate increases with cofactor concentration.

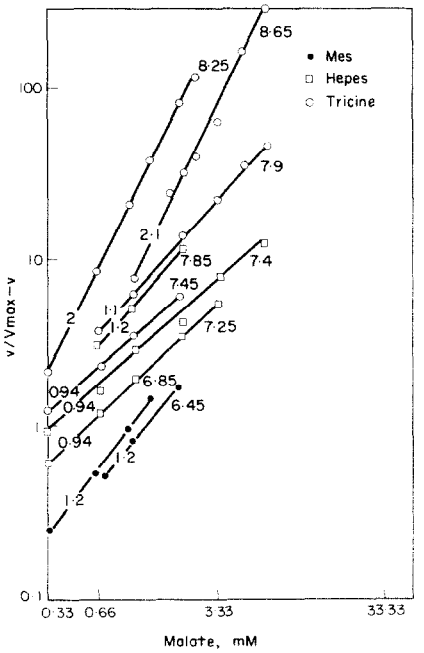

FIG. 3. EFFECT OF pH ON THE ACTIVITY OF MALIC ENZYME.
Log–log plots of v/V_{max}-v vs malate concentration. pH ranges from 6·45 to 8·65; NADP 0·33 mM; $MgCl_2$ 6·66 mM.

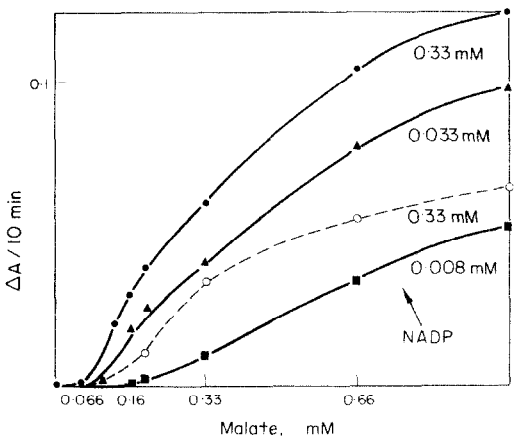

FIG. 4. EFFECT OF NADP AND $MgCl_2$ ON THE ACTIVITY OF MALIC ENZYME AS A FUNCTION OF MALATE CONCENTRATION.
Rate vs concentration in malate. NADP concentration ranges from 0·008 to 0·33 mM; $MgCl_2$ is 1·66 mM (– – –) or 6·66 (——); pH 6·9; temperature: 21°; K_m = 0·82 mM.

Effect of $MgCl_2$ and malate on the activity of malic enzyme as a function of NADP concentration. Kinetics show no sigmoidicity in the range of concentrations considered in Fig. 5. Changes in malate concentration do not affect the affinity for NADP (Fig. 6): K_m is 6 μM either in presence of 0·66 or 3·33 mM malate (6·66 mM $MgCl_2$, pH 6·9, 21°); at pH 7·9 and at 26° (6·66 mM $MgCl_2$), K_m is 20 μM. From reciprocal plots, it appears that the binding of NADP and of malate on the enzyme are independent. On the contrary, the binding of $MgCl_2$ and NADP would be ordered. n is about 1 for 6·66 mM $MgCl_2$ and 3·33 mM malate, and it reaches about 1·4 when the concentration in one of them is lowered. It therefore appears that in *Bryophyllum* the enzyme has two binding sites for NADP, and that binding of NADP and of malate are independent.

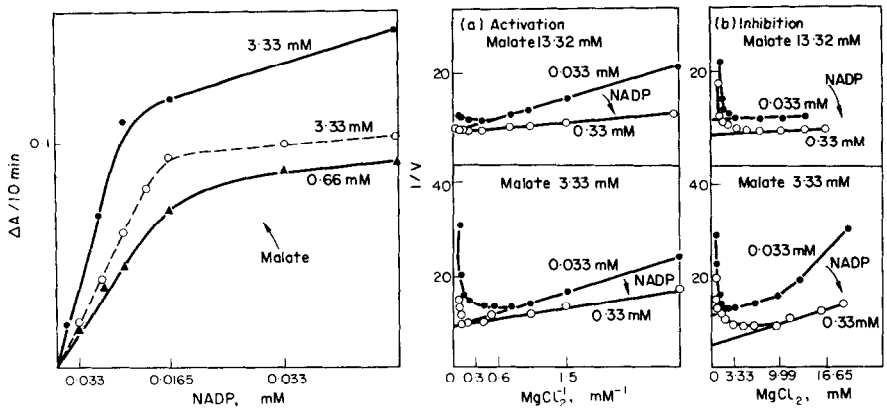

FIG. 5. EFFECT OF MALATE AND $MgCl_2$ ON THE ACTIVITY OF MALIC ENZYME AS A FUNCTION OF NADP CONCENTRATION.

Rate vs concentration in malate. Malate concentration ranges from 0·66 to 3·33 mM; $MgCl_2$ is 1·66 mM (– – – –) or 6·66 mM (——); pH 6·9, temperature: 21°; K_m = 0·006 mM.

FIG. 6. EFFECT OF NADP (AT A FIXED MALATE CONCENTRATION) ON THE ACTIVITY OF MALIC ENZYME AS A FUNCTION OF $MgCl_2$ CONCENTRATION.

(a) Activation, reciprocal of rate vs reciprocal of concentration in $MgCl_2$. (b) Inhibition, reciprocal of rate vs concentration in $MgCl_2$. pH 7·9, temperature: 26°.

Effect of NADP and malate on the activity of malic enzyme as a function of $MgCl_2$ concentration. Enzyme activity appears only when $MgCl_2$ concentration exceeds 0·66 mM in the presence of 0·66 mM Na_2–EDTA. The chelating agent would act as an inhibitor relative to the Mg^{2+} ion which appears as a necessary cofactor for the operation of the enzyme. Mg^{2+} would act as an activator at low concentrations and as an inhibitor at high concentrations; the range of optimum concentrations is more or less broad, depending on substrate and coenzyme concentrations (Figs. 6 and 7). Increase in malate concentration from 1·66 mM to 13·32 mM produces an increase in V_{max} (Fig. 7a) and delays the inhibitory effect of high $MgCl_2$ concentrations (Fig. 7b).

Activation phase: plots of $1/v$ in Fig. 6a, compared to the reciprocal plot of Fig. 5, suggest ordered binding of NADP and $MgCl_2$, with the cofactor binding on the enzyme-NADP complex. Concentration in malate would affect this binding (Fig. 7a). Inhibition phase: in the presence of high $MgCl_2$ concentrations, there would be competitive inhibition between malate, and the cofactor (Fig. 7b) and also non-competitive inhibition of the binding of $MgCl_2$ on the enzyme-NADP complex (Fig. 6b). Results reported here suggest

that the enzyme-NADP complex is formed prior to $MgCl_2$ binding and that there would be a competitive inhibition between malate and the cofactor.

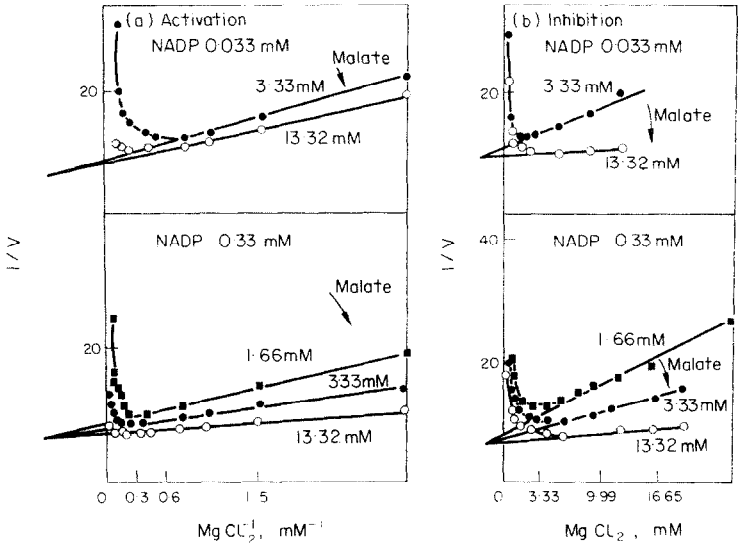

FIG. 7. EFFECT OF MALATE (AT A FIXED NADP CONCENTRATION) ON THE ACTIVITY OF MALIC ENZYME AS A FUNCTION OF $MgCl_2$ CONCENTRATION.
(a) Activation, reciprocal of rate vs reciprocal of concentration in $MgCl_2$. (b) Inhibition, reciprocal of rate vs concentration in $MgCl_2$. pH 7·9, temperature: 26°.

DISCUSSION

Malic enzyme is classically a manganese enzyme rather than a magnesium enzyme, but Mg^{2+} can replace Mn^{2+}.[9,10] Many authors used Mn^{2+} in their experiments,[5,7,8] but other authors showed that the malic enzyme of maize was 30% more active with Mg^{2+} than with Mn^{2+} at their respective optimum concentrations.[11] *B. tubiflorum* malic enzyme showed with Mg^{2+}, an activity which was 86% of the activity with Mn^{2+} in equimolar amount.[12] It must be noted that, for the two divalent cations, the optimum concentrations are not necessarily the same, as suggested by Johnson and Hatch.[11]

Malic enzyme is associated with chloroplasts[2] and products would be utilized in photosynthesis. Hence, it seemed of importance whether this enzyme is effective with Mg^{2+}, which was shown to be a cofactor with different effects: cofactor of phosphorylation,[13] factor increasing CO_2 fixation,[14,15] activator for ribulose-1,5-diphosphate carboxylase.[16,17] For these reasons, we used Mg^{2+} in our experiments. However, it should be

[9] OCHOA, S. (1955) in *Methods in Enzymology* (COLOWICK, S. P. and KAPLAN, N. O., eds.), Vol. 1, p. 739. Academic Press, New York.
[10] KUN, E. (1963) in *The Enzymes* (BOYER, P. D., LARDY, H. and MYRBÄCK, K., eds), Vol. 7, p. 157. Academic Press, New York.
[11] JOHNSON, H. S. and HATCH, M. D. (1970) *Biochem. J.* **119**, 273.
[12] BRANDON, P. C. and VAN BOECKEL-MOL, T. N. (1973) *European J. Biochem.* **35**, 62.
[13] WHATLEY, F. R., ALLEN, M. B. and ARNON, D. I. (1955) *Biochim. Biophys. Acta* **16**, 605.
[14] LIN, D. C. and PARK, S. N. (1971) *Arch. Biochem. Biophys.* **145**, 622.
[15] BALDRY, C. W. and COOMBS, J. (1973) *Z. Pflanzenphysiol.* **69**, 213.
[16] SUGIYAMA, T., NAKAYAMA, N. and AKAZAWA, T. (1968) *Biochim. Biophys. Res. Commun.* **30**, 118.
[17] BASSHAM, J. A., SHARP, P. and MORRIS, I. (1968) *Biochim. Biophys. Acta* **153**, 898.

noted that we previously observed,[2] that malic enzyme of *Bryophyllum daigremontianum* chloroplasts was effective with Mn^{2+}.

For all malate concentrations, we observed an optimum pH ranging from 6·85 to 7·25. With *Bryophyllum tubiflorum* enzyme also, maximum activity was obtained at pH 7·0–7·2.[12] In contrast, pH optimum for *Kalanchoe crenata* enzyme has been shown to depend on the malate concentration.[18] Optima around pH 6·5 were reported for the three isoenzymes of *Opuntia* stems.[5] Leaves of C_4 photosynthetic plants exhibited a more basic pH optimum: 8·0 for *Pennisetum purpureum* and, for *Zea Mays*, between 7·4 and 8·0 in presence of 0·1 to 1 mM malate and 8·5 in presence of 10 mM malate;[11] so, for *Zea mays*, the optimum pH is depending on the malate concentration. Reaction rates appear to be of the same order in *Bryophyllum daigremontianum* and in maize (1–5 μmol NADP reduced/min/mg protein). In *Bryophyllum tubiflorum*, the specific activity is 10 times higher.[12]

Affinity for NADP appears to be 100–130 times higher than affinity for malate: at pH 6·9 and 21°, K_m for malate is 0·82 mM and for NADP 6 μM; at pH 7·9 and 26°, values are 2·1 and 0·02 mM respectively. The K_m for malate values found here resemble K_m values of *Bryophyllum tubiflorum* enzyme (370 μM at pH 7·5 and 30°)[12] and *Kalanchoe crenata* enzyme (550 μM at pH 7·4);[18] but the affinity for NADP is only 25-fold higher than affinity for malate with *Bryophyllum tubiflorum*.[12] Reported values of K_m for *Opuntia*[5] are 1·5 mM for malate and 8 μM for NADP suggesting a 200-fold higher affinity for NADP. The ratio between affinity for NADP and for malate is about 90 in *Pennisetum purpureum* ($K_{m_{(NADP)}}$ = 3·7 μM, $K_{m_{(malate)}}$ = 340 μM)[6] and about 6 for maize ($K_{m_{(NADP)}}$ = 25 μM, $K_{m_{(malate)}}$ = 150 μM at pH 8).[11] This relatively strong affinity of the enzyme for NADP suggests that NADP would not be a control factor *in vivo*. Inhibition of malic enzyme by malate at pH 7·5 was shown in *Zea mays*,[11] this would not be the case with the enzyme of *Bryophyllum*.

Low NADP concentrations increase cooperativity between malate molecules, mainly at low substrate concentrations; such an effect is not observed with $MgCl_2$. The same relationships between malic enzyme and some ligands was reported for *Escherichia coli* and the same sequential association was observed: the enzyme-NADP complex is formed prior to $MgCl_2$ binding.[8]

From the present *in vitro* results, complex effects of $MgCl_2$ should be expected *in vivo*: changes in concentration could produce either increase or strong decrease in enzymic activity.

It appears that malate production *in vivo* would be controlled by its own amount in the tissues.[19] This is confirmed by recent results with *B. tubiflorum*[20] and by earlier data on malate variations in *Kalanchoe blossfeldiana*.[21] Circadian variations in malate concentration is basically the result of a two-step mechanism involving synthesis through a β-carboxylation reaction, occurring generally during the night and further depletion by decarboxylation during the day. The role of phospho-enolpyruvate carboxylase in the first step of this mechanism and its possible allosteric control by malate in a number of

[18] WALKER, D. A. (1960) *Biochem. J.* **74,** 216.
[19] DARDART, J. (1958) *Comptes Rendus Acad. Sc. Paris* **246,** 301, erratum 1785.
[20] KLUGE, M. (1969) *Planta* **88,** 113.
[21] QUEIROZ, O. (1966) *Physiol. Vég.* **4,** 323.
[22] BRADBEER, J. W., RANSON, S. L. and STILLER, M. (1958) *Plant Physiol.* **33,** 66.
[23] MOYSE, A. (1965) *Travaux Dédiés à Lucien Plantefol*, p. 21, Masson & Cie, Paris.
[24] QUEIROZ, O. (1968) *Physiol. Vég.* **6,** 117.
[25] MUKERJI, S. K. and TING, I. P. (1971) *Arch. Biochem. Biophys.* **143,** 293.
[26] COOMBS, J., BALDRY, C. W. and BUCKE, C. (1972) *Biochem. J.* **130,** 25 p.
[27] COOMBS, J., BALDRY, C. W. and BUCKE, C. (1973) *Planta* **110,** 95.

plants has been discussed by different authors.[22-27] The fact that malate depletion usually starts only in light even when maximum malate content has been attained a few hours earlier, probably implies a role of light either directly on the activity of the enzyme or indirectly on the availability of malate.[28] The amount of malate depleted during the day considered as a function of its total amount in the young leaves of *K. blossfeldiana*[21] and the daily variation in malic enzyme activity in these leaves[24] lead to the hypothesis that, besides the possible role of light, an activation of the enzyme by malate could control the mechanism.[28,29] The data reported in the present paper confirm this hypothesis. Hence in Crassulacean acid metabolism activation of malic enzyme by malate could be one of the factors contributing to the overall circadian variation in malate concentration.

EXPERIMENTAL

Extraction and purification of the enzyme. Methods are adapted from Johnson and Hatch.[11] Extracts from 3rd row leaves (120 g) of *Bryophyllum daigremontianum* grown in a greenhouse were prepared by grinding the tissues at high speed in a blender (3 × 10 sec) with 360 ml of Tris–HCl 0·1 M, calculated for a final pH between 7·5 and 8, containing 50 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 5 mM EDTA-di Na and 0·5% (w/v) PVP. The homogenate was filtered through cheesecloth and cotton and the filtrate was centrifuged at 20 000 *g* for 10 min. The supernatant was treated by $(NH_4)_2SO_4$ and the 50–55% saturation fraction was collected by centrifugation, after standing for a few hr. The ppt. was taken up in 20 ml of 50 mM Tris–HCl (pH 8) with 5 mM dithiothreitol and 2·5 mM Na_2–EDTA, brought to 70% saturation with $(NH_4)_2SO_4$. After centrifugation, the ppt. was dissolved in a small vol. of the same buffer. All operations were carried out at 4°.

A polyacrylamide (Biogel P_{200}, 100–200 mesh) column (23 × 1·1 cm) was equilibrated by washing with 25 mM Tris–HCl buffer (pH 8) containing 2·5 mM dithiothreitol and 2·5 mM Na_2–EDTA. The enzyme extract was filtered through the column with the same buffer. Fractions (1 ml) were collected and assayed for enzyme activity.

Enzyme assays. Activity was followed spectrophotometrically by measuring the reduction of NADP at 340 nm in a reaction mixture (3 ml) containing: enzyme extract (2·5–6 μg protein, 33 mM buffer (Tris–HCl, Mes–NaOH, Hepes–NaOH or tricine–NaOH, according to the desired pH), 0·66 mM Na_2–EDTA, 6·6 mM $MgCl_2$, 0·33 mM NADP and 3·3 mM malate. A blank without $MgCl_2$ allowed an evaluation of an eventual reducing activity of malate dehydrogenase.

NAD- and NADP-malate dehydrogenases and 6-P-gluconate dehydrogenase activity were measured in reaction media similar to that described above, except that appropriate substrates and nicotinamide adenine nucleotides were substituted for malate and NADP.

Protein was estimated by the method of Warburg and Christian.[30]

Acknowledgements—The authors wish to thank Dr. J. Garnier, Maître de Recherches at I.N.R.A., Laboratoire de Biochimie, Université de Paris XI, 91405-Orsay, France, for helpful discussions on the enzymatic data.

[28] QUEIROZ, O. (1972) in *Phytochrome* (MITRAKOS, M. and SHROPSHIRE, W., JR. eds), pp. 295, Academic Press, New York.
[29] MOREL, C., CELATI, C. and QUEIROZ, O. (1972) *Physiol. Vég.* **10**, 743.
[30] WARBURG, O. and CHRISTIAN, W. (1941) *Biochem. Z.* **310**, 384.