Mechanism of the Propionyl Carboxylase Reaction

II. ISOTOPIC EXCHANGE AND TRACER EXPERIMENTS*

YOSHIITO KAZIRO, L. F. HASS,† P. D. BOYER, AND SEVERO OCHEA

From the Department of Biochemistry, New York University School of Medicine, New York 16, New York, and the Department of Physiological Chemistry, University of Minnesota, Minneapolis 14, Minnesota

(Received for publication, January 11, 1962)

Previous work (1-3) has shown that propionyl carboxylase is a single enzyme that catalyzes a two-step reversible reaction (Reactions 1 and 2) resulting in the over-all Reaction 3.

\[
\begin{align*}
\text{ATP} + \text{CO}_2^- + \text{enzyme} & \overset{(\text{Mg}^{++})}{\longrightarrow} \text{ADP} + \text{P} + \text{CO}_2^- + \text{enzyme} \quad (1) \\
\text{CO}_2^- + \text{enzyme} + \text{propionyl CoA} & \Rightarrow \text{enzyme} + \text{methylmalonyl CoA} \quad (2)
\end{align*}
\]

\[
\text{Sum: ATP} + \text{CO}_2^- + \text{propionyl CoA} \overset{(\text{Mg}^{++})}{\longrightarrow} \text{ADP} + \text{P} + \text{methylmalonyl CoA} \quad (3)
\]

Experiments with bicarbonate-C\(^{14}\) or methylmalonyl coenzyme A-3-C\(^{14}\) and substrate amounts of crystalline propionyl carboxylase (1) demonstrated unequivocally that the over-all reaction involves carboxylation and decarboxylation of the enzyme according to Reactions 1 and 2. Reaction 1 itself had previously been believed (4-6) to consist of two separate steps. However, careful reinvestigation of the exchange reactions between adenosine triphosphate (ATP) and either radioactive inorganic phosphate (P\(_i\)) or adenosine diphosphate (ADP) (2) indicated that in all probability this reaction proceeds as a single step, possibly through a concerted mechanism.

Several problems required further study: (a) the reason for the slow exchange of P\(_{32}\) with ATP (2) even under optimal conditions, i.e. in the presence of appropriate amounts of ADP and bicarbonate; (b) the exchange of propionyl CoA with methylmalonil CoA which would be expected to occur via Reaction 2. Although it was reported from other laboratories (7, 8), we had previously failed (2) to obtain this exchange to a significant extent; (c) the source of oxygen (H\(_2\)O, CO\(_2\), or HCO\(_3^-\)) for ATP cleavage to ADP + P\(_i\) as well as the point of cleavage; and (d) the source of the carboxyl oxygen of methylmalonyl CoA.

The results to be presented in this paper show that (a) the P\(_{32}\)-ATP exchange proceeds at a rate slower than that of the over-all back reaction, which in turn is much slower than the forward reaction. The slow rate of exchange may be attributed to competition between ATP and ADP (which is required for the exchange), for ADP inhibits the over-all forward reaction and, above a given optimal concentration, also inhibits the P\(_{32}\)-ATP exchange; (b) the exchange between propionyl CoA-1-C\(^{14}\) and methylmalonyl CoA occurs at a rate well above that of the over-all back reaction and, like the transfer of CO\(_2\) from CO\(_2^-\) enzyme to propionyl CoA (1) or the carboxylation of the enzyme by methylmalonyl CoA, requires no Mg\(^{++}\) and is inhibited by avidin and by p-mercuribenzoate. Our previous failures with this exchange must be ascribed to impurities in the propionyl CoA-1-C\(^{14}\) used at that time; (c and d) the oxygen for ATP cleavage is derived from bicarbonate, 1 bicarbonate oxygen atom appearing in the released P\(_i\) and 2 in the free carboxyl group of methylmalonyl CoA. Other experiments bearing on the reaction mechanism, including the arsenolysis of ATP and the incorporation of "C\(^{14}\)O\(_2\)" into methylmalonyl CoA, are also reported.

EXPERIMENTAL PROCEDURE

**Methods**

Incorporation of P\(_{32}\) into ATP—Incorporation of P\(_{32}\) into ATP, as a result of either the over-all backward reaction or P\(_i\)-ATP exchange, was followed as in previous work (9). In the experiments in Fig. 1, organically-bound P\(_{32}\) was determined as the radioactivity remaining after removal of P\(_{32}\) by conversion to ammonium phosphomolybdate and extraction with isobutanol. For this purpose 0.2-ml portions of the reaction mixture were pipetted at various times into 0.8 ml of ice-cold 2% HClO\(_4\). After adding 1.0 ml of 20 X H\(_2\)SO\(_4\) and 1.0 ml of 5% ammonium molybdate, the mixture was extracted four times with 2.0 ml of isobutanol. Aliquots, 0.2 ml, of the aqueous phase were used for measurement of radioactivity. This was negligible in zero time samples. In the experiments in Fig. 3, the reaction was stopped with 0.2 ml of 20% trichloroacetic acid and after adding ADP to make its concentration equal in all samples, the nucleotides were absorbed on charcoal and
eluted with ammoniacal ethanol after repeated washing of the charcoal with water to ensure removal of $P_{18}$. Aliquots of the eluates were used to measure the radioactivity of the nucleotides. Although the recovery of nucleotides in the eluates was 50% or less, no correction for this was made in Fig. 3. $P_{18}$ radioactivity was measured with a thin window Geiger-Müller counter.

**Incorporation of Propionyl CoA 1-18C into Methylmalonyl CoA**

—This reaction was measured either through conversion to the hydroxamates, as in the experiments of Table III, or to the free acids, as in Fig. 4, followed by chromatographic separation and measurement of radioactivity. In the former case, the incubated samples (0.23 ml) were cooled in ice, treated with 0.1 ml of salt-free 2.0 M hydroxylamine, and kept for a further 10 minutes at 0°. Aliquots (0.05 ml) were then chromatographed on Whatman No. 1 filter paper in the isomyl alcohol-formic acid system with addition of authentic hydroxamates as carriers (10). The hydroxamate spots were located with a ferric chloride spray (11) and eluted overnight with 2.0 ml of water. Authentic hydroxamates were used as markers. Aliquots of the eluate, 0.2 ml, were used for measurement of radioactivity with a windowless gas flow counter. In preliminary runs the radioactivity was located on the paper chromatograms by use of a strip, gas flow counter equipped with an automatic recorder. Before incubation there was only one radioactive peak corresponding to propionohydroxamate; there were two radioactive peaks after incubation corresponding to the hydroxamates of propionic and methylmalonic acid. For separation of the free acids, in the experiments of Fig. 4, 0.1-ml portions of the reaction mixture were pipetted at various times into 0.1 ml of 0.6 N NaOH and heated for 1 minute at 100° to hydrolyze the thiol esters. Aliquots (0.05 ml), with 1.0 μ mole of methylmalonic acid added as carrier, were then chromatographed as above. Authentic methylmalonic acid was used as marker. The spots were located by spraying with 0.04% bromoresol green in 95% ethanol (adjusted to pH 8.0) and eluted overnight with 2.0 ml of water. Aliquots (0.2 ml) were used for radioactivity measurements. Although methylmalonic acid gave rise to a rather diffuse, large spot, duplicate runs always gave closely agreeing values. The recovery of hydroxamates or free acids by the above method varied from 62 to 76%, or an average of 69%, in several control runs. All values in Table III and Fig. 4 are corrected for this recovery.

**O18 Experiments**—For the experiments in Table VI the carboxylase reaction was carried out in the presence of either $H_{2}O^{18}$ or $HCO_{3}^{18}$, and the $O^{18}$ content of the $P_{1}$ and ADP formed was determined.

An aqueous solution containing the various reaction ingredients was lyophilized to dryness, and the resulting residue was dissolved in either $H_{2}O^{18}$ or $HCO_{3}^{18}$. When bicarbonate-$O^{18}$ was used, all ingredients were lyophilized except NaHCO$_3$ which was added as a solid to the reaction mixture shortly before the enzyme. The reaction was started by the addition of 0.1 ml of enzyme solution after allowing 2 minutes for temperature equilibration at 30°. The reaction was terminated by quick freezing in a solid CO$_2$ acetone bath, and $H_{2}O^{18}$ was recovered by lyophilization. The enzymatic activity was followed by removing a 0.1 ml aliquot from the reaction mixture, both before and after incubation, and measuring the production of ADP by colorimetric determination of the amount of pyruvate formed from phosphoenolpyruvate with pyruvic kinase (12). To determine the extent of exchange between $HCO_{3}^{-}$ and $H_{2}O^{18}$, appropriate aliquots of reaction mixture, with or without enzyme, were frozen after incubation in a suitable tube. A small amount of solid citric acid was then added, the tube evacuated, and the contents allowed to thaw to liberate the CO$_2$. The solution was quickly refrozen and the CO$_2$ collected for mass spectrometer analysis.

The lyophilized residue from the principal incubation was dispersed in cold 0.3 N HClO$_4$ both to drive off CO$_2$ and to inactivate the enzyme. An appropriate amount of carrier KH$_2$PO$_4$ (10 to 15 times the amount of $P_1$ formed) was added, the solution neutralized with 1.0 N KOH, and the insoluble KClO$_4$ removed by centrifugation. $P_1$ was then precipitated as MgNH$_4$PO$_4$, and the mixture was centrifuged. The supernatant solution was diluted 4-fold with water to decrease the salt concentration and lyophilized. The resulting residue was dissolved in 1.0 N HCl, heated for 20 minutes at 100° to hydrolyze the labile phosphate groups of ADP and ATP, and the $P_1$ precipitated as MgNH$_4$PO$_4$. The MgNH$_4$PO$_4$ samples, from the original $P_1$ and that released by hydrolysis of the nucleoside polyphosphates, were converted to KH$_2$PO$_4$ as described earlier (13) and analyzed for $O^{18}$ content by the guanidine hydrochloride procedure of Boyer et al. (14).

For the experiments in Table VII, the reaction was carried out in the presence of $HCO_{3}^{18}$ and the amount of $O^{18}$ appearing in the carboxyl group of the methylmalonyl CoA formed was determined. Since no $H_{2}O^{18}$ was used in these experiments, lyophilization of the reaction mixtures as described above was omitted. A small amount of Na$_2$C$_3$O$_4$ was added to the incubation medium along with the other components; however, NaHCO$_3^{18}$ was added just before the enzyme. The reaction was terminated by addition of 0.2 ml of 4.5 N HClO$_4$, the acidified solution was quickly frozen in a solid CO$_2$-acetone bath and stored in this condition for 3 to 4 hours. After thawing, carrier KH$_2$PO$_4$ was added, and the solution was adjusted to pH 4.0 with 1.0 N KOH. The insoluble KClO$_4$ was removed by centrifugation and washed once with water. The combined supernatant solution and waterwash were lyophilized to ensure removal of any $H_{2}O^{18}$ present. In the first two experiments the residue was dissolved in 5.0 ml of 0.1 N KOH and incubated for 30 minutes at 30° to hydrolyze methylmalonyl CoA and, conversely, unreacted propionyl CoA. Hydrolysis was found to be incomplete under these conditions, and in later experiments, solutions were adjusted to a final OH concentration of 0.1 M for this purpose. After acidifying the solution to pH 2.0 with 2.0 N HCl, methylmalonic and propionic acids were extracted three times with four volumes of reagent grade anhydrous ether. After evaporation of the ether under vacuum, the small amount of residue was dissolved in a minimal amount of water and lyophilized. The aqueous phase remaining after ether extraction was used for isolation and determination of the $O^{18}$ content of the $P_1$ formed in the reaction.

The flask containing the lyophilized ether extract was attached to a high vacuum line, evacuated to less than 5 μ, and heated with a soft flame in the first two experiments. Such heating gave considerable CO$_2$ from material (probably propionic acid) other than methylmalonic acid present in the dried ether extract. In later experiments, samples were decarboxylated by heating at 135-165° for 15 minutes in a sand bath; this avoided formation of extraneous CO$_2$. The evolved CO$_2$ was condensed in a manometer with use of liquid nitrogen. Approxima-
mately two volumes of tank CO₂ were added, a portion of the sample was trapped with Ba(OH)₂ for C₁⁴ determination, and the remainder used for mass 46/44 analysis. The C₁⁴ content of the CO₂ was analyzed as follows. A measured portion was covered, and all measurements were made with the same total Ba(OH)₂. After thawing the Ba(OH)₂ solution by immersing planchet; the planchet held a frozen solution of 10-fold excess bled into an evacuated 15 X 3-cm chamber which was immersed the remainder used for mass 46/44 analysis. The C₁⁴ content sample was trapped with Ba(OH)₂ for C₁⁴ determination, and approximately two volumes of tank CO₂ were added, a portion of the 1462 Mechanism of Propionyl Carboxylase Reaction. II. amount of BaC₀₃. NazCr₄O₃ in solution was analyzed in a similar manner by directly plating a suitable aliquot of the sample before mixing with a standard amount of Ba(OH)₂. Uniform plating of all samples was ensured by dispersing the Ba(OH)₂ in sufficient water so that the bottom surface of the planchet was completely covered, and all measurements were made with the same total amount of BaC₀₃.

Other Methods—Propionyl carboxylase, kept in the refrigerator as a suspension in 0.02 m potassium phosphate buffer, pH 6.5, 60% saturated with ammonium sulfate containing 0.001 m EDTA and 0.0005 m GSH, was harvested by centrifugation, washed with 60% saturated ammonium sulfate, and dissolved in an appropriate volume of 0.02 m Tris-Cl buffer, pH 7.5, containing 0.001 m EDTA and 0.0005 m GSH. Protein concentration was determined spectrophotometrically and enzyme activity measured by optical assay as previously described (9).

In the arsenolysis experiment in Table II, the liberation of P₃² from ATP labeled with P₃² in the terminal phosphate was determined by conversion to ammonium phosphomolybdate, extraction with isobutanol, and counting of an aliquot of the isobutanol extract. The procedure was otherwise as described for the P₃²-ATP exchange. For measuring incorporation of ATP₃² into ATP, the nucleotides were adsorbed on charcoal, eluted with ammoniacal ethanol and separated by paper chromatography as previously described (2).

Preparations

Propionyl CoA-1-C¹⁴ was prepared enzymatically as described by Flavin, Castro-Mendoza, and Ochoa (15). An amount of Clostridium kluyveri extract containing 32 mg of protein was incubated for 40 minutes at 30° with 54 μmoles of propionyl CoA and 27 μmoles of sodium propionate-1-C¹⁴ (2.83 μc per μmole). The crude propionyl CoA-1-C¹⁴ was purified by ion exchange chromatography essentially as previously described for methylmalonyl CoA-3-C¹⁴ (1). After removal of propionic acid by ether extraction, the acid aqueous solution was neutralized to pH 6.0 and passed through a 1.0 X 25-cm Dowex (X2, 200 to 400 mesh) column, formate form. Propionyl CoA was eluted a little ahead of methylmalonyl CoA, between Tubes 25 and 33 (cf. (1)) in the second elution range (4.0 formic acid-0.4 m ammonium formate). The yield after lyophilization, as determined from the release of -SH (16) by alkali was about 25.2 μmoles (specific radioactivity, 520,000 c.p.m. per μmole). Methylmalonyl CoA was prepared synthetically by the method of Beck, Flavin, and Ochoa (17). The preparation of methylmalonyl CoA-3-C¹⁴ was described earlier (1). Methylmalonic acid and methylmalon-monohydroxamate were prepared as described earlier (10) except that the former was repeatedly crystallized from benzene and ether (18). Propionohydroxamate was prepared by reaction of propionic anhydride with salt-free (19) hydroxylamine and propionyl CoA as in previous work (10). P₃²-labeled ADP (AMP-P₃²) and ATP (AMP-P₃²-P₃²) were prepared as previously described (2). Four times crystallized pig heart propionyl carboxylase, specific activity 17 (3), was used unless otherwise specified.

Sodium bicarbonate enriched with O¹⁸ was prepared by dissolving 60 mg of NaHCO₃ in 0.8 ml of H₂O¹⁸ containing 10 atoms % excess O¹⁸. The solution was gassed with CO₂, sealed in a tube under CO₂, and heated at 100° for approximately 4 hours. H₂O¹⁸ was recovered by lyophilization. The O¹⁸ content of the NaHCO₃ was determined through release of CO₄⁸ by pyrolysis.

Sodium propionate-1-C¹⁴ (2.83 μc per μmole) was purchased from the New England Nuclear Corporation under allocation from the Atomic Energy Commission. Methylmalonic diethyl-ester was obtained from the Sapon Laboratories. The sources of other commercial preparations were as previously given (2, 3, 9). We are indebted to Dr. D. O. Brummond, Radioisotope Unit, Veterans Administration Hospital, Cleveland, Ohio, for a gift of dried Clostridium kluyveri cells.

RESULTS

Rate of P₃²-ATP Exchange—In previous experiments (2) the exchange reactions between P₃² and ATP and between ADP-C₁⁴ (or ADP₃²) and ATP were found to be dependent on the presence of ADP and P₃, respectively. A requirement for bicarbonate was also apparent. These observations suggested the occurrence of Reaction 1 as a single partial step in the over-all reaction catalyzed by propionyl carboxylase (Reaction 3). However, the rate of the exchange reactions seemed slow not only in comparison to that of the over-all forward reaction but also in comparison to that of the much slower back reaction (9). Because of this discrepancy, a careful investigation of the rates of the P₃²-ATP exchange and the over-all back reaction was undertaken. The results of these experiments, in which both reactions were followed through the incorporation of P₃² into ATP, are shown in Fig. 1. It may be seen that the over-all back reaction (Reaction 3 from right to left) was faster than the P₃²-ATP exchange, even though the latter was allowed to take place under the most suitable conditions, i.e., in the presence of bicarbonate, ADP, and P₃. Whereas the back reaction reached equilibrium in about 15 minutes, the concentration of ATP₃² remaining constant thereafter, the exchange reaction proceeded linearly for at least 60 minutes. At 60 minutes the specific radioactivity of ATP in the exchange experiment was 37,800 c.p.m. per μmole. This is to be compared with a specific radioactivity of 175,000 c.p.m. per μmole for 100% exchange. The initial velocities were calculated to be approximately 0.023 and 0.003 μmole per minute per unit of enzyme at 30° for the back and the exchange reaction, respectively. The initial velocities at 25° of the over-all forward and backward reactions, the P₃-ATP exchange, and the propionyl CoA-methylmalonyl CoA exchange (the latter calculated from the data of Fig. 4) are listed in Table I. Correction to 25° is based on the observation that the over-all forward reaction is approximately 30% slower at 25° than at 30°.

Since the P₃²-ATP exchange requires the simultaneous presence of ADP and ATP, competition between these two reactants for a site on the enzyme might account for the low rate of exchange. This assumption received support from two findings: (a) ADP inhibited the over-all forward reaction, the inhibition being competitive with respect to ATP, and the latter, as ex-
petted, inhibited the over-all back reaction, and (b) ADP, which is required for the P_i-ATP exchange, became inhibitory above a certain optimal concentration. Competitive inhibition of the forward reaction by ADP is illustrated in Fig. 2, in which the reaction was measured by use of the C^{14}0_4 fixation assay described earlier (9). From these data, $K_a$ for ATP was calculated as $1.82 \times 10^{-4}$ M and $K_i$ for ADP as $5.72 \times 10^{-4}$ M. The inhibition of the over-all back reaction by ATP was shown by the following experiment in which the rate of incorporation of P_i into ATP was measured under conditions approximating both those used for measurement of the rate of the over-all back reaction and that of the P_i-ATP exchange (see legend for Fig. 1). The samples for the combined experiment contained the following components (in µmoles per ml). Tris-HCl buffer, pH 7.5, 100; MgCl_2, 6; GSH, 2; ADP, 2; ATP, 2; KHC_2O_4, 1; methylmalonyl CoA, 2; and crystalline enzyme, 0.41 unit. The back reaction samples lacked ATP and KHC_2O_4, whereas the exchange samples lacked methylmalonyl CoA. The following initial rates (in µmoles per minute per enzyme unit at 25°C) were obtained. Back reaction, 0.012; exchange, 0.002; combined experiment, 0.006. This provides further support for the view that under the same experimental conditions the over-all back reaction and the P_i-ATP exchange proceed at similar rates. Inhibition of the exchange when ADP exceeds an optimal concentration is shown in Fig. 3. It may be seen that, as previously reported (2), there was no significant exchange in the absence of added ADP. The rate of exchange increased with increasing concentration of ADP to reach a maximum at $2 \times 10^{-3}$ M ADP (ADP to ATP ratio, 2.0) and decrease markedly thereafter.

Supplementing previous observations on the inhibition of the over-all reaction by avidin or CMB and of the P_i-ATP and ADP-ATP exchanges by avidin (2), it has been found that the two exchange reactions are completely inhibited by $1 \times 10^{-4}$ M CMB.

Arsonolysis of ATP—Incubation of propionyl carboxylase with ATP labeled with P_32 in the two terminal phosphates, Mg^{2+} and arsenate, resulted in a slow liberation of P_32 which, as shown in Table II, required the presence of added ADP. As pointed out in the discussion, this observation provides further support for the view that Reaction 1 is a single, partial step of the over-all reaction. It might be mentioned in this connection that

![Fig. 1. Time course of over-all back reaction and of P_i-ATP exchange. For the back reaction (Curve 1), 1.0 ml of reaction mixture contained (in µmoles), Tris-HCl buffer, pH 7.5, 100; MgCl_2, 6; GSH, 2; ADP, 2; P_i (554,000 c.p.m. per µmole), 2; methylmalonyl CoA, 1.2; and crystalline enzyme, 0.01 unit. The back reaction samples lacked ATP and KHC_2O_4, whereas the exchange samples lacked methylmalonyl CoA. The following initial rates (in µmoles per minute per enzyme unit at 25°C) were obtained. Back reaction, 0.012; exchange, 0.002; combined experiment, 0.006. This provides further support for the view that under the same experimental conditions the over-all back reaction and the P_i-ATP exchange proceed at similar rates. Inhibition of the exchange when ADP exceeds an optimal concentration is shown in Fig. 3. It may be seen that, as previously reported (2), there was no significant exchange in the absence of added ADP. The rate of exchange increased with increasing concentration of ADP to reach a maximum at $2 \times 10^{-3}$ M ADP (ADP to ATP ratio, 2.0) and decrease markedly thereafter.

Supplementing previous observations on the inhibition of the over-all reaction by avidin or CMB and of the P_i-ATP and ADP-ATP exchanges by avidin (2), it has been found that the two exchange reactions are completely inhibited by $1 \times 10^{-4}$ M CMB.

![Fig. 2. Inhibition of forward propionyl carboxylase reaction by ADP. Lineweaver-Burk plots of reciprocal velocity ($v = \text{c.p.m. C}^{14}0_4$ fixed in 10 minutes) versus the reciprocal molar concentration of ATP. Samples contained (in µmoles), Tris-HCl buffer, pH 7.5, 100; MgCl_2, 6; GSH, 2; NaC_4O_4 (25,000 c.p.m. per µmole), 10; propionyl CoA, 1; crystalline enzyme, 0.03 unit; and varying amounts of ATP without or with ADP as indicated. Final volume, 1.0 ml. Incubation, 10 minutes at 30°C. C^{14}0_4 fixation assay as previously described (9) except that a window-less gas flow counter was used. Curve 1, no ADP; Curve 2, $1 \times 10^{-4}$ M ADP; Curve 3, $2 \times 10^{-3}$ M ADP.

![Fig. 3. P_i-ATP exchange as a function of the concentration of ADP. Samples contained (in µmoles), Tris-HCl buffer, pH 7.5, 100; MgCl_2, 6; GSH, 2; ATP, 1; P_i (322,000 c.p.m. per µmole), 1; KHC_2O_4, 0.5; ADP, as shown; and crystalline enzyme, 0.16 unit. Final volume, 1.0 ml. Incubation, 30 minutes at 30°C.}
arsenate cannot replace Pi for the exchange between ADP and ATP. Thus, in an experiment similar to those previously reported (2), incubation for 60 minutes at 30° of amorphous enzyme (specific activity 6, 0.16 unit) with Tris-HCl buffer, pH 7.5, 25 pmoles; and crystalline enzyme, Experiment 1, 0.225 unit; Experiment 2, 0.085 unit; and Experiment 3, 0.0225 unit. Incubation at 30°; Experiment 1, 30 minutes; Experiments 2 and 3, 10 minutes, led to radiation of 7550 c.p.m. in ATP, but only 330 c.p.m. were found in ATP with 1.0 pmole of Na2HAsO4 substituted for orthophosphate.

**TABLE II**

Arsonolysis of ATP

The complete system contained in (μmoles), Tris HCl buffer, pH 7.5, 100; MgCl2, 6; GSH, 2; KHCO3, 1; P32-labeled ATP (150,000 c.p.m. per μmole in terminal phosphate), 1; ADP, 1; Na2HAsO4, 2; and amorphous enzyme (specific activity, 6), 0.4 unit. Final volume, 1.0 ml. Incubation, 90 minutes at 30°.

<table>
<thead>
<tr>
<th>System</th>
<th>Phosphate liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>3620</td>
</tr>
<tr>
<td>No arsenate</td>
<td>440</td>
</tr>
<tr>
<td>No ADP</td>
<td>520</td>
</tr>
</tbody>
</table>

**TABLE III**

**Propionyl CoA-1-C14-methylmalonyl CoA exchange**

The complete system contained in all cases, Tris-HCl buffer, pH 7.5, 25 μmoles; and GSH, 0.5 μmole, in a final volume of 0.25 ml. Additions, other than those specified on the table, included: propionyl CoA-1-C14, Experiment 1, 0.25 μmole (130,000 c.p.m.); Experiments 2 and 3, 0.125 μmole (65,000 c.p.m.); methylmalonyl CoA, Experiment 1, 0.55 μmole; Experiments 2 and 3, 0.275 μmole; and crystalline enzyme, Experiment 1, 0.225 unit; Experiment 2, 0.085 unit; and Experiment 3, 0.0225 unit. Incubation at 30°; Experiment 1, 30 minutes; Experiments 2 and 3, 10 minutes.

Experiments 2 and 3, 0.125 pmole (65,000 c.p.m.); methylmalonyl CoA, Experiment 1, 0.55 pmole; Experiments 2 and 3, 0.275 pmole; and crystalline enzyme, Experiment 1, 0.225 unit; Experiment 2, 0.085 unit; and Experiment 3, 0.0225 unit. Incubation at 30°; Experiment 1, 30 minutes; Experiments 2 and 3, 10 minutes.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>System</th>
<th>Additions</th>
<th>Radioactivity of hydroxamates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Complete</td>
<td>No enzyme</td>
<td>Propionyl CoA</td>
</tr>
<tr>
<td></td>
<td>No methylmalonyl CoA</td>
<td>MgCl2, 1.5 μmoles</td>
<td>c.p.m.</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td></td>
<td>72,400</td>
</tr>
<tr>
<td></td>
<td>No enzyme</td>
<td>Avidin, 0.3 unit</td>
<td>111,700</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>Avidin, 0.3 unit + biotin, 720 μg</td>
<td>40,000</td>
</tr>
<tr>
<td>2</td>
<td>Complete</td>
<td>No enzyme</td>
<td>Propionyl CoA</td>
</tr>
<tr>
<td></td>
<td>No enzyme</td>
<td>Avidin, 0.3 unit</td>
<td>c.p.m.</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>Avidin, 0.3 unit + biotin, 720 μg</td>
<td>67,700</td>
</tr>
<tr>
<td></td>
<td>No enzyme</td>
<td>MgCl2, 1.5 μmoles</td>
<td>40,200</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>CMB, 2 × 10-4 M</td>
<td>65,000</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>EDTA, 5 μmoles</td>
<td>43,700</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>CMB, 1 × 10-4 M</td>
<td>73,500</td>
</tr>
<tr>
<td>3</td>
<td>Complete</td>
<td>No enzyme</td>
<td>Propionyl CoA</td>
</tr>
<tr>
<td></td>
<td>No enzyme</td>
<td>Avidin, 0.3 unit</td>
<td>c.p.m.</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>Avidin, 0.3 unit + biotin, 720 μg</td>
<td>40,200</td>
</tr>
<tr>
<td></td>
<td>No enzyme</td>
<td>MgCl2, 1.5 μmoles</td>
<td>65,000</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>CMB, 2 × 10-4 M</td>
<td>43,700</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>CMB, 1 × 10-4 M</td>
<td>73,500</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>EDTA, 5 μmoles</td>
<td>67,700</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>CMB, 2 × 10-4 M</td>
<td>73,500</td>
</tr>
</tbody>
</table>

* GSH was omitted in samples with added CMB.
† Avidin and biotin preincubated together before addition.

The results in Table III show that this exchange occurred readily and that, as expected from the earlier experiments on carboxylation of the enzyme (1), it required no Mg2+ and was inhibited by avidin and by CMB. Further experiments, given in Table IV, show that Reaction 2, as followed directly with substrate amounts of enzyme, is inhibited in either direction by avidin or CMB. From the data of Table III, the propionyl CoA-methylmalonyl CoA exchange appeared to be fairly rapid. The experi-

---

**TABLE IV**

Inhibition of reaction methylmalonyl CoA-3-C14 + enzyme → propionyl CoA + CO2 ~ enzyme by avidin and CMB

A. Samples containing (in μmoles) Tris-HCl buffer, pH 7.5, 10; GSH, 2; propionyl CoA-1-C14 (320,000 c.p.m. per μmole), 0.5; methylmalonyl CoA, 1; and crystalline enzyme, 0.0074 unit. Incubation at 30°. Values are given per ml of reaction mixture and are averages of duplicate experiments.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Enzyme radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) None</td>
<td>6620</td>
</tr>
<tr>
<td>(b) Avidin</td>
<td>6630</td>
</tr>
<tr>
<td>(c) CMB</td>
<td>6640</td>
</tr>
</tbody>
</table>

B. C1402N enzyme + propionyl CoA → enzyme + methylmalonyl CoA-3-C14

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Methylmalonyl CoA radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) None</td>
<td>6620</td>
</tr>
<tr>
<td>(b) Avidin</td>
<td>6630</td>
</tr>
<tr>
<td>(c) CMB</td>
<td>6640</td>
</tr>
</tbody>
</table>

* GSH was omitted in samples with added CMB.
Incorporation of "C\textsubscript{4}O\textsubscript{2}\" into Methylmalonyl CoA—This incorporation involves both Reactions 1 and 2, and in the absence of added ATP and propionyl CoA, it should require the presence of both ADP and P\textsubscript{i}. Table V shows that this was indeed the case in agreement with previous observations of Lane et al. (20). In contrast to their results, however, arsenate could not be substituted for P\textsubscript{i}. The reason for this discrepancy is not known, but no pointed out in the discussion, our result is in agreement with other observations reported in this paper.

\textit{O\textsuperscript{18} Experiments}—Experiments in which the over-all forward reaction was carried out in the absence of either H\textsubscript{2}O\textsubscript{18} or HCO\textsubscript{3}\textsuperscript{18} showed that the oxygen for ATP cleavage is derived from bicarbonate, 1 bicarbonate oxygen atom appearing in the liberated P\textsubscript{i} and 2 atoms in the free carboxyl group of methylmalonyl CoA. The results of measurements of the O\textsuperscript{18} content of ADP and P\textsubscript{i} when the reaction was carried with either H\textsubscript{2}O\textsubscript{18} or NaHCO\textsubscript{3}\textsuperscript{18} present are given in Table VI. They show that when ATP is cleaved, O\textsuperscript{18} is incorporated into the P\textsubscript{i} formed. Under our experimental conditions, a fairly rapid exchange of oxygen between H\textsubscript{2}O and bicarbonate was to be expected (21). This exchange does not significantly alter the isotope content of the water, which is present in large excess, but does appreciably change the isotope content of the bicarbonate. Appropriate correction for such exchange must therefore be made. Measurement of the amount of water oxygen present in the bicarbonate at the end of the incubation showed that approximately 45% of the bicarbonate oxygen had exchanged with water. Thus, the observed incorporation of O\textsuperscript{18} from H\textsubscript{2}O\textsubscript{18} into the P\textsubscript{i} resulted from exchange with the bicarbonate, and the amount of water oxygens found in the P\textsubscript{i} can be used to calculate the extent of exchange that had occurred with the bicarbonate that actually participated in the reaction. A correction made on this basis was used to calculate the number of oxygen atoms per molecule of P\textsubscript{i} derived from H\textsubscript{2}O or NaHCO\textsubscript{3} given in the last column of Table VI. Clearly, within the experimental error, the oxygen for cleavage came from bicarbonate.

Although the data in Table VI show that oxygen from bicarbonate is involved in the cleavage of ATP, they do not indicate whether the immediate oxygen donor is bicarbonate or CO\textsubscript{2} derived from the bicarbonate. A distinction may be made between HCO\textsubscript{3}\textsuperscript{18} and CO\textsubscript{2} as the active compound by analyzing the O\textsuperscript{18} content of the carboxyl groups of the methylmalonyl CoA formed. Bicarbonate could furnish one oxygen for cleavage of the ATP and the other two bicarbonate oxygens could appear in the free carboxyl group of methylmalonyl CoA. In contrast, if CO\textsubscript{2} furnished the oxygen for ATP cleavage, an oxygen from water would have to be picked up to give the methylmalonyl CoA carboxyl. The results of experiments on the extent of oxygen transfer from HCO\textsubscript{3}\textsuperscript{18} to the —COOH of methylmalonyl CoA are given in Table VII. In Experiments 1 and 2, considerable CO\textsubscript{2} was obtained from sources other than methylmalonic

---

**Table V**

<table>
<thead>
<tr>
<th>Isotope source</th>
<th>Atoms % excess O\textsuperscript{18} in compound added</th>
<th>Observed atoms % excess O\textsuperscript{18} in reaction products</th>
<th>Number of P\textsubscript{i} oxygens derived directly from original isotope source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H\textsubscript{2}O</td>
<td>2.90</td>
<td>0.00</td>
<td>0.27</td>
</tr>
<tr>
<td>NaHCO\textsubscript{3}</td>
<td>5.96</td>
<td>0.00</td>
<td>1.01</td>
</tr>
</tbody>
</table>

* Corrected for H\textsubscript{2}O-HCO\textsubscript{3} exchange as described in text.

**Table VII**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Observed atoms % excess O\textsuperscript{18}</th>
<th>Number of oxygen atoms* from NaHCO\textsubscript{3} appearing in methylmalonic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.96</td>
<td>1.13</td>
</tr>
<tr>
<td>2</td>
<td>5.96</td>
<td>1.35</td>
</tr>
<tr>
<td>3</td>
<td>3.70</td>
<td>0.76</td>
</tr>
</tbody>
</table>

* Corrected for 24%, 9%, and 18% exchange of oxygens between water and bicarbonate in Experiments 1, 2, and 3, respectively, as estimated from the observed O\textsuperscript{18} in the P\textsubscript{i} compared to the expected value of \( \frac{1}{4} \) of the atoms % excess in the NaHCO\textsubscript{3} used.
Mechanism of Propionyl Carboxylase Reaction. II.

DISCUSSION

The experiments reported in this paper provide additional evidence for the proposed two steps in the propionyl carboxylase reaction and throw further light on its mechanism. They also identify HCO$_3^-$ as the reactive species of CO$_2$.

The exchange reactions, predicted from Reactions 1 and 2, might be expected to occur at a rate at least as high as that of the over-all reaction (Reaction 3) in the direction in which this reaction is less rapid, in this case the backward direction. A glance at Table I shows that this is true for the propionyl CoA-methylmalonyl CoA exchange, predicted from Reaction 2, which is 40 times faster than the over-all back reaction and more than half as fast as the forward reaction. On the other hand, the bicinearbonate- and ADP-dependent P$_1$-ATP exchange (2), predicted from Reaction 1, proceeds at about one seventh the rate of the over-all back reaction and do not seem to fulfill the above expectation. However, since as shown in this paper ADP is a competitive inhibitor of the over-all forward reaction and, above a certain concentration, becomes an inhibitor of the P$_1$-ATP exchange, competition of ADP and ATP for a site on the enzyme may account for the slow rate of this exchange. This means that the rate of P$_1$-ATP exchange which requires the simultaneous presence of ATP and ADP will proceed more slowly than that of the over-all back reaction which is measured in the absence of ATP. It may be noted that in an experiment in which the exchange was measured under conditions approximating those of the back reaction, a rate of 0.006 pmole per minute per unit of enzyme was obtained. This was half the rate of the over-all back reaction and 3 times higher than that of the exchange, both determined under standard conditions in the same experiment. This experiment can be regarded as the over-all back reaction in the presence of ATP and bicarbonate, or the P$_1$-ATP exchange reaction in the presence of methylmalonyl CoA. The results show that in the presence of ATP, the rate of over-all back reaction becomes slower, whereas in the presence of methylmalonyl CoA, the P$_1$-ATP exchange proceeds more rapidly. It seems therefore justified to assume that under the same experimental conditions, the over-all back reaction and the P$_1$-ATP exchange would proceed at similar rates. As seen in Table I, the over-all forward reaction is almost 70 times faster than the back reaction. Since the propionyl CoA-methylmalonyl CoA exchange is quite rapid, it is probable that the rate-limiting step of the back reaction is the formation of ATP from CO$_2$-enzyme, ADP, and P$_1$, i.e. Reaction 1 in the backward direction.

We have no explanation for our previous failure (2) to obtain propionyl CoA-methylmalonyl CoA exchange other than the possible presence of inhibitory impurities in the preparations of propionyl CoA-1-C$^{14}$ then used. For the present experiments, the thiol ester was purified by ion-exchange chromatography. It may be pointed out that the rate of exchange reported by other workers was exceedingly low. Thus, the rate obtained by Halenz and Lane (7) with purified enzyme can be calculated approximately as 0.0013 pmole per minute per unit of enzyme (at 25°) and that measured by Friedman and Stern (6) with very crude enzyme as 0.084 µmole per minute per unit (at 25°). Propionyl CoA or enzyme impurities, or both, may be responsible for these results. In the present work (Table I) we obtained an initial rate of 0.6 µmole per minute per unit (at 25°). The observed inhibition of the propionyl CoA-methylmalonyl CoA exchange by avidin and by CMB is in agreement with earlier results (1) and with further experiments, shown in Table IV, with substrate amounts of enzyme.

The formulation of Reaction 1 as a single step is based on the requirement of P$_1$ for ADP-ATP exchange and that of ADP (and bicarbonate) for P$_1$-ATP exchange (2). The requirement of ADP for the arsenolysis of ATP reported in this paper (Table II) provides further support for a one-step mechanism for this reaction. This requirement could be explained by occurrence of the following reactions.

\[
\text{ATP} + "\text{CO}_2" + \text{enzyme} \rightarrow \text{ADP} + \text{P}_1 + \text{CO}_2-\text{enzyme} \quad (1)
\]

\[
\text{ADP} + \text{arsenate} + \text{CO}_2-\text{enzyme} \rightarrow \text{ADP-arsenate} + "\text{CO}_2" + \text{enzyme} \quad (4)
\]

\[
\text{ADP-arsenate} \rightarrow \text{ADP} + \text{arsenate} \quad (5)
\]

\[
\text{Sum: } \text{ATP} \rightarrow \text{ADP} + \text{P}_1 \quad (6)
\]

Should an ADP-enzyme intermediate be formed as a partial step of Reaction 1, as in ATP + enzyme $\rightarrow$ ADP-enzyme + P$_1$, no ADP requirement would be expected for the arsenolysis of ATP.

The incorporation or exchange of "C$^{14}$O$_2$" into methylmalonyl CoA requires both ADP and P$_1$. This is as expected from Reaction 3 and partial Reactions 1 and 2. Similar observations were...
The above results do not rule out a two-step mechanism for Reaction 1 in which carbonyl monophosphate, \(-O-C\sim O\), is formed first and is subsequently attacked by enzyme-biotin to give enzyme-biotin\(-CO_2\) and \(P_i\). Carbonyl monophosphate is probably incapable of independent existence in solution, but could conceivably have sufficient stability on the enzyme surface to allow for a two-step mechanism. However, this is made unlikely by the requirement of \(P_i\) for the ADP-ATP exchange in the presence of bicarbonate (2). Moreover, a concerted mechanism with reaction of bound and activated reactants, without appreciable shift in reactant position during the catalysis, appears more acceptable than the two-step mechanism (22).

The finding that both oxygens of the carbonyl group of methylmalonyl CoA as well as one oxygen of the \(P_i\) formed in the reaction come from bicarbonate conclusively demonstrates that bicarbonate, and not CO$_2$ arising from the bicarbonate, participates in the enzymatic catalysis. Probably a similar situation applies to other carboxylation reactions involving biotin.

**SUMMARY**

1. The rate of the isotopic exchanges predicted by the two-step mechanism of propionyl carboxylase action might be expected to be as fast as, or faster than, the slow component of the over-all reaction, i.e. the back reaction. Whereas this is true of the exchange of propionyl CoA-1-C$_{14}$ with methylmalonyl CoA, the bicarbonate- and adenosine diphosphate (ADP)-dependent exchange of \(^{32}\)P labeled orthophosphate with adenosine triphosphate (ATP) was found to be slower than the back reaction. However, ATP and ADP compete with each other for a site on the enzyme, and as the orthophosphate-ATP exchange requires the simultaneous presence of ATP and ADP, it must always be inhibited to a greater or lesser extent.

2. ATP undergoes arsenolysis, in the presence of enzyme, magnesium ions, bicarbonate, and ADP. Probably the CO$_2$-enzyme is cleaved by arsenate and ADP to form "CO$_2$-"enzyme, and ADP-arsenate which undergoes spontaneous hydrolysis to ADP and arsenate. These results provide additional support for the view that the formation of CO$_2$-enzyme, from ATP, bicarbonate, and enzyme, proceeds as a single-, rather than a two-step reaction, with simultaneous release of ADP and orthophosphate.

3. The exchange between propionyl CoA-1-C$_{14}$ and methylmalonyl CoA requires no magnesium ions, and like the over-all reaction, is inhibited by avidin and by p-mercuribenzoate. This is in line with previous work and with further experiments reported in this paper, in which the individual reactions were followed separately with use of substrate amounts of enzyme.

4. Experiments with \(H_2O_{18}\) and \(HCO_3^{-}_{18}\) show that the oxygen for ATP cleavage is derived from bicarbonate, one bicarbonate oxygen appearing in the released orthophosphate and two in the free carboxyl group of methylmalonyl CoA. These findings also afford unequivocal proof that \(HCO_3^{-}\) is the reactive species of CO$_2$ in the propionyl carboxylase reaction. A concerted reaction mechanism is proposed for the formation of CO$_2$-enzyme coupled to ATP cleavage.

**Acknowledgment**—Our thanks are due to Mr. Morton C. Schneider for help with the preparation of the enzyme.

**REFERENCES**

Mechanism of Propionyl Carboxylase Reaction. II.

Mechanism of the Propionyl Carboxylase Reaction: II. ISOTOPIC EXCHANGE AND TRACER EXPERIMENTS
Yoshito Kaziro, L. F. Hass, P. D. Boyer and Severo Ochoa