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Physiological Studies on Acid Metabolism

7. MALIC ENZYME FROM *KALANCHOE CRENATA*: EFFECTS OF CARBON DIOXIDE CONCENTRATION*

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Green parts of certain succulent plants, including many members of the Crassulaceae, show a striking diurnal fluctuation in their organic acid content. This is largely a result of an accumulation of malic acid by night and its disappearance by day (Bennet-Clark, 1933). Thomas (1947) proposed that carbon dioxide is a metabolite in acid synthesis in addition to being, as many earlier investigators believed, a product of acid breakdown. This view was substantiated by extensive physiological experiments (Thomas, 1947, 1949; Thomas & Beever, 1949; Thomas & Ranson, 1954) and by the demonstration that $^{14}\text{CO}_2$ was incorporated into a

number of acids, but principally into malate, by crassulacean plants in the dark (Thurlow & Bonner, 1948; Varner & Burrell, 1950; Thomas & Ranson, 1954).

A known reaction (1) in which carbon dioxide is fixed and malate is synthesized is that catalysed by the malic enzyme (Ochoa, Mehler & Kornberg, 1947):

(TPNH₂, reduced triphosphopyridine nucleotide, TPN, triphosphopyridine nucleotide.) This enzyme is of considerable interest in relation to crassulacean acid metabolism, for it is present in at least

* Part 6: Walker & Ranson (1958).

two crassulacean plants (Anderson, Stafford, Conn & Vennesland, 1952; Coles & Waygood, 1957). The present paper shows that it is present in cell-free extracts of *Kalanchoë crenata* and its properties are compared with those of the malic enzyme from pigeon liver (Ochoa *et al.* 1947; Rutter & Lardy, 1958) and wheat germ (Ochoa, 1955). However, although malic enzyme might be concerned in carbon dioxide fixation, evidence has been obtained (Moyse & Jolchine, 1956; Saltman, Kunitake, Spolter & Stitt, 1956; Walker, 1956, 1957; Walker & Brown, 1957) which suggests that in crassulacean plants the part played by reaction (1) in acidification might be less important than the part played by reaction (2).

The equilibrium constant for reaction (2), which is catalysed by phosphopyruvate carboxylase, has not been determined, but the reaction favours carboxylation to such an extent that it has been considered, for practical purposes, to be irreversible (Tchen & Vennesland, 1955). Part of the evidence in support of the role of phosphopyruvate carboxylase in crassulacean plants rests on the fact that this enzyme is inhibited by high concentrations of carbon dioxide (Walker & Brown, 1957). Carbon dioxide fixation by intact leaves of *K. crenata* (a member of the Crassulaceae) is similarly inhibited over a corresponding range of carbon dioxide concentration (Thomas & Ranson, 1954).

Malic enzyme from animal sources is capable of catalysing a rapid synthesis of malate in concentrations of carbon dioxide as high as 70% (Ochoa, 1951). If malic enzyme preparations from crassulacean plants behaved similarly this would suggest that this enzyme is not the main one concerned in fixation of carbon dioxide by such plants. On the other hand, if phosphopyruvate carboxylase rather than malic enzyme is the catalyst concerned this inconsistency would not arise. It seemed desirable therefore to investigate the effect of high concentrations of carbon dioxide on malic enzyme from a crassulacean plant. Extracts from *K. crenata* were selected for this purpose.

MATERIALS AND METHODS

Triphosphopyridine nucleotide (TPN), reduced triphosphopyridine nucleotide (TPNH), reduced diphosphopyridine nucleotide (DPNH), glucose 6-phosphate, glucose 6-phosphate dehydrogenase and oxaloacetic acid were from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). L- and DL-Malic acid, sodium pyruvate and lactic dehydrogenase were from L. Light and Co. Ltd., Bucks. Sodium [3-¹⁴C]-pyruvate and sodium [¹⁴C]bicarbonate were obtained from The Radiochemical Centre, Amersham, Bucks.

Enzyme. Cell-free extracts of *Kalanchoë* leaves were pre-

pared as before (Walker, 1957). The fraction of protein which precipitated between 50 and 60% saturation with (NH₄)₂SO₄ was redissolved in 0.1% 2-amino-2-hydroxy-methylpropane-1:3-diol (tris) buffer at pH 7.4 and dialysed in the cold for 24 hr. against the same buffer or against water before use. In addition to malic enzyme the enzyme preparation also contained detectable quantities of malic dehydrogenase, phosphopyruvate carboxylase and a TPNH oxidase. Of these only the TPNH oxidase interfered with the assay of malic enzyme under the experimental conditions used (see Results).

Assay. Spectrophotometric measurements of the oxidation and reduction of TPN in reaction (1) were made in a temperature-controlled room (25°) with a Unicam SP. 500 fitted with a constant-temperature cell housing maintained at 25° by water circulated from a bath. The spectrophotometer was also used to follow the oxidation of TPNH by the TPNH oxidase. Manometric measurements of oxaloacetic carboxylase activity were made with a Warburg respirometer at 25°. Reactions were started by the addition of the substrate from the side arm after equilibration for 5 min.

Pyruvate estimation. Reactions were stopped by adding mixtures to boiling water and boiling for 60 sec. The solutions were then centrifuged to remove precipitated protein. Pyruvate was determined by the oxidation of reduced DPN in the presence of lactic dehydrogenase. Lactic dehydrogenase is specific for lactate and pyruvate and under these conditions the equilibrium position is such that virtually all the pyruvate is reduced to lactate with the concomitant oxidation of an equimolar quantity of DPNH. In Fig. 1 (curve 1) the pyruvate estimated in this manner is expressed in terms of the extinction of an equimolar quantity of DPNH or TPNH to facilitate comparison with curve 2.

Estimation of triphosphopyridine nucleotide. The increase in extinction at 340 mμ after incubation with a relative excess of glucose 6-phosphate and glucose 6-phosphate dehydrogenase was used as a measure of the TPN added to reaction mixtures (see legend to Fig. 1).

Chromatography. Standard methods (see Ranson, 1955) were used. (a) Carboxylic acids: after ether extraction the acids were chromatographed on Whatman no. 1 paper with *tert.*-amyl alcohol (2-methylbutan-2-ol)-water-formic acid (3:3:1, by vol.) as the developing solution. (b) Keto acids: reactions were stopped by the addition of 2:4-dinitrophenylhydrazine in 5N-H₂SO₄. After incubation for 1 hr. the precipitated protein was centrifuged and the supernatant extracted with ethyl acetate. The ethyl acetate solution was in turn extracted with 2% (w/v) Na₂CO₃, which was then acidified and re-extracted with ethyl acetate. The final extract was evaporated to dryness *in vacuo* and redissolved in ethanol for chromatography with *tert.*-amyl alcohol-ethanol-water (50:10:40, by vol.) on Whatman no. 3 paper buffered at pH 6.2 with 0.2M-phosphate buffer. Spots were eluted in 0.2M-NaHCO₃ for determination of their u.v.-absorption spectra.

Carbon dioxide concentration. This was varied as required by the addition of CO₂-saturated NaHCO₃ at pH 7.4. Quantities were calculated from the Henderson-Hasselbalch equation (Umbreit, Burris & Stauffer, 1949) to give solution in equilibrium with the desired atmosphere of CO₂. In spectrophotometric experiments reactions were carried out in closed cells. The loss of CO₂ during the period of

measurement (2 min.) was negligible. In long-term experiments the appropriate percentage of CO_2 was used in the gas phase.

RESULTS

Oxidative decarboxylation of malate

Spectrophotometric assay. The oxidative decarboxylation catalysed by malic enzyme (the reverse of reaction 1) can be followed by measuring the increase in extinction at $340\text{ m}\mu$ associated with the reduction of TPN. Fig. 1 (curve 2) shows the course of this reaction catalysed by the cell-free extract from *Kalanchoë* leaves. Similar results have also been obtained with extracts from *Sedum acre*, *S. album* and *S. spectabile*. No reaction occurred if any one component of the reaction system was omitted, if fumarate was substituted for malate, if DPN was substituted for TPN or if the leaf extract had been boiled for 15 sec. When equimolar MgCl_2 was substituted for MnCl_2 the initial rate was decreased to 5% of that in the presence of MnCl_2 (increasing the MgCl_2 concentration fivefold increased this value to 40%). In a similar reaction mixture to that given for Fig. 1 but in the presence of a relative excess of enzyme the initial rate of the oxidative decarboxylation with DL-malate as substrate was half that with an equal

concentration of L-malate, indicating that the enzyme does not react to an appreciable extent with the D form.

Production of pyruvate. Fig. 1 (curve 1) illustrates the production of pyruvate during the oxidative decarboxylation (see Materials and Methods section). The apparent discrepancy between TPN reduction and pyruvate production during the latter part of the reaction results from the presence of a TPNH oxidase which reoxidizes a small quantity of the TPN reduced. This oxidase is TPN specific, shows diaphorase activity and is insensitive to cyanide; in these respects it is similar to a TPN diaphorase extracted from spinach chloroplasts by Avron & Jagendorf (1956, 1957). The malic enzyme in *Kalanchoë* extracts is not readily separated from this oxidase but, as Fig. 1 shows, the effect of this contaminant on the initial rate of TPNH oxidation was too small to be discernible.

The production of pyruvate during the course of the oxidative decarboxylation as indicated in Fig. 1 was also demonstrated by chemical methods. Phenylhydrazones were prepared from reaction mixtures (3 ml.) in which malate ($2\text{ }\mu\text{moles}$) had been incubated with TPN ($2\text{ }\mu\text{moles}$), MnCl_2 ($0.2\text{ }\mu\text{mole}$) and enzyme for 60 min. These were identified by their R_f values and their absorption spectra as the phenylhydrazones of pyruvic acid. No trace of oxaloacetic acid phenylhydrazones was detected.

Effect of pH. The rate of oxidative decarboxylation was determined in reaction mixtures (3 ml.) containing $1.5\text{ }\mu\text{moles}$ of L-malate, $3\text{ }\mu\text{moles}$ of MnCl_2 , $0.2\text{ }\mu\text{mole}$ (approx.) of TPN, $75\text{ }\mu\text{moles}$ of glycylglycine and $75\text{ }\mu\text{moles}$ of tris buffer at the desired pH. When the initial rate of TPN reduction was plotted against pH a sharp peak was obtained with a maximum at pH 7.2. The reaction mixtures used in these determinations were essentially similar to those used by other workers in investigating the effect of pH on malic enzyme from pigeon liver (Salles & Ochoa, 1950) and wheat germ (Harary, Korey & Ochoa, 1953). In the absence of buffer, in reaction mixtures in which the pH was maintained constant during readings by the presence of a larger quantity (10 mM) of DL-malate, adjusted to the required pH beforehand with KOH, an optimum value of 7.8 was obtained. Higher pH optima at higher substrate concentrations have also been noted with purified pigeon-liver enzyme (Rutter & Lardy, 1958).

Effects of substrate and enzyme concentration. Some effects of enzyme and substrate concentrations on the oxidative decarboxylation (reverse of reaction 1) are shown in Fig. 2, where the results are plotted as the double reciprocal (Dixon, 1953). The value of K_m obtained was 0.55 m-mole/l.

Fig. 1. Oxidative decarboxylation of malic acid. Curve 1, pyruvate produced; curve 2, TPN reduced. Each reaction mixture contained, in a final volume of 3 ml.: L-malate, $10\text{ }\mu\text{moles}$; MnCl_2 , $3\text{ }\mu\text{moles}$; TPN, to give a value of $\log I_0/I$ ($340\text{ m}\mu$) of 0.625 in the reduced state; 0.1 ml. of tris buffer (20%), pH 7.4; 0.3 ml. of enzyme. Each point on curve 1 represents the mean of four determinations.

Reductive carboxylation of pyruvate

Spectrophotometric assay. The oxidation of TPNH in reaction (1) can also be followed spectrophotometrically. In these circumstances the initial concentration of TPNH is high and its reoxidation by the TPNH oxidase in the absence of pyruvate (see, for example, Fig. 3, first 2 min.) was readily detected. However, the rate of TPNH oxidation was substantially increased when reaction (1) was started by the addition of pyruvate. Reaction (1) was readily reversed (Fig. 3, 10.5 min.) by the addition of malate.

Fig. 2. Some effects of substrate and enzyme concentration on the oxidative decarboxylation of malic acid. Each reaction mixture contained, in a final volume of 3 ml.: L-malate; TPN, $0.2 \mu\text{mole}$ (approx.); MnCl_2 , $3 \mu\text{moles}$; tris buffer, 0.1 ml. of 20% (w/v) at pH 7.4; enzyme (as indicated). S represents the concn. of L-malate in $\mu\text{moles}/3 \text{ ml.}$ and v the initial velocity.

Fig. 3. Reversibility of reaction (1). At the start the reaction mixture (3 ml.) contained: enzyme; MnCl_2 , $3 \mu\text{moles}$; TPNH, $0.2 \mu\text{mole}$ (approx.); 2.5 ml. of CO_2 -saturated sodium bicarbonate (0.32 M) at pH 7.4. Additions; at 2 min., 0.1 ml. of sodium pyruvate ($40 \mu\text{moles}$); at 10.5 min., 0.1 ml. of sodium L-malate ($10 \mu\text{moles}$).

Whilst the presence of the TPNH oxidase had no detectable effect on spectrophotometric measurements of the initial rate of oxidative decarboxylation, it did, however, preclude the accurate determination of initial rates of reductive carboxylation by similar methods. A reasonable approximation of such rates can be made if the contribution of the TPNH oxidase is allowed for (see Table 1).

Production of malate. Evidence of the production of malate during the reductive carboxylation (reaction 1) was also obtained. Radioactive pyruvate ($2 \mu\text{moles}$, $2 \mu\text{C}$) was incubated with TPNH ($2 \mu\text{moles}$), enzyme and various concentrations of CO_2 -bicarbonate in a total of 3 ml. After 40 min. the reactions were stopped by the addition of formic acid and the mixtures taken to dryness and acidified to pH 1.0 and extracted with ether. Co-chromatography of ether-soluble solids with malate then indicated the incorporation of radioactivity in this acid. Qualitative experiments were also carried out with similar reaction mixtures containing unlabelled pyruvate and labelled bicarbonate. Radioactivity was again located in the malate spot after isolation and chromatography of the end products.

Oxaloacetic carboxylase activity

Malic enzyme from pigeon liver and from some other sources has also been shown to catalyse the decarboxylation of oxaloacetate. Manganese is again required as a cofactor and the pH optimum is about 5 (Ochoa, 1955). *Kalanchoë* extracts containing malic enzyme, in the presence of Mn^{2+} ions, also brought about the production of CO_2 from oxaloacetate in excess of that produced by the Mn^{2+} -catalysed decarboxylation (Speck, 1949) alone. In reaction mixtures (3 ml.) containing *Kalanchoë* extract, oxaloacetate ($15 \mu\text{moles}$), MnCl_2 ($6 \mu\text{moles}$) and 1 ml. of 1% (w/v) cacodylic acid buffer at the required pH, the excess of decarboxylation due to the presence of a thermolabile catalyst reached a maximum at pH 5.5, when it was approx. 300% of that of the control which contained boiled extract but was otherwise identical.

Effects of carbon dioxide

Experiments by Ochoa (1951) with the pigeon-liver enzyme showed that the production of malate from pyruvate can be accelerated by increasing the concentration of CO_2 and that the highest rate of synthesis was achieved in the highest concentration (70%) of CO_2 used. In the present experiments the effects of CO_2 on both the forward and reverse reactions (1), as catalysed by malic enzyme from *K. crenata*, were studied.

Oxidative decarboxylation of malate. In Fig. 4 the reciprocals of the initial rates of the oxidative

decarboxylation are plotted against the reciprocals of malate concentration at six concentrations of CO_2 . The maximum velocity, i.e. the velocity at infinite substrate concentration, is seen to have been independent of CO_2 concentration and this would appear to rule out the possibility that CO_2 might act as a non-competitive inhibitor of malic enzyme. The family of straight lines shown in Fig. 4 is typical of the relationships which obtain in a competitive inhibition. In discussing competitive inhibition, Dixon & Webb (1958) distinguish between two types: wholly competitive and partially competitive. These are indistinguishable when the reciprocal of velocity ($1/v$) is plotted against the reciprocal of substrate concentration, but when $1/v$ is plotted against inhibitor concentration ($[I]$) the partially competitive type does not

Fig. 4. Effect of six levels of CO_2 and four levels of substrate on the oxidative decarboxylation of malate. Reaction mixtures (3 ml.) contained 10, 5, 2.5 or 1.25 μ -moles of L-malate; TPN, 0.2 μ mole (approx.); MnCl_2 , 3 μ moles; tris buffer, 0.1 ml. of 20% (w/v) at pH 7.4; 0.1 ml. of enzyme and CO_2 -saturated sodium bicarbonate at pH 7.4 to give the percentage of CO_2 indicated (see Materials and Methods section). S represents the substrate concentration in μ moles/3 ml. and v the initial velocity.

Fig. 5. Values from Fig. 1 replotted as the reciprocal of reaction rate against CO_2 concentration. $v = 10^3 \Delta \log I_0/I$ at 340 $m\mu$ between the first 30 and 60 sec. of reaction time. $[I] = \text{CO}_2$ percentage of an atmosphere.

give a straight line as is given in the fully competitive type. Fig. 5 shows the data in Fig. 4 replotted in this manner. The possible significance of these results is considered in the Discussion.

The question arises whether the effects reported in this and subsequent sections were caused by dissolved CO_2 , HCO_3^- ion or even Na^+ ion or high salt concentration. Some inhibition was observed in control experiments with KCl and NaCl, although this could never account for the total effect produced by equimolar bicarbonate saturated with CO_2 . For example, in Fig. 4 the inhibition produced by the highest concentration of CO_2 -bicarbonate, with 10 μ moles of malate as substrate, was 79%, but in similar reaction mixtures, equimolar (0.16M) NaCl and KCl both gave 39% inhibition. Whatever the nature of this latter effect it seems clear that CO_2 or bicarbonate produced a large response which could not be duplicated by either NaCl or KCl. It also follows from Fig. 4 that there was no additional salt effect of a non-competitive nature.

Reductive carboxylation of pyruvate. Accurate spectrophotometric measurements of the reductive carboxylation could not be made because of the presence of the TPNH oxidase in the extract. The oxidation of TPNH by the oxidase and malic enzyme acting together could be measured, however, and since the malic enzyme showed an absolute requirement for manganese the activity of the oxidase acting alone could be determined in reaction mixtures from which manganese was omitted. The results of such an experiment, carried out at several levels of CO_2 , are given in Table 1. The bottom row of figures, representing the difference between the two preceding rows, is believed to give reasonable approximations of the rates of the reductive carboxylation. These rates have also been plotted as a percentage of the maximum rate obtained (Fig. 6, curve 3). It will be seen that when the concentration of CO_2 was increased from 0.05% (laboratory air) to 30% the rate of carboxylation was increased correspondingly. Above 30%, further increases caused slight decrease in the rate which, at the highest CO_2 concentration used, fell to about 80% of the maximum.

The effect of CO_2 on reductive carboxylation was also measured (Table 2) by determining the quantity of labelled pyruvate incorporated into malate in a given time in the presence of the appropriate CO_2 -bicarbonate mixtures (see Materials and Methods section). This method involved chromatographic separation of the end products from the reaction mixture and the possibility of experimental error is large. On the basis of spectrophotometric experiments an incubation time of 40 min. was chosen which would provide a measurable change but would not permit any of the reactions to

Table 1. *Effects of carbon dioxide concentration on the oxidation of reduced triphosphopyridine nucleotide by malic enzyme and reduced triphosphopyridine nucleotide oxidase*

Each spectrophotometer cell contained 0.2 ml. of enzyme, 0.1 ml. of sodium pyruvate (40 μ moles) in tris buffer, pH 7.4, 0.1 ml. of TPNH (0.2 μ mole), 2.8 ml. of CO₂-saturated NaHCO₃ at pH 7.4 or water to give the required CO₂ level, in a final volume of 3.3 ml. For measurement of TPNH oxidase and malic enzyme together the mixture also contained 0.1 ml. of MnCl₂ (3 μ moles).

CO ₂ (%)	10 ³ × Decrease in log I ₀ /I (340 m μ) between 30 and 90 sec. from start									
	0-0.05	1.125	2.25	4.5	9	21	30	45	60	84
TPNH oxidase	24	24	23	22	20	20	13	11	10	8
TPNH oxidase + malic enzyme	27	38	58	68	78	90	84	80	72	66
Malic enzyme (by difference)	3	14	35	46	58	70	71	69	62	58

Table 2. *Effect of carbon dioxide concentration on the incorporation of labelled pyruvate into malate*

Each reaction mixture contained enzyme, MnCl₂ (0.3 μ mole), sodium pyruvate (2 μ c, 2 μ moles), TPNH (2 μ moles), tris buffer, pH 7.4 (1%, w/v), CO₂-saturated NaHCO₃ at pH 7.4 in equilibrium with the appropriate percentage of CO₂ in the gas phase. Mixtures were incubated for 40 min. at 25°.

CO ₂ (%)	0-0.05	1	9	30	45	60	75
10 ⁻³ × Radioactivity in malate (counts/min.)	8	21	36	62	78	115	108

Fig. 6. Comparison of the effects of CO₂ concentration on the accumulation of malate by crassulacean plants in the dark (curve 1), the carboxylation of phosphopyruvate by phosphopyruvate carboxylase (curve 2) and the reductive carboxylation of pyruvate by malic enzyme (curve 3). Curve 1, data from Thomas & Ranson (1954); curve 2, data from Walker & Brown (1957); curve 3, data from Table 1.

proceed to completion. The figures in Table 2 are therefore not in any sense accurate values of initial rates but clearly the general trend was again the same, that is, the rate of synthesis increased as the concentration of CO₂ increased, though a large excess of CO₂ produced a slight inhibition.

DISCUSSION

Malic enzyme from pigeon liver catalyses reaction (1), the reversible oxidative decarboxylation of L-malate to yield pyruvate and carbon dioxide (Ochoa *et al.* 1947). The enzyme (see Ochoa, 1955) is TPN specific, Mn²⁺ ion dependent and does not react with D-malate or fumarate. There is no evidence that free oxaloacetate is formed as an intermediate. The enzyme also catalyses the decarboxylation of oxaloacetate to yield pyruvate and carbon dioxide and the pH optimum of this latter reaction is 4.5. Malic enzyme from wheat germ (see Ochoa, 1955) is essentially similar and the enzyme again catalyses the decarboxylation of oxaloacetate with an optimum pH of 5.2. Evidence is presented in this paper which indicates that an enzyme from *Kalanchoë crenata* also catalysed reaction (1) and that it was specific with respect to Mn²⁺, TPN and L-malate. The *Kalanchoë* enzyme preparation also catalysed the decarboxylation of oxaloacetate, the optimum pH being 5.5.

Recent work with purified malic enzyme from pigeon liver (Rutter & Lardy, 1958) has established that, for the oxidative decarboxylation, the pH optimum varies with substrate concentration and the apparent Michaelis constant varies with pH. The pH optimum of the oxidative decarboxylation catalysed by the *Kalanchoë* enzyme was 7.2 (0.5 mM-malate) and this may be compared with values of 7.3 (0.5 mM-malate) for wheat-germ enzyme (Harary *et al.* 1953) and, for pigeon-liver enzyme, 7.2 (0.1 mM-malate) (Rutter & Lardy, 1958) and 7.4 (0.5 mM-malate) (Salles & Ochoa, 1950).

The value of K_m 0.55 m-mole/l. (pH 7.4) obtained with the *Kalanchoë* enzyme may be compared with 0.7 m-mole/l. (pH 7.4) for wheat-germ enzyme (Harary *et al.* 1953) and 0.39 (pH 7.5) (Rutter & Lardy, 1958) and 0.05 (pH 7.4) for pigeon-liver enzyme (Salles & Ochoa, 1950).

The reaction catalysed by malic enzyme (reaction 1) is freely reversible and *in vitro* it can therefore be driven in the direction of carboxylation by high concentrations of carbon dioxide. It would appear that all the effects of carbon dioxide on reaction 1 which are recorded in this paper can be related to the action of carbon dioxide as a substrate. There was no suggestion that carbon dioxide acted as a non-competitive inhibitor (cf. the effect of carbon dioxide on the enzymic catalysis of reaction 2, Walker & Brown, 1957).

The effects of carbon dioxide on the carboxylations catalysed by malic enzyme (reverse of reaction 1) and phosphopyruvate carboxylase (reaction 2) are illustrated in Fig. 6, where they are compared with the effect of carbon dioxide on acid formation by the intact leaf. There is a great deal of similarity between the ways in which the enzymic carboxylation of phosphoenolpyruvate (curve 2), and acidification *in vivo* (curve 1), are affected by concentration of carbon dioxide. On the other hand, the carboxylation of pyruvic acid by malic enzyme (curve 3) did not show this resemblance. It occurred only slowly in low concentrations of carbon dioxide and reached a maximum in the presence of concentrations of carbon dioxide which markedly inhibited the formation of acid by the intact leaf.

When the respective characteristics of phosphopyruvate carboxylase and malic enzyme are considered in their relation to the known facts of crassulacean acid metabolism (see also Walker, 1957; Walker & Brown, 1957) it seems probable that the former enzyme is concerned in acid synthesis and that the latter is concerned in acid breakdown. This view is strengthened by the present results, which confirm that the observed effects of carbon dioxide on the enzymic carboxylation of pyruvate by malic enzyme make it unlikely that this enzyme plays a major role in acid synthesis by crassulacean plants.

The consideration of the effects of carbon dioxide on the initial rate of the oxidative decarboxylation has provided additional information which may also be of interest. Theoretically it would not be possible to account, in terms of mass action, for an effect of carbon dioxide on the initial rate of the oxidative decarboxylation because of the absence of the other two products, pyruvate and TPNH. In practice, of course, it was necessary to allow the reaction to proceed for a finite time (60 sec.) to obtain rate measurements, and it might be argued

that sufficient pyruvate and TPNH could accumulate in this period to permit the reductive carboxylation to occur at a rate sufficiently rapid to account for the apparent decrease in the rate of the oxidative decarboxylation. If this was the case, however, it would follow that the rate of the oxidative decarboxylation would decrease rapidly with time, particularly in the presence of high concentrations of carbon dioxide, as the value of [pyruvate] [TPNH] increased from zero to finite levels. In actual fact the rate showed no tendency to decrease rapidly. For example, in the highest concentration of carbon dioxide used (46.8%) in a reaction mixture containing originally 5 μ moles of malate there was no detectable departure from linearity between 15 and 135 sec. from the start, although the level of TPNH (and therefore pyruvate) increased more than eightfold during this period. Reference to Figs. 4 and 5 will show that the kinetics of the oxidative decarboxylation in the presence of high concentrations of carbon dioxide (up to 46.8%) closely resembled those of a partially competitive inhibition (Dixon & Webb, 1958). Such results are consistent with the view that molecules of carbon dioxide, unable to participate in the reductive carboxylation because of insufficient TPNH and pyruvate, nevertheless occupied their usual sites on the enzyme and, in so doing, reduced the affinity of the enzyme for the substrates of the oxidative decarboxylation.

SUMMARY

1. Cell-free extracts of the crassulacean plant *Kalanchoë crenata* catalysed the reversible reductive carboxylation of pyruvate to yield malate.

2. The malic enzyme was specific with respect to L-malate, Mn^{2+} and triphosphopyridine nucleotide. In the presence of 0.5 mM substrate, the pH optimum of the oxidative decarboxylation was 7.2. K_m (malate) was 0.55 m-mole/l. (25°, pH 7.4). The extracts also catalysed the Mn^{2+} -dependent decarboxylation of oxaloacetate with an optimum pH of 5.5.

3. The characteristics of the enzyme are compared with those of malic enzyme from pigeon liver and wheat germ.

4. Carbon dioxide accelerated the reductive carboxylation of pyruvate and retarded the oxidative decarboxylation of malate.

5. Kinetically, the effects of carbon dioxide on the initial rates of the oxidative decarboxylation of malate resembled a partially competitive inhibition.

6. The results have been discussed in relation to the supposed role of malic enzyme in acid metabolism of crassulacean plants. An interpretation of the reaction kinetics has been suggested.

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Biochemical Investigation of a Case of Glycogen-Storage Disease (von Gierke's Disease)

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Glycogen-storage disease is characterized by the deposition of unusually large quantities of glycogen, particularly in the liver and kidneys. In a survey of a number of cases, Cori (1952-53) has recognized the existence of four types of the disease. In type 1 (also known as von Gierke's disease), the physical properties and the chemical structure of the glycogen appear to be normal and the biochemical defect lies in a striking deficiency of glucose 6-phosphatase both in the liver and kidney. Type 2 is shown by the generalized deposition of glycogen in many tissues, notably in heart and skeletal muscle. The glycogen has a normal

structure, glucose 6-phosphatase is present and the biochemical abnormality has not so far been defined. In types 3 and 4, of which few cases are known, the glycogen accumulated in the liver and kidney has an unusual structure; in type 3 it has very short outer chains and in type 4 the glycogen resembles amylopectin in having relatively long inner and outer branches.

The present studies are concerned with the investigation of a fatal case of the disease in which the molecular structure of the liver and kidney glycogen and the enzymic activity of the liver have been examined. The patient (A.K.) was a sibling of one (S.K.) investigated earlier (Manners, 1954), which corresponded to type 3 of Cori's classification. This

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