Effects of Acetyl CoA on the Pre-Steady-State Kinetics of the Biotin Carboxylation Reaction of Pyruvate Carboxylase†

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ABSTRACT: The approach to steady-state for the formation of the enzyme−carboxybiotin complex obeys first-order kinetics, with the proportion of the total enzyme present as the enzyme−carboxybiotin complex in the steady-state being about 60%. The approach to steady-state for ATP cleavage also obeys first-order kinetics. The apparent first-order rate constants for the approach to steady-state, in the presence and absence of acetyl CoA, respectively, are 6.6 and 0.028 s^{-1} for ATP cleavage and 6.1 and 0.028 s^{-1} for enzyme−carboxybiotin formation. The similarities of the values of the rate constants for the two reactions indicates that there is a common rate-limiting step. The large enhancement of these rate constants in the presence of acetyl CoA suggests that a major effect of acetyl CoA in the reaction is to enhance the rate of the step in which the putative carboxyphosphate complex is formed and in which ATP is cleaved. In addition, in the presence of acetyl CoA, the formation of the enzyme−carboxybiotin complex is much more tightly coupled to ATP cleavage in the presence of acetyl CoA than in its absence. Modeling studies were performed, and reaction schemes are proposed which give simulations similar to the experimental data. In the reaction schemes, the carboxyphosphate intermediate is able to undergo abortive decomposition without carboxylating biotin. The rate of this abortive reaction is greatly reduced in the presence of acetyl CoA.

Pyruvate carboxylase (EC 6.4.1.1) contains a covalently bound biotin prosthetic group, and the overall reaction that it catalyzes proceeds in two steps as follows:

$$\text{MgATP} + \text{HCO}_3^- + \text{E-biotin} \overset{\text{Mg}^{2+},\ \text{acetyl CoA}}{\rightleftharpoons} \text{MgADP} + \text{P}_\text{i} + \text{E-biotin-CO}_2^- \quad (1)$$

$$\text{E-biotin-CO}_2^- + \text{pyruvate} \rightleftharpoons \text{E-biotin} + \text{oxaloacetate} \quad (2)$$

In reaction 1 ATP cleavage occurs and is accompanied by carboxylation of the biotin moiety. In reaction 2, this carboxyl group is transferred to pyruvate to form oxaloacetate. Free Mg^{2+} and acetyl CoA are only required in reaction 1, but the degree of dependence on acetyl CoA for activity depends on the source of the enzyme [for a review see Attwood (1995)]. In the present work, chicken liver pyruvate carboxylase was used, and this shows an almost complete dependence on acetyl CoA for activity. Climent and Rubio (1986) proposed that enzymes that use HCO_3^- for carboxylation proceed via a common mechanism in which a carboxyphosphate intermediate is formed. On the basis of currently available evidence, the most likely way in which reaction 1 is thought to proceed is via such a carboxyphosphate intermediate as shown in reaction 3 [see Knowles (1989) and Attwood (1995)].

Scrutton et al. (1965) showed that incubation of pyruvate carboxylase with Mg^{2+}, MgATP, $H^{14}CO_3^-$ and acetyl CoA resulted in the formation of the enzyme−[^{14}C]carboxybiotin complex. Later, Phillips et al. (1992) found that if the above reaction was performed in the absence of acetyl CoA, a [^{14}C]-

$$\begin{array}{l} \text{MgATP} + \text{HCO}_3^- + \text{E-biotinH} \rightleftharpoons \underset{\displaystyle{}^{-2}\text{O}_3\text{POCO}_2^-}{\text{E-biotinH}} \rightleftharpoons \underset{\displaystyle\text{MgADP}}{\text{MgADP} +} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \text{P}_\text{i} + \text{E-biotin-CO}_2^- \end{array} \quad (3)$$

carboxyenzyme complex was formed that had greatly reduced capacity to carboxylate pyruvate in the absence of acetyl CoA. These authors suggested that this complex was in fact the enzyme−[^{14}C]carboxyphosphate complex and that acetyl CoA facilitated the transfer of the carboxyl group to biotin. However, Attwood (1993) showed that the apparent dependence on acetyl CoA for the complex to carboxylate pyruvate was an artefact of the reaction system and that in fact the [^{14}C]carboxyenzyme complex behaved in the same way as the enzyme−[^{14}C]carboxybiotin complex in its ability to carboxylate pyruvate in the absence of any other substrates or effectors. Thus the putative major locus of action of acetyl CoA in facilitating the transfer of the carboxyl group from carboxyphosphate to biotin remains unproven.

Attwood and Graneri (1984) showed that pyruvate carboxylase was capable of catalyzing the release of phosphate from MgATP in the presence of HCO_3^- and Mg^{2+} in the absence of pyruvate and in the presence or absence of acetyl CoA. However, these steady-state reactions proceeded at less than 0.1% of the rate of the full pyruvate carboxylation reaction and the authors suggested that the rate-limiting step in the catalytic cycle in the absence of pyruvate was the decarboxylation of the carboxyenzyme complex.

Few studies in which rate constants have been measured for individual parts of the reaction catalyzed by pyruvate carboxylase have been performed. One early study was concerned with substrate binding (McGurk & Spivey, 1978), another with the carboxylation of pyruvate by the enzyme−carboxybiotin complex (Attwood et al., 1984), and recently,

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nucleotide binding was examined (Geeves et al., 1995). The present study set out to measure the rate constants for the approach to steady-state of the formation of the carboxy-enzyme complexes capable of carboxylating pyruvate and for ATP cleavage and to examine the effects of acetyl CoA on these kinetics to determine which step in reaction 3 acetyl CoA was exerting its major effect.

MATERIALS AND METHODS

Preparation of Chicken Liver Pyruvate Carboxylase. Chicken liver pyruvate carboxylase was prepared to an average specific activity of 25−33 units/mg of protein as described by Goss et al. (1979) except that a DEAE-Sepharose CL-6B column was used in place of the DEAE-Sephadex column (1 unit of enzymic activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol of oxaloacetate/min under saturating substrate conditions at 30 °C). The enzyme was stored at −80 °C in a storage solution composed of 0.1 M Tris-HCl, pH 7.2, 1.6 M sucrose, and 40 mM $(NH_4)_2SO_4$. Prior to experiments, the enzyme was transferred from the storage solution to 0.1 M Tris-HCl, pH 7.8, by centrifuging through Sephadex G-25 as described by Helmerhorst and Stokes (1980). The purity of the enzyme preparations used was estimated to be between 86% and 91% by SDS gel capillary electrophoresis.

Other Materials. From Amersham Australia Pty. Ltd., $NaH^{14}CO_3$ was obtained as an aqueous solution at a specific radioactivity of 55 mCi/mmol and [^{14}C]biotin was obtained as a solid at a specific radioactivity of 50−62 mCi/mmol. [γ-^{32}P]ATP was obtained as an aqueous solution of the triethylammonium salt at a specific radioactivity of approximately 3000 Ci/mmol from either Du Pont NEN or Bresatec Pty. Ltd. All other materials were high-purity preparations from commercial suppliers.

Spectrophotometric Pyruvate Carboxylase Assays. The conditions for this assay were as described by Attwood and Cleland (1986), except that the buffer used was 0.1 M Tris-HCl, pH 7.8. After transfer of the enzyme from storage buffer to 0.1 M Tris-HCl, pH 7.8, assays were performed in triplicate at 30 °C. For the determination of the turnover number of the enzyme the assay was performed as above except the temperature was 20 °C and the concentration of HCO_3^- was 15 mM, as in the quenched-flow experiments.

Biotin Determinations. After removal from storage solution, aliquots of the enzyme solutions were set aside and stored at −80 °C for later determination of biotin content. The enzyme solutions were incubated with 0.2% (w/v) chymotrypsin at 37 °C for 24 h. Pronase was then added to a final concentration of 0.45% (w/v), and the solutions incubated for an additional 72 h at 37 °C. The solutions were then heated for 15 min at 100 °C before being used in the biotin assay described by Rylatt et al. (1977).

ATP Cleavage Experiments in the Absence of Acetyl CoA. An enzyme solution was prepared containing 70 units of pyruvate carboxylase/mL, 4.5 mM $MgCl_2$, 15 mM $NaHCO_3$, and 0.1 M Tris-HCl, pH 7.8. A substrate solution was prepared containing 5 mM [γ-^{32}P]ATP (approximately 30 μCi/μmol), 9.5 mM $MgCl_2$, 15 mM Na HCO_3, and 0.1 M Tris-HCl, pH 7.8. The solutions were equilibrated at 20 °C, and then equal volumes were mixed to start the reaction. At various times after starting the reaction, 25 μL aliquots of the reaction mixture were withdrawn and added to 50 μL of ice-cold 2.5 M trichloroacetic acid. After 10 min on ice, the tubes containing the reaction mixture plus trichloroacetic acid were centrifuged in a bench centrifuge for 5 min to pellet the protein precipitate. A 50 μL aliquot from each tube was then spotted onto a 2.5 cm square of Whatman 31ET CHR filter that had been pretreated with a molybdate precipitating solution according to the method of Reimann and Umfleet (1978). The filters were then washed to remove any [γ-^{32}P]-ATP as described previously (Reimann & Umfleet, 1978), they were then dried and placed in scintillation fluid to count the radioactivity corresponding to [^{32}P]phosphate retained on the filters that was released from [γ-^{32}P]ATP. The exact specific activity of the [γ-^{32}P]ATP was determined by placing small aliquots of the substrate solution directly into scintillation fluid for counting. Controls were also performed in triplicate in which a 12.5 μL aliquot of the enzyme solution was added to 50 μL of the ice-cold trichloroacetic acid solution followed by 12.5 μL of the substrate solution. These controls were then processed in the same way as the samples. The radioactivity in the controls was then subtracted from the radioactivity of the samples. The data were then corrected for HCO_3^--independent ATP cleavage by subtracting P_i-released in HCO_3^--independent ATP cleavage. This was calculated by measuring ATP cleavage by pyruvate carboxylase that had been incubated with a 10-fold excess of avidin such that it exhibited no pyruvate carboxylating activity (Attwood & Graneri, 1992). Attwood and Graneri (1992) have previously shown that the avidin-treatment of chicken liver pyruvate carboxylase completely abolished the HCO_3^--dependent cleavage of ATP by enzyme preparations.

ATP Cleavage in the Presence of Acetyl CoA. Enzyme and substrate solutions were prepared in the way described in the previous section except that the enzyme solution contained 210 units of pyruvate carboxylase/mL, the substrate solution contained 5 mM [γ-^{32}P]ATP (approximately 17 μCi/μmol), and both solutions contained 250 μM acetyl CoA. The reactions were performed by mixing equal volumes of the enzyme and substrate solutions at 20 °C in a Hi-Tech PQ/SF 53 preparative quench/stopped-flow spectrofluorimeter. After a preset reaction time, the reaction mixture was quenched by mixing with 2 M HCl (1.25 vol of reaction solution) and expelled from the instrument with water (1.25 vol of reaction solution), the samples were collected into tubes, and to each tube was added 100% (w/v) ice-cold trichloroacetic acid to a final concentration of 4.5%. After 10 min on ice the samples were centrifuged at 14 000 rpm for 5 min in a bench centrifuge and 3 × 50 μL aliquots of the each supernatant spotted on to 2.5 cm squares of Whatman 3ET CHR filter. These filters were then treated as described in the previous section. Controls were performed in which the pyruvate carboxylase was omitted from the enzyme solution.

Carboxyenzyme Formation in the Absence of Acetyl CoA. The solutions used in these experiments were the same as those used in similar experiments to measure ATP cleavage except that the substrate solution contained no [γ-^{32}P]ATP and both the substrate and enzyme solutions contained 12.5 mM $NaHCO_3$ and 2.5 mM $NaH^{14}CO_3$. The reactions were performed as described for the ATP cleavage experiments except that the reactions were quenched by addition of 25 μL aliquots of the reaction mixture to 25 μL of a quench solution used previously (Attwood, 1993) which contained 200 mM Pyruvate, 0.4 mM NADH, 22 units of malate dehydrogenase/mL, 200 mM ethylenediaminetetraacetic acid

(EDTA), and 400 mM $NaHCO_3$ adjusted to pH 7.8. It was demonstrated that this quench solution effectively prevented further catalytic cycles of the enzyme leading to [^{14}C]malate formation. Using this quench, the carboxyl groups from the carboxyenzyme complex formed in the reaction are transferred to pyruvate to form oxaloacetate which is then converted to malate. Given the relative stability of the enzyme−carboxybiotin complex and its very rapid reaction with pyruvate (Attwood et al., 1984), it is expected that all of the carboxyl groups from enzyme−carboxybiotin complex will be transferred to pyruvate. After 2−3 min, 25 μL of 6 M HCl saturated with semicarbazide-HCl was added. The tubes containing the quenched solutions were then left uncapped in the fume hood for 10−15 min to allow the $^{14}CO_2$ released on acidification to escape. After this time 50 μL was spotted on to a 2.5 cm square of Whatman 3MM filter and oven-dried at 105 °C. The filters were then placed in scintillation fluid, and the radioactivity due to [^{14}C]malate on the filters was counted. Controls were performed in which the pyruvate was omitted from the quench solution, and the radioactivity measured in these controls was subtracted from the samples. In order to correlate the radioactivity measured with the amount of malate formed a spectrophotometric end point assay of a solution of pyruvate was performed using pyruvate carboxylase and malate dehydrogenase, under the spectrophotometric assay conditions described above. However, the assay contained $NaHCO_3$ and $Na^{14}CO_3$ at the concentrations used in the kinetic experiments to measure [^{14}C]carboxyenzyme formation. From the change in absorbance at 340 nm due to NADH oxidation the amount of malate formed was calculated. Aliquots of the reaction mixture were then added to equal volumes of the quench solution from which pyruvate had been omitted. The samples were then processed as described above and the radioactivity due to [^{14}C]malate on the filters counted and the dpm used to calculate the specific activity of the [^{14}C]malate (between 12 000 and 15 000 dpm/nmol).

[^{14}C]Carboxyenzyme Formation in the Presence of Acetyl CoA. These measurements were performed on the quenched-flow instrument as described for the ATP cleavage experiments performed in the presence of acetyl CoA. The reaction solutions and quench solutions contained 250 μM acetyl CoA but were otherwise as described above for the experiments to measure [^{14}C]carboxyenzyme formation in the absence of acetyl CoA. Two minutes after the collection of the samples, 0.7 vol of 6 M HCl saturated with semicarbazide-HCl was added to each; the samples were then left in the fumehood for 10 min to allow the $^{14}CO_2$ to escape. Aliquots of 100 μL were then spotted onto 3.5 cm squares of Whatman 3MM filter. The filters were then dried and counted as described above.

Data Analysis. The kinetic data were analyzed using nonlinear least-squares regression analysis to fit one of the following equations to the data as detailed in the text:

$$y = A(1 - e^{-kt}) \quad \text{(i)}$$

$$y = A(1 - e^{-kt}) + rt \quad \text{(ii)}$$

$$y = A(1 - e^{-kt}) + B \quad \text{(iii)}$$

where y is either mol of carboxyenzyme formed/mol of biotin or mol of P_i released/mol biotin; A is the amplitude of the approach to steady-state; r is the steady-state turnover number, and B is any residual at time zero.

RESULTS

Figure 1 shows the approach to steady-state of the formation of the carboxyenzyme complex in the absence (a) and presence (b) of acetyl CoA. As can be seen from the fitted curves, the reactions are apparently first-order with rate constants shown in Table 1. When eq iii (see Experimental Procedures) was fitted to the data shown in Figure 1a, the value obtained for B was not significantly different from zero. This indicates that the quenching procedure is not only efficient at preventing further catalytic cycles of the enzyme over long periods of time but is also rapid in its effect, on a millisecond time scale. The final proportion of the total enzyme in the steady-state in the form of carboxyenzyme under both reaction conditions was similar (see Table 1). The ratio of the two rate constants of 226 indicates that acetyl CoA enhances the rate of this reaction by over 2 orders of magnitude. The amplitudes of the approach to steady-state are similar both in the presence and absence of acetyl CoA

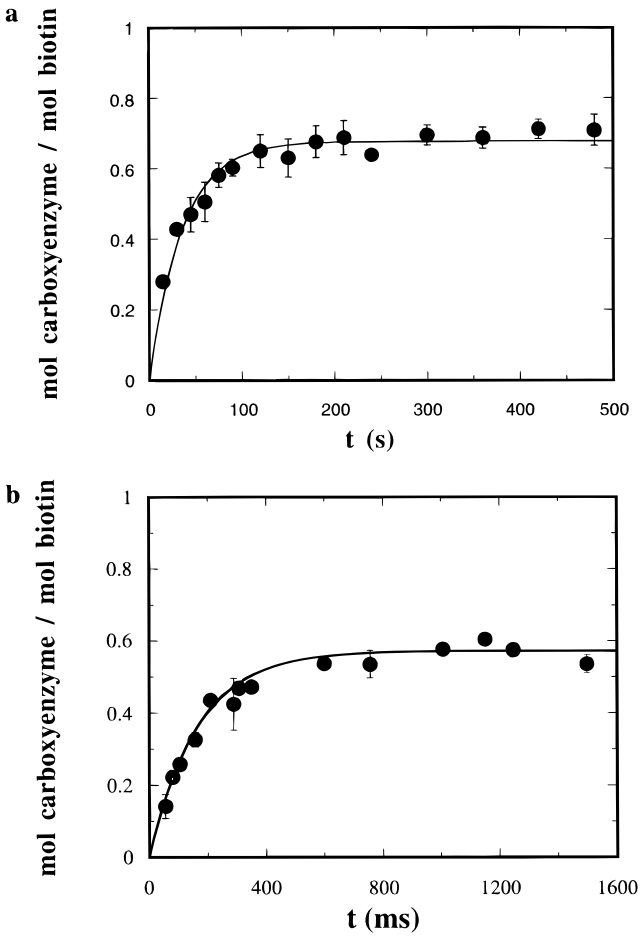


FIGURE 1: The kinetics of carboxyenzyme formation were measured in the absence (a) and the presence (b) of 0.25 mM acetyl CoA at 20 °C in 0.1 M Tris-HCl, pH 7.8, with 7 mM $MgCl_2$, 2.5 mM ATP, 12.5 mM $NaHCO_3$, and 2.5 mM $NaH^{14}CO_3$. The solid lines represent nonlinear least-squares fits to the data of eq i. In a, the data points represent the mean and the error bars represent the standard deviation of three separate measurements from three separate time-course experiments. In b, the data points represent the mean and the error bars represent the standard deviation derived from three determinations of the radioactivity present as carboxyenzyme at a particular time point. The data as a whole in b comes from two separate time-course experiments.

Table 1: Kinetic Parameters of Carboxyenzyme Formation and ATP Cleavage Reactions in the Presence and Absence of Acetyl CoA Derived from Nonlinear Least-Squares Analysis of the Data Shown in Figures 1 and 2 as Described in Materials and Methods[a]

	amplitude of approach to steady-state (mol/mol of biotin)	apparent first-order rate constant (s^{-1})	steady-state turnover number (s^{-1})
carboxyenzyme formation			
+ acetyl CoA	0.57 (± 0.01)	6.1 (± 0.3)	
− acetyl CoA	0.68 (± 0.01)	0.028 (± 0.002)	
ATP cleavage			
+ acetyl CoA	1.15 (± 0.05)	6.6 (± 0.7)	0.0158 (± 0.0009)[b]
− acetyl CoA	7.4 (± 0.4)	0.028 (± 0.004)	0.009 (± 0.001)

[a] Figures in brackets are the standard errors of the estimates of the kinetic parameters derived from the nonlinear least-squares regression analysis of the data unless otherwise indicated. [b] Mean and standard deviation of three determinations.

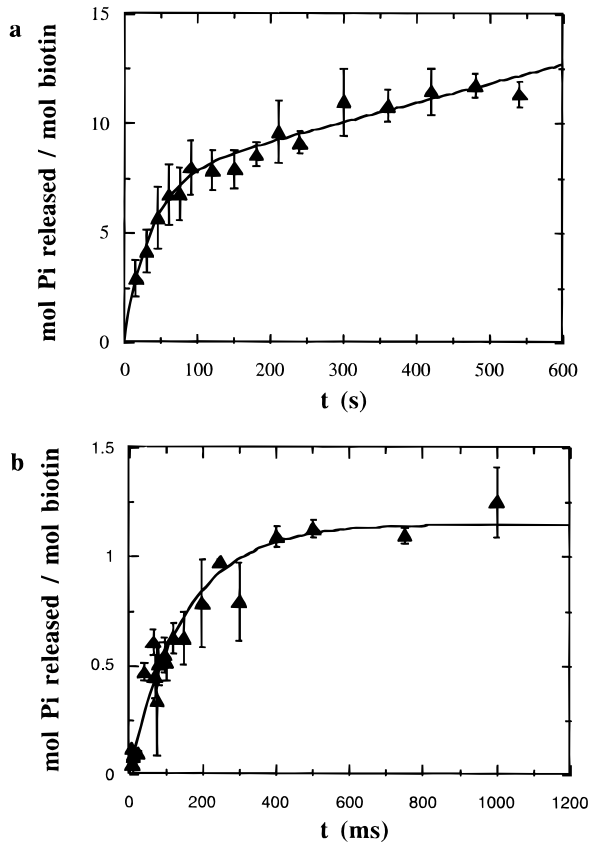


FIGURE 2: The kinetics of ATP cleavage were measured in the absence (a) and the presence (b) of 0.25 mM acetyl CoA at 20 °C in 0.1 M Tris-HCl, pH 7.8 with 7 mM $MgCl_2$, 2.5 mM [γ^{32}P]ATP, 15 mM $NaHCO_3$. P_i released/mol of biotin corresponds the total mol of ATP cleaved/mol of biotin. The solid lines represent nonlinear least-squares fits to the data of eq ii except that in b the value of *r* was set at the value for the steady-state turnover number measured manually, i.e., 0.0158 s^{-1}. In a, the data points represent the mean and the error bars represent the standard deviation of three separate measurements from three separate time-course experiments. In b, the data points represent the mean and the error bars represent the standard deviation derived from three determinations of the radioactivity present as carboxyenzyme at a particular time point. The data as a whole in b comes from three separate time-course experiments. All data have been corrected for HCO_3^- independent ATP as detailed in Materials and Methods.

and indicate that in the steady-state, between 57% and 68% of the enzyme is in the form of carboxyenzyme capable of carboxylating pyruvate.

Figure 2 shows the kinetics of the approach to steady-state of the ATP cleavage reaction in the absence (a) and presence (b) of acetyl CoA. Both reactions were apparently first-order with rate constants as shown in Table 1. The ratio of the two rate constants of 236 indicates that acetyl CoA enhances the rate of approach to steady-state by over 2 orders of magnitude. The amplitudes of the burst phases in the approach to steady-state are shown in Table 1. In the presence of acetyl CoA, the amplitude of this phase and the rate constant for the approach to steady-state are similar to those for the formation of the carboxyenzyme complex. However, in the absence of acetyl CoA, while the rate constants for the approach to steady-state in each type of reaction are similar, the amplitude of the burst phase is much greater for ATP cleavage than for carboxyenzyme formation and is in large excess of 1 mol of ATP cleaved per mol of biotin. The steady-state turnover number for ATP cleavage in the absence of acetyl CoA was determined from the nonlinear least-squares fit to the data of eq ii shown in Figure 2a (see Table 1). The steady-state turnover number for ATP cleavage in the presence of acetyl CoA was determined in manual experiments as described by Attwood and Graneri (1992) and is shown in Table 1. This shows that the steady-state cleavage of ATP is of the same order of magnitude in the absence of acetyl CoA compared to that in its presence.

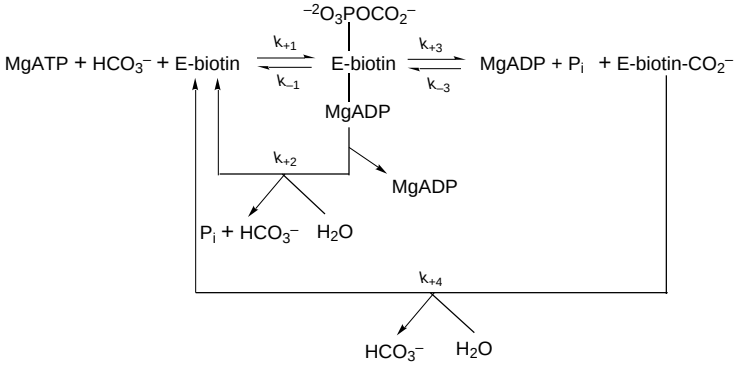
The amplitudes of the approach to steady-state phase of the reactions are in both cases higher than those observed in the carboxyenzyme formation reactions (Table 1). The ratio of the amplitudes for the ATP cleavage reaction to that of the carboxyenzyme formation reaction in the presence of acetyl CoA is 2.0. The same ratio in the absence of acetyl CoA is 10.9.

The steady-state turnover number for the pyruvate carboxylation reaction in the presence of acetyl CoA under identical conditions of temperature and substrate concentrations as those used in the quenched-flow measurements of carboxyenzyme formation and ATP cleavage was determined to be 11.0 (± 0.3) s^{-1} (mean and standard deviation of three determinations).

DISCUSSION

The first point that has to be addressed is what is actually being measured in the above experiments? If we accept that the biotin carboxylation reaction proceeds via a carboxyphosphate intermediate, then the simplest model of the reaction is that shown in reaction 3. Given the lability of carboxyphosphate (Sauers et al., 1975), when the reaction is quenched with acid, as in the ATP cleavage experiments, this intermediate will decompose resulting in the formation of P_i and CO_2. Thus the ATP cleavage experiments actually measure the rate of formation of the enzyme−carboxyphosphate complex. In the case of the experiments to measure carboxyenzyme formation, the method of detection is to transfer any activated [^{14}C]carboxyl groups from the enzyme

Scheme 1: Reaction 4



to pyruvate to initially form [^{14}C]oxaloacetate and ultimately [^{14}C]malate. Thus "carboxyenzyme" may consist of both the enzyme−carboxyphosphate complex and the enzyme−carboxybiotin complex, provided that the carboxyl group from carboxyphosphate can be efficiently transferred to biotin and hence to pyruvate under the conditions of the quench. If this were the case, one would expect the apparent rate constants for the approach to steady-state of both ATP cleavage and carboxyenzyme formation to be the same. Indeed, our results show that in both the presence and absence of acetyl CoA, the apparent first-order rate constants for the approach to steady-state of carboxyenzyme formation and ATP cleavage are similar.

Examination of the amplitudes of the approaches to steady-state, however, shows that there are differences between carboxyenzyme formation and ATP cleavage. In both the presence and absence of acetyl CoA, the amplitudes for ATP cleavage are higher than those for carboxyenzyme formation. In order to produce the degree of carboxylation of the enzyme in the steady-state in the presence of acetyl CoA which is similar to that in its absence, 7.4 mol of ATP per mol of biotin was cleaved compared to just over 1 mol of ATP per mol of biotin in the presence of acetyl CoA. This suggests that in the absence of acetyl CoA, carboxyenzyme capable of carboxylating pyruvate accumulates more slowly and inefficiently compared to ATP cleavage. If the first step in the biotin−carboxylation reaction is the formation of carboxyphosphate, then each ATP cleaved should correspond to the formation of a carboxyenzyme intermediate. This is clearly not the case in the absence of acetyl CoA. If the mechanism shown in reaction 3 is correct, then how can multiple ATP cleavage events occur before steady-state is reached in which about two-thirds of the total enzyme is in the carboxyenzyme form? This could occur if some of the carboxyphosphate is lost in an abortive reaction rather than carboxylating biotin to form carboxybiotin. This possibility is shown in Reaction 4 (see Scheme 1).

Reaction 4 shows the catalytic cycles that may occur in the absence of acetyl CoA. The stability of the enzyme−carboxybiotin complex has been studied by Attwood and Wallace (1986). From an extrapolation of the Arrhenius plot presented in this work (Attwood & Wallace, 1986) and the effects of Mg^{2+} present in the reaction mixtures (Attwood et al., 1984), the decarboxylation of the enzyme−carboxybiotin complex would be expected to have an apparent first-order rate constant of less than 0.002 s^{-1} at 20 °C. The reaction given by k_{+2} is meant to represent a series of steps which result in the decomposition of carboxyphosphate, formation of P_i and HCO_3^-, release of MgADP, and return of the enzyme to the form ready for the next catalytic cycle. The abortive decomposition of carboxyphosphate as represented in the reaction steps governed by k_{+2} may occur in several ways: the carboxyphosphate may dissociate from the enzyme active site before hydrolysis or decarboxylation occurs, or hydrolysis or decarboxylation (as has been proposed to occur in the reaction pathway itself; Knowles, 1989) may occur in the active site, followed by the release of the products into solution. If the enzyme−carboxyphosphate complex decomposed much more rapidly than the carboxylation of biotin occurred and at the same time the stability of the enzyme−carboxyphosphate complex was much lower than that of the enzyme−carboxybiotin complex, then there could be several cycles of formation and decarboxylation of the enzyme−carboxyphosphate complex before the formation of a molecule of enzyme−carboxybiotin complex. Since the enzyme−carboxybiotin complex is relatively stable, this would slowly accumulate until a steady-state was achieved, with enzyme−carboxyphosphate being present at steady-state at only low levels.

To test whether this type of mechanism was feasible, a simplified form of Scheme 1 was simulated using the computer program KSIM in which the rate equations are solved by numerical integration using the Gear (1971) algorithm. In the interests of simplicity, in the simulation of reaction 4, k_{-1} and k_{-3} were set to zero. In reaction 4 the step represented by k_{+3} includes release of MgADP and P_i, which under these initial conditions would be essentially irreversible and hence setting k_{-3} to zero is not unreasonable. The value of k_{+4} was set at 0.00115 s^{-1} (see above). It was found that by setting k_{+2} and k_{+3} in the ratio of 7.1:1 (i.e., close to the estimated amplitude of the approach to steady-state of the ATP cleavage reaction, within the error of the estimate), on nonlinear least-squares analysis of the simulated data, that this gave an amplitude of approach to steady-state of 7.4 (see Table 2). When k_{+2} was set $\geq$1.00 s^{-1} and k_{+1} was between 0.225 and 0.27 s^{-1} simulated reaction time courses similar to those shown in Figures 3a,b were produced. Regression analysis of the data shown in Figure 3 gave the kinetic parameters listed in Table 2. At lower values of k_{+2}, k_{+2} starts to become rate-limiting in the catalytic cycle involving carboxyphosphate decarboxylation. Compensation by increasing k_{+1} results in a rapid burst of P_i formation associated with initial enzyme−carboxyphosphate complex formation that is not seen in the experimental data.

Table 2: Kinetic Parameters Obtained from Nonlinear Least-Squares Regression Analysis of the Simulated Data Presented in Figures 3 and 4[a]

	amplitude of approach to steady-state (mol/mol of biotin)	apparent first-order rate constant (s^{-1})	steady-state turnover number (s^{-1})
ATP cleavage			
Figure 3a	7.409 (± 0.005)	0.02843 (± 0.00005)	0.00899 (± 0.00001)
Figure 4	0.9895 (± 0.0005)	6.381 (± 0.008)	0.01653 (± 0.0005)
enzyme−carboxybiotin formation			
Figure 3b	0.961 (± 0.001)	0.02710 (± 0.00009)	
Figure 4	0.5758 (± 0.0002)	6.204 (± 0.010)	

[a] Figures in brackets are the standard errors of the estimates of the kinetic parameters derived from the nonlinear least-squares regression analysis of the data.

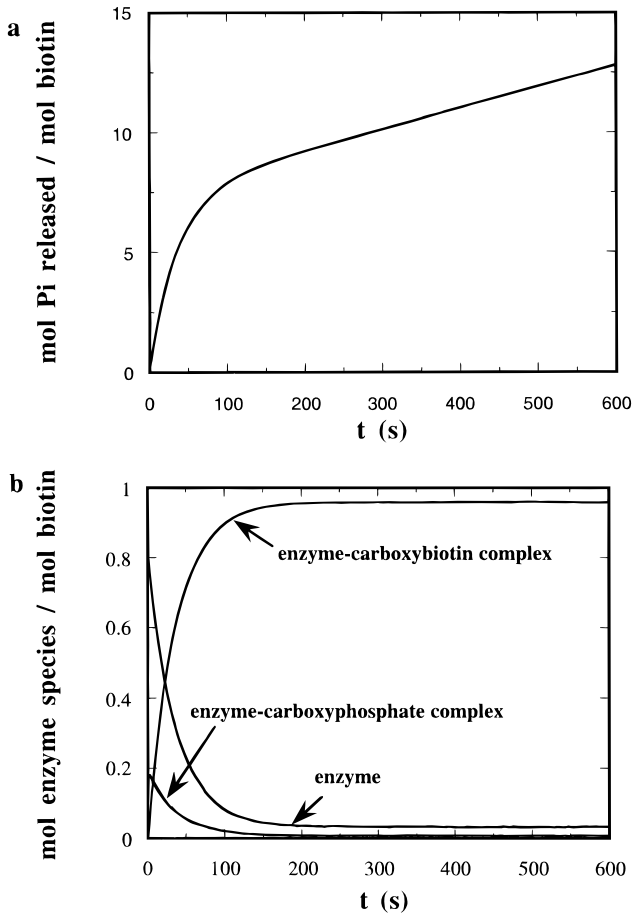


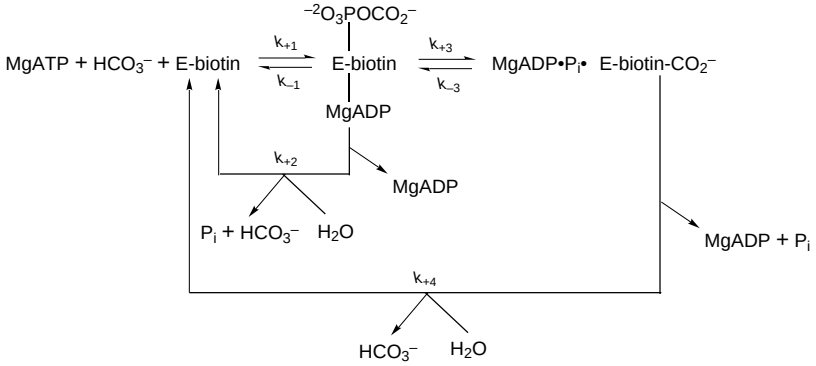
FIGURE 3: Simulations of reaction time-courses based on reaction 4. Simulations were performed using the program KSIM in which the rate equations are solved by the numerical integration using the Gear (1971) algorithm. k_{-1} and k_{-3} were set to zero, and the values of the other rate constants were set as follows: $k_{+1} = 0.27$ s^{-1} (assuming this step to be governed by a first-order reaction); $k_{+2} = 1.00$ s^{-1}; $k_{+3} = 0.141$ s^{-1}; $k_{+4} = 0.00115$ s^{-1}. In a, mol of P_i released/mol of biotin corresponds to total mol of ATP cleaved/mol of biotin. In b, the time-courses of interconversion of enzyme species versus time were plotted as indicated on the figure.

Figure 3b shows that in the simulation, a transient burst of enzyme−carboxyphosphate production occurs, peaking at about 0.2; this was not detected in the experimental kinetics of carboxyenzyme formation. However, this is not surprising since according to the model, when the reaction is quenched, only about one-eighth of the enzyme−carboxyphosphate complex present at any time would transfer its carboxyl group to biotin and hence onto pyruvate while the rest would decarboxylate. This would thus be not detectable as a burst of carboxyenzyme formation.

As can also be seen from Figure 3b and Table 2, the steady-state level of enzyme−carboxybiotin complex produced by this simulation is 0.96 relative to total enzyme. This is significantly higher than the experimental steady-state level of 0.68; however, it should be noted that the experimental values are based on biotin measurements with an implicit assumption that one biotin molecule represents one *active* enzyme-active site. There is likely to be some inactive enzyme present, and thus the biotin measurements represent *maximum* estimates of active enzyme-active sites. Hence the ratios of carboxyenzyme formed and P_i formed to total enzyme shown in figures 1 and 2 are *minimum* estimates with respect to active enzyme. Therefore the experimental steady-state level of carboxyenzyme may in fact be closer to the value obtained for enzyme−carboxybiotin complex from the simulations if active enzyme is considered. Another possible contributing factor for the somewhat low accumulation of the enzyme−carboxybiotin complex in the steady-state is that enzyme is accumulating in another form or forms. In an attempt to test this idea, simulations were performed in which there was an equilibrium between the enzyme−carboxyphosphate and enzyme−carboxybiotin complex at steady-state. In these simulations, however, the large release of P_i in the approach to steady-state could not be matched to a steady-state turnover number that matched the experimental data. Another possibility is that a form of enzyme is accumulating between the enzyme−carboxybiotin complex and the enzyme form that participates in the next catalytic cycle, for example, a conformer of the enzyme in which the biotin is at the site of reaction 2 (Attwood et al., 1984). However, in the simulations including the irreversible formation and decay of this enzyme species, the amplitude of formation of enzyme−carboxybiotin complex in the approach to steady-state could only be reduced to about 0.84 mol of enzyme−carboxybiotin complex/mol of biotin without noticeably affecting the other kinetic parameters of the reaction.

In the context of reaction 4, in the presence of acetyl CoA, the ratio of k_{+2}/k_{+3} would be reduced so as to more tightly couple ATP cleavage with the formation of enzyme−carboxybiotin complex. The presence of acetyl CoA could change the ratio of k_{+2}/k_{+3} by increasing k_{+3} (enhancing carboxybiotin formation) or by reducing k_{+2} (by reducing the exposure of the carboxyphosphate to the aqueous solvent) or a combination of both. Modeling based on reaction 4 showed that in order to produce simulations with similar kinetics to the experimental data, it was necessary to increase k_{+4} to 0.01 s^{-1} in order to obtain the steady-state turnover number for ATP cleavage measured experimentally. There is no evidence that decarboxylation of the enzyme−carboxybiotin complex proceeds more rapidly in the presence of acetyl CoA compared to in its absence. Indeed, Attwood and Wallace (1986) found that acetyl CoA reduced the rate

Scheme 2: Reaction 5



constants for decarboxylation of the enzyme−carboxybiotin complex by about 25%. In addition, in simulations based on the model for the reaction in the absence of acetyl CoA, the end result is that most of the enzyme accumulates in the form of the enzyme−carboxybiotin complex in the steady-state. Thus even in the presence of acetyl CoA, a large degree of uncoupling between ATP cleavage and carboxybiotin formation would have to occur in which about two ATPs were cleaved for every carboxybiotin formed. This is clearly not the case in the overall reaction, where, in the presence of saturating pyruvate concentrations, the stoichiometry of the formation of oxaloacetate to ATP cleavage is 1:1 (Easterbrook-Smith et al., 1976). These observations make it unlikely that merely changing the values of the rate constants in the model used for the reaction in the absence of acetyl CoA can produce simulations that match the experimental data obtained in the presence of acetyl CoA.

Reaction 5 (see Scheme 2) is a proposed scheme for the reaction in the presence of acetyl CoA. It is very similar to reaction 4, except that in this case in the step represented by k_{+3}, carboxyphosphate is formed but there is no release of P_i. The release of P_i occurs in the slow step(s) associated with the decarboxylation of the enzyme−carboxybiotin complex (in reaction 5, MgADP release is shown also to occur with P_i release; however, this is not essential and this could occur after the formation of the enzyme−carboxyphosphate complex). Thus the interconversion of the enzyme−carboxyphosphate and enzyme−carboxybiotin complexes becomes reversible. For the sake of simplicity k_{-1} was set at zero, although in any case this rate constant would have to be small in comparison to k_{+1} so as to attain the large amplitude of P_i formation seen in the experimental data, k_{+4} remained set at 0.00115 s^{-1}. When $k_{+3} \geq 30$ s^{-1}, $K_3 = 1.36$ (k_{+3}/k_{-3}) and the value k_{+2} was between 0.023 and 0.032 s^{-1}, simulations similar to those shown in Figure 4 resulted. The kinetic parameters derived by regression analysis of the simulations shown in Figure 4 are given in Table 2. Below a value of k_{+3} of 30 s^{-1}, the lag in the formation of carboxybiotin becomes very noticeable. Thus as long as carboxyphosphate formation is rate-limiting in the formation of carboxybiotin, the model works reasonably well. The amplitude of P_i formation is close to 1 mol of P_i formed/mol of biotin in the approach to steady-state and is thus somewhat lower than the experimental value, but at 1200 ms this value just exceeds 1 and is closer to the experimental value. The assumption that is inherent in comparing this model with the experimental data is that the enzyme−carboxybiotin complex in the model corresponds to the experimental carboxyenzyme and implies that the carboxyl group from the enzyme−carboxyphosphate complex is not transferred to pyruvate on quenching. The binding of pyruvate to the enzyme is known to induce the movement of biotin to the site of reaction 2 (Goodall at al., 1981; Attwood et al., 1984). In the case where the carboxyenzyme was in the form of the enzyme−carboxyphosphate complex, this may leave the carboxyphosphate at the site of reaction 1 exposed to the aqueous solvent and thus induce its rapid decarboxylation while the carboxyl group from carboxybiotin would be trnasferred to pyruvate.

In both the presence and absence of acetyl CoA, if schemes similar to those in reactions 4 and 5 apply, the reverse reaction commitment of the enzyme−carboxyphosphate complex would be small. This small reverse reaction commitment could explain the failure to observe positional isotope exchange using [^{18}O]ATP (Knowles, 1989).

The turnover number of the overall pyruvate carboxylation reaction in the presence of acetyl CoA is 11 s^{-1} compared to the 6.6 and 6.1 s^{-1} for the approach to steady-state of ATP cleavage and carboxyenzyme formation, respectively, and to the value of k_{+1} used in the simulations of 6.5 s^{-1}. This suggests that the ATP cleavage (and putatively, carboxyphosphate formation) step is likely to be the rate-limiting step in the overall reaction. From the modeling studies, it would appear that acetyl CoA acts to increase the rate constant for this step by about 30-fold, and this is likely to be an effect on the catalytic step rather than ATP binding,

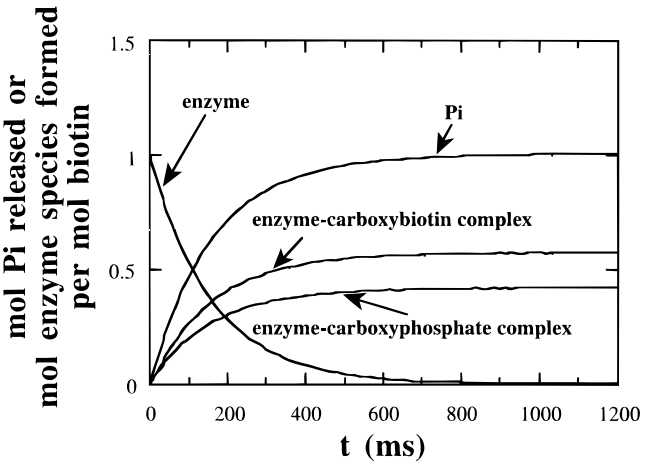


FIGURE 4: Simulations of reaction time-courses based on reaction 5. Simulations were performed using the program KSIM in which the rate equations are solved by the numerical integration using the Gear (1971) algorithm. k_{-1} was set to zero, and the values of the other rate constants were set as follows: $k_{+1} = 6.5$ s^{-1} (assuming this step to be governed by a first-order reaction); $k_{+2} = 0.025$ s^{-1}; $k_{+3} = 300$ s^{-1}; $k_{-3} = 220$ s^{-1}; $k_{+4} = 0.00115$ s^{-1}.

since ATP binding is fast relative to the turnover number of the overall reaction and is not greatly affected by acetyl CoA (Geeves et al., 1995). Pyruvate has been suggested to lower the K_m of HCO_3^- (Ashman & Keech, 1975; McClure et al., 1975), and thus in the measurement of the turnover number, the enzyme may be more saturated with HCO_3^- than in the experiments in the absence of pyruvate, and this would result in a higher rate apparent constant for the overall reaction compared to that for ATP cleavage in the absence of pyruvate. The rate constant for carboxylation of pyruvate by enzyme−carboxybiotin was found to be 9 s^{-1} at 0 °C (Attwood et al., 1984), and thus at 20 °C, this might be expected to be 36 s^{-1}, assuming a minimal Q_{10} for this reaction of 2. However, Attwood and Wallace (1986) found that using the pyruvate analogue, 2-oxobutyrate, the Q_{10} of this reaction was about 5 and thus the estimate of rate constant for the carboxylation of pyruvate by enzyme−carboxybiotin at 20 °C could be much higher than 36 s^{-1}. Therefore, the carboxylation of pyruvate by enzyme−carboxybiotin is unlikely to be rate-limiting in the overall reaction.

Schemes 1 and 2 represent the simplest schemes that explain the data. In his review, Knowles (1989) considered another reaction pathway in which there is initial formation of *O*-phosphobiotin followed by a pericyclic reaction with HCO_3^- to form carboxybiotin and P_i. Reactions 4 and 5 can also be applied to this reaction pathway, by simply replacing the enzyme−carboxyphosphate complex by *O*-phosphobiotin. In this case, the step represented by k_{+2} would be dephosphorylation of *O*-phosphobiotin and that represented by k_{+3} would be the pericyclic reaction between *O*-phosphobiotin and HCO_3^-.

In summary, acetyl CoA has been found to increase the rate constants for the approach to steady-state of both ATP cleavage and carboxyenzyme formation by 226−236-fold. In addition, acetyl CoA greatly enhanced the degree of coupling of ATP cleavage and carboxyenzyme formation. Reaction models have been proposed that can explain these observations, and a major feature of these models is that carboxyphosphate may either decompose (before or after release from the active site) or carboxylate biotin to form carboxybiotin. In the absence of acetyl CoA, the decomposition of carboxyphosphate is rapid relative to carboxybiotin formation, whereas in the presence of acetyl CoA this is not the case. In the presence of acetyl CoA, the rate-limiting step in the formation of carboxybiotin appears to be ATP cleavage (i.e., carboxyphosphate formation). Acetyl CoA acts to increase the rate constant for ATP cleavage somewhere in the order of 30-fold (from the simulations) although it is not clear if the rate constant for the step in which carboxybiotin is formed is also increased.

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REFERENCES

Ashman, L. K., & Keech, D. B. (1975) *J. Biol. Chem. 250*, 14−21.
Attwood, P. V. (1993) *Biochemistry 32*, 12736−12742.
Attwood, P. V. (1995) *Int. J. Biochem. Cell Biol. 27*, 231−249.
Attwood, P. V., & Cleland, W. W. (1986) *Biochemistry 25*, 8191−8196.
Attwood, P. V., & Wallace, J. C. (1986) *Biochem. J. 235*, 359−364.
Attwood, P. V., & Graneri, B. D. L. A. (1992) *Biochem. J. 287*, 1011−1017.
Attwood, P. V., Wallace, J. C., & Keech, D. B. (1984) *Biochem. J. 219*, 243−251.
Attwood, P. V., Mayer, F., & Wallace, J. C. (1986) *FEBS Lett. 203*, 191−196.
Climent, I., & Rubio, V. (1986) *Arch. Biochem. Biophys. 251*, 465−470.
Easterbrook-Smith, S. B., Hudson, P. J., Goss, N. H., Keech, D. B., & Wallace, J. C. (1976) *Arch. Biochem. Biophys. 176*, 709−720.
Gear, C. W. (1971) *Commun. ACM 14*, 176−190.
Geeves, M. A., Branson, J. P., & Attwood, P. V. (1995) *Biochemistry 34*, 11846−11854.
Goodall, G. J., Baldwin, G. S., Wallace, J. C., & Keech, D. B. (1981) *Biochem. J. 199*, 603−609.
Goss, N. H., Dyer, P. Y., Keech, D. B., & Wallace, J. C. (1979) *J. Biol. Chem. 254*, 1734−1739.
Helmerhorst, E., & Stokes, G. B. (1980) *Anal. Biochem. 104*, 130−135.
Knowles, J. R. (1989) *Annu. Rev. Biochem. 58*, 195−221.
McClure, W. R., Lardy, H. A., Wagner, M., & Cleland, W. W. (1975) *J. Biol. Chem. 246*, 3569−3578.
McGurk, K. S., & Spivey, H. O. (1978) *Biochem. J. 177*, 697−705.
Phillips, N. F. B., Snoswell, M. A., Chapman-Smith, A., Keech, D. B., & Wallace, J. C. (1992) *Biochemistry 31*, 9445−9450.
Reimann, E. M., & Umfleet, R. A. (1978) *Biochim. Biophys. Acta 523*, 516−521.
Rylatt, D. B., Keech, D. B., & Wallace, J. C. (1977) *Arch. Biochem. Biophys. 183*, 113−122.
Sauers, C. K., Jencks, W. P., & Groh, S. (1975) *J. Am. Chem. Soc. 97*, 5546−5553.
Scrutton, M. C., Keech, D. B., & Utter, M. F. (1965) *J. Biol. Chem. 240*, 574−581.
Wallace, J. C., Phillips, N. F. B., Snoswell, M. A., Goodall, G. J., Attwood, P. V., & Keech, D. B. (1985) *Ann. N. Y. Acad. Sci. 447*, 169−188.

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