The Mechanisms of Reductive Carboxylation Reactions

CARBON DIOXIDE OR BICARBONATE AS SUBSTRATE OF NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE-LINKED ISOCITRATE DEHYDROGENASE AND 'MALIC' ENZYME

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1. A simple kinetic method was devised to show whether dissolved CO_2 or HCO_3^- ion is the substrate in enzyme-catalysed carboxylation reactions. 2. The time-course of the reductive carboxylation of 2-oxoglutarate by NADPH, catalysed by isocitrate dehydrogenase, was studied by a sensitive fluorimetric method at pH 7.3 and pH 6.4, with large concentrations of substrate and coenzyme and small carbon dioxide concentrations. 3. Reaction was initiated by the addition of carbon dioxide in one of three forms: (i) as the dissolved gas in equilibrium with bicarbonate; (ii) as unbuffered bicarbonate solution; (iii) as the gas or as an unbuffered solution of the gas in water. Different progress curves were obtained in the three cases. 4. The results show that dissolved CO_2 is the primary substrate of the enzyme, and that HCO_3^- ion is at best a very poor substrate. The progress curves are in quantitative agreement with this conclusion and with the known rates of the reversible hydration of CO_2 under the conditions of the experiments. The effects of carbonic anhydrase confirm the conclusions. 5. Similar experiments on the reductive carboxylation of pyruvate catalysed by the 'malic' enzyme show that dissolved CO_2 is the primary substrate of this enzyme also. 6. The results are discussed in relation to the mechanisms of these enzymes, and the effects of pH on the reactions. 7. The advantages of the method and its possible applications to other enzymes involved in carbon dioxide metabolism are discussed.

In considering the mechanisms of enzymes involved in carbon dioxide metabolism, the question arises as to which of the three forms that exist in equilibrium in solution under physiological conditions, CO_2 , H_2CO_3 and HCO_3^- , is the immediate substrate or product. Krebs & Roughton (1948) showed that CO_2 is the chief primary product of the yeast carboxylase and urease reactions, by manometric measurements of the rates of CO_2 evolution and the effects of carbonic anhydrase upon them. For several pyridoxal phosphate-dependent amino acid decarboxylases, CO_2 also appears to be the immediate product, as shown by the absence of significant isotope enrichment in the gas produced by the reaction in H_2^{18}O (Rothberg & Steinberg, 1957). On the other hand, studies of carboxylation with $\text{NaHC}^{18}\text{O}_3$ indicate that HCO_3^- (or H_2CO_3) is the substrate of the biotin enzyme propionyl-CoA carboxylase (Kaziro, Hass, Boyer & Ochoa, 1962) and of phosphoenolpyruvate carboxylase (Maruyama & Lane, 1962).

To answer this question for the nicotinamide nucleotide-linked oxidative decarboxylases, which

do not appear to have been studied from this point of view, a simpler and more direct method than those used previously was devised. The method involves measurements of the rate of reductive carboxylation after initiation by the addition of CO_2 or HCO_3^- , and requires only a sensitive means of recording progress curves for the early stages of the reaction under suitable conditions. This group of enzymes, which includes isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase and the 'malic' enzyme, catalyses reactions of the general type:

or

A recording fluorimeter (Dalziel, 1962) provides a sensitive method for following the oxidation of NADPH. A preliminary account of the work

described here has been published (Londesborough & Dalziel, 1968a).

THEORETICAL CONSIDERATIONS

In aqueous solutions of carbon dioxide at pH 5.5–7.5 the following equilibria exist:

The ionization reaction is effectively instantaneous, but the hydration and dehydration reactions are relatively slow in the absence of catalysts. The velocity constants and temperature coefficients have been estimated by several workers using different techniques, with good agreement (Roughton & Clark, 1951; Dalziel, 1953; Sirs, 1958). The hydration reaction in this pH range can be described by:

$$-\frac{d[\text{CO}_2]}{dt} = k_{+1}[\text{CO}_2] - k_{-1}[\text{H}_2\text{CO}_3]$$

Values at 17° for k_{+1} , calculated from data at three temperatures given by Roughton & Clark (1951), and for k_{-1} , from the value at 18° and the activation energy (Dalziel, 1953), are shown in Table 1. From the ratio of these velocity constants, and the pK_1' value 6.40 (Harned & Davis, 1943) for the apparent first ionization constant of H_2CO_3 :

$$K_1' = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{CO}_2] + [\text{H}_2\text{CO}_3]}$$

the true first ionization constant:

$$K_1 = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]}$$

is $3.0 \times 10^{-4} \text{ M}$ at 17°. The concentration of H_2CO_3 is therefore negligible compared with those of CO_2 and HCO_3^- , and at any fixed pH in the range considered the hydration and dehydration reactions can be represented as a reversible first-order system:

and described by:

$$-\frac{d[\text{CO}_2]}{dt} = k_{+1}[\text{CO}_2] - k'_{-1}[\text{HCO}_3^-]$$

where $k'_{-1} = k_{-1}[\text{H}^+]/K_1$. Calculated values for k'_{-1} at pH 7.3 and pH 6.4 are also given in Table 1.

Roughton & Booth (1938) showed that weak acid anions

catalyse the reactions, and that in phosphate buffer the velocity constants are increased by a factor $(1 + 8[\text{HPO}_4^{2-}])$. For 0.1 M-phosphate buffer, this factor is 1.22 at pH 6.4 and 1.6 at pH 7.3, and values for the velocity constants in this buffer are given in Table 1. Triethanolamine buffer was also used in the present work. The results, compared with those obtained in phosphate buffer, indicate that this cationic buffer, like other straight-chain nitrogenous bases tested by Roughton & Booth (1938), does not catalyse the reactions.

For such a reversible first-order reaction, the integrated rate equation for the approach to equilibrium from either side is:

$$(k_{+1} + k'_{-1})t = 2.3 \log [x_e / (x_e - x_t)]$$

where x_e is the equilibrium concentration of product and x_t its concentration at time t . The half-reaction time is therefore $t_{0.5} = 0.69 / (k_{+1} + k'_{-1})$, and the time required to reach 90% of the equilibrium concentration is $t_{0.9} = 2.3 / (k_{+1} + k'_{-1})$. These times are also shown in Table 1.

The ratio $[\text{CO}_2]/[\text{HCO}_3^-]$ at equilibrium is 8 at pH 7.3 and 1 at pH 6.4, from the Henderson-Hasselbalch equation with $pK_1' 6.4$.

Principle of the method. Reductive carboxylation can be initiated in a buffered solution containing the enzyme and other substrates by adding carbon dioxide as (i) pre-equilibrated $\text{NaHCO}_3\text{-CO}_2$ solution at the pH of the reaction mixture, or (ii) unbuffered NaHCO_3 solution, or (iii) unbuffered CO_2 solution or CO_2 gas. (HCO_3^- and H_2CO_3 cannot be distinguished, and in the subsequent discussion only the two dominant forms of carbon dioxide in solution, CO_2 and HCO_3^- , will be considered.) If only one of the two forms can serve as substrate, the time-course of reductive carboxylation during the first 2 min. will be different in the three cases, and for cases (ii) and (iii) can be predicted from the progress curves for case (i) and the rates of the hydration and dehydration reactions in Table 1. For unequivocal results, certain experimental conditions must be realized. First, the coenzyme and keto acid concentrations should be large, and the carbon dioxide concentrations small, compared with their K_m values, so that the rate of carboxylation is sensitive to (ideally, proportional to) the carbon dioxide concentration. Secondly, the rate of carboxylation, determined by the enzyme concentration, should be small, so that the amount of carbon dioxide used up in the first 2 min. is negligible compared with the amount added. Thirdly, the amounts of unbuffered CO_2 or NaHCO_3 added should be small enough,

Table 1. *Velocity constants for the reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ at 17°*

The constants are defined by:

$$-d[\text{CO}_2]/dt = k_{+1}[\text{CO}_2] - k_{-1}[\text{H}_2\text{CO}_3] = k_{+1}[\text{CO}_2] - k'_{-1}[\text{HCO}_3^-] \quad \text{and} \quad k'_{-1} = k_{-1}[\text{H}^+]/K_1$$

where $K_1 = 3.0 \times 10^{-4} \text{ M}$ is the true first ionization constant of H_2CO_3 . The data are from Roughton & Clark (1951) and Dalziel (1953).

Buffer	pH	k_{+1} (sec. ⁻¹)	k_{-1} (sec. ⁻¹)	k'_{-1} (sec. ⁻¹)	$t_{0.5}$ (sec.)	$t_{0.9}$ (sec.)
None, or triethanolamine	6.4	0.015	11.1	0.015	23	77
	7.3	0.015	11.1	0.002	41	136
0.1 M-Phosphate	6.4	0.018	13.6	0.018	19	63
	7.3	0.024	17.8	0.003	26	85

and the buffer capacity of the reaction mixture large enough, to prevent significant pH changes. All these conditions make demands on the sensitivity of the method of rate measurement that will vary with the properties of the enzyme under study.

If these conditions are satisfied, the progress curves obtained with CO_2 - NaHCO_3 solution should be almost linear. On the other hand, when unbuffered CO_2 or NaHCO_3 solution is used, the rate of carboxylation, and therefore the slope of the progress curve, should increase or decrease during the first 2 min. as the concentration of the true substrate increases or decreases in accordance with the data of Table 1.

For example, consider the results to be expected in 0.1 M-phosphate buffer when reaction is initiated by unbuffered NaHCO_3 solution. If CO_2 is the sole substrate, there will be a lag phase; the slope of the progress curve will increase from zero to a practically constant value in 85 sec. at pH 7.3 and 63 sec. at pH 6.4 (Table 1). The slope should reach half the final value in 26 sec. and 19 sec. respectively. Then 10% and 50% of the added HCO_3^- should be converted into CO_2 at equilibrium and the final slopes should be the same as those of linear progress curves obtained with the appropriate amounts of CO_2 - NaHCO_3 solution. On the other hand, if HCO_3^- is the sole substrate, the slope of the progress curve should decrease. At pH 7.3, the effect will be negligible, since the equilibrium concentration of HCO_3^- will be 90% of the initial concentration. At pH 6.4 the equilibrium HCO_3^- concentration, and therefore the final slope after 63 sec., should be about half the initial value.

Similarly, when reaction is initiated by addition of unbuffered CO_2 solution or by a rapid injection of CO_2 gas into the reaction mixture, the slope of the progress curve will decrease if CO_2 is the substrate, and increase if HCO_3^- is the substrate. In the former case, the rate at pH 7.3 especially should decrease markedly, since the final CO_2 concentration will be only 10% of the initial value.

The conclusions may be confirmed by adding carbonic anhydrase. With enough of this enzyme, all forms of carbon dioxide substrate should give identical progress curves for the same amount of total carbon dioxide.

It was indicated above that the hydration or dehydration reactions that follow the addition of unbuffered CO_2 or HCO_3^- will be accompanied by changes of pH according to eqn. (3). If the intrinsic enzyme activity changes significantly with pH, the slope of the progress curve may change owing to this effect, with the same time-course as that predicted for changes in CO_2 or HCO_3^- concentration. The change of pH resulting from the addition of a known amount of CO_2 or HCO_3^- can easily be calculated for any given buffer concentration. An arbitrary upper limit of 0.05 pH unit was selected as the permissible pH change. With both 0.1 M-phosphate and 0.1 M-triethanolamine buffers at pH 7.3, initial concentrations of 0.018 M- HCO_3^- and 0.002 M- CO_2 are needed to change the pH by this amount. In 0.1 M-phosphate buffer, pH 6.4, the upper limits for the initial concentrations by this criterion are 0.0056 M- HCO_3^- or - CO_2 . Measurable rates were easily obtained with these concentrations, but in some experiments in which reaction was initiated with CO_2 gas these limits were exceeded. As a further check, therefore, the final pH of reaction mixtures was measured, and the variation of enzyme activity with pH was studied.

MATERIALS AND METHODS

Enzymes. Isocitrate dehydrogenase [*threo*-D₃-isocitrate-NADP⁺ oxidoreductase (decarboxylating), EC 1.1.1.42] was prepared from ox heart mitochondria (Londesborough & Dalziel, 1968b), and was free from glutamate dehydrogenase activity.

'Malic' enzyme [L-malate-NADP⁺ oxidoreductase (decarboxylating), EC 1.1.1.40] was prepared from acetone-dried powder of wheat germ by acid treatment, $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel filtration with Sephadex G-200 and chromatography on CM-Sephadex C-50. The specific activity was 3 μmoles of NADP⁺/min./mg. of protein in the assay described by Harary, Korey & Ochoa (1953), and is about twice that of the preparation described by these workers. The product was free from lactate dehydrogenase and NADPH oxidase activities.

Carbonic anhydrase was prepared by the method of Meldrum & Roughton (1933). To 30 ml. of citrated ox blood diluted with 30 ml. of water, 20 ml. of ethanol and 20 ml. of chloroform were added and the mixture was shaken for 3 min. Then 100 ml. of water was added, and after 10 min. the denatured haemoglobin was removed by centrifugation. The aqueous upper layer containing carbonic anhydrase was almost colourless, and was diluted as required with water. The enzyme was stable for several weeks in the cold.

Coenzymes and substrates. NADPH and sodium pyruvate were purchased from Boehringer Corp. (London) Ltd. (London, W. 5), and 2-oxoglutaric acid was from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). Stock solutions were 1 mM-NADPH in buffer solution, assayed by the extinction at 340 μm , 1.0 M-sodium pyruvate in water and 0.1 M-2-oxoglutaric acid neutralized with NaOH.

Saturated solutions of CO_2 in water or in NaHCO_3 solution were prepared and stored in a stoppered aspirator, immersed in a water bath at 17° and connected to a stoppered Thermos flask containing solid CO_2 , which provided a convenient steady stream of the pure gas. The concentration of the solutions in water and in NaHCO_3 solution was estimated, with sufficient accuracy for the present work, as 40 (± 2) mM- CO_2 at 17° and the existing atmospheric pressure, from the solubility data of Harned & Davis (1943). Saturated solutions of CO_2 in 0.32 M- NaHCO_3 and 0.04 M- NaHCO_3 were therefore prepared to give equilibrium mixtures at pH 7.3 and pH 6.4 respectively.

Buffers. With the 'malic' enzyme, the rate of the carboxylation reaction in sodium phosphate buffers was found to be too small to give satisfactory results. Phosphate also has the disadvantage of catalysing the CO_2 -hydration reaction. Glycylglycine and other primary amines do not, and the rate of reaction in glycylglycine was greater than in phosphate. Nevertheless, such buffers were regarded as unsuitable because they react with carbon dioxide to give carbamino compounds. Veronal was not sufficiently soluble to give the required buffer capacity. 0.1 M-Triethanolamine hydrochloride-NaOH buffer, pH 7.3 (Hems, 1959), proved satisfactory for the 'malic' enzyme. The hydrochloride was purchased from Boehringer Corp. (London) Ltd.

Most of the experiments with isocitrate dehydrogenase were done in 0.1 M-sodium phosphate buffers, pH 6.4 and pH 7.3, but some experiments were also made in triethanolamine buffer.

Rate measurements. Progress curves for the carboxylation reactions at 17° were obtained by measurements of the

decrease of fluorescence accompanying NADPH oxidation with a recording fluorimeter (Dalziel, 1962). The initial fluorescence of NADPH (approx. $10\mu\text{M}$) was largely backed off, so that large amplifications could be used, and a small fraction of the reaction to equilibrium occupied the whole width of the recorder chart. With precautions against interference from dust and debris in the solutions (cf. Dalziel, 1962), rates down to $0.1\mu\text{M-NADPH/min.}$ could be measured accurately.

Reaction mixtures were made up in fluorimeter tubes. For the 'malic' enzyme, they contained 2.0ml. of 0.2M-triethanolamine buffer, 0.05ml. of 1mM-NADPH, 0.1ml. of 1.0M-pyruvate, 0.12ml. of 0.05M-MnCl₂ and 0.16ml. of water, to give a total volume of 4ml. after the subsequent addition of 0.1ml. of enzyme solution and the desired volume of carbon dioxide solution. For isocitrate dehydrogenase, the reaction mixtures contained 2.0ml. of 0.2M-phosphate or triethanolamine buffer, 1.0ml. of 0.1M-2-oxoglutarate, 0.05ml. of 1mM-NADPH, 0.1ml. of 0.02M-MgSO₄ and 0.085ml. of water.

To minimize the blank reaction without added carbon dioxide, the stock buffer solutions and water were largely freed from CO₂ by twice passing a rapid stream of CO₂-free N₂ for 5min. and then shaking under vacuum. After temperature equilibration of each reaction mixture in the fluorimeter, and immediately before the addition of enzyme and carbon dioxide substrate, a brisk stream of CO₂-free N₂ was passed for 2min. With these precautions, blank rates recorded after the addition of enzyme and before addition of carbon dioxide were usually negligible.

When carbon dioxide was added as a saturated solution of CO₂ in water or in NaHCO₃ solution, a few millilitres of the stock solution, stored in an aspirator under CO₂, were withdrawn into a test tube; the required volume was at once pipetted into the reaction mixture, and after gentle mixing with a stirrer the progress curve was recorded immediately. In some experiments, CO₂ gas was introduced directly into the reaction mixture. A sintered-glass filter fused into the end of a glass tube connected to a Thermos flask of solid CO₂ provided a stream of small gas bubbles. It was plunged to the bottom of the fluorimeter tube and withdrawn after 1–2sec. The concentration of CO₂ introduced in this way, estimated from the rate of carboxylation after 2 min., was about 2–10mm.

RESULTS

'Malic' enzyme. Progress curves for the reductive carboxylation of pyruvate in 0.1M-triethanolamine buffer, pH 7.3, were recorded with several concentrations of carbon dioxide, added as a saturated solution of CO₂ in sodium hydrogen carbonate at pH 7.3 (0.04M-CO₂, 0.32M-HCO₃⁻). The concentrations of NADPH and pyruvate were $12\mu\text{M}$ and 25mM respectively. From a plot of reciprocal of the initial rate against reciprocal of the CO₂ concentration (Fig. 1) the apparent K_m under these conditions is $3.8 \times 10^{-3}\text{M}$ for CO₂ and $30 \times 10^{-3}\text{M}$ for HCO₃⁻. There is some indication of substrate inhibition with the highest concentration used, but this could be a salt effect, since the ionic strength is more than doubled by the large

sodium hydrogen carbonate concentration of 0.128M.

The progress curve with 2mM-CO₂-16mM-HCO₃⁻, shown in Fig. 2 (curve A), is practically linear for the first 2min. With sodium hydrogen carbonate solution as substrate, however, the progress curve with 16mM-HCO₃⁻ shows a distinct lag phase (Fig. 2, curves B and D). The slope increases to a

Fig. 1. Variation of the reciprocal of the initial rate of reductive carboxylation of pyruvate at 17°, catalysed by the 'malic' enzyme, with the reciprocal of the dissolved CO₂ concentration. Reaction mixtures contained 25mM-pyruvate, $12\mu\text{M-NADPH}$, 1.5mM-MnCl₂ and 0.1M-triethanolamine buffer, pH 7.3. The CO₂ was added as a saturated solution (0.04M) in NaHCO₃ (0.32M), pH 7.3. Rate measurements made in two separate experiments are shown (○ and Δ).

Fig. 2. Progress curves for the reductive carboxylation of pyruvate catalysed by the 'malic' enzyme. For clarity, curves have been displaced with respect to the ordinate, which shows the fluorescence of the reaction mixture in recorder-chart units ($10\text{ units} \equiv 0.166\mu\text{M-NADPH}$). Reaction was initiated at zero time by adding 2mM-CO₂-16mM-NaHCO₃ (curve A) or 16mM-NaHCO₃ (curves B, C and D). For curve C carbonic anhydrase was present in the reaction mixture. Other conditions were as in Fig. 1.

Fig. 3. Progress curves for the reductive carboxylation of pyruvate catalysed by the 'malic' enzyme. Reaction was initiated in every case by bubbling CO_2 into the reaction mixtures for 1–2 sec. at time zero. For curve *C* carbonic anhydrase was present in the reaction mixture. For curves *D* and *E* a larger amount of carbonic anhydrase was added 15 sec. after the introduction of CO_2 . Other conditions were as in Fig. 1.

constant value, equal to that obtained with 2 mM- CO_2 –16 mM- HCO_3^- , in about 2 min. This result is consistent with the conversion of 10% of the added HCO_3^- , essentially inactive as substrate, into reactive dissolved CO_2 , at the rate predicted from kinetic data for the dehydration of H_2CO_3 (Table 1). The effect of adding carbonic anhydrase to the reaction mixture immediately before the addition of 16 mM-sodium hydrogen carbonate, shown in curve *C* of Fig. 2, is to eliminate the lag phase; from a few seconds after the addition of sodium hydrogen carbonate, the progress curve is practically identical with curve *A*. It was shown that carbonic anhydrase did not affect progress curves obtained with an equilibrium mixture of CO_2 and HCO_3^- as substrate.

Progress curves obtained when reaction was initiated by bubbling CO_2 gas into the reaction mixture for 1–2 sec. are shown in Fig. 3 (curves *A* and *B*). In contrast with the almost linear progress curves obtained with CO_2 – HCO_3^- solution, the reaction rate decreases rapidly during the first 2 min. When a small amount of carbonic anhydrase is present in the reaction mixture, the initial rate is lower and a constant rate is reached more quickly (curve *C*). The effect of adding a larger amount of carbonic anhydrase about 15 sec. after the reaction was started by CO_2 gas (shown in curves *D* and *E*) is to decrease the rapid initial rate of carboxylation at once to a much smaller, constant, value.

In control experiments, it was found that progress curves obtained with CO_2 – HCO_3^- solution as substrate were unaffected by bubbling nitrogen gas into the reaction mixture, containing 'malic' enzyme, immediately before the addition of the CO_2 – HCO_3^- substrate.

Since 90% of the added CO_2 will be converted into HCO_3^- in these experiments, qualitative consideration of these results shows clearly that CO_2 is the primary substrate of the enzyme, and that HCO_3^- is at best a much poorer substrate. Approximate quantitative analysis supports this conclusion. The constant rates reached after 2 min., or after the addition of carbonic anhydrase, range from 0.10 μM - to 0.16 μM -NADPH/min. and correspond to initial rates obtained with about 0.55–0.85 mM- CO_2 added as an equilibrium mixture with sodium hydrogen carbonate, from Fig. 1. The total amount of CO_2 gas introduced in these experiments was therefore about 5–7.7 mM, and from the results of Fig. 1 the initial rates should be in the region of 0.55–0.60 μM -NADPH/min. The actual initial rates in the experiments of Fig. 3 are 0.55–0.65 μM -NADPH/min.

The conversion of CO_2 into HCO_3^- is accompanied by the production of an equivalent of H^+ ions, and the pH of reaction mixtures in the experiments with CO_2 gas falls by 0.1–0.2 unit. It is important to show that the decline of the rate of carboxylation with time is not due to a decrease of enzyme activity with decrease of pH, though it is unlikely that such a small pH change could alter the enzyme activity fivefold. The initial rate of carboxylation was measured in 0.1 M-triethanolamine buffer, pH 7.0, with the same enzyme, NADPH and pyruvate concentrations as before, and with a saturated solution of CO_2 (0.04 M) in sodium hydrogen carbonate (0.16 M), pH 7.0, as substrate. With 2 mM- CO_2 –8 mM- HCO_3^- the rate was 0.61 μM -NADPH/min. compared with 0.30 μM -NADPH/min. at pH 7.3 with 2 mM- CO_2 –16 mM- HCO_3^- (Fig. 1). Thus the activity of the enzyme increases with decrease of pH in this region, and the decrease of rate that accompanies conversion of CO_2 into HCO_3^- cannot be ascribed to the accompanying pH change.

Isocitrate dehydrogenase. The results of initial-rate studies of the carboxylation of 2-oxoglutarate in 0.1 M-phosphate buffer pH 7.3, with 11 μM -NADPH, 25 mM-2-oxoglutarate and several concentrations of dissolved carbon dioxide are shown in Fig. 4. The carbon dioxide was added as a saturated solution of CO_2 (0.04 M) in equilibrium with sodium hydrogen carbonate (0.32 M) at pH 7.3. The apparent K_m is 1.6×10^{-3} M for CO_2 and 12.8×10^{-3} M for HCO_3^- .

With 0.2 mM- CO_2 –1.6 mM- HCO_3^- the progress curve for the reaction (Fig. 5, curve *A*) is linear.

Fig. 4. Variation of the reciprocal initial rate of reductive carboxylation of 2-oxoglutarate at 17° , catalysed by isocitrate dehydrogenase, with the reciprocal of the dissolved CO_2 concentration. Reaction mixtures contained $11\mu\text{M}$ -NADPH, 25mM -2-oxoglutarate, 0.5mM - MgSO_4 and 0.1M -phosphate buffer, pH 7.3. The CO_2 was added as a saturated solution (0.04M) in equilibrium with NaHCO_3 (0.32M), pH 7.3.

Fig. 5. Progress curves for the reductive carboxylation of 2-oxoglutarate. Reaction was initiated by 0.2mM - CO_2 - 1.6mM - NaHCO_3 (curve A) or 2mM - CO_2 (curves B and C). For curve B carbonic anhydrase was present in the reaction mixture. Other conditions were as in Fig. 4. The ordinate shows the fluorescence of the reaction mixture in recorder-chart units ($10\text{ units} \equiv 0.082\mu\text{M}$ -NADPH).

Curve C of Fig. 5 is a progress curve obtained under the same conditions but with a saturated solution of CO_2 in water as substrate. The initial dissolved CO_2 concentration was 2mM . The rate of carboxylation decreases with a time-course similar to that for the hydration of CO_2 (Table 1), reaching a constant value equal to that of curve A after about $1\frac{1}{2}\text{min.}$ Curve B shows the result of a similar experiment in which carbonic anhydrase was present in the reaction mixture.

In these experiments the conversion of 1.8mM -

Fig. 6. Progress curves for the reductive carboxylation of 2-oxoglutarate. Reaction was initiated by bubbling CO_2 into the reaction mixture for 1–2 sec. For curve B carbonic anhydrase was added 15 sec. after the introduction of CO_2 . Other conditions were as in Fig. 4.

CO_2 into HCO_3^- is accompanied by a fall of 0.05pH unit, but control experiments showed that the activity of isocitrate dehydrogenase is not significantly affected by this pH change.

The initial rate of carboxylation was measured at pH 7.0 and pH 7.6, with saturated solutions of CO_2 in 0.16M - and 0.71M -sodium hydrogen carbonate respectively as substrate. The other conditions were the same as in the experiments at pH 7.3. With an initial concentration of dissolved CO_2 of 2mM , the initial rates at pH 7.0 and pH 7.6 were the same as at pH 7.2, within the experimental error ($\pm 5\%$). Thus the activity of the enzyme, under the conditions of these experiments, does not vary significantly from pH 7.0 to pH 7.6, and the changes of rate with time observed when unbuffered CO_2 and HCO_3^- are used as substrates at pH 7.3 cannot be accounted for by the small changes of pH that accompany the hydration and dehydration reactions.

In Fig. 6, curve A is a progress curve obtained after initiation of reaction by the direct introduction of CO_2 gas, and the effect of adding carbonic anhydrase 15 sec. after reaction was started in this way is shown in curve B.

In Fig. 7, progress curves obtained when reaction was started with 4mM -sodium hydrogen carbonate, in the presence and absence of carbonic anhydrase, are compared with that obtained with 4mM -sodium hydrogen carbonate pre-equilibrated with dissolved CO_2 at pH 7.3. The lag phase in the former case is evident. The time taken to reach a constant rate is shorter than that predicted from the rate of H_2CO_3 hydration in Table 2. This is consistent with the

Fig. 7. Progress curves for the reductive carboxylation of 2-oxoglutarate. Reaction was initiated by the addition of 0.5 mM- CO_2 -4 mM- NaHCO_3 (curve A) or 4 mM- NaHCO_3 (curves B and C). For curve C carbonic anhydrase was present. Other conditions were as in Fig. 4. The ordinate shows the fluorescence of the reaction mixture in recorder-chart units (10 units $\equiv$ 0.165 μM -NADPH).

slight curvature, in the opposite sense, of the progress curve A for pre-equilibrated CO_2 - HCO_3^- under these conditions.

Experiments of the same kind in 0.1 M-triethanolamine buffer, pH 7.3, gave similar results, except that the time required to reach a constant rate was longer, indicating that this buffer does not catalyse H_2CO_3 hydration, as does phosphate.

Experiments in 0.1 M-phosphate buffer, pH 6.4, confirmed the conclusion that dissolved CO_2 is the true substrate of isocitrate dehydrogenase. The differences between the progress curves were less dramatic because at this pH the H_2CO_3 reaction reaches equilibrium more rapidly (Table 1) and the equilibrium concentrations of dissolved CO_2 and HCO_3^- are equal. The rates of the enzymic reaction at pH 6.4 and pH 7.3 with the same concentrations of dissolved CO_2 and other reactants did not differ significantly, and the apparent K_m for CO_2 at pH 6.4 was $2 \times 10^{-3}\text{M}$.

DISCUSSION

These experiments seem to show in a simple and unequivocal manner that dissolved CO_2 is the real substrate of NADP-linked isocitrate dehydrogenase of ox heart mitochondria and of 'malic' enzyme from wheat germ. The HCO_3^- ion (or H_2CO_3) is either a very poor substrate or not a substrate at all. It is unlikely that the same mechanisms of carboxylation would serve for these very different molecular species, or that an enzyme would use both species in different mechanisms. The apparent K_m values for dissolved CO_2 at pH 7.3 and 17°, with large but not saturating concentrations of the other reactants,

are $3.8 \times 10^{-3}\text{M}$ for 'malic' enzyme and $1.6 \times 10^{-3}\text{M}$ for isocitrate dehydrogenase, of the same order as the physiological concentration of 1.5 mM. It follows from the principle of microscopic reversibility that CO_2 is also the primary product of oxidative decarboxylation by these enzymes.

These findings are in accordance with present views of the mechanism of non-enzymic decarboxylation of 3-oxo acids (Bender & Breslow, 1962) and with other work on the mechanism of the isocitrate dehydrogenase reaction. Isocitrate dehydrogenase from pig heart catalyses the labilization of a single tritium atom in 2-oxo[3- ^3H]glutarate, which suggests that the immediate product of oxidative decarboxylation is an enzyme-bound enolate of 2-oxoglutarate (Rose, 1960). NADPH and Mg^{2+} ions are required for the exchange reaction, and are presumed to play a role in binding of the 2-oxoglutarate to the enzyme. Carbon dioxide is not required, and is therefore likely to be the last of the substrates to react in the carboxylation. From studies with stereospecifically labelled 2-oxo[3- ^3H]glutarate, prepared by enzymic oxidative decarboxylation of isocitrate in tritiated water, it was shown that the same hydrogen atom is involved in the overall oxidative decarboxylation reaction and in the exchange reaction, and in the former reaction replaces the carboxyl group with retention of configuration (Leinhard & Rose, 1964). Simultaneous protonation at C-3 and removal of the carboxyl group is therefore improbable, and it was concluded that decarboxylation gives first a carbanion intermediate, and that the enzyme-bound enolate of 2-oxoglutarate formed by electronic redistribution is subsequently stereospecifically protonated and liberated from the enzyme as the keto form. Thus, in the carboxylation reaction, the last step would be the conversion of the carbanion intermediate into oxalosuccinate by electrophilic attack by CO_2 .

Although isocitrate dehydrogenase catalyses the decarboxylation of oxalosuccinate, the latter has never been detected as an intermediate in the overall reductive carboxylation, and is thought to be directly reduced to isocitrate without dissociation from the enzyme (Siebert, Dubuc, Warner & Plaut, 1957). The present experiments are consistent with this view in that the rate of NADPH oxidation accurately reflects the CO_2 concentration in solution. The experiments of Leinhard & Rose (1964) also seem to show that the enzyme-bound oxalosuccinate must be in the keto form, since randomly labelled 2-oxo[^3H]glutarate would be formed by oxidative decarboxylation of isocitrate in tritiated water if the enol of oxalosuccinate were formed. This is in accord with present views of the mechanism of metal ion-catalysed decarboxylation of 3-oxo acids (Steinberger & Westheimer, 1951).

'Malic' enzyme of wheat germ has not been subjected to such detailed study. Chicken liver 'malic' enzyme is reported to catalyse labilization of a 3-tritium atom from pyruvate only in the presence of all the reactants for the carboxylation reaction, including carbon dioxide (Rose, 1960), and the mechanism may therefore be different from that of isocitrate dehydrogenase. Studies of the chicken liver enzyme, which has been crystallized (Hsu & Lardy, 1967), by the method described here are desirable. Results already obtained for 6-phosphogluconate dehydrogenase from sheep liver show that dissolved CO_2 is the true substrate for this enzyme also (R. H. Villet & K. Dalziel, unpublished work).

The information that dissolved CO_2 is the true substrate of reductive carboxylation shows that change of pH does not directly affect the equilibrium of the overall reactions catalysed by these enzymes (eqn. 1), in contrast with most reactions involving nicotinamide nucleotides, and permits the estimation of the true equilibrium constant and free-energy change (Ochoa, 1948; Harary *et al.* 1953; Londesborough & Dalziel, 1968b). It also makes it possible to interpret pH effects on the reaction rates. With isocitrate dehydrogenase, the rate of carboxylation with a fixed total carbon dioxide concentration increases with decrease of pH (Siebert *et al.* 1957), and the present experiments show that this is largely due to increase in the proportion of dissolved CO_2 . From preliminary experiments with arbitrary reactant concentrations, the rates at pH 6.4 and 7.4 with the same dissolved CO_2 concentration are practically the same, and the apparent K_m values for CO_2 are also similar. In contrast, the 'malic' enzyme shows a true pH effect, the rate at pH 7.0 being twice that at pH 7.4 with the same dissolved CO_2 and other reactant concentrations.

The kinetic method for the identification of the true substrate of carboxylases has advantages over both isotopic and manometric methods. Isotopic experiments must be carried out under conditions such that $\text{CO}_2\text{--HCO}_3^-$ exchange is minimized. For example, studies of the carboxylation of propionyl-CoA with $\text{NaHC}^{18}\text{O}_3$, which showed that all three oxygen atoms of the latter appear in the products and therefore that HCO_3^- is the substrate of propionyl-CoA carboxylase, were made in alkaline solution and at low temperatures, with minimal reaction times and large reactant concentrations, and it was necessary to correct for isotope exchange with the medium through H_2CO_3 dehydration by control experiments (Kaziro *et al.* 1962). The manometric method of Krebs & Roughton (1948) requires large concentrations of enzyme and any cofactor so that a small amount of substrate can be rapidly decarboxylated. A few experiments by this method with

isocitrate dehydrogenase provided some support for the conclusion that CO_2 is the substrate, but the amounts of enzyme and NADP^+ needed prohibited a proper study.

The kinetic method could, of course, be applied to any carboxylase if a sufficiently sensitive means of recording progress curves is available. For nicotinamide nucleotide-linked carboxylases a recording fluorimeter provides such a means and it may be possible to apply it to other enzymes by coupled assays, for example to pyruvate carboxylase and phosphoenolpyruvate carboxykinase by coupling with malate dehydrogenase, and to ribulose diphosphate carboxylase coupled with phosphoglycerate kinase and triose phosphate dehydrogenase.

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