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THE EFFECT OF UNIVALENT CATIONS ON ACTIVITIES CATALYZED BY BOVINE-LIVER PROPIONYL-CoA CARBOXYLASE*

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SUMMARY

The propionyl-CoA carboxylase (propionyl-CoA:CO_2 ligase (ADP), EC 6.4.1.3) activity of a highly purified preparation (1400 units/mg of protein at pH 8.5 and 37°) was activated by K^+, NH_4^+, Rb^+, or Cs^+; Na^+, Li^+, or tetramethyl ammonium ion showed little or no activation. The maximal velocity of carboxylation was enhanced 7- to 9-fold in the presence of K^+; however, the values of K_m for propionyl-CoA, bicarbonate, or Mg^{2+} were the same in the absence or presence of K^+. The P_i–ATP and ADP–ATP exchange reactions and transcarboxylase activities catalyzed by the enzyme were also stimulated by K^+.

The propionyl-CoA carboxylase activity was activated maximally by Mg^{2+} or Mn^{2+} and was optimal at pH 8 to pH 8.5. However, the P_i–ATP and ADP–ATP exchange activities of the enzyme were activated by Mn^{2+}; Mg^{2+} was much less effective than Mn^{2+}, and the optimal rates of the exchange reactions occurred between pH 6.8 and pH 7.0. These properties of the exchange activities of the enzyme and certain other features reported here suggest that the 'exchange enzyme' of CHIGA AND PLAUT[3] is identical with propionyl-CoA carboxylase.

A method of separation of propionyl-CoA and methylmalonyl-CoA by thin-layer chromatography has been described.

INTRODUCTION

During an investigation of propionyl-CoA carboxylase (propionyl-CoA:CO_2 ligase (ADP), EC 6.4.1.3) from bovine liver a lack of proportionality between enzyme concentration and activity in assays of preparations at various stages of purification was noted. These variations were found to be attributable to different concentrations of NH_4^+ introduced from preparations of the enzyme into the assay medium. It was

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observed that NH_4^+, as well as a number of other univalent cations, stimulates markedly the activity of this enzyme.

An enhancement of activity by certain monovalent cations of propionyl-CoA carboxylase was reported previously by NEUJAHR[1] for the enzyme from porcine heart. It became of interest to know whether the monovalent cations affect the reaction catalyzed by liver propionyl-CoA carboxylase at a specific stage. The influence of the univalent cations on the various partial reactions catalyzed by the enzyme, *i.e.*, P_i–ATP and ADP–ATP exchange reactions and transcarboxylase activity was therefore investigated. It appears that all of these reactions require the presence of univalent cations for maximal activity.

The properties of a purified enzyme, from extracts of hog-liver mitochondria, catalyzing exchange reactions of P_i with ADP and of ADP with ATP[2,3] and of propionyl-CoA carboxylase from bovine liver were compared in parallel experiments. The results suggest that all of these activities are attributable to a single protein, namely propionyl-CoA carboxylase.

An investigation of the effect of monovalent cations and other agents on various kinetic parameters of highly purified propionyl-CoA carboxylase from bovine liver prepared by a modification of the method of HALENZ *et al.*[4] is the subject of this article.

EXPERIMENTAL PROCEDURES

Materials

The following reagent-grade chemicals were purchased from the commercial suppliers indicated: NaCl, KCl, KBr, $MnCl_2$, $MgCl_2$, from Mallinckrodt; RbCl, CsCl, and tetramethyl ammonium bromide from Matheson, Coleman, and Bell. CoA, Na_2ADP, Na_2ATP, reduced glutathione, and Tris (Sigma 121 enzyme grade) were obtained from Sigma Chemical Co.; $NaH^{14}CO_3$ and sodium [2-^{14}C]propionate from New England Nuclear Corporation; imidazole from Eastman Kodak Corp.; (+)-biotin from Merck and Co; avidin from Nutritional Biochemicals Co.; ^{14}C-labeled ADP from Schwarz BioResearch Inc.; and [^{32}P]orthophosphate from Oak Ridge National Laboratories or New England Nuclear Corp. Before use, the solutions of carrier-free [^{32}P]orthophosphate were heated in 2 M HCl at 94° for 60 min and neutralized with NaOH to pH 6.5. They were then diluted with appropriate amounts of unlabeled phosphate. ^{32}P-labeled ADP and ATP were prepared as described previously[3].

$(NH_4)_2SO_4$ (J. T. Baker Co., reagent grade) used for fractionation of the enzyme was recrystallized from an aqueous solution of 0.03% Na_2EDTA. Acetone (Mallinckrodt, reagent grade) was redistilled from glass. DEAE-cellulose (BioRad Corp., Cellex-D) was purified by a modification of the method of PETERSON AND SOBER[5]. The cellulose was washed sequentially in 1.0 M NaOH–0.5 M NaCl–0.1% EDTA, 1.0 M HCl, and 1.0 M NaOH–0.5 M NaCl–0.1% EDTA, then washed free of alkali with distilled water. A suspension of DEAE-cellulose in 0.05 M potassium phosphate at pH 7.0 was prepared, allowed to settle for 1 h, and the 'fines' poured off. This step was repeated until the supernatant fraction was essentially clear after settling. The purified DEAE-cellulose was stored in a refrigerator as a suspension containing 1 g of DEAE-cellulose per 60 ml of 0.05 M potassium phosphate at pH 7.0.

Sephadex G-200 (Pharmacia Fine Chemicals, Inc.) was suspended in 20 vols. of 4% acetic acid and stored in the refrigerator. Before use, the material was filtered, washed by suspension in 10 vols. of glass-distilled water, 5 vols. of 1% NaEDTA at pH 8, and 100 vols. of glass-distilled water. The gel was then suspended in 0.01 M potassium phosphate at pH 7.0.

Propionyl-CoA and [2-^{14}C]propionyl-CoA were prepared from propionic anhydride and CoA by a modification of the method of SIMON AND SHEMIN[6]. Racemic methylmalonyl-CoA was synthesized by the mixed anhydride method of FLAVIN[7]. The acyl-CoA derivatives were freed of excess anhydrides and carboxylic acids by treatment of the acidified aqueous solution with ether. The acyl-CoA contents of the solution was determined by the method of LIPMANN AND TUTTLE[8] and absence of unreacted reduced CoA was ascertained by the nitroprusside test.

[2-^{14}C]Propionic anhydride was formed from 0.0998 g of fused sodium [2-^{14}C]-propionate (New England Nuclear Corp.) and 0.090 ml of propionyl chloride (Aldrich Chemical Co., Inc.). The reaction mixture was heated at 145–150° for 15 min in a sealed thick-wall glass tube, the tube was cooled, and the contents suspended in ether with the aid of a dental vibrator. The suspension was filtered and the ether solution of [2-^{14}C]propionic anhydride (yield about 70%) was used for the acylation of CoA.

ADP and ATP were purified further before use by chromatography on columns of Dowex 1-X4 (chloride form) (BioRad Corp.).

Glassware was soaked in 2 M HCl overnight, rinsed with tap water, 1% Na_2EDTA at pH 8.0, and finally 6 times with glass-distilled water.

Water redistilled from glass was used for the preparation of all solutions. Adjustments of acidity to appropriate values of pH were made with NaOH or HCl.

METHODS

Assay of propionyl-CoA carboxylase activity

The incubation mixtures contained 81 mM Tris–HCl (pH 8.5), 12.5 mM $NaH^{14}CO_3$ (specific radioactivity 65 000–85 000 counts/min per μmole), 2.7 mM Na_2ATP, 3.3 mM reduced glutathione, 6.7 mM $MgCl_2$, 1.35 mM propionyl-CoA, 100 mM KCl, and enzyme in a final volume of 0.75 ml. All components of the assay media, excepting the enzyme, were placed in 1 cm × 15 cm thick-wall tubes. The vessels were placed into a 37° water bath for 5 min and the reactions were started by the addition of enzyme. After an incubation period of 10 min the reactions were stopped by the addition of 0.30 ml of 7% trichloroacetic acid and gassed vigorously with CO_2 to remove unreacted $^{14}CO_2$. Aliquots (0.2 ml) of this solution were transferred to scintillation vials, mixed with 15 ml of fluor solution, and counted.

One unit of enzyme is defined as that amount which catalyzes the fixation of ^{14}C from 1 μmole of [^{14}C]bicarbonate per h at 37° under the conditions of the assay. The assay gave a linear response with time when up to 0.25–0.30 unit of enzyme was used. Specific activities of enzyme preparations are expressed as units per mg of protein. Protein was determined by the method of WARBURG AND CHRISTIAN[9].

Deviations from the conditions of assay above have been indicated in the text, tables, and figures.

Assay of other activities

Exchange reactions between P_i and ATP and of ADP with ATP and transcarboxylase activity were measured as described previously[3,4]. Specific adaptations of these procedures are described in detail at the appropriate places in this paper.

Determination of radioactivity

Radioactivity of ^{14}C-containing compounds was determined routinely in a Nuclear Chicago Model 720 spectrometer.

The liquid scintillation fluor used contained 4.0 g of 2,5-diphenyloxazole and 0.05 g of [*p*-bis-{2-(5-phenyloxazolyl)}-benzene] per l of solvent (0.68 l of toluene and 0.32 l of ethanol). The appropriate corrections for quenching of counts were made.

Nucleotides containing ^{14}C or ^{32}P were separated by paper electrophoresis and counted in a scintillation spectrometer by cutting out the appropriate zones and placing the papers into scintillation vials containing counting fluid without ethanol. In some cases ^{32}P-containing nucleotides on paper were counted in a low-background gas-flow counter.

Methods of separation

Methylmalonyl-CoA and propionyl-CoA were separated by spotting 0.005 ml of the reaction mixture from the transcarboxylase assay on a sheet bearing a thin layer of silica (Eastman Chromagram sheet K-301-R, Distillation Products Industries), 'activated' before use by heating at 100° for 15 min. The chromatograms were developed in the Eastman Chromagram-Developing Apparatus overnight at room temperature in a system containing 57 ml of isobutyric acid, 4.0 ml conc. NH_4OH, 3.3 ml of 1% Na_2EDTA at pH 8.0, and 30.0 ml of water. The acyl-CoA derivatives were located under ultraviolet light. The spots were cut out, and propionyl-CoA (R_F 0.63) and methylmalonyl-CoA (R_F 0.48)* were eluted from the silica by suspending in 0.5-ml portions of 1 M HCl in 25-ml test tubes with a dental vibrator (Toothmaster). The content of each tube was mixed with 15 ml of scintillation fluor and counted. Recoveries of radioactivity applied to the chromatograms ranged from 65–75%.

ATP and ADP were separated by electrophoresis on EDTA-washed Whatman 3 MM paper in 0.03 M sodium citrate–citric acid buffer (pH 3.1)[3] or in 20% acetic acid.

Preparation of the enzyme

All operations were performed at 4° unless otherwise indicated.

Preparation of acetone powder. Particulate preparations enriched in mitochondria were prepared from bovine liver and desiccated with acetone as described previously[3].

Extraction. Ten g of acetone powder were added slowly to 200 ml of a solution of 0.05 M Tris–HCl at pH 7.5 and 0.002 M reduced glutathione. The suspension was stirred for 30 min with a magnetic stirrer, and centrifuged at 4500 $\times g$ for 30 min. The residue was discarded (Step 1).

* Free CoA migrated on the chromatogram at R_F 0.39.

Aging step. The solution was treated with 1 ml of toluene, flushed with N_2, and allowed to stand in a closed container overnight at 25°. A dense precipitate appeared which was removed by centrifugation at 4500 × *g* for 30 min (Step 2).

DEAE-cellulose steps. The solution from the previous step was mixed with a suspension of DEAE-cellulose (1 g DEAE-cellulose per 60 ml of 0.05 M potassium phosphate buffer (pH 7.0)) to give a ratio of weight of DEAE-cellulose to protein of 3:1, the suspension was diluted with water to a phosphate concentration of 0.021 M and mixed with a magnetic stirrer for 30 min*. The slurry was then filtered by gravity through a 4 cm × 60 cm chromatography column fitted with a sintered-glass plate.

The residue in the column was washed with 100 ml of a solution containing 0.021 M potassium phosphate at pH 7.0 and 0.002 M reduced glutathione; the effluent was discarded. The enzyme was eluted from the column with 100 ml of a solution of 0.10 M potassium phosphate (pH 7.0) and 0.002 M reduced glutathione (Step 3a).

The solution (100 ml) from the previous step was mixed with a suspension of 1 g DEAE-cellulose in 0.05 M potassium phosphate buffer at pH 7.0 and diluted with water to a final concentration of 0.04 M phosphate. The slurry was stirred for 30 min and filtered through a 2 cm × 40 cm column fitted with a sintered-glass plate. The enzyme activity was eluted from the DEAE-cellulose column with 100 ml of 0.002 M reduced glutathione in 0.1 M potassium phosphate at pH 7.0 (Step 3b).

*$(NH_4)_2SO_4$ fractionation***

The enzyme solution was placed in a dialysis sac and dialyzed against 4 vols. of 50% satd. $(NH_4)_2SO_4$ containing 0.003 M reduced glutathione at pH 4.5 for 48 h with stirring; the precipitate formed was removed by centrifugation. The supernatant solution was then dialyzed with stirring against 4 vols. of 65% satd. $(NH_4)_2SO_4$ at pH 7.5 for 24 h to raise the final $(NH_4)_2SO_4$ concentration from 40% to 60% satn. The suspension was centrifuged and the residue dissolved in 0.01 M potassium phosphate (pH 7.0)–0.003 M reduced glutathione yielding 4.2 ml of a solution containing 11 mg of protein per ml (Step 4).

Sephadex G-200 chromatography

A 40-cm column of Sephadex G-200 was prepared by pouring a suspension of the gel in 0.01 M potassium phosphate buffer (pH 7.0) into a 1.5-cm diameter column plugged with glass wool. The column was washed with 10–20 bed vols. of 0.01 M potassium phosphate (pH 7.0)–0.003 M glutathione. The solution from Step 4 was applied to the column and the enzyme was displaced from the gel with the 0.01 M potassium phosphate–0.003 M glutathione buffer. Activity first appeared after 35–40 ml of buffer had passed through the column; the bulk of the enzyme was recovered in the subsequent 8-ml portion of effluent (Step 5). A summary of the purification procedure is given in Table I.

The enzyme was stored at 4°, usually in 0.01 M potassium phosphate–0.003 M

* The acidity of the suspension at this step should be at pH 7.3–7.4.
** Concentrations of $(NH_4)_2SO_4$ refer to saturation at 25°.

TABLE I

PURIFICATION OF PROPIONYL-CoA CARBOXYLASE FROM BOVINE LIVER ACETONE POWDER
10 g of powder was used.

Step No.	*Treatment*	*Volume (ml)*	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Yield (%)*
1	Extract	180	31 500	2320	13.5	100
2	Aged extract	172	25 200	2220	11.3	80
3a	1st DEAE-cellulose step	100	18 000	220	82	58
3b	2nd DEAE-cellulose step	101	13 100	104	126	42
4	$(NH_4)_2SO_4$ fractionation	4.2	5 850	46	128	18
5	Sephadex G-200 chromatography	7.8	5 000	3.6	1400	16

reduced glutathione (pH 7.0). When studying the effects of univalent cations, enzyme solutions were dialyzed overnight against 1000 vols. of a solution containing 0.025 M Tris–HCl (pH 7.3) and 0.003 M reduced glutathione. The enzyme showed comparable stabilities in the Tris and the phosphate buffers, retaining about 70% of the initial activity after 3–4 weeks of storage.

The present method of purification gave routinely an enzyme preparation with a specific activity of 1300–1500 units per mg of protein. Preliminary investigations in the ultracentrifuge* showed a major component which possesses enzyme activity with a sedimentation coefficient ($s_{20,w}$) of 18.3 S. The preparation of HALENZ *et al.*[4] from the same source and assayed by the same procedure had a specific activity of 992 units per mg of protein, a sedimentation coefficient ($s_{20,w}$) of 19.0 S, and was estimated by them to be 70% pure.

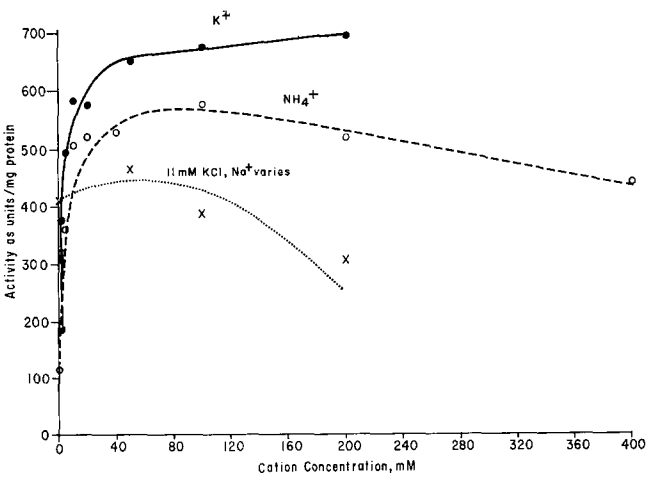

Fig. 1. Response of propionyl-CoA carboxylase activity to varying concentrations of NH_4^+, K^+, and Na^+. The carboxylase system described under EXPERIMENTAL PROCEDURES was used excepting that 100 mM KCl was replaced by varying quantities of KCl (●—●), $(NH_4)_2SO_4$ (○— —○), or by 11 mM KCl in the presence of varying concentrations of NaCl (× · · · · · ×). The reaction was initiated with 1.9 μg of enzyme protein (specific activity 500); all components were in a vol. of 1.5 ml.

* We wish to thank Mr. R. H. YUE for making these determinations.

RESULTS

The effect of univalent cations

During the course of purification of propionyl-CoA carboxylase it was noted that a non-linear increase in response was obtained with increasing volumes of certain fractions of the enzyme solution. These fractions contained substantial amounts of $(NH_4)_2SO_4$. Experiments with enzyme solutions dialyzed against a buffer containing 0.025 M Tris–HCl and 0.003 M GSH at pH 7.3 showed increased propionyl-CoA carboxylase activity proportional to varying concentrations of $(NH_4)_2SO_4$ (Fig. 1) and other ammonium salts.

The enhancement of propionyl-CoA carboxylase activity by NH_4^+ was reminiscent of the stimulatory effect of certain monovalent cations on pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) (for review see BOYER[10]). Further experiments showed that K^+, Rb^+, and Cs^+ enhanced propionyl-CoA carboxylase activity to about the same extent, but Li^+, Na^+, and tetramethyl ammonium ion were inactive (Table II). The effect is therefore specific for particular cations and is

TABLE II

THE EFFECT OF VARIOUS UNIVALENT CATIONS ON PROPIONYL-CoA CARBOXYLASE ACTIVITY

The conditions of incubation were as described under EXPERIMENTAL PROCEDURES except that KCl was omitted from the basic reaction mixture and replaced as indicated. Solutions of individual reagents were adjusted to the appropriate pH with NaOH or HCl. The specific radioactivity of $NaH^{14}CO_3$ was 70 400 counts/min per μmole and 0.09 unit of enzyme (specific activity 1500) was present in a final vol. of 0.75 ml.

Components added	*Concn. (mM)*	*Enzyme specific activity (units/mg of protein)*
None	—	237
LiCl	100	264
NaCl	100	219
KCl	100	1490
RbCl	100	1960
CsCl	100	1310
$(CH_3)_4NBr$	100	231
KBr	100	1060
KBr	5	645
$(CH_3)_4NBr$ *plus* KBr	95 5	864

not attributable merely to a salt effect. A value of the apparent K_m of $1.5 \cdot 10^{-3}$ M was calculated for NH_4^+ and K^+ from the data shown in Fig. 1. K^+ does not merely counteract an inhibitory effect of Na^+ since the reaction rate in the presence of a nearly optimal concentration of K^+ (10 mM) was not depressed by 50 mM Na^+; however, higher concentration of Na^+ caused some inhibition (Fig. 1). In a similar experiment with 5 mM KBr the presence of 95 mM tetramethyl ammonium bromide resulted in a slight increase and not a decline of activity (Table II).

The activation by K^+ is not due to stabilization of the enzyme. Thus, when Na^+ was present in the medium the carboxylation proceeded in a linear fashion over

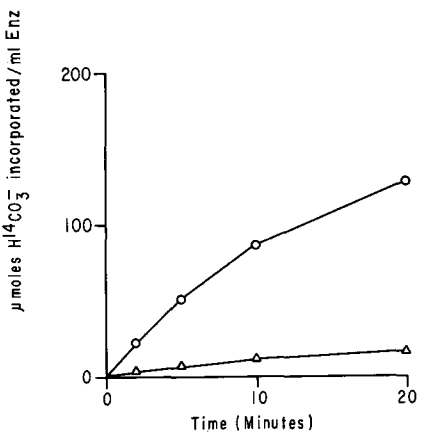

Fig. 2. The time course of carboxylation of propionyl-CoA in the presence of K^+ or Na^+. The carboxylase assay system described under EXPERIMENTAL PROCEDURES was used except that the incubation mixtures in a final vol. of 3.0 ml contained either 100 mM KCl (○—○) or 100 mM NaCl (△—△). Reactions were initiated with 1.56 μg of enzyme protein (specific activity 1150) incubated at 37°, and stopped with trichloroacetic acid at intervals of 2, 5, 10, and 20 min.

a 20-min incubation period (Fig. 2); when Na^+ was replaced by an equivalent concentration of K^+ the reaction was accelerated and proceeded at a linear rate in the initial 5 min and then declined. The enzyme also showed equal stability whether stored in the presence of Na^+, K^+, or NH_4^+ salts.

The effect of monovalent cations on partial reactions catalyzed by the enzyme

In previous studies on crystalline propionyl-CoA carboxylase from heart the following mechanism for the reaction was suggested[11]:

$$E\text{–biotin} + \text{ATP} + HCO_3^- \overset{Me^{2+}}{\rightleftharpoons} E\text{–biotin–}CO_2 + \text{ADP} + P_i \quad (1)$$

$$E\text{–biotin–}CO_2 + \text{propionyl-CoA} \rightleftharpoons \text{Methylmalonyl-CoA} + \text{E–biotin} \quad (2)$$

$$\text{Sum: Propionyl-CoA} + CO_2 + \text{ATP} \rightleftharpoons \text{Methylmalonyl-CoA} + \text{ADP} + P_i \quad (3)$$

In support of this mechanism it has been shown that the enzyme from heart and liver catalyzes a number of partial reactions, *i.e.*, exchange reactions between P_i and ATP[12], ADP with ATP[12,13], and transcarboxylations[4,14–17], *e.g.*,

$$\text{Methylmalonyl-CoA} + [^{14}C]\text{propionyl-CoA} \rightleftharpoons [^{14}C]\text{Methylmalonyl-CoA} + \text{propionyl-CoA} \quad (4)$$

$$\text{Methylmalonyl-CoA} + \text{butyryl-CoA} \rightleftharpoons \text{Ethylmalonyl-CoA} + \text{propionyl-CoA} \quad (5)$$

It became significant to know the effect of monovalent cations on the partial reactions, and whether they are required for these reactions.

Added K^+ enhanced markedly the exchange reactions of P_i with ATP and of ADP with ATP (Table III). Separate experiments, measuring the time course of the activity, showed that the effect of K^+ was not due to stabilization of the enzyme.

The effect of K^+ on the transcarboxylase activity of the enzyme was measured by determining the transfer of ^{14}C from ^{14}C-labeled methylmalonyl-CoA to butyryl-CoA to form ethylmalonyl-CoA (Eqn. 5)* and the transfer of label from propionyl-

* A. J. GIORGIO AND G. W. E. PLAUT, unpublished observations (1965).

TABLE III

THE EFFECT OF K^+ ON P_i–ATP AND ADP–ATP EXCHANGE REACTIONS

P_i–ATP exchange: The incubation mixtures contained 40 mM imidazole–HCl at pH 6.7, 0.3 mM sodium ATP, 1.5 mM sodium ADP, 2 mM $MnSO_4$, 1.5 mM sodium [^{32}P]phosphate, and 16 units of propionyl-CoA carboxylase (specific activity 1100) in a final vol. of 2.0 ml. The tubes were incubated at 25° and the reaction was stopped with trichloroacetic acid after 30 min. Organically bound ^{32}P was determined as described previously[2]. *ADP–ATP exchange:* The incubation mixtures contained 1.0 mM sodium ATP, 1.5 mM sodium [^{14}C]ADP (104 000 counts/min per μmole), 1.0 mM $MnSO_4$, 1.5 mM sodium phosphate, 40 mM imidazole–HCl at pH 6.7, and 20 units of propionyl-CoA carboxylase (specific activity 1000) in a final vol. of 0.5 ml. Incubation was done at 25° and the reaction was stopped after 30 min by immersing the tubes in an oil bath at 105° for 3 min. Nucleotides were separated by electrophoresis on EDTA-washed Whatman 3 MM paper in a 0.03 M sodium citrate–citric acid buffer (pH 3.1) by applying 4000 V for 25 min. The appropriate zones on the paper were cut out and radioactivity was determined in a scintillation counter as described under EXPERIMENTAL PROCEDURES.

Components added	*Incorporation of label into ATP (μmoles/mg of protein per h)*
$^{32}P_i$–ATP exchange	
None	0.03
100 mM KCl	1.68
100 mM KCl + 2 mM $MgCl_2$*	0.10
[^{14}C]ADP–ATP exchange	
None	0.28
100 mM KCl	1.96

* $MnCl_2$ was absent from the incubation mixture.

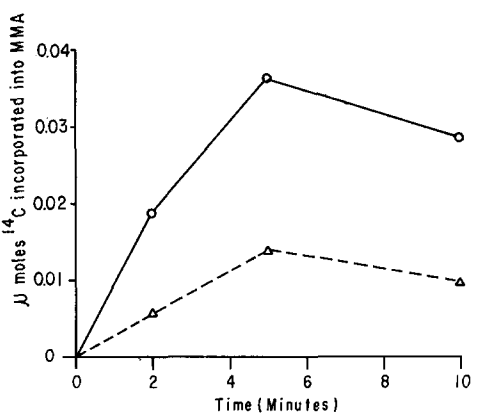

Fig. 3. The effect of K^+ on transcarboxylation between [2-^{14}C]propionyl-CoA and methylmalonyl-CoA (MMA). Incubation mixtures contained 100 mM Tris–HCl at pH 8.5, 0.55 mM [2-^{14}C]propionyl-CoA (460 000 counts/min per μmole), 1.4 mM racemic methylmalonyl-CoA, 2.8 mM reduced glutathione, and one unit of propionyl-CoA carboxylase (specific activity 1300) in a vol. of 0.9 ml. KCl (100 mM) was present (○—○) or absent (△- - -△) from the incubation media. Reactions were incubated at 37° and stopped with *p*-chloromercuribenzene sulfonate (final concn. 0.001 M) at intervals of 2, 5, and 10 min. Acyl-CoA derivatives were separated by thin-layer chromatography and counted as described under EXPERIMENTAL PROCEDURES.

[2-^{14}C]CoA to methylmalonyl-CoA (Eqn. 4). This partial reaction occurs without added divalent metal ions, but requires K^+ for maximal activity (Fig. 3).

The influence of K^+ on K_m and v_{max}

On closer examination it became apparent that K^+ affects maximal velocity of the propionyl-CoA carboxylase activity (Eqn. 3), but not the apparent affinity of the enzyme for substrates and Mg^{2+} (Figs. 4–6). In these experiments all components of the incubation mixture, except those varied separately, were used at or near saturating concentrations. The results were plotted by the method of DIXON[18]. With this procedure and at the concentrations used a linear relationship between the reciprocals of velocity and concentration was found. Comparison of maximal velocities in the absence and presence of 100 mM K^+ revealed 7–9-fold enhancement by K^+

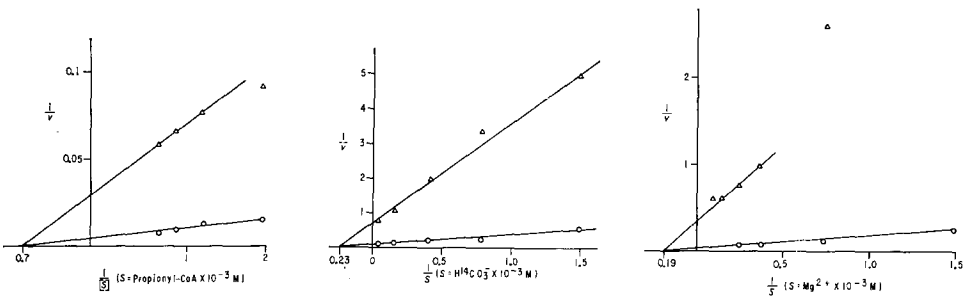

Fig. 4. K_m and v_{max} of propionyl-CoA in the presence and absence of K^+. The conditions of assay of carboxylase activity were as described under EXPERIMENTAL PROCEDURES except that KCl (100 mM) was either absent (△—△) or present (○—○) and propionyl-CoA was added at the levels indicated. The specific radioactivity of $NaH^{14}CO_3$ was 84 000 counts/min per μmole and 0.047 unit of enzyme (specific activity 850) was present in a vol. of 0.75 ml. v is expressed as mμmoles ^{14}C incorporated into methylmalonyl-CoA per h.

Fig. 5. K_m and v_{max} of HCO_3^- in the presence and absence of K^+. The conditions of assay of carboxylase activity were as described under EXPERIMENTAL PROCEDURES except that KCl (100 mM) was either absent (△—△) or present (○—○). $NaH^{14}CO_3$ (65 000 counts/min per μmole) was added at the levels indicated and 0.7 unit of enzyme (specific activity 900) was present in a vol. of 0.75 ml. v is expressed as units per ml of reaction mixture.

Fig. 6. K_m and v_{max} of Mg^{2+} in the presence and absence of K^+. The conditions of assay of carboxylase activity were as described under EXPERIMENTAL PROCEDURES except that KCl (100 mM) was either absent (△—△) or present (○—○) and that the concentrations of $MgCl_2$ varied as indicated. Each incubation mixture contained 0.65 unit of enzyme (specific activity 800) per 0.75 ml. v is expressed as units per ml of reaction mixture.

when the concentrations of propionyl-CoA (Fig. 4), bicarbonate (Fig. 5), and Mg^{2+} (Fig. 6) were varied. However, the value of K_m for each of the substrates tested was the same regardless of the absence or presence of K^+. With propionyl-CoA, bicarbonate, and Mg^{2+} they were $1.5 \cdot 10^{-3}$ M, $4 \cdot 10^{-3}$ M, and $5 \cdot 10^{-3}$ M, respectively, when calculated from the results shown in Figs. 4–6.

Comparison of properties of propionyl-CoA carboxylase and the 'exchange enzyme'

Previous studies in this laboratory led to the purification of an activity ('ex-

change enzyme') from porcine-liver mitochondria capable of catalyzing the exchange of $^{32}P_i$ with ATP and of ADP (labeled with ^{32}P in the terminal phosphate group) with ATP[3]. The P_i–ATP exchange required the presence of ADP whereas P_i was required for the exchange reaction between ADP and ATP. Subsequent experiments with purified 'exchange enzyme' showed that the best preparations still catalyzed the carboxylation of propionyl-CoA, and that the exchange reaction of P_i with ATP and of ADP with ATP could be inhibited by avidin*; this inhibition could be prevented by appropriate quantities of biotin when present with the enzyme before adding the egg protein. Avidin inhibits the carboxylase activity of the present preparation of highly purified propionyl-CoA carboxylase from mitochondria of bovine liver and, as can be seen in Table IV, the exchange reactions of P_i with ATP and of ADP with ATP.

The exchange enzyme[3] and all of the activities of various preparations of propionyl-CoA carboxylase[4,15] are inhibited by low concentrations of *p*-mercuribenzoate or *p*-chloromercuriphenyl sulfonate.

TABLE IV

INHIBITION BY AVIDIN OF EXCHANGE REACTIONS CATALYZED BY PROPIONYL-CoA CARBOXYLASE

P_i–ATP exchange: Propionyl-CoA carboxylase (10.8 μg of protein, specific activity 1100) and varying quantities of avidin were incubated in 0.87 ml of solution for 10 min at 0°. Each of these solutions was then diluted to a final vol. of 2.0 ml, the reaction mixture yielding the final composition: 1.5 mM ADP, 0.3 mM ATP, 1.5 mM potassium [^{32}P]phosphate ($6.5 \cdot 10^4$ counts/min per μmole), 2 mM $MnSO_4$, 50 mM KCl, 1 mM $NaHCO_3$, and 40 mM imidazole–HCl at pH 6.7. The mixtures were incubated at 25° and the reactions were terminated after 30 min with trichloroacetic acid. Organically bound ^{32}P was determined as described previously[2]. *ADP–ATP exchange:* Enzyme protein (5.4 μg, specific activity 1500) was added to each of three Tris-buffered solutions (0.06 M, pH 7.3), one containing buffer alone, another 10.8 μg of avidin, and a third 10.8 μg of avidin and 2 μg of (+)-biotin, all in a final vol. of 0.1 ml. After incubation for 30 min at 23° 0.03 ml of each of the treated enzyme solutions was added to reaction mixtures containing 1.5 mM ^{32}P-labeled ADP, 1.0 mM ATP, 1.5 mM potassium phosphate, 2 mM $MnSO_4$, 100 mM KCl, and 40 mM imidazole–HCl at pH 6.7, all in a final vol. of 0.10 ml. The solutions were incubated at 25° and the reactions were terminated after 30 min by immersing the tubes in an oil bath at 90° for 3 min. Nucleotides were separated by electrophoresis on EDTA-washed Whatman 3 MM paper in 0.03 M sodium citrate–citric acid buffer (pH 3.1), applying 3500 V for 30 min. The radioactive zones were located by radioautography, cut out, and counted in a low-background gas-flow counter.

Additions (μg protein/ml reaction mixture)		*Exchange reaction (μmoles ^{32}P incorporated into ATP/mg protein per h)*
Avidin	*Enzyme*	
P_i–ATP exchange		
None	5.4	3.7
25	5.4	2.4
50	5.4	1.0
150	5.4	0.0
ADP–ATP exchange		
None	16.2	7.5
32	16.2	2.2
32 *plus* 6 μg biotin	16.2	8.5

* M. CHIGA, T. AOGAICHI AND G. W. E. PLAUT, unpublished observations (1963).

The similarity of these properties suggested that propionyl-CoA carboxylase and the exchange enzyme represent activities which belong to the same protein. However, these activities seemed to differ in two important respects. Thus, the exchange enzyme exhibits a sharp pH optimum at pH 6.7 and is inactive above pH 8 (ref. 2) while the optimum of propionyl-CoA carboxylase from heart is at pH 8.0–8.5 (ref. 19) and that from bovine liver is at pH 8.0–8.3 (ref. 20). Exchange reactions between P_i and ATP and of ADP with ATP have been carried out with such propionyl-CoA carboxylase preparations at pH values ranging from pH 6.2 to 8.5 (refs. 12, 13, 15)

Furthermore, Mg^{2+} and Mn^{2+} can lead to maximal activation of the propionyl-CoA carboxylase activity of the enzyme from heart and liver, and Mg^{2+} has been used as the activator in studies on the exchange reactions between P_i and ATP, and ADP with ATP catalyzed by these propionyl carboxylase preparations[12,13,15]. In contrast, Mn^{2+} was the activator of choice for reactions catalyzed by the exchange

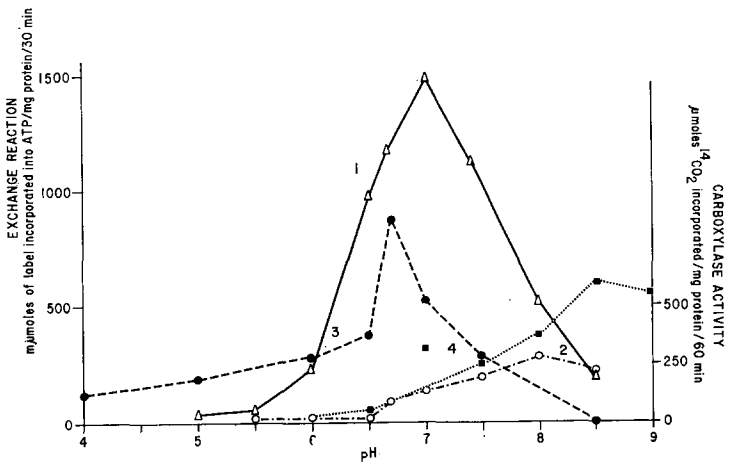

Fig. 7. The effect of pH on the carboxylase activity and the ADP–ATP and P_i–ATP exchange reactions of propionyl-CoA carboxylase. Buffers (exchange reactions): Sodium acetate–acetic acid (40 mM) at pH 4.0–5.0; imidazole–HCl (40 mM) at pH 6.0–7.0; Tris–HCl (40 mM) at pH 7.5–8.5. (1) P_i–ATP exchange: The reaction mixtures contained 1.5 mM potassium [^{32}P]phosphate (320 000 counts/min per μmole), 1 mM $MnSO_4$, 1.5 mM sodium ADP, 1.0 mM sodium ATP, and 6 units of propionyl-CoA carboxylase (specific activity 595) in a vol. of 2.0 ml. The reactions were brought to the pH indicated with the buffers described above and incubated for 30 min at 25°. Bound ^{32}P was determined as described previously[2]. (2) P_i–ATP exchange: The conditions of incubation were identical with those described for Curve 1 except that $MnSO_4$ was replaced by 6.7 mM $MgCl_2$. (3) ADP–ATP exchange: The reaction mixtures contained 1.5 mM sodium [^{14}C]-ADP (104 000 counts/min per μmole), 1 mM sodium ATP, 1.5 mM sodium phosphate, 2 mM $MnSO_4$, 100 mM KCl, and 0.45 unit of propionyl-CoA carboxylase (specific activity 1150) in a final vol. of 0.10 ml. The solutions were brought to the pH indicated and incubated at 25°. The reactions were terminated after 30 min by immersing the tubes in an oil bath at 105° for 3 min. Nucleotides were separated by electrophoresis on EDTA-washed Whatman 3 MM paper in 20% acetic acid by exposure to 4000 V for 25 min. The appropriate zones on the paper were cut out and radioactivity was determined in a scintillation counter as described under EXPERIMENTAL PROCEDURES. (4) Carboxylase activity: The conditions of incubation were as described under EXPERIMENTAL PROCEDURES except that 115 mM $(NH_4)_2SO_4$ replaced KCl and the following buffers were used to adjust the solutions to the acidity indicated: 46 mM imidazole–HCl for the range pH 6.5–7.0 and Tris–HCl for the range pH 7.5–9.0. Each reaction mixture in a final vol. of 1.3 ml contained 1.1 units of enzyme (specific activity 595) and was incubated for 20 min at 37°.

enzyme; Mg^{2+} had some activity at high concentrations, but did not give the same maximal activity as Mn^{2+} (ref. 3).

The carboxylase activity of the present propionyl-CoA carboxylase preparation from bovine liver could be activated equally well by Mg^{2+} or Mn^{2+}, although lower concentrations of Mn^{2+} than Mg^{2+} were needed to reach maximal activity. The optimal carboxylase activity was found to occur at around pH 8.5, in accord with values reported for other propionyl-CoA carboxylase preparations[19,20]. However, the optimal conditions for the exchange reactions with the present preparation differed markedly from those of its carboxylase activity. The optimal values in the presence of Mn^{2+} for the exchange reaction between P_i and ATP and of ATP with ADP were reached at pH 7.0 and pH 6.8, respectively (Fig. 7). At pH 8.5, which is optimal for the carboxylation reaction, there was essentially no ADP–ATP exchange (Fig. 7) and the P_i–ATP reaction proceeded at 14% of that obtained at pH 7.0. The P_i–ATP exchange activity in the presence of Mg^{2+} between pH 5.0 and pH 8.4 was markedly lower than that obtained under optimal conditions with Mn^{2+} (Fig. 7). The pH profiles and requirements for divalent metal ions of these exchange reactions with propionyl-CoA carboxylase from bovine liver are thus remarkably similar to those obtained with the exchange enzyme from porcine liver[2,3].

DISCUSSION

The requirement for K^+ or other suitable univalent ions for maximal propionyl-CoA carboxylase activity for the enzyme from bovine liver is in agreement qualitatively with studies of the enzyme from porcine heart[1]. NEUJAHR[1] obtained a stimulation by K^+ of around 2-fold of carboxylase activity with the crystalline enzyme from cardiac muscle while a more pronounced enhancement by K^+ (7–9-fold) was found with the present preparation from bovine liver. It is not known whether the liver enzyme has an absolute requirement for a monovalent cation for activity. It is possible that the lower rates obtained in the absence of added K^+ or NH_4^+ are attributable to the presence of small amounts of Na^+, since NaOH was used to neutralize certain reagents (*e.g.*, ATP and ADP) introduced into the reaction mixture. However, if Na^+ had the ability to activate at a low rate and had substantial affinity for the enzyme one could have expected a severe inhibition by increasing concentrations of sodium salt in the presence of constant amounts of K^+; inhibition occurred but at rather high concentrations of Na^+ (Fig. 1). Whenever the requirements for univalent cation were examined the enzyme preparations were dialyzed extensively against Tris buffer before use; nevertheless, enough protein-bound K^+ or some other activating cation could have been retained to account for the low level of activity observed; the contents of K^+ of the dialyzed preparations was not determined.

The results show that K^+ is needed not only for maximal activity of the overall reaction (Eqn. 3), but also for partial reactions which are a part of the mechanism of the carboxylase, *i.e.*, exchange reactions between P_i and ATP, ADP with ATP (Table III) and transcarboxylations (Fig. 3). The requirement for an activating univalent cation for all of these activities contrasts to the requirement for divalent metal ions which are necessary for the carboxylase activity and the exchange reactions with ATP, but are not for the transcarboxylation reaction (Eqns. 4 and 5) catalyzed by the enzyme from porcine heart[14] or bovine liver (Fig. 3).

The action of univalent cations on propionyl-CoA carboxylase is reminiscent of their effect on the activity of pyruvate kinase. The same cations, *i.e.*, NH_4^+, K^+, and Rb^+, are active with both enzymes[21] (Table II). Furthermore, KACHMAR AND BOYER[21] have shown that the K_m for the substrate phosphoenolpyruvate is essentially unchanged in the presence of varying concentrations of K^+. It has been observed with the present enzyme that while maximal velocity is enhanced in the presence of K^+, the values of K_m for propionyl-CoA, bicarbonate, or Mg^{2+} are unchanged regardless of whether or not K^+ has been added (Figs. 4–6).

The properties of the P_i–ATP and ADP–ATP exchange activities of propionyl-CoA carboxylase from bovine liver are essentially in agreement with those reported by KAZIRO *et al.*[12,15] for the crystalline enzyme from porcine heart, although they seem to differ in certain details*. However, there are some substantial differences between bovine liver propionyl-CoA carboxylase as studied here and the published observations of LANE and co-workers with enzyme from the same source. Thus, HALENZ AND LANE[22] failed to detect a P_i–ATP exchange and LANE *et al.*[13] did not obtain inhibition by avidin of the ADP–ATP exchange. Avidin sensitive exchange reactions of P_i with ATP and of ADP with ATP were found here (Table IV). In the light of the present results, it would appear that the conditions of incubation used by these workers (pH 8.5 and Mg^{2+} as activator) were favorable for demonstration of the propionyl-CoA carboxylase activity but unfavorable for study of the P_i–ATP and ADP–ATP exchange reactions with the liver enzyme (Fig. 7 and Table III). The avidin insensitive ADP–ATP exchange reported[13] was probably not an expression of the activity of propionyl-CoA carboxylase, but possibly due to the presence in the earlier enzyme preparations of nucleoside diphosphokinase which can catalyze an ADP–ATP exchange[3,23]. Nucleoside diphosphokinase and ATP–AMP transphosphorylase[24] are absent from the present preparation.

The difference in optimal conditions for the carboxylase activity (pH 8–8.5, activation by Mg^{2+} or Mn^{2+}) and the P_i–ATP and ADP–ATP exchange reactions (pH 6.8–7.0, activation by Mn^{2+}, but only weakly by Mg^{2+}) presents an enigma since the exchanges reflect partial reactions (Eqn. 1) of the overall carboxylase activity (Eqn. 3). The qualitative differences in requirement of divalent metal ions for various activities of propionyl-CoA carboxylase bear some resemblance to properties of pyruvate kinase as studied with certain substrates. Thus, it was found by KUPIECKI AND COON[25] that a crystalline enzyme preparation from rabbit muscle exhibits pyruvate kinase and ATP–fluoride transphosphorylase activities in the presence of Mg^{2+} but not of Zn^{2+}, whereas the phosphorylation of hydroxylamine by ATP is activated by Zn^{2+} but not by Mg^{2+}. A number of the components of the medium for assay of exchange reactions of propionyl-CoA carboxylase were therefore varied to determine their effect on activation by Mg^{2+}. Replacement of K^+ by NH_4^+ or Na^+, change of pH (Fig. 7), or variations of the bicarbonate content of the medium** did not produce

* A requirement for Mg^{2+} for the exchange reactions catalyzed by the enzyme from porcine heart has been demonstrated[12], however, a comparison of the relative effectiveness of Mg^{2+} and Mn^{2+} as activators has not been reported. Furthermore, the exchange reactions were examined at pH's 6.2 and 7.5 (ref. 12), but apparently not at pH 8–8.5 which is optimal for the carboxylase activity[19]. In the absence of this information a detailed comparison of the properties of the enzyme from heart and liver is not possible.

** The P_i–ATP and ADP–ATP exchange reactions of propionyl-CoA carboxylase from liver are dependent on the presence of bicarbonate. This is in agreement with the properties of the enzyme from heart[12].

a change in the relative effects as activators of Mg^{2+} and Mn^{2+}*. Preliminary experiments indicated an increased effectiveness of Mg^{2+} activation of P_i–ATP exchange in the presence of acetyl-CoA; however, it is not certain whether this effect is due to a change in properties of the protein by the acyl-CoA, *e.g.*, by enhancing its reactivity with Mg^{2+}, or whether it is merely due to the reversal of the overall reaction which can occur when the acyl-CoA derivative is also present*,**.

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** The carboxylation of acetyl-CoA by the enzyme from bovine liver has been reported by HALENZ *et al.*[4] to proceed at 1% the rate of that of propionyl-CoA; however, the carboxylation of acetyl-CoA and its reversal cannot be neglected when compared to the P_i–ATP exchange. It can be calculated from the data in Table III that under optimal conditions the velocity of the exchange reaction is only 0.2% of that of the carboxylation of propionyl-CoA.