Physiological Studies on Acid Metabolism

4. PHOSPHOENOLPYRUVIC CARBOXYLASE ACTIVITY IN EXTRACTS OF CRASSULACEAN PLANTS*

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A large increase in titratable acidity at night, and a concomitant decrease in carbohydrate, has long been recognized as a peculiar feature of the green tissues of Crassulacean and some other succulent plants (Wolf, 1939; Bennet-Clark, 1949). The increase in acidity in the dark is a result of the accumulation of malic acid, which in leaves (in air) may be as much as 800 mg./100 g. of fresh tissue during a single night (Vickery, 1953). It has also been shown, in earlier papers in this series (Thomas 1949; Thomas & Beevers, 1949; Thomas & Ranson, 1954; Ranson & Brown, in preparation) that this increase in acidity is accompanied by the fixation of respiratory carbon dioxide and may be augmented by supplying additional carbon dioxide. In the light, respiratory carbon dioxide is preferentially utilized in photosynthesis, but an increase in acidity in the light may be induced by the addition of carbon dioxide in excess of the photosynthetic requirement. It is clear then that the green tissues of Crassulacean plants possess unusually strong powers of carboxylation and provide suitable material for the study of the enzymes concerned in this process. Earlier investigations were concerned principally with malic and isocitric enzymes, and these were extended to include phosphoenolpyruvic carboxylase after its discovery in extracts of spinach leaves (Bandurski & Greiner, 1953). This, and an essentially similar enzyme in wheat germ (Tchen & Vennesland, 1955; Tchen, Loewus & Vennesland, 1955) catalyses the reaction

Phosphoenolpyruvic carboxylase has been suggested (Tchen & Vennesland, 1955; Bandurski, 1955) to be an important enzyme in dicarboxylic acid accumulation in plant tissues, particularly in the Crassulaceae. As reported briefly elsewhere (Walker, 1956), this enzyme has now been obtained in crude extracts from Crassulacean plants and shown to function synthetically *in vitro*. Since this paper was prepared independent evidence of phos-

phoenolpyruvic carboxylase activity in homogenates of a Crassulacean plant has been reported by Saltman, Kunitake, Spolter & Stitts (1956). Details of the extraction and of the characteristics of the enzyme are given in this paper, and some aspects of the reaction are discussed in relation to the formation of acids in the dark.

MATERIALS AND METHODS

The barium-silver salt of phosphoenolpyruvic acid, adenosine diphosphate and triphosphate (ADP, ATP), inosine diphosphate and triphosphate (IDP, ITP), guanosine diphosphate and triphosphate (GDP, GTP) and reduced diphosphopyridine nucleotide (DPNH) were obtained from the Sigma Chemical Corporation, St Louis, Missouri. *Kalanchoë crenata* and *Sedum spectabile* were grown by Mr M. Matheson in the garden of the Botany Department at Newcastle.

Preparation of enzyme extracts. Leaves were cut in the afternoon when their titratable-acid content was known to be at a minimum, and chilled at -5° for 30–60 min. Meanwhile a weighed sample of the leaves was shredded into boiling water, which was then cooled and titrated against standard alkali. The titratable-acid value (ml. of 0.1N-alkali/100 g. of leaf) thus derived was used to calculate the weight of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) necessary to bring 100 g. of macerated leaf to pH 7.4. This quantity of tris was dissolved in 100 ml. of 1% (w/v) NaCl and the ice-cold solution placed in a chilled Waring Blendor. Chilled leaf (100 g.) was then finely and rapidly (within 10 sec.) shredded with a razor, quickly added to the Blendor and homogenized for 1 min. The homogenate was strained through muslin and centrifuged at 20 000 g for 10 min. All the preparative manipulations were carried out in a cold room at -5° within a period of 15 min.

Partial purification of extracts. The protein in the supernatant was precipitated by the addition of $(\text{NH}_4)_2\text{SO}_4$, at pH 7, to 60% saturation and the precipitate redissolved in, and dialysed against, 0.2% tris at pH 7.4. Alternatively, if the protein was resuspended in saturated $(\text{NH}_4)_2\text{SO}_4$ and stored at -30° it was found to keep indefinitely without loss of activity. It could then be centrifuged down and redissolved as before.

Assay. Except where stated, reactions were followed with a Unicam SP. 500 spectrophotometer by measuring the change in optical density at 340 m μ associated with the oxidation of DPNH. Reactions were carried out in cells with a light path of 1 cm. in a temperature-controlled room at 25° . Each reaction mixture was made up to a final volume of 3 ml.

* Part 3: Thomas & Ranson (1954).

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to prevent the slight oxidation of DPNH in the absence of added phosphoenolpyruvate seen in Fig. 2. The requirement for added magnesium (Figs. 2, 3) could also be more clearly demonstrated after such treatment (see Table 2).

Typically, 100 g. fresh wt. of *Kalanchoë* leaf yielded 25 ml. of partly purified extract, in which the respective activities ($V_{\max}$) of malic dehydrogenase and phosphoenolpyruvic carboxylase were of the order of 10 and $0.5 \mu\text{moles}$ of substrate utilized/min./ml. respectively.

Substrate and coenzyme requirement. Phosphoenolpyruvic carboxylase had been found (Tchen & Vennesland, 1955) in extracts of wheat germ to be associated with an enzyme which catalyses the reaction: phosphoenolpyruvate + CO_2 + ADP or IDP $\rightleftharpoons$ oxaloacetate + ATP or ITP. This 'phosphorylating decarboxylase' seems essentially similar to one in avian liver (Utter, Kurahashi & Rose, 1954;

Utter & Kurahashi, 1954) for which GDP and GTP are even more effective than the corresponding inosine nucleotides (Kurahashi & Utter, 1955). The reaction is reversible but it also appears to differ from that catalysed by phosphoenolpyruvic carboxylase in that its principal role is probably in oxidative phosphorylation rather than carboxylation (Utter & Kurahashi, 1954; Tchen & Vennesland, 1955; Bartley, 1955). Added nucleotides were without effect on the *Kalanchoë* enzyme (Table 1) and it has already been seen that inorganic phosphate was produced during the course of the reaction (Fig. 1).

No reaction occurred if potassium pyruvate (either alone or together with ATP, or ITP or GTP, in tris or phosphate buffer at pH 7) was substituted for phosphoenolpyruvate.

Cofactor requirement. The rate of oxidation of DPNH when manganese and other cations were substituted for magnesium in the reaction mixture is shown in Table 2. Manganese is seen to have been as effective as magnesium, although other results

Fig. 2. Reaction (3) catalysed by *Kalanchoë* extracts. Each reaction mixture (3 ml.) contained *Kalanchoë* enzyme preparation, 1% (w/v) tris buffer at pH 7.4, DPNH (approx. $0.2 \mu\text{mole}$), sodium phosphoenolpyruvate ($1 \mu\text{mole}$), MgCl_2 ($3 \mu\text{moles}$), except for the omissions indicated. The reaction was started by the addition of phosphoenolpyruvate (PEP) and DPNH, except where the former was omitted. The curves have been equated to the same starting point.

Fig. 3. Reaction (3) catalysed by *Sedum* extracts. The reaction mixture (3 ml.) contained *Sedum spectabile* enzyme preparation, 1% (w/v) tris buffer at pH 7.4, DPNH (approx. $0.3 \mu\text{mole}$) added at zero time, sodium phosphoenolpyruvate (PEP; $1 \mu\text{mole}$) and MgCl_2 ($3 \mu\text{moles}$) added as indicated.

have indicated that this may not be the case after prolonged dialysis. Except for cobalt and cadmium the other cations were not only ineffective but even inhibitory when used in addition to magnesium (see Table 2).

Effect of pH. In phosphate buffer the linked reaction (3) was most rapid at about pH 7. At either side of this pH value the reaction is less rapid, falling to about 75 % of the maximum at pH 6 and 8. The rate of the dehydrogenase reaction alone, acting in the direction of malate synthesis, falls steadily over this range as the pH rises, which suggests that the optimum of the carboxylase is, if anything, above pH 7.

Effect of inorganic phosphate. Tehen & Vennesland

(1955) found that low concentrations of inorganic phosphate accelerated the reaction. Table 3 illustrates a similar effect with the *Kalanchoë* enzyme. It will be seen, however, that above an optimum concentration further increases in phosphate concentration brought about a progressive inhibition.

Substrate concentration. Increases in either the concentration of phosphoenolpyruvate or carbon dioxide brought about corresponding increases in the reaction rate. Laidler & Socquet (1950) have derived an equation for the initial velocity of a two-substrate enzyme system in which each type of substrate molecule occupies its own site on the enzyme surface. For relatively small concentrations

Table 1. *Effect of several coenzymes on the rate of reaction (3)*

Each spectrophotometer cell contained enzyme, MgCl_2 (3 μmoles), DPNH (0.2 μmole), tris buffer (1 %, w/v) at pH 7.4, and sodium phosphoenolpyruvate (2 μmoles) together with the additions indicated, in a volume of 3 ml. The rate given is the change in $\log I_0/I$ (340 $\text{m}\mu$) $\times 1000$ in the first 60 sec. of the reaction.

		Additions						
	Control (no additions)	Cocarcboxylase (2 μ moles)	IDP	ITP	GDP	GTP	Inorganic phosphate (2.5 μ moles)	IDP + inorganic phosphate (2.5 μ moles)
Rate	88	85	87	88	86	84	97	93

Table 2. *Effect of several cations on the rate of reaction (3)*

Each spectrophotometer cell contained enzyme, DPNH (0.2 μmole), tris buffer (1 %, w/v) at pH 7.4, and sodium phosphoenolpyruvate (2 μmoles) together with the additions indicated, in a final volume of 3 ml. The rate given is the change in $\log I_0/I$ (340 $\text{m}\mu$) $\times 1000$ in the first 60 sec. of the reaction.

Rate	Control (no additions)	Added cations (3 μmoles)								
		Mg^{2+}	Mn^{2+}	Cd^{2+}	Co^{2+}	Ca^{2+}	Ni^{2+}	Zn^{2+}	Cu^{2+}	Fe^{2+}
	7	90	100	31	28	5	4	2	Precipitation	

Inhibition (%) by cations (3 μmoles) in the presence of magnesium (3 μmoles)

	%
Fe^{2+}	100 precipitating protein
Cu^{2+}	100 precipitating protein
Zn^{2+}	98
Ni^{2+}	82
Ca^{2+}	30

Table 3. *Effect of orthophosphate concentration on the rate of reaction (3)*

Each spectrophotometer cell contained enzyme, sodium phosphoenolpyruvate (2 μmoles), MgCl_2 (3 μmoles), DPNH (0.2 μmole) and tris buffer (1 %, w/v) at pH 7.4, and the quantities of orthophosphate (also at pH 7.4) indicated, in a final volume of 3 ml. The rate given is the change in $\log I_0/I$ (340 $\text{m}\mu$) $\times 1000$ in the first 60 sec. of the reaction. In the absence of added phosphate the reaction mixture contained less than 0.05 μmole of free inorganic phosphate, and the observed change in optical density due to the reaction corresponds to an increase of about 0.04 μmole .

Rate	Phosphate added (μmoles)							
	0	1	2	3	15	30	150	300
	112	120	128	152	144	116	68	36

their equation may be simplified and rewritten (Schwert & Hakala, 1952) as

$$\frac{1}{V} = \frac{K_a}{V_{\max}} \left[\frac{K_b}{[b]} + 1 \right] \frac{1}{[a]} + \frac{1}{V_{\max}} \left[\frac{K_b}{[b]} + 1 \right],$$

where a and b are the two substrates and K_a and K_b the respective dissociation constants. In this form the expression is linear for $1/V$ against $1/[a]$ at constant $[b]$. Alternatively, written

$$\frac{1}{V} = \frac{K_b}{V_{\max}} \left[\frac{K_a}{[a]} + 1 \right] \frac{1}{[b]} + \frac{1}{V_{\max}} \left[\frac{K_a}{[a]} + 1 \right],$$

the expression is linear for $1/V$ against $1/[b]$ at constant $[a]$.

On this basis the kinetic constant (K_a) for phosphoenolpyruvate was evaluated by plotting the reciprocals of velocity against the reciprocals of phosphoenolpyruvate concentration at constant carbon dioxide concentration.

The straight line so obtained was then extrapolated (cf. Dixon, 1953) to provide the value of $-1/K_a$ at its intersection with the x axis (Fig. 4). The value of K_a derived in this way was 1.94×10^{-4} mole/l., and a value of 1.5×10^{-4} was similarly obtained from measurements of inorganic phosphate accumulation in reaction 1. The evaluation of reaction rates by inorganic phosphate determinations was rather less accurate than by the spectrophotometric method, and the discrepancy between the two results is not considered to be greater than experimental error. The fact that the rates of reactions 1 and 3 clearly corresponded closely over a wide range of concentrations justifies the use of the spectrophotometric method of obtaining kinetic constants.

Fig. 4. Increase in the rate of reaction (3) with increasing phosphoenolpyruvate concentration. Each spectrophotometer cell contained *Kalanchoë* enzyme preparation, 1% (w/v) tris buffer at pH 7.4, DPNH ($0.2 \mu\text{mole}$), MgCl_2 ($3 \mu\text{moles}$) and sodium phosphoenolpyruvate in the concentrations ($\mu\text{moles/ml.}$) indicated by the reciprocal, in a final volume of 3 ml.

The value of K_a is of the same order as that of 5×10^{-4} obtained by Bandurski (1955) under slightly different conditions. Similar results were obtained when the carbon dioxide concentration was varied at constant phosphoenolpyruvate concentration (Walker & Brown, 1957); the value of the dissociation constant (K_b) was found in this case to be 2.2×10^{-4} mole/l.

Enzyme concentration. Within the limits of measurement on the spectrophotometer, the rate of reaction was directly proportional to the enzyme concentration, being doubled as the enzyme concentration was doubled.

Synthesis of malate in the linked reaction

The fixation of carbon dioxide in oxaloacetate in reaction mixtures containing the *Kalanchoë* enzyme preparation has been described at the beginning of this section. If DPNH also was added to these solutions the oxaloacetate was reduced to malate (reaction 3) and the concomitant oxidation of DPNH could be followed in the spectrophotometer. Chromatographic analysis of such reaction mixtures gave spots which had the same R_f values as malic acid chromatographed on the same sheets. When $50 \mu\text{C}$ of radioactive bicarbonate was also added to the reactants the malate synthesized became highly active. After co-chromatography with malate the identity of the synthesized acid was then confirmed by radioautographs, which showed the major part of the activity to emanate from the malate spots. Some activity (less than 1% of the total) was found to be associated with the fumaric spots, probably as a result of a weak fumarase activity in the enzyme preparation. When active malate was eluted from the chromatograms and degraded by *Lactobacillus* (see Methods) 99.7% of the counts were recovered in carbon dioxide originating from the β -carboxyl group. The residue, which would contain all the activity associated with the other carbon atoms, together with any activity not released by the *Lactobacillus*, contained only 0.3% of the total counts.

DISCUSSION

If it is accepted that the synthesis of malate which occurs in Crassulacean plants during the night is brought about at the expense of carbohydrate breakdown, it may be assumed that phosphoenolpyruvate is an intermediate since this substance is produced directly by the Embden-Meyerhof-Parnas pathway and also indirectly by the pentose phosphate carboxylating cycle. If carboxylation occurs after the formation of phosphoenolpyruvate the most likely system would involve either malic enzyme or phosphoenolpyruvic carboxylase.

If the former system operates the sequence would involve reactions (4) and (5):

(TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.) Malic enzyme, which catalyses reaction (5), has frequently been suggested as an important agent in carboxylation. The equilibrium position is unfavourable, but the reaction may be driven in the direction of reductive carboxylation if it is linked with a system which generates reduced TPN (Ochoa, 1951). Harary, Korey & Ochoa (1953) have shown that at pH 7 and at 22° in an atmosphere containing 5% of carbon dioxide and equimolar concentrations of oxidized and reduced TPN, the ratio pyruvate:malate would be approx. 1:0.035. The actual ratio in the *Kalanchoë* leaf, on the other hand, is probably never less than 1:100 (Bradbeer, 1954). It seems reasonable to assume therefore that in any explanation of acidification (in *Kalanchoë*) based upon the malic enzyme, a place must be found, not only for the regeneration of reduced TPN but also for some other system by which malate is removed from the site of synthesis at an extremely rapid rate.

The alternative sequence would involve reactions (1) and (2). According to Tchen & Vennesland (1955), the equilibrium position of reaction (1) is such that for all practical purposes the reaction is irreversible. From the value of the equilibrium constant of reaction (2) (Stern, Ochoa & Lynen, 1952) it may be calculated that, at pH 7.4 in the presence of equimolar concentrations of oxidized and reduced DPN, the ratio oxaloacetate:malate at equilibrium would be about 1:43 000. Moreover, the value of the equilibrium constant is strongly dependent on pH, and under the acid conditions in the leaf the actual ratio might be much higher. Acidification via this sequence could therefore occur at near maximal rates even if malate was not rapidly removed from the site of synthesis.

Whichever system operates the total free-energy changes are of the same order, but there are important differences in the way in which this energy

is conserved. Thus it appears that in reaction (1) the large negative free energy associated with the hydrolysis could be directly utilized in the carboxylation. Results obtained by Bandurski (1955) indicate that the reversible formation of ortho-phosphate and a pyruvate-enzyme complex is unlikely. Moreover, though Tchen *et al.* (1955) do not rule out oxaloacetyl phosphate as a possible intermediate, they have clearly shown that the phosphoenol P-O bond of phosphoenolpyruvate must be broken before, or simultaneously with, the addition of carbon dioxide. J. G. Buchanan (private communication) has suggested that the latter possibility might be essentially similar to that postulated by Srinivasan, Katagiri & Sprinson (1955) for the synthesis of shikimic acid (see Fig. 5).

If the malic enzyme system operates, the direct utilization of energy in this way would not be possible, although the ATP generated in reaction (4) might bring about the regeneration of reduced TPN required for reaction (5) (Krebs, 1954).

If both systems operate in the leaf it might be thought that competition would occur between reactions (1) and (4). Then the course of acidification would depend both on the amounts of the respective enzymes present and on their respective affinities for phosphoenolpyruvate. There would also be competition between reactions (1) and (5) for the available carbon dioxide. The importance of this will be discussed more fully in a second paper (Walker & Brown, 1957).

SUMMARY

1. The β -carboxylation of phosphoenolpyruvate to yield oxaloacetate and inorganic phosphate has been catalysed by cell-free extracts of Crassulacean plants.
2. The phosphoenolpyruvic carboxylase and malic dehydrogenase in these extracts have been linked to bring about malate synthesis.
3. The conditions under which the extracts bring about the catalysis of the linked reaction have been studied and the effect of substrate concentration has been determined.
4. Some aspects of the linked reaction and of the other reactions which bring about the conversion of phosphoenolpyruvate into malate have been considered in relation to the increase in acidity in the dark in Crassulacean plants.

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Fig. 5. Suggested mechanism of reaction (1).

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Physiological Studies on Acid Metabolism

5. EFFECTS OF CARBON DIOXIDE CONCENTRATION ON PHOSPHOENOLPYRUVIC CARBOXYLASE ACTIVITY*

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The demonstration that the increase in acidity in leaves of Crassulacean plants in the dark is accompanied by the fixation of respiratory carbon dioxide and can be augmented by feeding with this gas (Thomas, 1947, 1949; Thomas & Beevers, 1949) made it highly probable that the concentration of carbon dioxide would affect the rate of acid synthesis. In parallel work, Bonner & Bonner (1948), though not actually demonstrating carbon dioxide fixation, observed a sharp increase in the rate of acid synthesis in *Bryophyllum* leaves on increasing the external carbon dioxide concentration from zero to 0.1 %, and a further slow rise to a maximum at higher concentrations.

Ranson's measurements of acidification and carbon dioxide fixation in *Kalanchoë* leaves reported by Thomas (1951) and by Thomas & Ranson (1954) covered a more extensive range of concentration, and disclosed that beyond 3 % further increases in carbon dioxide depressed the rate of acidification.

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From the earlier work it could be inferred that one or other of the enzymes responsible for malate synthesis in *Kalanchoë* leaves should (a) be able to carboxylate a carbohydrate cleavage product; (b) have a high affinity for carbon dioxide. And, in addition, that one or more of the reactions involved should be (c) accelerated by augmenting carbon dioxide concentration at low levels; (d) inhibited by high concentrations of carbon dioxide.

In the earlier paper (Walker, 1957) phosphoenolpyruvic carboxylase extracted from *Kalanchoë* leaves has been shown to fulfil the first of these requirements. In the present paper the other requirements are also shown to be satisfied and these findings are contrasted with the known characteristics of malic enzyme. Some aspects of the observed carbon dioxide inhibition are also discussed.

So far as is known this is the first time that a direct inhibitory effect of high carbon dioxide concentrations on an enzyme has been observed *in vitro*, although Thomas (1925) virtually inferred that carbon dioxide can inhibit the aerobic enzymic consumption of a zymase cleavage product.