REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Bandurski, R. S. (1955). *J. biol. Chem.* **217**, 131.
Bandurski, R. S. & Greiner, C. M. (1953). *J. biol. Chem.* **204**, 781.
Bartley, W. (1955). *Biochem. J.* **61**, 555.
Bennet-Clark, T. A. (1949). *Annu. Rev. Biochem.* **18**, 639.
Bradbeer, J. W. (1954). Ph.D. Thesis: University of Durham.
Dixon, M. (1953). *Biochem. J.* **55**, 170.
Harary, I., Korey, S. R. & Ochoa, S. (1953). *J. biol. Chem.* **203**, 595.
Krebs, H. A. (1954). *Johns Hopk. Hosp. Bull.* **95**, 19.
Kurahashi, K. & Utter, M. F. (1955). *Fed. Proc.* **14**, 240.
Laidler, K. J. & Socquet, I. M. (1950). *J. phys. Chem.* **54**, 530.
Nossal, P. M. (1951). *Biochem. J.* **49**, 407.
Ochoa, S. (1951). In *The Enzymes*, vol. 2, part 2, p. 929. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press.
Ranson, S. L. (1955). In *Modern Methods of Plant Analysis*, vol. 2, p. 539. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.
Saltman, P., Kunitake, G., Spolter, H. & Stitts, C. (1956). *Plant Physiol.* **31**, 464.
Schwert, G. W. & Hakala, M. T. (1952). *Arch. Biochem. Biophys.* **38**, 55.
Speck, J. F. (1949). *J. biol. Chem.* **178**, 315.
Srinivasan, P. R., Katagiri, M. & Sprinson, D. B. (1955). *J. Amer. chem. Soc.* **77**, 4943.
Stern, J. R., Ochoa, S. & Lynen, F. (1952). *J. biol. Chem.* **198**, 313.
Tchen, T. T., Loewus, F. A. & Vennesland, B. (1955). *J. biol. Chem.* **213**, 547.
Tchen, T. T. & Vennesland, B. (1955). *J. biol. Chem.* **213**, 533.
Thomas, M. (1949). *New Phytol.* **48**, 390.
Thomas, M. & Beevers, H. (1949). *New Phytol.* **48**, 421.
Thomas, M. & Ranson, S. L. (1954). *New Phytol.* **53**, 1.
Utter, M. F. & Kurahashi, K. (1954). *J. biol. Chem.* **207**, 821.
Utter, M. F., Kurahashi, K. & Rose, I. A. (1954). *J. biol. Chem.* **207**, 803.
Vickery, H. B. (1953). *J. biol. Chem.* **205**, 369.
Walker, D. A. (1956). *Nature, Lond.*, **178**, 593.
Walker, D. A. & Brown, J. M. A. (1957). *Biochem. J.* **67**, 79.
Wolf, J. (1939). *Planta*, **29**, 314.

Physiological Studies on Acid Metabolism

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

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(*Received* 15 *January* 1957)

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.

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.

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MATERIALS AND METHODS

All solutions, other than those containing the enzyme, were prepared in boiled mineral-free water which had been allowed to cool in a stoppered vessel containing no air. Residual carbon dioxide which remained in the reaction mixtures despite these precautions was measured manometrically as follows. Immediately after each reaction (i.e. within 3 min. of the start) the contents of the spectrophotometer cell were tipped into a Warburg flask. After equilibration for 2 min., acid was added from the side arm and the volume of evolved carbon dioxide was recorded.

The concentration of added carbon dioxide was varied as required by the addition of carbon dioxide-saturated bicarbonate at pH 7·4. Quantities were calculated from the Henderson–Hasselbalch equation (Umbreit, Burris & Stauffer, 1949) to give solutions in equilibrium with atmospheres containing the percentage of carbon dioxide required. For low concentrations, the total of added and residual carbon dioxide was measured manometrically as above. Totals obtained in this way were found to be in excellent agreement with the calculated figures if the residual carbon dioxide was taken to be constant in each case.

Materials and other methods were as described in the earlier paper (Walker, 1957).

RESULTS

Both the carboxylation of phosphoenolpyruvate

$$\text{Phosphoenolpyruvate} + CO_2 \rightarrow \text{oxaloacetate} + \text{phosphate} \quad (1)$$

and the reduction of oxaloacetate

$$\text{Oxaloacetate} + \text{DPNH} \rightarrow \text{malate} + \text{DPN} \quad (2)$$

are catalysed by cell-free extracts of *Kalanchoë crenata* (Walker, 1957), and the two reactions may be linked to give the overall reaction

$$\text{Phosphoenolpyruvate} + CO_2 + \text{DPNH} \rightarrow \text{malate} + \text{phosphate} + \text{DPN}. \quad (3)$$

(DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.) Reaction (3) was found to proceed at a rapid rate, even when precautions were taken to reduce to a minimum the carbon dioxide present in the reagents. The source of carbon dioxide was then believed to be a small residue which could not be readily removed from the enzyme preparation. Fig. 1 shows results of an experiment in which initial reaction rates were determined at several low carbon dioxide concentrations. Rate is plotted against the sum of residual and added carbon dioxide; this total was determined manometrically (see Methods).

In this experiment the residual carbon dioxide was found to be 18·3 μl. in 3 ml., and evidently the reaction was able to proceed initially at this concentration at almost two-thirds of the maximal rate.

It will be seen that the addition of further carbon dioxide brought about increases in the reaction rate but it appears that the system became saturated at a relatively low level. The curve is seen to permit a reasonable extrapolation to zero rate at zero carbon dioxide concentration. Plotted as the double

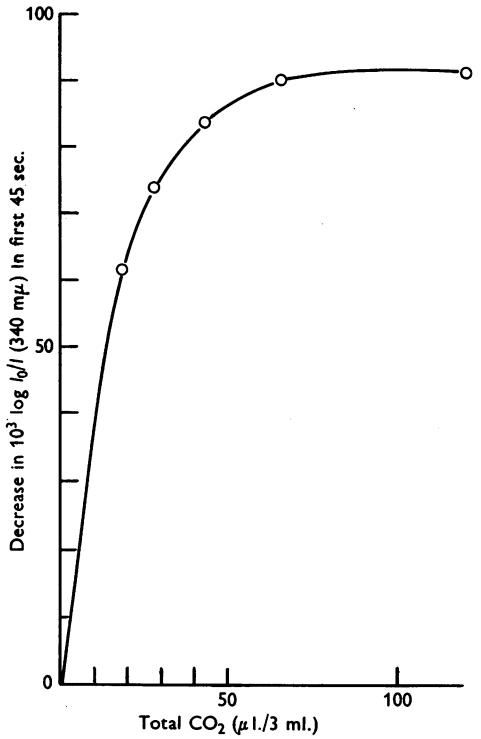

Fig. 1. Increases in the rate of reaction (3) brought about by small increases in the carbon dioxide concentration. Each spectrophotometer cell contained *Kalanchoë* enzyme preparation, 1% (w/v) tris buffer at pH 7·4, $MgCl_2$ (3 μmoles), sodium phosphoenolpyruvate (1 μmole), DPNH (0·2 μmole) and carbon dioxide-saturated bicarbonate at pH 7·4 to yield the carbon dioxide content indicated (see Methods), in a total volume of 3 ml.

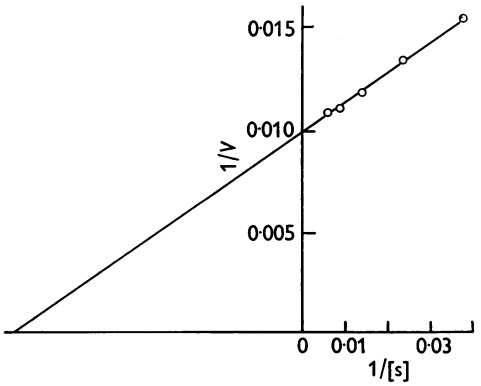

Fig. 2. Double reciprocal plot of the values in Fig. 1.

Table 1. *Effects of carbon dioxide concentration on phosphoenolpyruvic carboxylase activity and on the accumulation of malic acid in leaves in the dark*

For the spectrophotometric measurements (line 2) each cell contained *Kalanchoë* enzyme preparation, 1% (w/v) tris buffer at pH 7·4, $MgCl_2$ (3 μmoles), sodium phosphoenolpyruvate (1 μmole), DPNH (0·2 μmole) and carbon dioxide-saturated bicarbonate at pH 7·4 (see Methods), in a total volume of 3 ml. Reactions were started by the addition of phosphoenolpyruvate, and the figures refer to the decrease in log I_0/I (340 mμ) × 1000 in the period between 15 and 60 sec. after this addition. All figures in the third line (taken from Thomas & Ranson, 1954) are mean values for a number of experiments, except those which are underlined. These latter were derived from single experiments only, and the value 37 would appear to be an aberrant.

CO_2 (%)	0–0·05	0·05	0·125	0·25	0·5	1·0	2·0	3·0	5	10	15	30	50	70
Inorganic phosphate produced in 12 min. (see text) (μmoles)	0·59	—	—	—	1·55	1·2	—	—	1·15	1·03	—	0·43	0·30	0·2
Decrease in log I_0/I (340 mμ) in 45 sec. (see text)	64	74	84	90	91	84	—	79	—	66	—	36	18	11
Accumulation of malic acid in *Kalanchoë* leaf (mg./hr./100 g. fresh wt.)	19	22	—	—	—	60	62	67	45	24	16	37	4	1

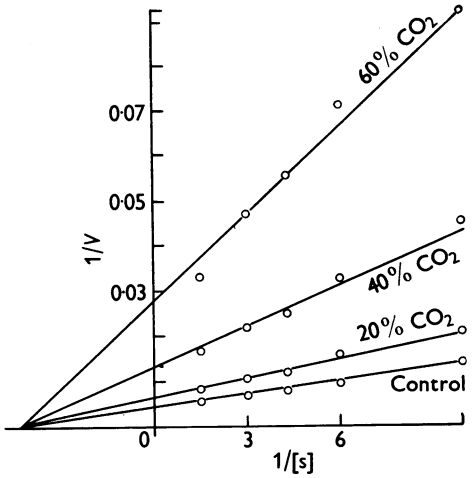

Fig. 3. 'Non-competitive' nature of carbon dioxide inhibition of reaction 3. Each spectrophotometer cell contained *Kalanchoë* enzyme preparation, 1% (w/v) tris buffer at pH 7·4, $MgCl_2$ (3 μmoles), DPNH (0·2 μmole) and sodium phosphoenolpyruvate (PEP) in the concentrations (μmoles/ml.) indicated by the reciprocal 1/[s]. Carbon dioxide concentration was adjusted as described (see Methods).

reciprocal (Lineweaver & Burk, 1934) these figures yield points which approximate to a straight line. This has been extrapolated to obtain a value of $-1/K_s$ at its intersection with the x axis (Fig. 2). The value of the constant derived in this way is $K_s = 2·2 \times 10^{-4}$ mole/l. (also see Walker, 1957).

Increases in carbon dioxide concentration above the saturation level and to about 3% did not affect the reaction rate to any large extent, but further increases were progressively inhibitory (see Table 1, line 2).

In another experiment initial reaction rates were measured at several concentrations of phosphoenolpyruvate in the presence of each of three such inhibitory levels of carbon dioxide. Fig. 3 shows the results plotted as the double reciprocals (cf. Lineweaver & Burk, 1934). A family of straight lines is obtained and these lines coincide at a point on the x axis equivalent to $-1/K_s$. This is clearly indicative of a purely non-competitive type of inhibition as defined by Friedenwald & Maengwyn-Davies (1954).

A number of control experiments were carried out which showed that carbon dioxide (at several concentrations between 0·05 and 90%) did not alter the rate of the reaction catalysed by malic dehydrogenase (reaction 2). That it was, in fact, the phosphoenolpyruvic carboxylase reaction that was affected was confirmed by direct measurement. In the absence of reduced DPN this reaction does not proceed beyond the production of oxaloacetate and inorganic phosphate (reaction 1). Table 1 (line 1) shows the amount of phosphate produced when reaction mixtures containing enzyme, 2 μmoles of phosphoenolpyruvate, 3 μmoles of $MgCl_2$ and carbon dioxide as indicated were incubated in 2% (w/v) tris buffer at pH 7·4 for periods of 12 min. The response to changes in the carbon dioxide concentration is in the same direction as that observed when the oxidation of reduced DPN was followed in the linked reaction (Table 1, line 2). For the phosphate measurement the 'residual' carbon dioxide (which was present in the reaction mixture despite attempts to exclude it) was not evaluated but is expressed as 0–0·05%.

DISCUSSION

In a previous paper (Walker, 1957) two enzymic systems have been discussed which might bring about the conversion of phosphoenolpyruvate into malate during the increase in acidity in the dark in Crassulacean plants (see criterion (*a*) in the introduction). These involve reactions (1) and (2) or, alternatively, reactions (4) and (5):

$$\text{Phosphoenolpyruvate} + \text{ADP} \rightleftharpoons \text{pyruvate} + \text{ATP}, \quad (4)$$

$$\text{Pyruvate} + CO_2 + \text{TPNH} \rightleftharpoons \text{malate} + \text{TPN}. \quad (5)$$

(TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.) One of the main criticisms of the malic enzyme reaction (5) as an important carboxylating system is its high carbon dioxide requirement. Thus from Ochoa's figures (Ochoa, 1951) it is seen that the highest rate of synthesis was achieved in the highest concentration (70 %) of carbon dioxide used, whereas in the presence of 5 % carbon dioxide this rate had fallen to about 27 % of the maximum. Assuming a linear decline in activity, we may infer that the rate of synthesis would have fallen to a very low level had this concentration been reduced still further. Yet rapid increase in acidity in the dark, at the expense of respiratory carbon dioxide, has been shown to occur (Ranson, reported by Thomas, 1951; Wood, 1952; Thomas & Ranson, 1954) in the leaves of Crassulacean plants maintained in atmospheres containing no carbon dioxide. In fact the affinity for respiratory carbon dioxide is so high that, for leaves enclosed in ordinary air, the value of the apparent respiratory quotient (carbon dioxide actually evolved/oxygen absorbed), as has long been known (Thomas, 1949; Wolf, 1938), is at first very low and may be zero. In one experiment on *Opuntia* the darkened shoot organs removed all the carbon dioxide from the surrounding air in addition to consuming respiratory carbon dioxide (Thomas & Ranson, 1954).

No doubt it can be very misleading to attach too much importance to the equilibrium constants of enzymes which are functioning in a dynamic system. Many overall reactions are known in which at least one step proceeds in a direction contrary to that which might have been expected from its equilibrium constant. In fact reaction (5) *in vitro* has often been driven in the direction for carboxylation by coupling with other reactions which regenerate reduced TPN and by reactions which tend to remove the malate which is synthesized. Nevertheless, *in vivo* the concentration of pyruvate available is probably never more than 5 mg./100 g. fresh wt. of leaf at any one time (Bradbeer, 1954), and it has been seen that rapid acidification can occur in leaves exposed to conditions which would not be expected to favour high levels of carbon dioxide. Since the equilibrium position of reaction (5) favours decarboxylation to a very large extent it seems improbable that this system, acting under these conditions, could bring about the large synthesis of malic acid which is known to occur. On this basis alone the facts point to a reaction which favours carboxylation and to an enzyme with a very great affinity for carbon dioxide. Phosphoenolpyruvic carboxylase fulfils both of these requirements. Both Tchen & Vennesland (1955) and Bandurski (1955) have commented on the high affinity for carbon dioxide exhibited by this enzyme (see introduction criterion (*b*)). Figures given in this paper indicate that reaction (1) *in vitro* functions at near maximal rates in the presence of very small quantities of carbon dioxide. This suggests not only that this enzyme system might bring about acidification at low carbon dioxide tensions, it implies also that *in vivo* this enzyme might offer strong competition to other carboxylating systems. Thus an active phosphoenolpyruvic carboxylase could preferentially utilize substrates of both reactions (4) and (5) of the alternative sequence.

Additional evidence that phosphoenolpyruvic carboxylase might play a role in the increase in acidity occurring in the dark is given by its behaviour in high carbon dioxide concentrations. It has been seen that below 0·5 % increases in carbon dioxide concentration caused concomitant increases in the rate of the linked reaction [see introduction, criterion (*c*)]. Between 0·5 and 10 % the rate falls slowly to about two-thirds of the maximum, and above 10 % further increases brought about a progressive inhibition [see introduction, criterion (*d*)]. In Table 1 (line 3) are given results obtained by Thomas & Ranson (1954), which show the effect of carbon dioxide on increase in acidity in the intact leaf in the dark. Comparison with the results *in vitro* (Table 1, lines 1 and 2) will show that in both instances there is an initial acceleration, a broad plateau and finally a depression or inhibition. Though in no way conclusive, this parallelism is certainly consistent with the view that phosphoenolpyruvic carboxylase is concerned in the increase in acidity in whole leaves in the dark.

There is little in the present data which can be used to define the actual nature of the observed inhibition by carbon dioxide. The kinetic picture clearly corresponds to that outlined by Friedenwald & Maengwyn-Davies (1954) for purely non-competitive inhibition. This casts doubt on the view that carbon dioxide could compete with phosphoenolpyruvate for the site at which the latter becomes attached to the enzyme surface. If each substrate molecule has its own point of attachment to the enzyme then steric interference with its own activa-

tion, by an excess of carbon dioxide acting at its own site, seems to us the most feasible of the possibilities which could produce the observed kinetic behaviour.

SUMMARY

1. Two different methods have been used to determine the effects of carbon dioxide on the rate of the reaction catalysed by phosphoenolpyruvic carboxylase.

2. The kinetics of the carbon dioxide effects have been studied with one of these methods in a reaction in which the carboxylase was linked with malic dehydrogenase.

3. The results have been discussed in relation to factors which are known to affect the rate of increase of acidity in the dark in Crassulacean plants.

The authors wish to express their indebtedness to Professor M. Thomas, F.R.S., at whose suggestion this work was undertaken, and to thank Miss J. Moyle, Dr R. Hill, F.R.S., and Dr S. L. Ranson for their invaluable advice and criticism. They are also grateful to Dr G. Maengwyn-Davies for her comments on specific points. Part of this work was carried out under grants from the D.S.I.R., and part during the tenure of an I.C.I. Fellowship held by one of us (D. A. W.).

REFERENCES

Bandurski, R. S. (1955). *J. biol. Chem.* **217**, 131.
Bonner, J. & Bonner, W. (1948). *Amer. J. Bot.* **35**, 113.
Bradbeer, J. W. (1954). Ph.D. Thesis: University of Durham.
Friedenwald, J. S. & Maengwyn-Davies, G. D. (1954). In *The Mechanism of Enzyme Action*, p. 154. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Ochoa, S. (1951). In *The Enzymes*, vol. 2, part 2, p. 929. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press.
Tchen, T. T. & Vennesland, B. (1955). *J. biol. Chem.* **213**, 533.
Thomas, M. (1925). *Biochem. J.* **19**, 927.
Thomas, M. (1947). *Plant Physiology*. London: Churchill.
Thomas, M. (1949). *New Phytol.* **48**, 390.
Thomas, M. (1951). *Symp. Soc. exp. Biol.* **5**, 72.
Thomas, M. & Beevers, H. (1949). *New Phytol.* **48**, 421.
Thomas, M. & Ranson, S. L. (1954). *New Phytol.* **53**, 1.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, p. 25. Minneapolis: Burgess Publ. Co.
Walker, D. A. (1957). *Biochem. J.* **67**, 73.
Wolf, J. (1938). *Planta*, **28**, 71.
Wood, W. M. L. (1952). *J. exp. Bot.* **3**, 26.

The Amino Acid Composition of Amphibian, Reptile and Avian Gelatins

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(*Received* 4 *March* 1957)

The present paper completes an investigation of the variation in amino acid composition found among the collagens and derived gelatins of the more important groups of vertebrates. Results are given for avian, reptilian and amphibian gelatins for comparison with the two previous sections of the work on mammalian (Eastoe, 1955) and fish (Eastoe, 1957) materials. Neuman (1949) included both chicken tendon and the subcutaneous membrane of the turtle in his survey, in which microbiological methods were used. No values for amphibian proteins of this type appear to have been published previously.

It has already been demonstrated (Eastoe, 1955) that, within the limits of experimental error, a carefully prepared gelatin adequately represents the composition of its collagen precursor, except in amide nitrogen. This is especially valuable when non-collagenous impurities, which cannot readily be removed, are present in a tissue and would introduce errors if the whole tissue were analysed. The materials selected for analysis were gelatins prepared from toad, python and crocodile skins and from chicken-leg tendon. In this way representative values for the gelatins and collagens of three further classes of vertebrates were obtained by ion-exchange chromatography (Moore & Stein, 1951), a technique identical with that of the previous papers being used.

Following the suggestion of Gustavson (1953) that shrinkage temperatures are a measure of structural stability, these have been measured for each of the collagens studied.

EXPERIMENTAL

Materials

Python-skin gelatin. Python (probably *Python sebae*). The skin was limed at 0° for 2 months, washed, and gelatin extracted from it by heating with water for 2 hr. at 60° and pH 7.