The carboxybiotin complex of pyruvate carboxylase

A kinetic analysis of the effects of Mg^{2+} ions on its stability and on its reaction with pyruvate

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The enzyme-[^{14}C]carboxybiotin complex of sheep liver pyruvate carboxylase was isolated and the reaction between this and pyruvate was studied by using the quenched-flow rapid-reaction technique. At $0.5^{\circ}C$ the reaction was 80% complete within 180 ms. The reaction was monophasic and obeyed pseudo-first-order kinetics. Increasing concentrations of Mg^{2+} caused a decrease in the magnitude of the observed pseudo-first-order rate constant. Throughout the carboxylation of pyruvate, the rate-limiting step of the reaction occurred after the dissociation of carboxybiotin from the first sub-site, whereas in the slow phase of the reaction with 2-oxobutyrate this dissociation is the rate-limiting step. It is possible, from the reaction scheme proposed, that the inhibition of overall enzymic activity by high concentrations of Mg^{2+} could be caused by the transfer of the carboxy group from biotin to pyruvate becoming rate-limiting. The efficacy of a substrate as a signal for the movement of carboxybiotin from the first sub-site is reflected by the amount that the effective affinity of the enzyme-carboxybiotin complex for Mg^{2+} is lowered. In the presence of the substrates tested, the affinities of the carboxybiotin complex can be arranged in order of increasing magnitude, i.e.:

The kinetics of the decay of the enzyme-[^{14}C]carboxybiotin complex at $0^{\circ}C$ in the absence of substrates are similar to the reaction with pyruvate except that the carboxybiotin is also unstable in the first sub-site, to some degree. This similarity allows for the proposal of a general scheme for the decarboxylation of the enzyme-carboxybiotin complex in the presence or in the absence of substrates.

The current view of the active site of the biotin-dependent enzyme pyruvate carboxylase (EC 6.4.1.1) is that it consists of two spatially distinct sub-sites. The overall reaction catalysed by the enzyme occurs as two partial reactions (1 and 2); in the first sub-site the biotin prosthetic group is carboxylated with the concurrent cleavage of ATP, in a reaction requiring the presence of free Mg^{2+} and acetyl-CoA:

The carboxybiotin thus formed moves to the site of the second partial reaction (2), where pyruvate binds and the carboxy group is transferred from

biotin to pyruvate to form oxaloacetate:

Easterbrook-Smith *et al.* (1976) showed that pyruvate acts as a signal to summon carboxybiotin from the first sub-site to the second. More recently Goodall *et al.* (1981) were able to examine the translocation of carboxybiotin between the sub-sites by using a substrate analogue of pyruvate, namely 2-oxobutyrate. With the use of this compound it was possible to measure part of the time course of carboxylation of 2-oxobutyrate by the isolated enzyme-[^{14}C]carboxybiotin complex, by using conventional methods. The reaction with pyruvate, however, occurred too rapidly to be

measured by these means and required the use of rapid-reaction technology.

In the present investigation the quenched-flow technique was used to examine the carboxylation of pyruvate by the isolated enzyme-carboxybiotin complex. In addition, the effects of Mg^{2+} on the kinetics of both this reaction and the spontaneous decay of the enzyme-carboxybiotin complex were investigated.

Materials and methods

Materials

MgCl_2 was prepared from spectroscopically pure Mg and standardized by atomic absorption spectroscopy. *N*-Ethylmorpholine (redistilled) and sodium pyruvate were as previously described by Easterbrook-Smith *et al.* (1978). $\text{NaH}^{14}\text{CO}_3$ was obtained as an aqueous solution of 2 mCi/ml at a specific radioactivity of 55 mCi/mmol from Amersham Australia Pty. Ltd.

Preparation of pyruvate carboxylase

Pyruvate carboxylase was prepared from freeze-dried sheep liver mitochondria by the procedure described by Goss *et al.* (1979), except that a DEAE-Sephadex column was used in place of the DEAE-Sephadex column. The enzyme was purified to specific activities in the range 15–25 units/mg of protein, where 1 unit of enzyme catalysed the formation of 1 μmol of oxaloacetate/min at 30°C. The enzyme was stored at –80°C in a solution composed of 1.6 M-sucrose/0.1 M-*N*-ethylmorpholine/acetate buffer, pH 7.0, containing 40 mM- $(\text{NH}_4)_2\text{SO}_4$.

Isolation of the enzyme-[^{14}C]carboxybiotin complex

The enzyme was transferred from storage buffer to 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.8, by centrifuging through Sephadex G-25 as described by Helmerhorst & Stokes (1980). To the enzyme solution, various additions were made to form a final solution of 0.5 ml volume containing 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.8, 5 mM- MgCl_2 , 0.25 mM-acetyl-CoA, 7.3 mM- $\text{NaH}^{14}\text{CO}_3$ and 2.5 mM-ATP. The reaction was started by the addition of ATP, and after 4 min at room temperature the reaction mixture was placed on ice for 10 min. The reaction was terminated by the addition of 0.5 ml of ice-cold 0.2 M-EDTA solution, pH 7.0. After standing for 10 min at 0°C the enzyme-[^{14}C]carboxybiotin complex was transferred to a solution of 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.8, by centrifugation through Sephadex G-25, as described above.

Quenched-flow apparatus

A simple quenched-flow apparatus was constructed composed of two thermostatically con-

trolled 1 ml glass reactant syringes, plastic loading taps, a Perspex mixing chamber and interchangeable reaction tubes of various lengths made of Teflon. The ends of the reaction tubes dip into the quenching solutions contained in glass vials. The reactant syringes are emptied by means of a piston, driven by gas pressure, which pushes against the plungers of the syringes. The gas pressure to the piston is regulated by an electrically operated solenoid switch. Attached to the piston is a variable potentiometer, and the speed and motion of the piston during an experiment were monitored, initially, with a storage oscilloscope, by observing the voltage change across the potentiometer as the piston moved. Later, this monitoring function was performed by a timing circuit, interfaced to a Commodore CBM 4016 computer, which was also used to control the solenoid switch.

The reaction time is varied by changing the length of the reaction tube or by changing the gas pressure used to drive the piston. Each reaction tube was calibrated by using the hydrolysis of 2,4-dinitrophenyl acetate by NaOH as a test reaction, in the way described by Gutfreund (1967) and Eccleston *et al.* (1980). The apparatus was found to have an effective range of reaction times between 10 ms and 210 ms.

In the experiments described in the present paper the quenching solution used was 4 ml of 0.2 M-semicarbazide hydrochloride in 1 M-HCl. The end of the reaction tube being used was positioned in the centre of the vial at a distance of about 3 mm from the bottom of the vial to ensure rapid mixing of the reaction solution with the quencher.

Quenched-flow experiments

The prepared enzyme-[^{14}C]carboxybiotin complex was diluted down to 1–2 units/ml at 0°C with *N*-ethylmorpholine/HCl buffer, pH 7.8, containing the required concentration of MgCl_2 . A 1 ml portion of this solution was used to fill one of the reactant syringes in the quenched-flow apparatus, and the other syringe was filled with a solution containing 20 mM-sodium pyruvate and the required concentration of MgCl_2 . A glass vial was weighed, 4 ml of quencher was then added and the vial was re-weighed. The reactants were then mixed and quenched, the time at which the reaction was performed was noted and the vial was weighed again. From the solution in the vial, 4 ml was transferred to a tube and the vial was weighed for the last time. The contents of the tube were then freeze-dried and the resulting solid was re-dissolved in 0.2 ml of distilled water. Of this solution, 50 μl was applied to a 2.5 cm $\times$ 2.5 cm square of Whatman 3MM paper, which was then dried at 105°C for 5 min. The radioactivity of the paper was

counted in 3ml of toluene scintillation fluid (Bousquet & Christian, 1960).

As the enzyme- ^{14}C carboxybiotin complex is relatively unstable, a significant proportion of it could decay during the approx. 50 min required for a complete time-course experiment. The residual ^{14}C carboxybiotin available for transfer to pyruvate was measured at intervals during the experiment by mixing the reactants as described above and allowing the reaction to go to completion (approx. 1 min) before adding the 4ml of the quenching solution to the receiving vial. These controls were then treated in exactly the same way as the experimental solutions.

From the calculated weights of reaction mixtures, the radioactivity for each time point and each control was expressed as c.p.m./g of reaction mixture. The control values were fitted to an exponential decay curve as described below, and values from this curve were used to determine the total available c.p.m./g of reaction mixture for each experimental time point. In this way the percentage of the total transferable radioactivity transferred to pyruvate during the reaction time was calculated.

Stability of the enzyme- ^{14}C carboxybiotin complex

The complex was prepared as described above and divided equally among six tubes containing 0.1M-*N*-ethylmorpholine/HCl buffer, pH 7.8, and various concentrations of MgCl_2 in a final volume of 6.5 ml. The enzyme- ^{14}C carboxybiotin complex solutions (approx. 1.5 units/ml) were incubated at 0°C , and at various times 0.8 ml samples were removed, each to be added to 0.8 ml of 20 mM-sodium pyruvate in the *N*-ethylmorpholine/HCl buffer, pH 7.8. After at least 1 min to ensure completion of the reaction, 3 ml of 0.2M-semicarbazide hydrochloride in 1M-HCl was added. The solutions were then freeze-dried and treated in the same way as the quenched-flow samples. The small but significant acid-stable radioactivity that is evident at the end of the decay time courses was accounted for by subtracting from each sample's radioactivity (c.p.m.) the value obtained for the sample without added MgCl_2 . The net results were then fitted to a single exponential decay curve.

Data analysis

All lines and curves were fitted to the data on the CBM4016 computer, by using the non-linear regression program written by Duggleby (1981).

Results and discussion

Carboxylation of pyruvate from the enzyme- ^{14}C carboxybiotin complex

Fig. 1 shows the time course of the decay of the enzyme- ^{14}C carboxybiotin complex in the pres-

Fig. 1. Time course of the transfer of ^{14}C -labelled carboxy group from the enzyme- ^{14}C carboxybiotin complex at 0.5°C ($\pm 0.5^\circ\text{C}$).

The reaction was carried out in 0.1M-*N*-ethylmorpholine/HCl buffer, pH 7.8, containing 10 mM-sodium pyruvate. The data represent the computer-averaged results of five separate experiments. The continuous line is the computer-fitted curve of the data to the equation:

At time t

$$\%[\text{Enz-biotin-}^{14}\text{CO}_2^-] = \%[\text{Enz-biotin-}^{14}\text{CO}_2^-]_0 \cdot e^{-k_{\text{obs}} t}$$

where $\%[\text{Enz-biotin-}^{14}\text{CO}_2^-]_0 = \% \text{ at } t = 0$.

ence of 10 mM-sodium pyruvate at 0.5°C . The reaction obeys pseudo-first-order kinetics, with the fitted curve passing very close (101%) to 100% at zero time and approaching 0% at infinite time. The measured pseudo-first-order rate constant had a value of $10.55 \pm 0.93 \text{ s}^{-1}$, and this did not change significantly when the reaction was carried out in the presence of 30 mM-pyruvate.

In addition to the differences in time scale, these time courses of reaction with pyruvate differ from those observed by Goodall *et al.* (1981), where 2-oxobutyrate was used as a substrate, in that with this substrate analogue the kinetics were biphasic, with the measurable slow phase being preceded by a fast 'burst' phase. These investigators interpreted the existence of these two phases to indicate that the enzyme- ^{14}C carboxybiotin complex exists in two forms that are in equilibrium with each other before the addition of substrate, i.e.:

On the addition of 2-oxobutyrate, all the enzyme-[^{14}C]carboxybiotin complex in the form of State II reacted rapidly in the fast phase, which was then followed by a slower reaction where the rate-limiting step was the conversion of State I into State II, i.e.:

where $k_{+3} \gg k_{+2}$.

If the existence of two such states is accepted, then they must be taken into account when considering a model scheme for the reaction with pyruvate. In order to account for the monophasic nature of the reaction with pyruvate, which indicates that there is no apparent change in the rate-limiting step throughout the course of the reaction, it must be concluded that $k_{+3} < k_{+2}$. However, it is known that the rate of the overall reaction with pyruvate is much higher than the rate of the slow phase of the reaction with 2-oxobutyrate, which is governed by k_{+2} . This implies that the size of k_{+2} must depend on the substrate being used. In both sets of experiments the concentrations of substrates being used were saturating, and it is known that pyruvate binds to the enzyme in a rapid equilibrium (Mildvan & Scrutton, 1967). There is also no known reason why the substrates cannot bind to all forms of the enzyme. Thus the reaction schemes for pyruvate and 2-oxobutyrate may be re-written as follows:

where S may be either pyruvate or 2-oxobutyrate and it is assumed that the substrate has bound very rapidly to each form of the enzyme-[^{14}C]carboxybiotin complex. Here the magnitudes of the rate constants k_{+2} and k_{-2} are dependent on the substrate.

Effect of Mg^{2+} on the transcarboxylation reaction

Fig. 2 shows examples of the effect of Mg^{2+} on the transfer of the carboxy group from the enzyme-[^{14}C]carboxybiotin complex to pyruvate. The kinetics of these reactions were similar to those in the absence of Mg^{2+} , except that increasing concentrations of Mg^{2+} caused the magnitude of the observed pseudo-first-order rate constant to decrease (Table 1).

Goodall *et al.* (1981) also found that increasing Mg^{2+} concentrations decreased the magnitude of the observed pseudo-first-order rate constant. In the case of 2-oxobutyrate, the effect of Mg^{2+} was observed on the slow phase of the reaction, i.e. the

Fig. 2. Effects of various concentrations of Mg^{2+} on the rates of carboxylation of pyruvate by enzyme-[^{14}C]carboxybiotin complex

The lines are drawn in accordance with sets of the data, in the way described in the legend to Fig. 1. Concentrations of Mg^{2+} were as follows: $\square$, 0 mM; $\blacksquare$, 10 mM; $\circ$, 40 mM; $\bullet$, 80 mM.

Table 1. Pseudo-first-order rate constants for the transfer of the ^{14}C -labelled carboxy group from the enzyme-carboxybiotin complex to pyruvate in the presence and in the absence of various concentrations of MgCl_2

The values given are derived from multiple observations for each concentration of Mg^{2+} (one to five time courses each comprised of seven to 12 observations), with the raw data being computer-averaged and subjected to a single curve-fitting process. The standard errors are calculated from the computer fit of the rate equation to the data, as described by Duggleby (1981).

$[\text{MgCl}_2]$ (mM)	$k_{\text{app.}}$ (s^{-1})
0	10.55 ± 0.93
2	10.53 ± 1.65
5	9.09 ± 1.50
10	7.87 ± 0.65
15	6.74 ± 0.86
20	6.72 ± 1.18
40	4.02 ± 0.05
80	2.34 ± 0.21

conversion of State I into State II, where k_{+2} governs the rate-limiting step. With pyruvate, however, k_{+3} is the rate-limiting step throughout the reaction, and to account for the experimental

Scheme 1. Model A

In this model k_{+2} and k_{-2} are both $> k_{+3}$, and thus throughout the reaction State I is in equilibrium with State II.

Abbreviation: Pyr, pyruvate.

Pyr

Pyr

Scheme 2. Model B

In this model k_{+1} , k_{-1} , k_{+2} and k_{-2} are all $> k_{+4}$. Abbreviation: Pyr, pyruvate.

data we propose model A (Scheme 1) for the reaction with pyruvate in the presence of Mg^{2+} .

Model A was developed mathematically in the same way as Goodall *et al.* (1981), and details of the derivation of $k_{\text{obs.}}$, the observed pseudo-first-order rate constant, are given in the Appendix.

The observed pseudo-first-order rate constant, $k_{\text{obs.}}$, is:

$$k_{\text{obs.}} = \frac{k_{+3}}{\left(\frac{k_{-1} \cdot k_{-2} [\text{Mg}^{2+}]}{k_{+1} \cdot k_{+2}} + \frac{k_{-2}}{k_{+2}} + 1 \right)}$$

which may be expressed:

$$k_{\text{obs.}} = \frac{1}{(A \cdot [\text{Mg}^{2+}] + B)}$$

or

$$\frac{1}{k_{\text{obs.}}} = A \cdot [\text{Mg}^{2+}] + B$$

where

$$A = \frac{k_{-1} \cdot k_{-2}}{k_{+1} \cdot k_{+2} \cdot k_{+3}}$$

and

$$B = \frac{1}{k_{+3}} \left(\frac{k_{-2}}{k_{+2}} + 1 \right)$$

Fig. 3 shows a plot of $k_{\text{obs.}}^{-1}$ versus $[\text{Mg}^{2+}]$, which fits the prediction of linearity well. One of the main points that results from this model of the carboxylation of pyruvate is the obligatory dissociation of Mg^{2+} from the enzyme during the catalytic cycle.

Fig. 3. Plot of $k_{\text{obs.}}^{-1}$ versus $[\text{Mg}^{2+}]$ for the carboxylation of pyruvate by enzyme- ^{14}C carboxybiotin complex. The line is drawn in accordance with the equation derived from model A (Scheme 1). The error bars represent ± 1 S.E.

This may explain the observed inhibition of overall enzymic activity at high Mg^{2+} concentrations (Goodall, 1981), when the rate-limiting step of the overall reaction could become the carboxy transfer reaction from carboxybiotin to pyruvate. A scheme was considered where carboxy transfer could occur without dissociation of Mg^{2+} , i.e. model B (Scheme 2).

The pseudo-first order rate constant for model B is given by a relationship:

$$k_{\text{obs.}} = \frac{1}{(C \cdot [\text{Mg}^{2+}] + D)} + k_{+4}$$

where

$$C = \frac{\frac{k_{-1} \cdot k_{-2}}{k_{+1} \cdot k_{+2}}}{k_{+3} - k_{+4} \left(1 + \frac{k_{-2}}{k_{+2}}\right)}$$

and

$$D = \frac{\left(1 + \frac{k_{-2}}{k_{+2}}\right)}{k_{+3} - k_{+4} \left(1 + \frac{k_{-2}}{k_{+2}}\right)}$$

However, when the data were fitted to this equation, the value of k_{+4} was small and negative, indicating that k_{+4} is close to zero and that model A is an adequate representation of the reaction scheme.

One difference between the reaction with 2-oxobutyrate and that with pyruvate is that with the latter substrate the rate-limiting step throughout the reaction is either the movement of carboxybiotin to the part of the active site where carboxylation of pyruvate occurs or the carboxylation reaction itself.

To explain the 'hydrolytic leak' observed at low pyruvate concentrations, Easterbrook-Smith *et al.* (1976) proposed that pyruvate binds to the second sub-site and thus signals the movement of carboxybiotin from the first sub-site to the second. However, before the carboxybiotin reaches the second site, the pyruvate dissociates and, as the carboxybiotin is unstable in the second site, it is hydrolysed. These proposals agree with the experimental observations reported by Cheung & Walsh (1976) and Goodall *et al.* (1981). One of the conclusions to be drawn from these proposals is that the hydrolysis of the carboxybiotin in the second sub-site must occur rapidly before pyruvate can re-bind or the carboxybiotin can return to the first sub-site. This implies that the rate-limiting step in the carboxylation of pyruvate from the enzyme-carboxybiotin complex is the movement of the carboxybiotin from the vicinity of the first sub-site to the second sub-site.

When considering what States I and II represent in terms of the position of carboxybiotin within the active site, Goodall *et al.* (1981) proposed that in State II the carboxybiotin is not bound but is in the

vicinity of the site of the first partial reaction. It was not considered likely that the carboxybiotin would be in the site of the second partial reaction, since it is known to be unstable there (Easterbrook-Smith *et al.*, 1976). Thus one way that pyruvate could act as a better signal than 2-oxobutyrate for the movement of carboxybiotin from the first sub-site to the second would be by enhancing the rate of the dissociation of the carboxybiotin from the first sub-site, i.e. increasing the rate of interconversion between States I and II. Thus in the absence of Mg^{2+} the observed first-order rate constant is:

$$k_{\text{obs.}} = \frac{k_{+3}}{\left(1 + \frac{k_{-2}}{k_{+2}}\right)} = 10.55 \pm 0.93 \text{ s}^{-1}$$

and the apparent dissociation constant for the Mg^{2+} -enzyme-carboxybiotin complex is:

$$\begin{aligned} & \frac{[\text{Mg}^{2+}] \left(\left[\begin{array}{c} \text{State I} \\ \text{Pyr} \end{array} \right] + \left[\begin{array}{c} \text{State II} \\ \text{Pyr} \end{array} \right] \right)}{\left[\begin{array}{c} \text{Mg}^{2+} \cdot \text{State I} \\ \text{Pyr} \end{array} \right]} = \frac{\left(\frac{k_{-2}}{k_{+2}} + 1 \right)}{\frac{k_{-1} \cdot k_{-2}}{k_{+1} \cdot k_{+2}}} \\ & = (1 + K_2) K_1 \\ & = \frac{B}{A} \\ & = 22.6 \pm 0.7 \text{ mM} \end{aligned}$$

Effect of Mg^{2+} on the stability of the enzyme-[^{14}C]carboxybiotin complex

Fig. 4 shows the time courses for the decay of the enzyme-[^{14}C]carboxybiotin complex in the absence of substrates and in the presence of various concentrations of Mg^{2+} . As can be seen, the decay curves follow pseudo-first-order kinetics and the effect of increasing concentrations of Mg^{2+} is to decrease the magnitude of the observed first-order rate constant. In these respects the kinetics are very similar to the reaction with pyruvate and therefore the similar models C and D (Schemes 3 and 4) may be considered for the decay of the enzyme-[^{14}C]carboxybiotin complex. A significant value was obtained for k_{+4} ($0.0038 \pm 0.0003 \text{ min}^{-1}$) from the fit to model D, and this model can be seen to be a better fit to the data than model C.

This indicates that carboxybiotin is, to some degree, unstable in the site of the first partial reaction, but the rate constant for this process, k_{+4} , is approximately 5 times smaller than the rate constant for the decay of the enzyme-[^{14}C]carboxybiotin complex in the absence of Mg^{2+} , i.e.

$k_{+3}/[1 + (k_{-2}/k_{+2})] = 0.0202 \text{ min}^{-1}$. The other feature of the decay kinetics that is important is that the rate-limiting step is the same as in the reaction with pyruvate, that is the movement of carboxybiotin to the second sub-site.

Fig. 4. Effects of various concentrations of Mg^{2+} on the rates of decay of the enzyme- ^{14}C carboxybiotin complex in the absence of substrates at 0°C

The reaction was carried out in 0.1 M - N -ethylmorpholine/HCl buffer, pH 7.8, and the lines are drawn in accordance with computer fits of the data as described in the legend to Fig. 1. Concentrations of Mg^{2+} were as follows: Δ , 0 mM ; $\blacktriangle$, 2 mM ; $\circ$, 5 mM ; $\bullet$, 10 mM ; $\square$, 15 mM ; $\blacksquare$, 20 mM .

The apparent dissociation constant of the Mg^{2+} ·enzyme-carboxybiotin complex measured from the stability data is $1.24 \pm 0.47 \text{ mM}$. That measured by Goodall *et al.* (1981) for the

Mg^{2+} ·enzyme-carboxybiotin complex

2-oxobutyrate

was 9.35 mM , and, as shown above, that for

Mg^{2+} ·enzyme-carboxybiotin complex

pyruvate

Fig. 5. Plot of k_{obs} versus $[\text{Mg}^{2+}]$ for the decay of the enzyme- ^{14}C carboxybiotin complex

The lines represent computer fits of the data to equations derived from model C (Scheme 3) (.....) and model D (Scheme 4) (—). The error bars represent $\pm 1 \text{ s.e.}$ The value determined for k_{+4} from the fit to model D was $0.0038 \pm 0.0003 \text{ min}^{-1}$.

Scheme 3. Model C

Scheme 4. Model D

was 22.6 mM. In the stability experiments, there is no substrate to act as a signal for the movement of carboxybiotin from the site of the first partial reaction to the site of the second. 2-Oxobutyrate is a substrate analogue of pyruvate and would not be expected to be as good a signal as pyruvate. Thus in general terms it would appear that the efficacy of a substrate as a signal depends on how much it can diminish the effective affinity of the enzyme-carboxybiotin complex for Mg^{2+} . As concluded above, there is an effect on k_{+2} and k_{-2} , but effects as well on k_{+1} and k_{-1} cannot be excluded.

It should be noted that the half-lives of the enzyme- $[^{14}C]$ carboxybiotin complex in the absence of substrates, cited by Goodall *et al.* (1981), actually refer to the complex formed with pyruvate carboxylase from chicken livers. The rate constants measured in the present paper are in good agreement with the half-lives noted by Goodall (1981) for the enzyme from sheep livers. In those measurements Goodall (1981) used enzyme concentrations of about 20 units/ml, whereas in the present work enzyme concentrations of between 1 and 2 units/ml were used. It thus appears that the phenomenon of dilution inactivation of overall enzymic activity, which is apparent below about 4 units/ml (Ashman *et al.*, 1972), has no effect on the decay of the enzyme- $[^{14}C]$ carboxybiotin complex and, in view of the similarity between the kinetics, no effect on the carboxylation of pyruvate. This would agree with the observations made in this laboratory (Clements, 1979) that the pyruvate/oxaloacetate exchange reaction is not affected by dilution.

The present paper has shown the application of a rapid-reaction technique to the study of the mechanism of pyruvate carboxylase with the use of the natural substrate, enabling the measurement of certain rate constants for reaction steps within the

mechanism that can be related to other parts of the overall mechanism. It is hoped that by continued use of this technique that the mechanism of the second partial reaction may be further elucidated and that the first partial reaction may also be studied.

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APPENDIX

Derivation of the rate equation for the reaction of the enzyme-carboxybiotin complex with pyruvate in the presence of Mg^{2+}

In accordance with Scheme 1, model A, and assuming that k_{+1} and k_{-1} are both $> k_{+3}$, and that, as $[Mg^{2+}] \gg \left[\begin{smallmatrix} \text{Enzyme-}[^{14}C]\text{carboxybiotin} \\ \text{Pyr} \end{smallmatrix} \right]$ (where Pyr represents pyruvate), $[Mg^{2+}]$ is constant, then at time t :

$$\left[\begin{smallmatrix} \text{State I} \\ \text{Pyr} \end{smallmatrix} \right] = \frac{k_{-2}}{k_{+2}} \left[\begin{smallmatrix} \text{State II} \\ \text{Pyr} \end{smallmatrix} \right]$$

$$\left[\begin{smallmatrix} Mg^{2+} \cdot \text{State I} \\ \text{Pyr} \end{smallmatrix} \right] = \frac{k_{-1} \cdot [Mg^{2+}] \cdot k_{-2}}{k_{+1} \cdot k_{+2}} \left[\begin{smallmatrix} \text{State II} \\ \text{Pyr} \end{smallmatrix} \right]$$

$$\begin{aligned}
 \therefore \left[\begin{array}{c} \text{Enzyme-}^{14}\text{C} \text{carboxybiotin} \\ \text{Pyr} \end{array} \right] &= \left[\begin{array}{c} \text{Mg}^{2+} \cdot \text{State I} \\ \text{Pyr} \end{array} \right] + \left[\begin{array}{c} \text{State I} \\ \text{Pyr} \end{array} \right] + \left[\begin{array}{c} \text{State II} \\ \text{Pyr} \end{array} \right] \\
 &= \left[\begin{array}{c} \text{State II} \\ \text{Pyr} \end{array} \right] \left(\frac{k_{-1} \cdot [\text{Mg}^{2+}] \cdot k_{-2}}{k_{+1} \cdot k_{+2}} + \frac{k_{-2}}{k_{+2}} + 1 \right) \\
 - \frac{d}{dt} \left[\begin{array}{c} \text{Enzyme-}^{14}\text{C} \text{carboxybiotin} \\ \text{Pyr} \end{array} \right] &= k_{+3} \left[\begin{array}{c} \text{State II} \\ \text{Pyr} \end{array} \right] \\
 &= \frac{k_{+3} \left[\begin{array}{c} \text{Enzyme-}^{14}\text{C} \text{carboxybiotin} \\ \text{Pyr} \end{array} \right]}{\left(\frac{k_{-1} \cdot [\text{Mg}^{2+}] \cdot k_{-2}}{k_{+1} \cdot k_{+2}} + \frac{k_{-2}}{k_{+2}} + 1 \right)}
 \end{aligned}$$