STUDIES ON THE MECHANISM OF ACTION OF PROPIONYL-CoA CARBOXYLASE

by

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III. INTRODUCTION AND LITERATURE REVIEW

The carboxylation of propionyl-CoA by bovine liver mitochondrial propionyl-CoA carboxylase[1-5] or by pig heart propionyl-CoA carboxylase[6-8] has been demonstrated to involve at least the two partial reactions shown below:

(1) Enzyme + HCO_3^- + ATP^{4-} $\overset{Mg^{2+}}{\rightleftharpoons}$ Enzyme-CO_2^- + ADP^{3-} + Pi^{2-} + H^+

(2) Enzyme-CO_2^- + CH_3-CH_2-CO-SCoA $\rightleftharpoons$ Enzyme + CH_3-$CH(COO^-)$-CO-SCoA

The first reaction has been studied extensively with pig heart propionyl carboxylase[7,8], β-methylcrotonyl-CoA carboxylase[9-12], bovine liver propionyl-CoA carboxylase[1-5,13], and acetyl-CoA carboxylase[14,15]. Kaziro *et al*[16] have shown through O^{18} studies that the oxygen for ATP cleavage is derived from bicarbonate, and that the remaining two oxygen atoms of HCO_3^{18-} appear in the free carboxyl group of methylmalonyl-CoA. These findings prove that HCO_3^-, and not CO_2, is the reactive species in reaction 1 above.

These same workers have confirmed earlier observations[4,17] of a bicarbonate-and ADP-dependent Pi-ATP exchange catalyzed by the enzyme, have shown ADP to be a competitive inhibitor with respect to ATP of the overall forward reaction, and have proposed a concerted mechanism for the formation of enzyme-CO_2. Enzyme-CO_2 has been isolated[8], its stability properties investigated, and it has been shown to transfer its CO_2 quantitatively to the acyl-CoA acceptor in the absence of ADP and Pi. The CO_2 content of enzyme-CO_2 has been shown to be exactly

equivalent to the biotin content of the enzyme. Considerable attention has recently been given to clarifying the nature of the enzyme-CO_2 complex.

Waite and Wakil[14,15], using acetyl-CoA carboxylase, have presented evidence which led them to postulate that the ureido carbon of biotin is itself the active carbon of enzyme-CO_2. Work done in other laboratories[18,19,20] has failed to support this contention, and has instead implicated 1'N-(carboxy-(+)-biotinyl)-lysyl-enzyme as the active enzyme-CO_2. Kosow and Lane[21], Wood, Lochmuller, and Lynen[18], and Lane and Lynen[20] have shown by enzymatic degradation of various carboxylases that biotin and biotin-CO_2 are covalently bound to the enzymes through the ε-amino group of a lysyl residue of the enzyme.

The second of these two partial reactions was first investigated by Lynen et al[9], who found that β-methylcrotonyl-CoA carboxylase catalyzes an exchange reaction between C^{14}-β-methylcrotonyl-CoA and β-methylglutaconyl-CoA. Halenz and Lane[4] and Friedman and Stern[17] have demonstrated a similar exchange reaction between C^{14}-propionyl-CoA and methylmalonyl-CoA catalyzed by propionyl-CoA carboxylase. Magnesium ion, orthophosphate and adenine nucleotides are unnecessary for this exchange. Propionyl-CoA carboxylase also catalyzes[3,5] reversible net transcarboxylation between ethylmalonyl-CoA and propionyl-CoA to form methylmalonyl-CoA and butyryl-CoA. This reaction does not require adenine nucleotides and is avidin sensitive. Hatch and Stumpf[22] have reported similar transcarboxylations catalyzed by wheat germ acetyl-CoA carboxylase. These transcarboxylations are apparently a result of the reversible operation

of reaction 2. Jaenicke and Lynen[23] have proposed a concerted mechanism for carboxylations at nucleophillic centers involving a resonance stabilized carbanion. Lynen[24] has shown that both the thioethanolamine structure and the acylated nitrogen are absolutely necessary for the activity of a CoA model such as pantetheine.

Halenz et al[25] have reported some of the properties of bovine liver mitochondrial propionyl-CoA carboxylase which support the above mechanism. They found the enzyme to be inhibited by sulfhydryl reagents such as chloromercuribenzoate, iodoacetamide, and N-ethylmaleimide. Coenzyme A and valeryl-CoA, but not propionyl-pantetheine, were found to be competitive inhibitors of the enzyme. They also reported[1,25] that bovine liver propionyl-CoA carboxylase catalyzes the α-carboxylation of butyryl-CoA, acetyl-CoA, valeryl-CoA and isobutyryl-CoA at rates greatly reduced from that found for propionyl-CoA. The enzyme was not active[25] toward phenyl-substituted acyl-CoA derivatives.

In this paper, some investigations bearing on the nature of the enzyme-propionyl-CoA association and the mechanism of the transfer of CO_2 from enzyme-biotin-CO_2 to propionyl-CoA will be reported.

IV. MATERIALS AND METHODS

Propionyl-CoA carboxylase was prepared by an extension of the method described by Halenz et al[25]. The precipitate from the "second ammonium sulfate fractionation" was redissolved in 6.0 ml of 0.005 M phosphate buffer at pH 7.0 containing 60 mg of neutralized GSH and 5 ml of 0.1 M EDTA, pH 7.0, per liter. The redissolved precipitate was dialyzed for 4 hours against one liter of this same buffer. The dialyzed enzyme was applied to a 2.5 x 5 cm column of hydroxyapatite previously equilibrated with 0.005 M phosphate, pH 7.0 containing 0.2 micromoles of β-mercaptoethanol per ml. The column was eluted at a flow rate of 0.33 ml per minute with a phosphate gradient formed by allowing 300 ml of 0.25 M potassium phosphate to flow into a closed mixing vessel containing 100 ml of 0.005 M phosphate. Both buffers were pH 7.0 and contained 0.2 micromoles of β-mercaptoethanol per ml. Aliquots from each tube were assayed for propionyl-CoA carboxylase activity, and all active fractions were pooled. Figure I shows the ultraviolet transmittance (254 mμ) of the column effluent in relation to enzyme activity. The enzyme was recovered from the pooled hydroxyapatite fractions (to which 70 mg of neutralized GSH had been added) by dialyzing in ammonium sulfate, pH 7.5, to achieve 57% saturation at equilibrium. The precipitated hydroxyapatite-purified enzyme was then redissolved in 2.0 ml of 0.1 M phosphate, pH 7.0, and

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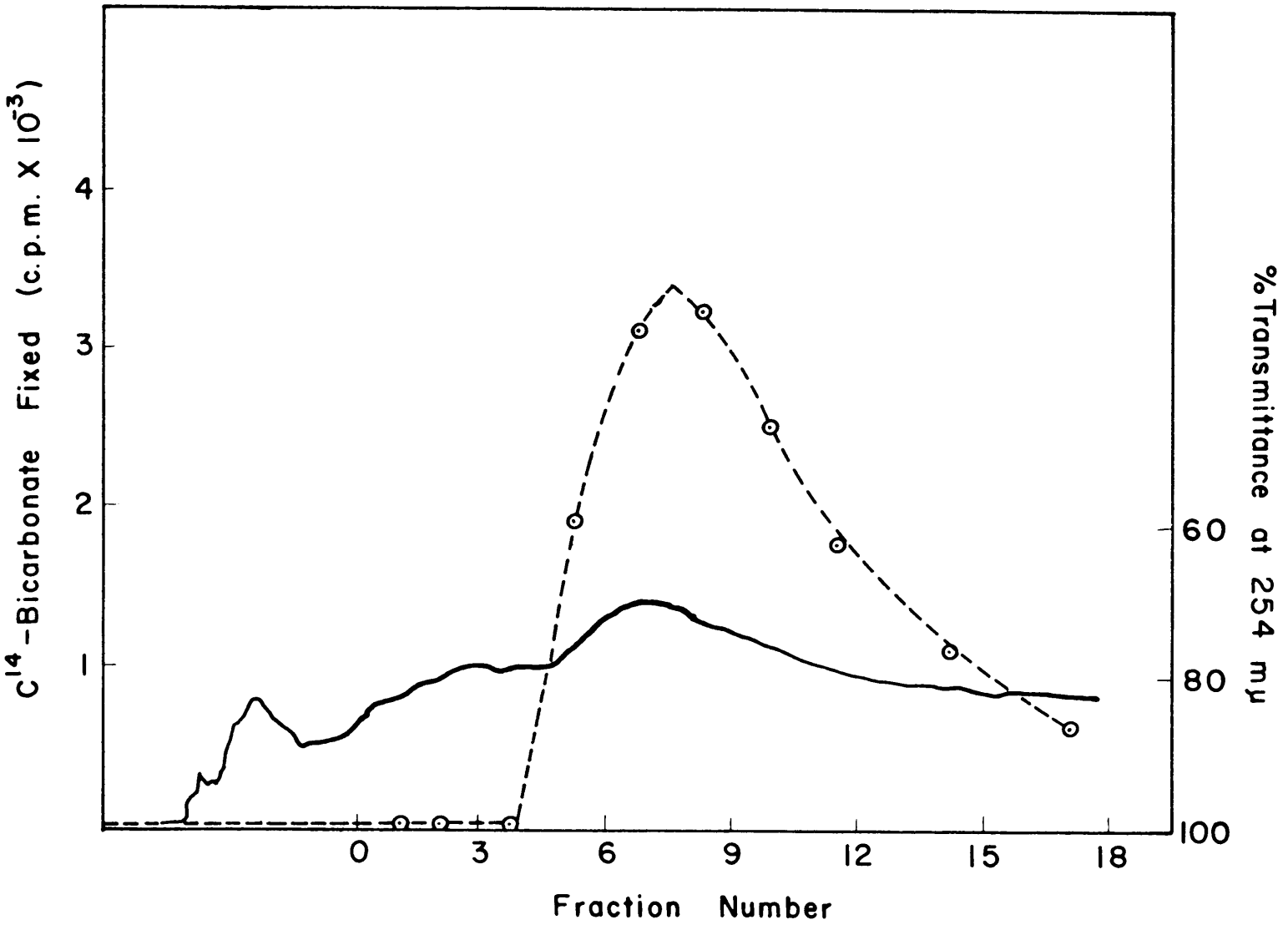
Abbreviations: PCMB, chloromercuribenzoate; TNBS, 2,4,6,-trinitrobenzenesulfonic acid; DPCoA, 3'-dephospho-Coenzyme A; pr-, propionyl-; -pant, -pantetheine; PEP, phosphoenolpyruvic acid.

FIGURE I

HYDROXYAPATITE CHROMATOGRAPHIC PURIFICATION OF MITOCHONDRIAL PROPIONYL-CoA CARBOXYLASE

Enzyme activity of the fractions (dotted line) is plotted as c.p.m. propionyl-CoA-dependent C^{14}-bicarbonate fixation per 0.005 ml aliquot per hour.

Protein content of the fractions (solid line) is plotted as % transmittance at 254 mμ.


C14-Bicarbonate Fixed (c.p.m. X 10-3)
4
3
2
1
0
3
6
9
12
15
18
Fraction Number
% Transmittance at 254 mμ
60
80
100

applied to a 2.5 x 25 cm column of Sephadex-G-200 previously equilibrated with the same buffer containing 0.2 micromoles of β-mercaptoethanol per ml. Elution was carried out with this buffer at a flow rate of 0.3 ml per minute. The ultraviolet transmittance pattern showed only slight resolution had been achieved, therefore all active fractions were pooled and precipitated by dialysis to 56% ammonium sulfate saturation as described above. The precipitate was resuspended in 5.0 ml of 60% saturated ammonium sulfate, pH 7.5, 0.005 M with respect to GSH. This precipitate showed a specific activity of 27 units per mg of protein (a 314-fold purification when compared to the aged acetone powder extract) and represented 4% of the units present in this extract. Enzyme prepared in this manner is more than 90% pure as judged by ultracentrifugal analysis. Preparations used in the following experiments varied in specific activity between 18.7 and 16.4 units per mg of protein, one unit being defined as that quantity of enzyme required to catalyze the propionyl-CoA-dependent fixation of 1 micromole of HCO_3^- per minute under specified conditions[1]. Except as otherwise noted, assays reported in this paper were conducted using 1.16×10^{-3} - 1.98×10^{-3} units of carboxylase in each assay tube. The standard assay mixture contained (in micromoles): GSH, 2.5; ATP, 2; $MgCl_2$, 2; propionyl-CoA, 0.5; tris, pH 8.5, 50; $KHC^{14}O_3$, 10 in a final volume of 0.765 ml. Adenine, ATP, reduced coenzyme A, 5'-AMP, adenosine, cytosine, hypoxanthine, xanthine, CTP, GTP, UTP, ATP, and ITP were obtained from Pabst Laboratories as the sodium salts. Aniline was redistilled, and fractions boiling between 180° and 185° were used. Reduced glutathione, D-pantethine, type II pyruvic kinase, type I lactic dehydrogenase, type

III hexokinase, DPNH, PCMB and phosphoenolpyruvate (trisodium salt) were obtained from Sigma Chemical Company. Adenosine-3'-phosphate and adenosine-2'-phosphate were obtained from Schwarz Bioresearch Inc. Pantetheine was prepared by the method of Wittle et al[26], and N-acetyl-cysteamine was prepared as described by Kuhn and Quadbeck[27]. Uracil was obtained from California Corporation for Biochemical Research. Potassium bicarbonate-C^{14}, and tritiated water were obtained from New England Nuclear Corp. Sodium propionate-1-C^{14} was obtained from Research Specialties Co. Trinitrobenzenesulfonic acid was prepared by Dr. Tanaka of the Tokyo Metropolitan University. Propionyl-1-C^{14}-CoA was prepared as previously reported[28]. Propionyl-thioesters were prepared by the method of Simon and Shemin[29], and assayed by the hydroxamate method[30]. The absorbancy at 540 mμ (1 cm) was divided[31] by 0.35 in the case of methylmalonyl monohydroxamate, and by 0.855 in the case of propionyl hydroxamate to determine the concentration in micromoles per ml. Methyl-malonyl-CoA was prepared by carboxylation of propionyl-CoA using large amounts of propionyl-CoA carboxylase.

Propionyl-dephospho-CoA was prepared by incubating 80 micromoles of propionyl-CoA with 130 units* of 3'nucleotidase (prepared through the first calcium phosphate adsorption step of the method of Shuster and Kaplan[32]) together with 40 micromoles of tris, pH 7.2, and 3.2 micromoles of $CaCl_2$

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*One unit, as defined by Shuster and Kaplan, causes the release of one micromole of inorganic phosphate per hour under the conditions of assay.

in a volume of 10.6 ml for one hour at 37°. The reaction was terminated by the addition of 0.5 ml of 2 N isobutyric acid. The reaction mixture was lyophyllized, redissolved in 3 ml of water and streaked across a 24 cm wide sheet of Whatman 3MM paper. The chromatograms were developed descending with the isobutyric acid-ammonia solvent system. The propionyl-dephospho-CoA band was eluted with water and assayed by the hydroxamate method.

Thioesters, CoA, and DPCoA were separated from reaction mixtures by descending chromatography for 15 hours on Whatman 3MM paper using the isobutyric acid-concentrated ammonia (28%)-water (66:1:33) system[33]. These compounds were located by ultraviolet absorbance and by spraying strips with 2 M neutralized hydroxylamine hydrochloride followed by 1.67% $FeCl_3$ in 3 N HCl. The free acid carboxylation products of propionyl-pantetheine and propionyl-DPCoA were identified by methods previously reported[2]. Rf's observed in the isobutyric acid-ammonia system were: CoA, 0.58; propionyl-CoA, 0.70; propionyl-DPCoA, 0.83; methylmalonyl-CoA, 0.40; methylmalonyl-DPCoA, 0.52; and methylmalonyl-pantetheine, 0.69.

Radioactivity of liquid samples was determined by liquid scintillation counting, while solid samples were counted using an end-window Geiger Müeller counter. All protein determinations were done spectrophotometrically[34] with appropriate controls to correct for the absorption by GSH at 260 mμ. Column eluates were monitored for ultraviolet absorbance with an LKB 4701 A Recording Ultraviolet Absorptiometer using a filter having maximum transmittance at 254 mμ.

V. EXPERIMENTAL

Introduction

The electrophillic attack by enzyme-CO_2 on the α-carbon of an acyl-CoA may proceed by either an SE_2 or an SE_1 type mechanism. That is, the CO_2 may add in a concerted manner with simultaneous expulsion of a proton; or proton abstraction may precede CO_2 addition leaving a strongly nucleophillic α-carbon which is subsequently attacked by enzyme CO_2. Since propionyl carboxylase has been shown[35] to produce only one enantiomorph of methylmalonyl-CoA, the addition of CO_2 to propionyl-CoA must proceed with either retention or inversion of the configuration about the α-carbon, and the enzyme must exert some control over which of the two chemically equivalent hydrogens will be replaced. In the case of an SE_2 mechanism, these limitations imply a close and specific spatial relationship between enzyme and the α-carbon such that attack by enzyme-CO_2 can occur from only one direction. In the case of an SE_1 mechanism, the carbanion formed would be readily stabilized by resonance and would adopt a planar configuration leading to a racemic product unless proximity to the enzyme surface permits addition from only one face. In the absence of enzyme-CO_2, tritium or deuterium exchange with the α-hydrogen would occur. An unstabilized carbanion must be asymmetrically solvated by enzyme or, under the influence of enzyme, by water. In these cases too, the α-carbon must be closely associated with enzyme in order to retain the stereospecificity of the reaction.

If an unstabilized carbanion intermediate asymmetrically solvated by water were to exist on the enzyme surface; due to hinderance by the enzyme bulk, CO_2 addition would have to take place with retention of configuration. In the absence of enzyme-CO_2, tritium or deuterium would exchange with the α-hydrogen. If solvation of the carbanion were by enzyme itself, no tritium or deuterium exchange would be expected, and the reaction would proceed with inversion.

T_2O Exchange With Propionyl-CoA

The possibility of a stabilized carbanion intermediate, or of an asymmetric water solvated carbanion intermediate, was tested. Propionyl-CoA was incubated in the presence (A) and absence, (B) of enzyme and in the presence, (C) of avidin-treated enzyme, in reaction mixtures enriched with T_2O to permit hydrogen exchange between T_2O and the α-carbon to be detected. In addition, enzymatically-synthesized methylmalonyl-CoA was enzymatically decarboxylated (D) in T_2O to check the stereospecificity of the reaction, as well as to give some estimate of the magnitude of the isotope effect involved. The tritiated propionyl-CoA samples isolated from these incubations were then analyzed in three ways. First, aliquots were counted to determine total incorporated tritium. Second, aliquots were converted to hydroxamates which were isolated and counted as one measure of tritium incorporated into the propionyl moiety. Third, aliquots were incubated with or without enzyme in a complete reaction mixture, and the tritium released to the water of the incubation mixture was determined as a measure of the enzymatic exchangeability of the tritium incorporated in the first incubation. The details of the procedures summarized above

are as follows: The basic reaction mixture for tritiation contained, in micromoles; tris, pH 8.5, 100; GSH, 5; and $MgCl_2$, 10. Thirty micromoles of glucose and 2 mg* of hexokinase were added to assure the absence of ATP and to force the reverse reaction in tube D to completion. Tubes A, B, and C received 6.3 micromoles of propionyl-CoA, and tube D received 11.7 micromoles of enzymatically-synthesized methylmalonyl-CoA. Ten micromoles of ADP and of orthophosphate were added to tube D to permit the reverse reaction. Tubes A, B, and D received 9.5 units of propionyl carboxylase, while tube C received 9.5 units of the enzyme which had been treated with 0.675 mg of avidin (sufficient to bind 1.7 mγ of biotin). All components except the enzymes were lyophyllized and redissolved in 0.2 ml of tritiated water, and enzymes were added to a total volume of 0.4 ml T_2O (final specific activity 1.22×10^6 d.p.m. per microatom of hydrogen). The reaction mixtures were incubated for one hour at 37°, and the reactions were terminated by addition 0.2 ml of 2 N isobutyric acid. The four acidified reaction mixtures were streaked across 14 cm wide sheets of Whatman 3MM paper and chromatographed descending using the isobutyric acid-ammonia solvent system. The propionyl-CoA (Rf, 0.70) was recovered from each sheet by elution with water. Separate aliquots of each eluted sample were counted, and assayed by the hydroxamate method. Aliquots of each sample were retained for carboxylation, and the remaining volumes of each were converted to propionyl hydroxamate by reaction with 200 micromoles of neutralized hydroxylamine for 20 minutes at room

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*Sufficient to convert 6 micromoles per minute.

temperature, streaked across 4 cm wide strips of Whatman 3MM, and chromatographed for 20 hours ascending in isoamyl alcohol saturated with 4 N formic acid. The propionyl hydroxamates (Rf 0.82) were located by spraying side strips with the ferric chloride reagent, eluted with water, and separate aliquots of each sample were counted, and assayed colorimetrically after addition of the ferric chloride reagent. Radioactivity remaining in the isolated hydroxamates is presumed to have been incorporated at the α-carbon of propionyl-CoA. The tritiated propionyl-CoA samples which had been retained were incubated with and without 0.32 units of carboxylase in the presence of 0.1 mg of pyruvic kinase*, 0.1 mg of lactic dehydrogenase** (to force the forward reaction to completion), and the following components, in micromoles: tris, pH 8.5, 120; ATP, 4; PEP, 4; DPNH, 3; $MgCl_2$, 4; GSH, 2.5; and $KHCO_3$, 10, in a total volume of 1.4 ml. The incubation was continued for 20 minutes at 37°, and terminated by the addition of 400 micromoles of NaOH. The samples were hydrolyzed at room temperature for 30 minutes to allow the formation of the less volatile sodium salts in place of the thioesters.

The water of the reaction mixture was in each case recovered by lyophyllization, and aliquots were counted. Tritium recovered as water from tritiated propionyl-CoA samples incubated without carboxylase originated from non-enzymatically exchangeable hydrogen in the substrate molecule. The difference in tritium recovery between enzyme-treated and untreated

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* Sufficient to convert 5 micromoles per minute.
** Sufficient to convert 4 micromoles per minute.

samples is the non-exchangeable hydrogen incorporated at the α-carbon of propionyl-CoA as a result of enzyme action. The results of this experiment are summarized in Table I. The differences between enzymatically incorporated tritium per micromole of propionyl-CoA as measured by the specific activity of the isolated hydroxamate when compared with values obtained by the enzymatic method may arise from the acid conditions of the hydroxamate isolation which would tend to repress the ionization of the hydroxyl group and thus inhibit the dilution of exchangeable hydrogen expected. The slight incorporation by treatment A as judged by the enzymatic method is well within experimental error and is not considered significant. The quantitative recovery of tritium incorporated in the reverse reaction (treatment D) upon recarboxylation is further evidence of the absolute stereospecificity of the reaction. If either the forward or the reverse reaction, or both, were not stereospecific with respect to the α-carbon, only 50% of the enzymatically incorporated tritium could have been recovered. A very great isotope effect was observed. The non-exchangeable tritium content of the propionyl-CoA isolated from treatment D might be expected to approach the specific activity of the water (1.22×10^6 d.p.m. per microatom of hydrogen). Instead, a value of 4.3×10^4 d.p.m. per micromole was observed--a 150-fold difference.

Propionyl-CoA--Pantetheine Exchange

The finding that the enzyme is inhibited by sulfhydryl reagents[25] led to the consideration of the possibility that propionyl-S-enzyme or methylmalonyl-S-enzyme is formed with the release of free CoA during

T A B L E I

DISTRIBUTION OF TRITIUM FROM T_2O IN PROPIONYL-CoA

Condition* of incorporation	d.p.m. per micromole of propionyl-CoA carboxylated	d.p.m. per micromole of propionyl hydroxamate	Total d.p.m.** carboxylated	Total d.p.m. recovered as T_2O		Enzymatically incorporated d.p.m. per micromole of propionyl-CoA
				+ enzyme	- enzyme	
A	34,000	8,000	16,300 (0.48)	16,200	15,000	2,500
B	28,000	4,800	14,600 (0.52)	16,100	16,400	-
C	40,000	2,800	15,500 (0.39)	15,600	15,300	-
D	77,000	56,000	29,300 (0.38)	29,300	16,100	43,000

* For conditions of tritium incorporation (A, B, C, D) see text.

** Values in parentheses are micromoles carboxylated.

propionyl-CoA carboxylation. This possibility was tested by use of the technique employed by Lynen[36] to demonstrate that fatty acid synthetase catalyzed malonyl transfer from malonyl-CoA to pantetheine. Large amounts (1.3 units) of carboxylase were incubated with either propionyl-1-C^{14}-CoA or methylmalonyl-1-C^{14}-CoA and 5.2 micromoles of pantetheine in the presence and absence of cofactors for the carboxylation reaction. In no case was C^{14} propionyl-pantetheine or C^{14}-methylmalonyl-pantetheine formed enzymatically, indicating that propionyl carboxylase does not behave as a propionyl- or methylmalonyl-transacylase. Thus the binding of propionyl-CoA to enzyme does not involve a covalent acyl-S-enzyme bond.

Attempted Isolation of Enzyme-propionyl-CoA

Another direct approach to the demonstration of enzyme-propionyl-CoA complex formation was tried. Propionyl-CoA carboxylase, 39 units, having a specific activity of 10.3 units per mg of protein was incubated for 15 minutes at 2° with the following components in micromoles: tris, pH 8.5, 80; GSH, 0.25; and propionyl-CoA, 1.5; in a final volume of 0.51 ml to give a propionyl-CoA concentration (2.95×10^{-3} M) equivalent to ten times the Km value for this substrate. Since CO_2 transfer from enzyme-CO_2 to propionyl-CoA takes place in the absence of $MgCl_2$, it was not likely that this cofactor would be required for the binding of propionyl-CoA to enzyme, and it was omitted from the reaction mixture. The reaction mixture was applied to a 2.5 x 18 cm column of Sephadex-G-50 which had been previously equilibrated with 0.2 M tris, pH 8.5 containing 0.5 micromoles of GSH per ml. The column was eluted with the same buffer

at a flow rate of 1 ml per minute. Eight 2 ml fractions were collected over the range of ultraviolet absorbing eluate as shown in Figure II. Each fraction was divided into three equal portions by allowing the eluate to drop serially into each of three previously prepared assay tubes. Tube A (enzyme assay) contained the complete basic reaction mixture with the exception of enzyme. Tube B (propionyl-CoA assay) contained the complete basic reaction mixture supplemented with 6.44×10^{-2} units of carboxylase; and the regular assay bicarbonate (0.05 microcuries per micromole) was replaced by 5 microcuries of C^{14}-bicarbonate having a specific activity of 21.8 microcuries per micromole. Tube C (enzyme-propionyl-CoA assay) was identical to B except that the additional enzyme was omitted. The volume of each reaction mixture was 0.43 ml, to which 0.66 ml of column eluate was added. Immediately after collection, the tubes were incubated for 20 minutes at 37°, and the reaction was terminated with 0.3 ml of 20% TCA. C^{14}-bicarbonate not fixed in these incubations was removed by gassing with N_2, and 1 ml samples were mixed with 15 ml of naphthalene-dioxane-PPO-POPOP cocktail in counting vials. These vials were stored open over 10% KOH in a dessicator and counted periodically until the radioactivity of negative control samples (no column eluate) had reached a constant minimum of about 400 c.p.m.

Figure II shows the propionyl-CoA dependent fixation of C^{14}-bicarbonate caused by each fraction when enzyme (A), propionyl-CoA (B), and both enzyme and propionyl-CoA (C) were limiting. The binding and subsequent carboxylation of as little as 1.7×10^{-4} micromoles of propionyl-CoA per unit of enzyme (about 1/4 saturation) would be expected

FIGURE II

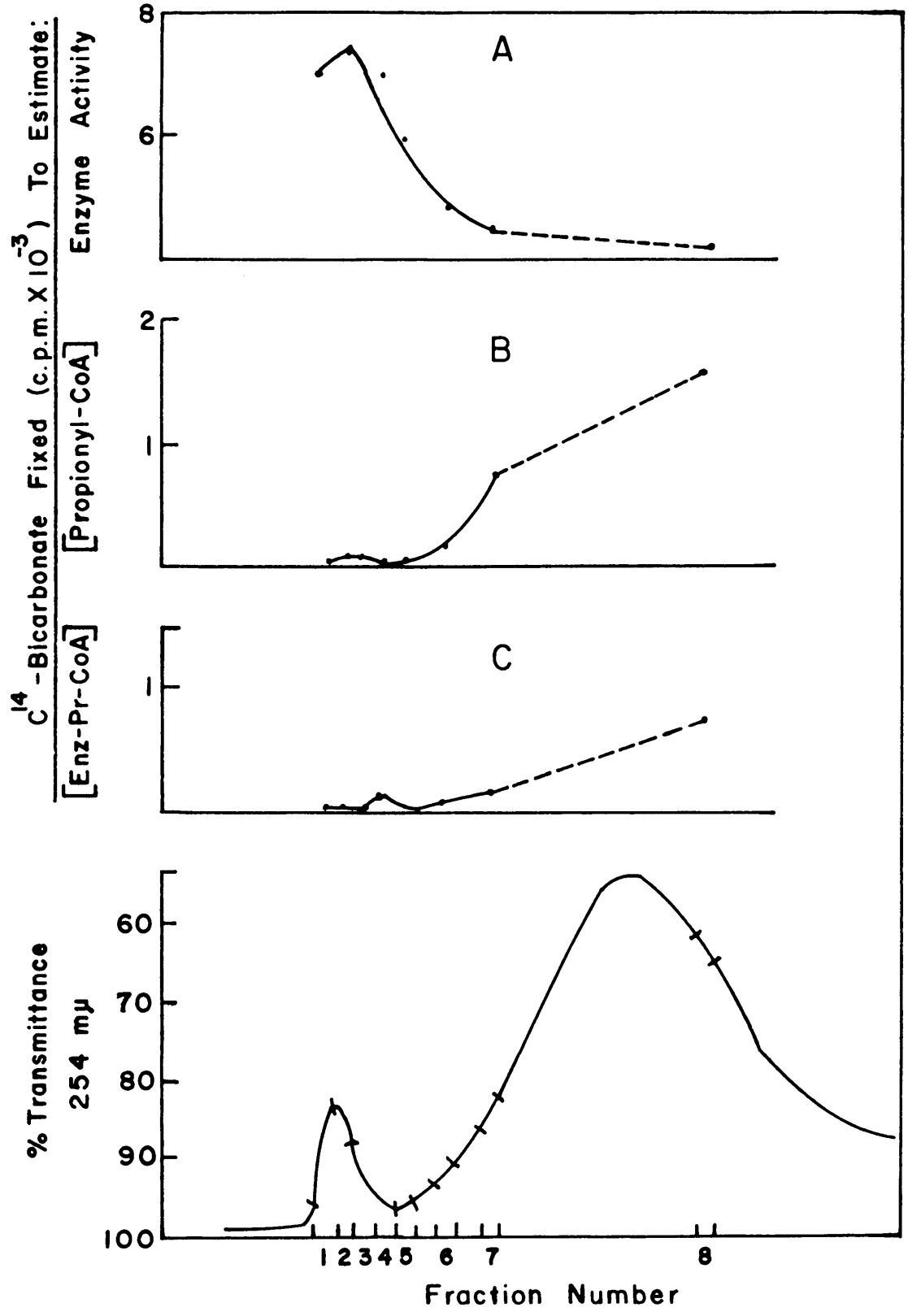
SEPHADEX GEL FILTRATION OF PROPIONYL-CoA-TREATED MITOCHONDRIAL PROPIONYL-CoA CARBOXYLASE

Ultraviolet absorbance of the fractions (lower curve) is plotted as % transmittance at 254 mμ.

Enzyme activity of the fractions (curve A) is plotted as c.p.m. propionyl-CoA dependent C^{14}-bicarbonate fixation per aliquot assayed in the presence of added propionyl-CoA.

Propionyl-CoA content of the fractions (curve B) is plotted as c.p.m. propionyl-CoA dependent C^{14}-bicarbonate fixation per aliquot determined in the presence of excess enzyme.

Enzyme-propionyl-CoA content of the fractions (curve C) is expressed as c.p.m. propionyl-CoA dependent C^{14}-bicarbonate fixation per aliquot in the absence of added propionyl-CoA.


C^{14}-Bicarbonate Fixed (c.p.m. X 10^{-3}) To Estimate:
Enzyme Activity
[Propionyl-CoA]
[Enz-Pr-CoA]
A
B
C
8
6
2
1
1
% Transmittance
254 mμ
60
70
80
90
100
1 2 3 4 5 6 7
8
Fraction Number

to give 5 x 10^4 c.p.m. total in the region of protein elution (fractions 1-5) under carboxylation conditions (C). From these considerations it is clear that no detectable amounts of propionyl-CoA were isolated. This again indicates that propionyl-CoA binding is not of a covalent nature as is the case with enzyme-CO_2. A readily dissociable complex would not be isolable under the conditions employed here since propionyl-CoA would be continually removed. In this attempted isolation, the ionic strength of the eluting buffer closely approximated that of the reaction mixture. In similar experiments, the ionic strength of the eluting buffer was varied both above and below that of the reaction mixture without change in the result.

Carboxylation of Propionyl-CoA, Propionyl-pantetheine, and Propionyl-N-acetylcysteamine

It has been previously suggested[25] that propionyl-CoA is bound or oriented with respect to the enzyme surface largely through the CoA moiety. It was therefore of interest to determine whether modification of the CoA moiety of propionyl-CoA by removal of the 3'-phosphate group, the entire adenosine-3'-phosphate-5'-pyrophosphate group, or of all except the N-acetylcysteamine group would affect the ability of the resulting thioesters (propionyl-DPCoA, propionyl-pantetheine, and propionyl-N-acetylcysteamine) to serve as substrates. These propionyl thioesters were prepared as described in "Materials and Methods." In the standard assay mixture containing 1.3 x 10^{-2} units of enzyme and C^{14}-bicarbonate having a specific activity of 4.7 microcuries per micromole, propionyl-pantetheine (5.1 micromoles) and propionyl-DPCoA (12.6 micromoles) but

not propionyl-N-acetylcysteamine (2.2 micromoles) were carboxylated. The free acid carboxylation products of these first two were identified paper chromatographically as methylmalonic acid. Table II shows an interesting comparison of carboxylation rates and Km's that have been found for the active propionyl-thioesters. The kinetic constants presented indicate that the binding of propionyl-CoA to enzyme involves the 3'-phosphate and some portion of the pantoic acid moiety.

Inhibitory Effects of Various Compounds

As another approach to the study of the nature of the enzyme-propionyl-CoA association, a series of compounds possessing structural features and functional groups similar to those of coenzyme A were tested as possible inhibitors of the overall carboxylation reaction. All compounds tested were dissolved in water and adjusted to as near pH 7 as was compatible with their solubilities. In a dummy reaction mixture containing 86 micromoles of tris, pH 8.5, and no bicarbonate, adenine, adenosine, and hypoxanthine at levels of 1×10^{-2} M caused pH changes of -0.21, -0.13, and +0.12 pH units respectively. The other compounds caused pH changes of $\pm$0.06 pH units or less. Table III shows the results of this study. It will be noted that adenine, adenosine, and the nucleoside mono- and triphosphates were the most effective inhibitors at levels of 1×10^{-2} M.

In addition, aniline (1×10^{-2} M) and puromycin (1.7×10^{-2} M) were found to inhibit to the extent of 48% and 59%, respectively. However, since aniline is so readily oxidized, it is possible that the inhibition

T A B L E I I

KINETIC CONSTANTS OF MITOCHONDRIAL PROPIONYL-CoA CARBOXYLASE

Reactant	Km (M)	Vm per unit of enzyme (μmoles $HC^{14}O_3$ fixed per minute per unit of enzyme)
Propionyl-CoA[1]	2.6×10^{-4}	1.4
Propionyl-DP-CoA[2]	2.8×10^{-3}	0.4
Propionyl-pentetheine[1]	2.4×10^{-2}	0.02
Propionyl-N-acetyl-cysteamine	-	0

[1] From Halenz et al (20).

[2] Calculated from limited data.

T A B L E I I I

EFFECT OF VARIOUS COMPOUNDS ON PROPIONYL-CoA CARBOXYLATION*

Compound (1×10^{-2} M)	% Inhibition**
None	0
adenine	71
adenosine	83
cytosine	0
hypoxanthine	16
uracil	2
xanthine	12
CTP-$MgCl_2$, 1:1	17
GTP-$MgCl_2$, 1:1	28
UTP-$MgCl_2$, 1:1	28
ITP-$MgCl_2$, 1:1	29
5'-AMP	39
3'-AMP	27
2'-AMP	22
β-alanine	2
L-tryptophan	8

* The assay mixture was the standard assay mixture containing 86, rather than 50, micromoles of tris, pH 8.5.

**Average of duplicate assays.

observed may be due to oxidation products rather than to aniline itself. It has been previously reported[25] that PCMB at levels of 1×10^{-6} M inhibits to the extent of 42% and that this inhibition can be reversed by the addition of GSH. These results were confirmed.

Citrate Inhibition

The observation by other workers[37,38] that citrate and isocitrate stimulate fatty acid biosynthesis suggested testing the effect on highly purified propionyl-CoA carboxylase. Under the usual assay conditions and using 0.5 micromoles of propionyl-CoA or acetyl-CoA, 1×10^{-2} M citrate, pH 8.3, was found to inhibit propionyl-CoA carboxylation 50% and acetyl-CoA carboxylation 60% rather than stimulating. The only free acid carboxylation products isolated from the carboxylation of propionyl-CoA or acetyl-CoA in the presence of 1×10^{-2} M citrate using C^{14}-bicarbonate having a specific activity of 3.6 microcuries per micromole were identified paper chromatographically as methylmalonic acid and malonic acid respectively. Thus, citrate was not carboxylated. The possibility still exists that citrate inhibition was due to binding of Mg^{++} by this compound, though such an electrostatic effect is unlikely to be so great as to mask stimulation by inhibition.

TNBS Inhibition

Propionyl-CoA carboxylase was found to be inhibited slowly by preincubation with 1×10^{-3} M 2,4,6-trinitrobenzenesulfonic acid, an amino group specific reagent[39,40], at 2° in potassium phosphate buffer, pH 7.5.

Although the inhibition by TNBS occurs more rapidly at pH 8.5, use of this pH was obviated by the fact that the two buffers having adequate buffering capacity around pH 8.5 (borate and tris) could not be used. Borate is itself inhibitory, probably due to its tendency to tie up Mg^{++}, and tris appears to react with TNBS since TNBS solutions in 0.1 M tris rapidly lose activity on storage. This instability is not found with TNBS solutions in 0.1 M phosphate. To demonstrate TNBS inhibition of propionyl-CoA carboxylase, 5.6×10^{-3} units of enzyme were preincubated at 0° for varying lengths of time from 0.5 to 4.0 hours in 0.2 ml of 0.05 M phosphate buffer, pH 7.5 with or without 1×10^{-3} M TNBS. No pH change resulted from the addition of TNBS. After preincubation, the carboxylation reaction was initiated by adding the remaining components of the complete assay mixture in a volume of 0.565 ml to each tube, 50 micromoles of potassium phosphate, pH 7.5, being substituted for tris. The remainder of the enzyme assay was carried out in the usual manner. Figure III shows clearly that TNBS is inhibitory, inhibition increasing with enzyme-inhibitor contact time. Essentially no loss in enzyme activity with time was noted in the presence of phosphate alone.

Protection Against PCMB Inhibition

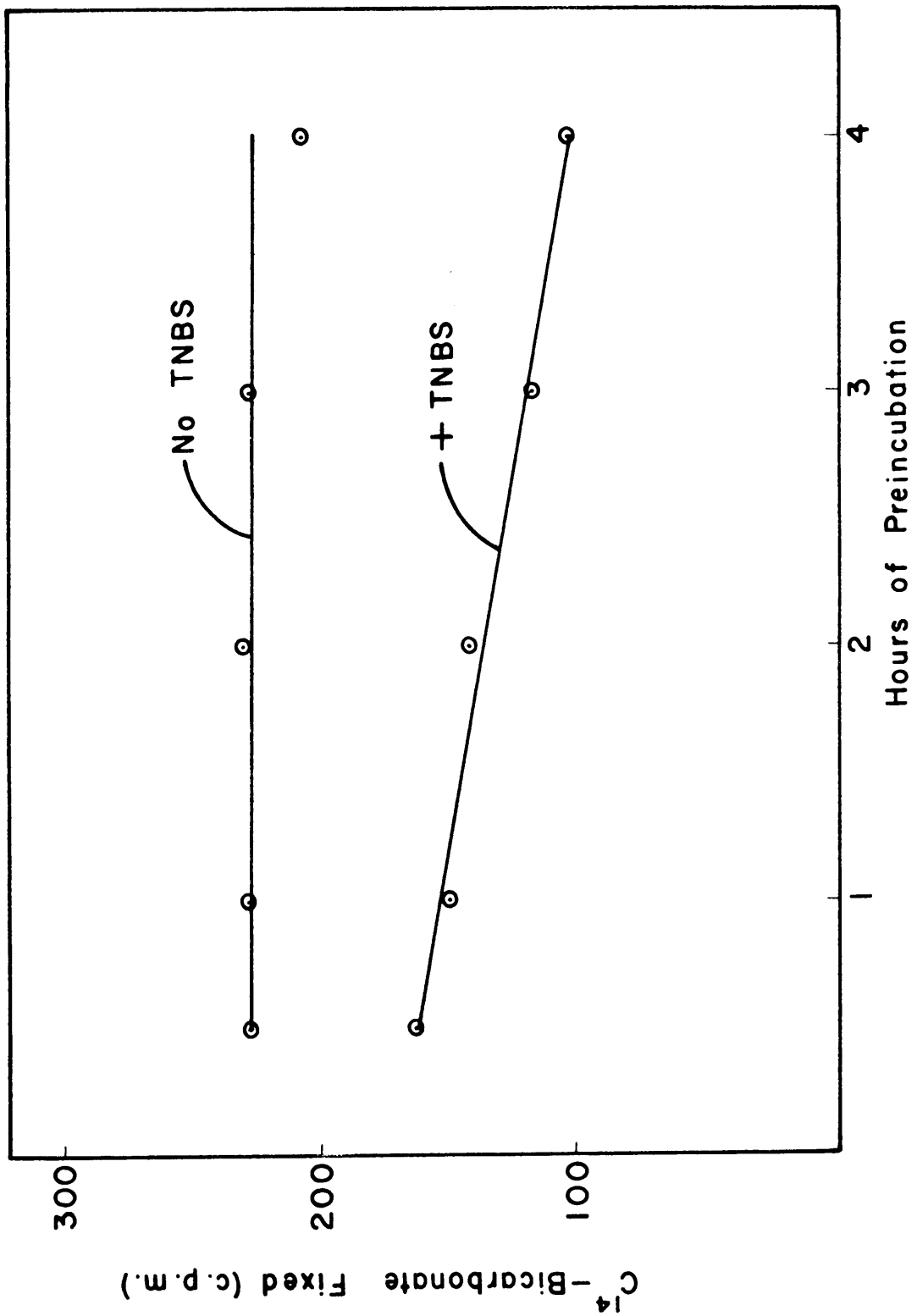
1) Experiment I

In order to test the possibility that enzyme sulfhydryl groups are involved in the binding of propionyl-CoA, or of ATP; the effects of propionyl-CoA, ATP, propionyl-pantetheine, and 3'-AMP on the extent of PCMB inhibition were studied. PCMB inhibition was accomplished in all

FIGURE III

TNBS INHIBITION OF MITOCHONDRIAL PROPIONYL-CoA CARBOXYLASE

Enzyme activity remaining after the indicated times of preincubation is plotted as c.p.m. propionyl-CoA dependent C^{14}-bicarbonate fixation per assay tube.


No TNBS
+ TNBS
300
200
100
1
2
3
4
Hours of Preincubation
C14-Bicarbonate Fixed (c.p.m.)

cases by preincubating enzyme for 10 minutes at 2° with or without 7.65×10^{-4} micromoles of PCMB in the presence of 60 micromoles of tris, pH 8.5, and 10 micromoles of C^{14}-bicarbonate, specific activity 5×10^{-2} microcuries per micromole. In Experiment I, propionyl-CoA, 0.5 micromoles; GSH, 0.5 micromoles; or ATP+$MgCl_2$, 2.0 micromoles each; as well as PCMB were added to the preincubation mixture as indicated in Table IV with sufficient water to bring the preincubation volume to 0.565 ml. When two additions were made to the preincubation, they were made one minute apart in the order given in the table. After 10 minutes preincubation in the presence of PCMB, the remaining components of the reaction mixture were added together, as noted in the table, in a volume of 0.2 ml. The tubes were incubated at 37° for 20 minutes, the remainder of the carboxylase assay being conducted in the usual manner.

Table IV shows that propionyl-CoA, at the level used, protects the enzyme to a very great extent against inhibition by PCMB when present during the preincubation. Presumably this protective effect also explains the low degree of inhibition observed when PCMB was not added until initiation of the carboxylation reaction. The protective effect of propionyl-CoA is apparently not due to the presence of contaminating sulfhydryl compounds since 0.5 micromoles of GSH (equivalent to all the propionyl-CoA added) did not afford comparable protection. Also, propionyl-CoA, unlike GSH, does not appear to reverse the inhibition. Furthermore, inhibition is greater when PCMB is added to the preincubation mixture prior to propionyl-CoA than when the order of addition is reversed, although the total contact time of propionyl-CoA and PCMB is the same in

T A B L E I V

EFFECT OF PROPIONYL-CoA AND ATP + $MgCl_2$ ON PCMB INHIBITION

(Experiment I)

Additions to Preincubation	Additions to Incubation	% Inhibition*
None	Pr-CoA+ATP+$MgCl_2$	0
Pr-CoA	ATP + $MgCl_2$	0
ATP + $MgCl_2$	Pr-CoA	0
PCMB	Pr-CoA + ATP + $MgCl_2$	67
None	PCMB + Pr-CoA + ATP + $MgCl_2$	4
PCMB, then Pr-CoA	ATP + $MgCl_2$	17
Pr-CoA, then PCMB	ATP + $MgCl_2$	11
GSH, then PCMB	Pr-CoA + ATP + $MgCl_2$	32
ATP + $MgCl_2$, then PCMB	Pr-CoA	82

* Average of duplicates.

both cases. In contrast to propionyl-CoA, ATP+$MgCl_2$ appears to facilitate inhibition by PCMB, and definitely does not protect. Both protection by propionyl-CoA and facilitation by ATP+$MgCl_2$ of PCMB inhibition have been observed in other experiments. It is not known whether or not the presence of bicarbonate is essential for ATP+$MgCl_2$ facilitation. When enzyme was treated with propionyl-CoA, and the propionyl-CoA subsequently removed as described in the section under the subheading "Attempted Isolation of Enzyme-Propionyl-CoA," no difference was found in the PCMB sensitivity of enzyme so treated and untreated enzyme.

The above results were taken as an indication that propionyl-CoA is bound to the enzyme surface in such a way as to hinder the PCMB sensitive site, but not necessarily through the agency of such a site. Under these conditions of preincubation, the presence of ATP+$MgCl_2$ would permit the formation of enzyme-CO_2. Thus, enzyme-CO_2 may adopt a configuration in which sulfhydryl groups are more accessible to PCMB than they are in native enzyme.

2) Experiment II

Since the entire propionyl-CoA molecule was found to protect against PCMB inhibition, it was then of interest to see whether propionyl-pantetheine or 3'-AMP would also protect and thus further define the site of propionyl-CoA--sulfhydryl interaction. Accordingly, 5.3 micromoles of propionyl-pantetheine or 4.8×10^{-1} micromoles of 3'-AMP were used as propionyl-CoA was in Experiment I to protect against PCMB inhibition. This experiment was conducted in the same manner as was Experiment I, and additions were made as noted in Table V.

T A B L E V

EFFECT OF PROPIONYL-PANTETHEINE AND 3'-AMP ON PCMB INHIBITION

(Experiment II)

Additions to Preincubation**	Additions to Incubation**	% Inhibition*
None	Pr-CoA + ATP + $MgCl_2$	0
Pr-pant	Pr-CoA + ATP + $MgCl_2$	36
None	Pr-pant + Pr-CoA + ATP + $MgCl_2$	34
PCMB	Pr-CoA + ATP + $MgCl_2$	46
PCMB + Pr-pant	Pr-CoA + ATP + $MgCl_2$	39 (63)***
PCMB	Pr-pant + Pr-CoA + ATP + $MgCl_2$	47 (63)
None	Pr-CoA + ATP + $MgCl_2$	0
3'-AMP	Pr-CoA + ATP + $MgCl_2$	29
None	3'-AMP + Pr-CoA + ATP + $MgCl_2$	5
PCMB	Pr-CoA + ATP + $MgCl_2$	37
3'-AMP + PCMB	Pr-CoA + ATP + $MgCl_2$	54 (55)
PCMB	3'-AMP + Pr-CoA + ATP + $MgCl_2$	42 (40)

*Average of duplicates.
**The first three treatments in each group serve as controls.
***Figures in parentheses are values predicted for independent inhibition--see text.

For two inhibitors simultaneously present, and acting independently, the observed inhibition should be greater than with either alone, but not the arithmetic sum of them. That is, inhibition of one enzyme molecule by both inhibitors will be expressed only once rather than twice. The same is true if one inhibitor is allowed to act for a period of time before the addition of the other. In both cases, the reduction in enzyme activity to be expected in the presence of both inhibitors can be calculated from the following expression:

$$\% I_T = 100 - \left[100 - \% I_1 - \frac{\% I_2 (100 - \% I_1)}{100}\right]$$

Percent I_T is the percentage inhibition to be expected in the presence of both inhibitors; and $\% I_1$ and $\% I_2$ are the percentages inhibition observed for each inhibitor acting alone under the same conditions of time and temperature that will exist for each when they are allowed to act together. The % inhibition values predicted on the basis of such a computation carried out for 3'-AMP and PCMB and for propionyl-pantetheine and PCMB appear in parentheses beside the values observed.

When considered in this way, the data presented in Table V show that inhibition by propionyl-pantetheine and by PCMB are mutually exclusive. When both are present, propionyl-pantetheine (like propionyl-CoA) is preferentially bound. This suggests that both propionyl-pantetheine and PCMB are acting by binding to the same enzyme sulfhydryl groups or to groups located so close to one another that the binding of one reagent sterically hinders the binding of the other. PCMB and 3'-AMP on the other hand appear to be inhibiting independently. The difference in

3'-AMP inhibition observed when 3'-AMP is added to the preincubation mixture rather than when incubation is started has not been explained, but must be taken into consideration when interpreting these data. Since propionyl-pantetheine (but not 3'-AMP) like propionyl-CoA appears to protect against PCMB inhibition, but not to reverse it, it seems likely that the pantetheine rather than the 3'-AMP moiety of propionyl-CoA is involved with enzyme sulfhydryl in an enzyme propionyl-CoA complex. Some evidence that propionyl-pantetheine may indeed have an affinity for sulfhydryl groups will be presented in the next section of this report.

Kinetic Study of Inhibitors

The types of inhibition caused by some of the portions of the CoA molecule examined earlier were studied by determining their effects on Km and Vm for both propionyl-CoA and ATP using the double reciprocal plot method of Lineweaver and Burke[41]. The carboxylation rate was determined by the method of Halenz and Lane[1]. The basic reaction mixture was modified by varying the concentration of the reactant under study, and by the inclusion of 86 micromoles of tris rather than the usual 50 micromoles to assure pH control. Six propionyl-CoA levels ranging from 6.7×10^{-5} M to 1.34×10^{-4} M were used in the presence of 2.7×10^{-3} M ATP and $MgCl_2$. ATP concentrations were varied at 6 levels between 6.4×10^{-5} M and 2.7×10^{-3} M in the presence of 2.7×10^{-3} M $MgCl_2$ and 6.5×10^{-4} M propionyl-CoA. The inhibitors tested were: 3'-AMP, 2×10^{-2} M; CoA, 1.5×10^{-3} M; 5'-AMP, 1×10^{-2} M; 2'-AMP, 2×10^{-2} M; adenine, 5×10^{-3} M; adenosine, 5×10^{-3} M; and pantetheine, 4.6×10^{-3} M.

In examining the kinetics of the propionyl-CoA carboxylase reaction it will be noted that the system is composed of three substrates; ATP + $MgCl_2$, the bicarbonate anion, and propionyl-CoA as well as enzyme. The kinetics of such a system are considerably more complex than those found for the one-substrate Michaelis type.

At saturating levels of all substrates except the one under investigation, Michaelis-Menten kinetics would apply. If more than one substrate were at subsaturating levels, interpretation of the effects of an inhibitor on reciprocal plots determined with respect to one of them becomes very complex. In these studies with propionyl-CoA carboxylase, saturating levels of ATP were approached by using approximately 100 x Km ATP when studying the effect of inhibitors on propionyl-CoA. On the other hand, propionyl-CoA levels of approximately 2 x Km were used in studies on the effect of these inhibitors on ATP. For these reasons, no attempt will be made to interpret reciprocal plots obtained with respect to ATP in terms of the site of inhibitor action.

1) 3'-AMP and CoASH

Figures IV and V show that 3'-AMP and CoA are acting as competitive inhibitors with respect to propionyl-CoA, both exhibiting Ki's of 5.5×10^{-3}. In this case, Ki may or may not be a reflection of the enzyme-inhibitor dissociation constant, but the agreement of the values obtained for CoA and 3'-AMP is considered significant, and indicates an important role for the 3'-phosphate in propionyl-CoA binding. Both 3'-AMP and CoA show uncompetitive or mixed-competitive inhibition with respect to ATP (Figures VI and VII).

FIGURE IV

LINEWEAVER-BURKE PLOTS FOR PROPIONYL-CoA IN THE PRESENCE AND ABSENCE OF 2×10^{-2} M 3'-AMP

V is expressed as micromoles of propionyl-CoA-dependent bicarbonate fixation per hour under standard propionyl-CoA carboxylase assay conditions, and propionyl-CoA is expressed in molar concentration.

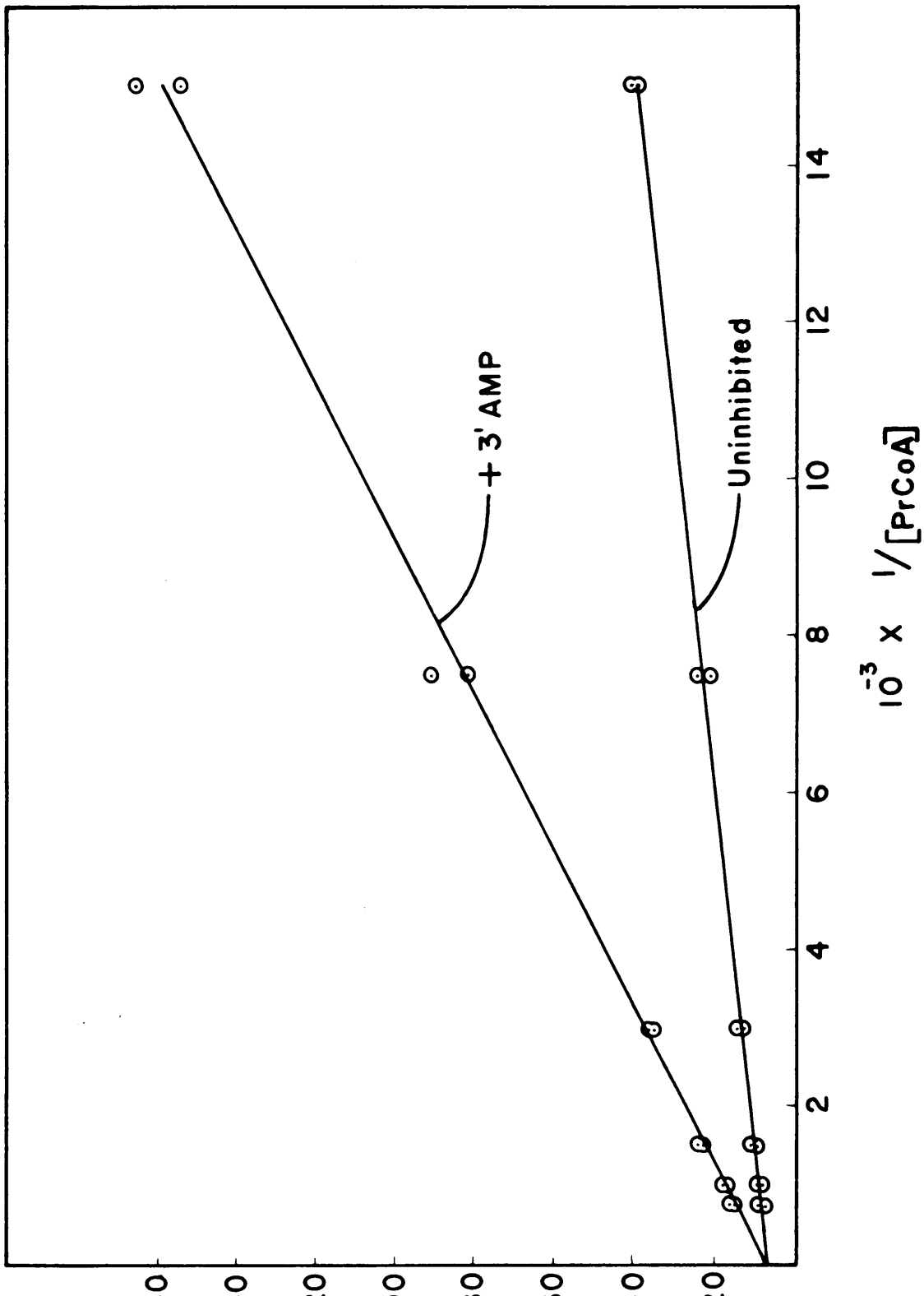

1/v
160
140
120
100
80
60
40
20
2
4
6
8
10
12
14
10^{-3} x 1/[PrCoA]
+ 3' AMP
Uninhibited

FIGURE V

LINEWEAVER-BURKE PLOTS FOR PROPIONYL-CoA IN THE PRESENCE AND ABSENCE OF 1.5×10^{-3} M COENZYME A

V is expressed as micromoles of propionyl-CoA dependent bicarbonate fixation per hour under standard propionyl-CoA carboxylase assay conditions, and propionyl-CoA is expressed in molar concentration.

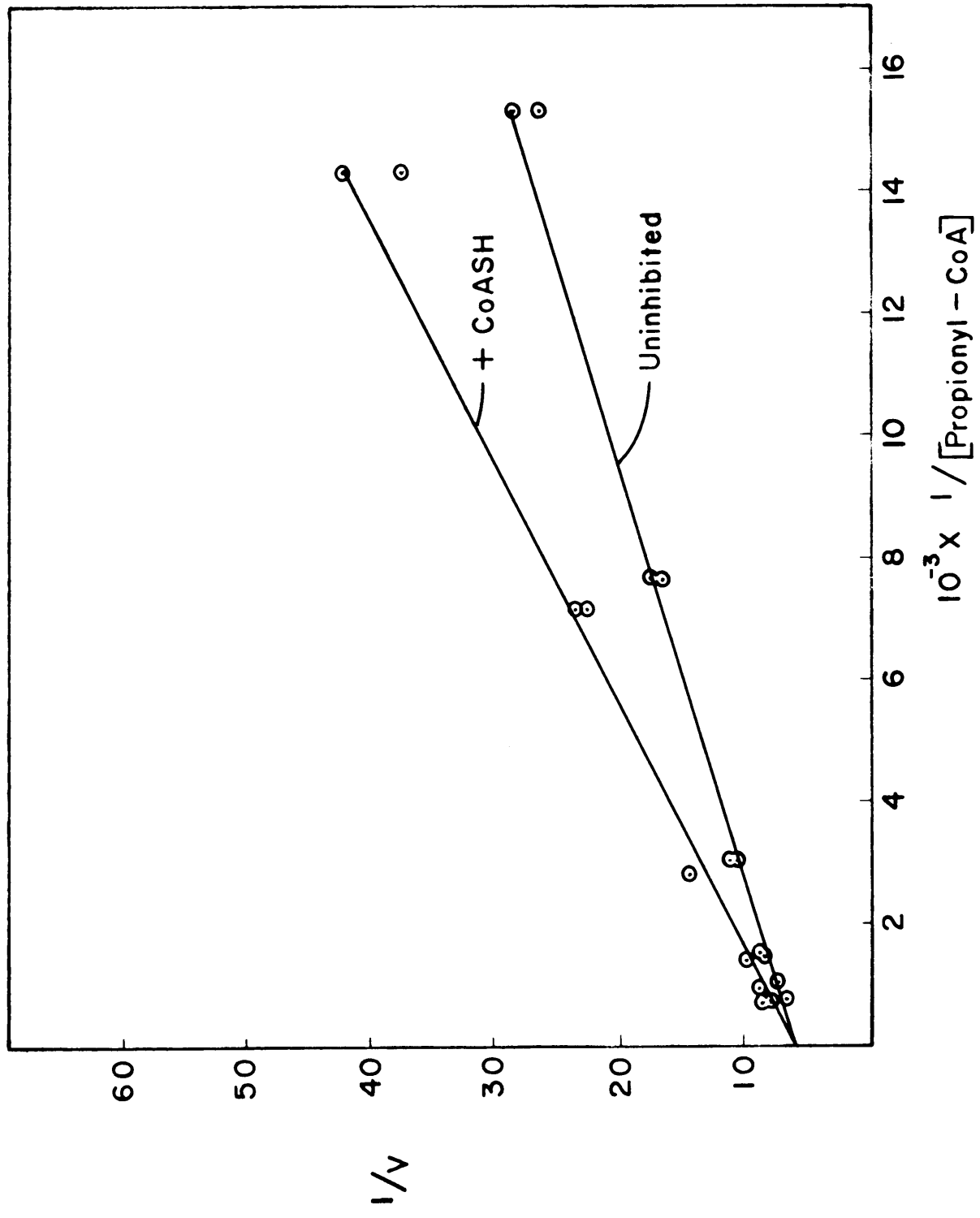

1/v
60
50
40
30
20
10
+ CoASH
Uninhibited
2
4
6
8
10
12
14
16
$10^{-3} \times 1/[\text{Propionyl-CoA}]$

FIGURE VI

LINEWEAVER-BURKE PLOTS FOR ATP IN THE PRESENCE AND ABSENCE OF 2×10^{-2} M 3'-AMP

V is expressed as micromoles of propionyl-CoA-dependent bicarbonate fixation per hour under standard propionyl-CoA carboxylase assay conditions and in the presence of 2.7×10^{-3} M $MgCl_2$. ATP is expressed in molar concentration.

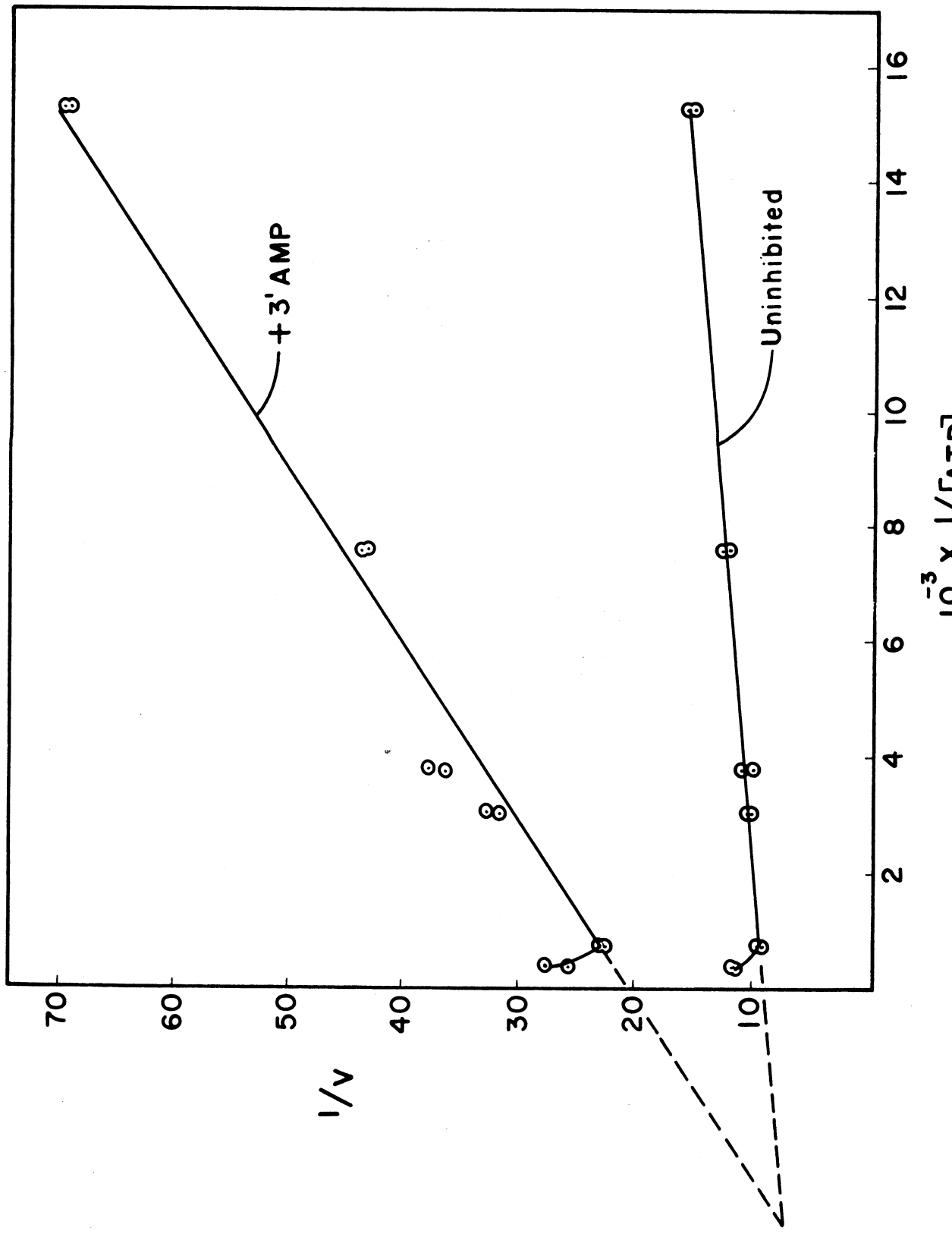
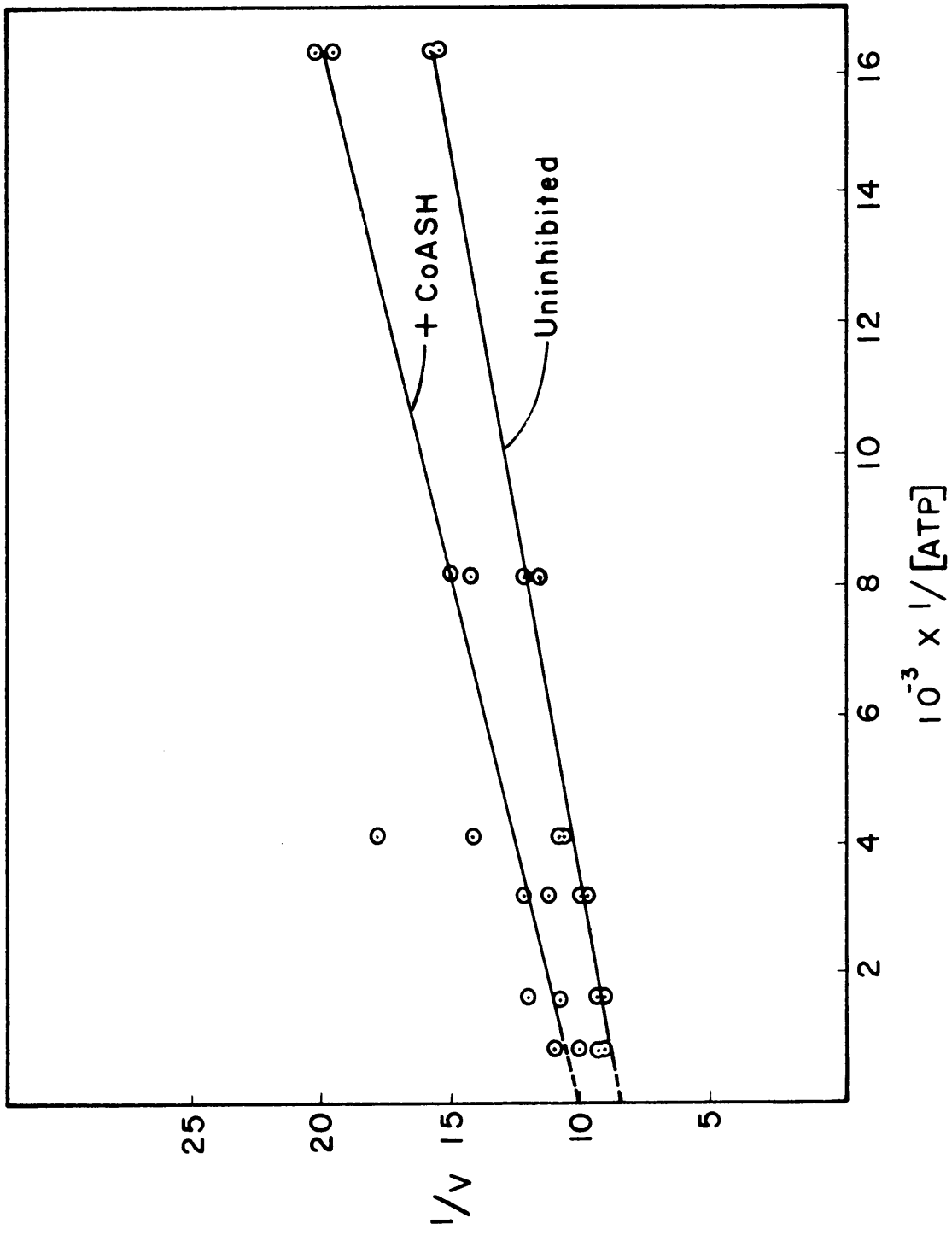

+3'AMP
Uninhibited
1/v
10^{-3} x 1/[ATP]
70
60
50
40
30
20
10
2
4
6
8
10
12
14
16

FIGURE VII

LINEWEAVER-BURKE PLOTS FOR ATP IN THE PRESENCE AND ABSENCE OF 1.5×10^{-3} M COENZYME A

V is expressed as micromoles of propionyl-CoA-dependent bicarbonate fixation per hour under standard propionyl-CoA carboxylase assay conditions and in the presence of 2.7×10^{-3} M $MgCl_2$. ATP is expressed in molar concentration.


+ CoASH
Uninhibited
1/v
25
20
15
10
5
2
4
6
8
10
12
14
16
10^{-3} x 1/[ATP]

2) 2'-AMP and 5'-AMP

Figures VIII and IX show that both 2'-AMP and 5'-AMP exhibit inhibition of the uncompetitive or mixed-competitive type. This type of inhibition indicates that propionyl-CoA can only partially overcome inhibition by these reagents. Such behavior could result from several situations. First, the inhibitor could be non-competitively occluding only one of several loci at which a single molecule of propionyl-CoA is attached as well as acting competitively at another such locus. Second, the inhibitor could be acting non-competitively near, as well as competitively at, any of the loci for propionyl-CoA attachment thus exerting steric hinderance. Third, the inhibitor could be acting competitively at the propionyl-CoA site and in addition be affecting enzyme-CO_2 formation by either competitive or non-competitive inhibition at the ATP site. Of these three possibilities, the first is most reasonable when the competitive mode of 3'-AMP and CoA inhibition is considered, since in the latter two cases propionyl-CoA should be unable to completely reverse inhibition by either 3'-AMP or CoA. In the first case propionyl-CoA may be considered to have reactive groups spatially distributed in such a manner as to permit effective competition with all reactive groups of 3'-AMP or CoA, but not of 2'-AMP or 5'-AMP. Again, 5'-AMP and 2'-AMP exhibit mixed competitive inhibition with respect to ATP (not shown).

3) Adenine and Adenosine

Figures X and XI show an unusual mode of inhibition by adenine and adenosine with respect to propionyl-CoA. The apparent reduction of

FIGURE VIII

LINEWEAVER-BURKE PLOTS FOR PROPIONYL-CoA IN THE PRESENCE AND ABSENCE OF 2×10^{-2} M 2'-AMP

V is expressed as micromoles of propionyl-CoA-dependent bicarbonate fixation per hour under standard propionyl-CoA carboxylase assay conditions, and propionyl-CoA is expressed in molar concentration.

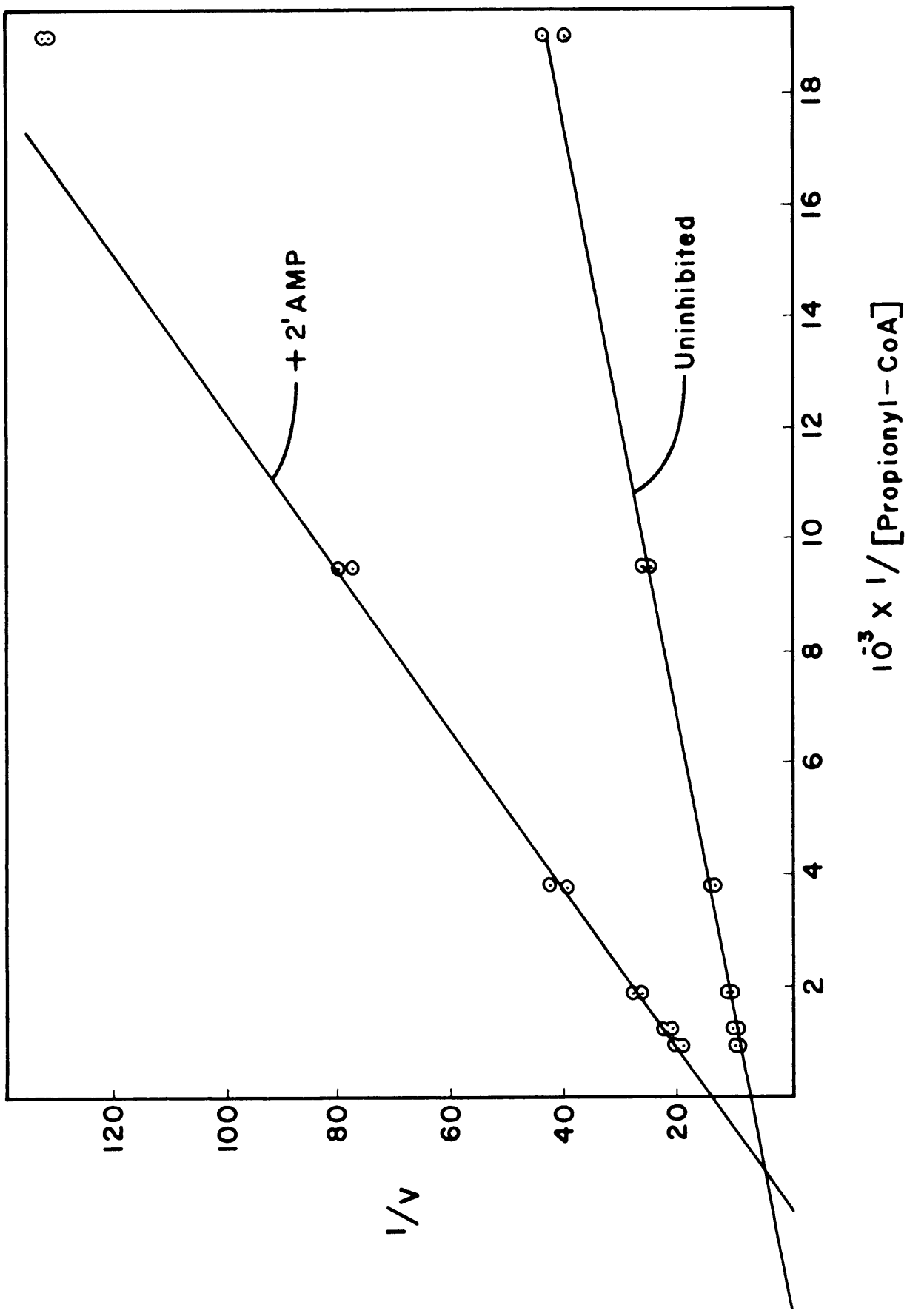

+ 2'AMP
Uninhibited
1/v
120
100
80
60
40
20
2
4
6
8
10
12
14
16
18
10^{-3} x 1/[Propionyl-CoA]

FIGURE IX

LINEWEAVER-BURKE PLOTS FOR PROPIONYL-CoA IN THE PRESENCE AND ABSENCE OF 1×10^{-2} M 5'-AMP

V is expressed as micromoles of propionyl-CoA-dependent bicarbonate fixation per hour under standard propionyl-CoA carboxylase assay conditions, and propionyl-CoA is expressed in molar concentration.

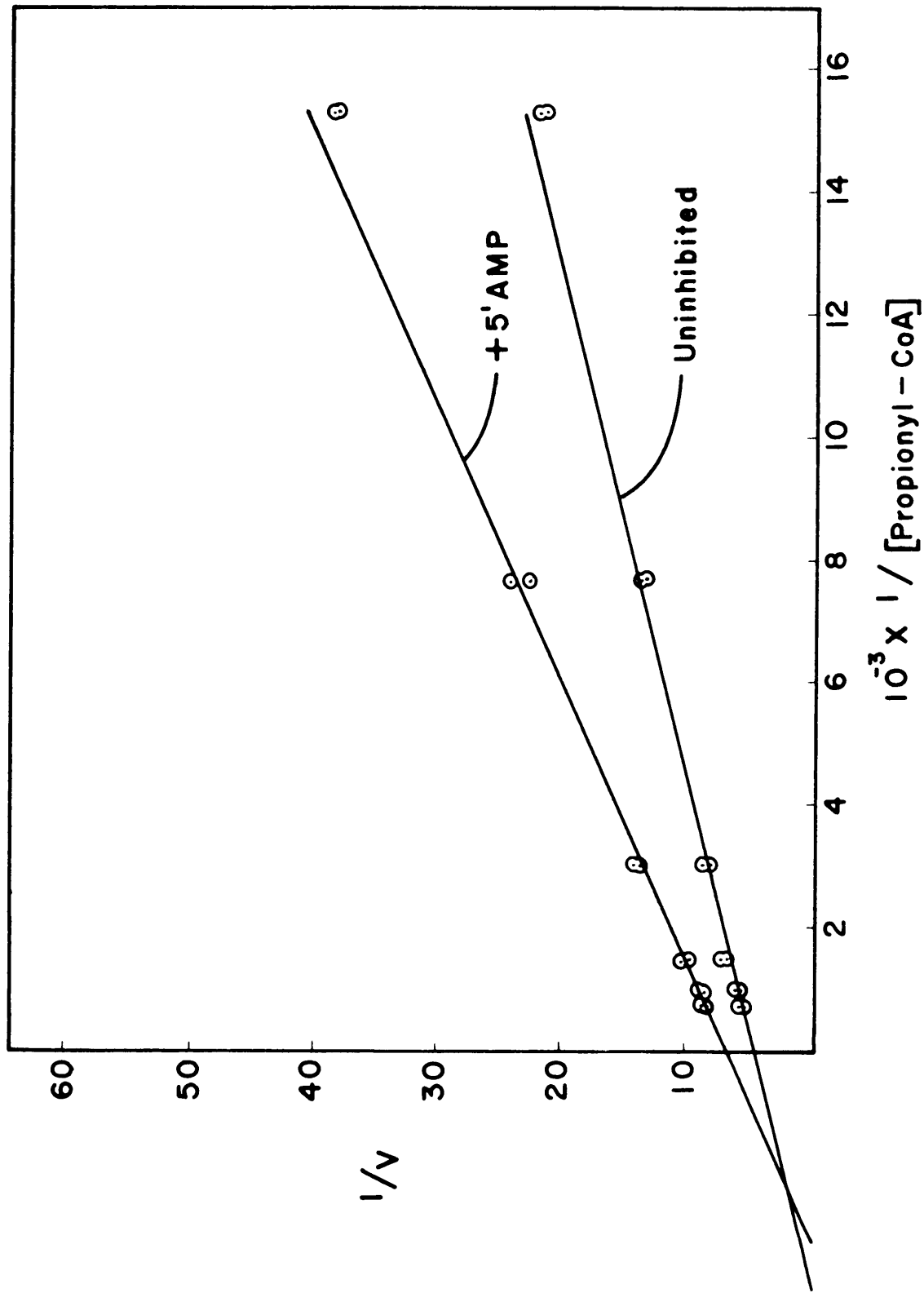

+5'AMP
Uninhibited
1/v
60
50
40
30
20
10
2
4
6
8
10
12
14
16
10^{-3} x 1 / [Propionyl – CoA]

FIGURE X

LINEWEAVER-BURKE PLOTS FOR PROPIONYL-CoA IN THE PRESENCE AND ABSENCE OF 5×10^{-3} M ADENINE

V is expressed as micromoles of propionyl-CoA-dependent bicarbonate fixation per hour under standard propionyl-CoA carboxylase assay conditions, and propionyl-CoA is expressed in molar concentration.

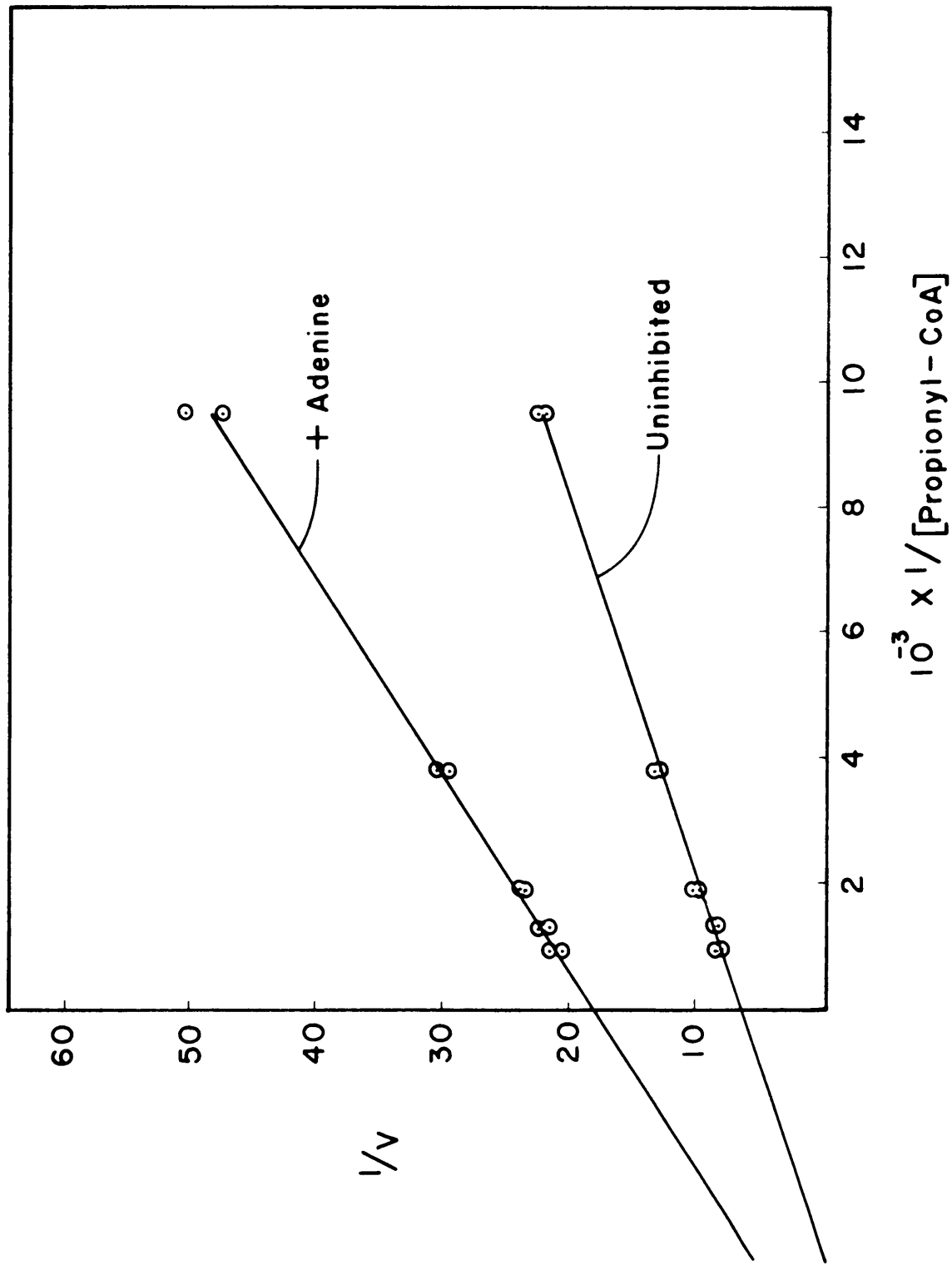
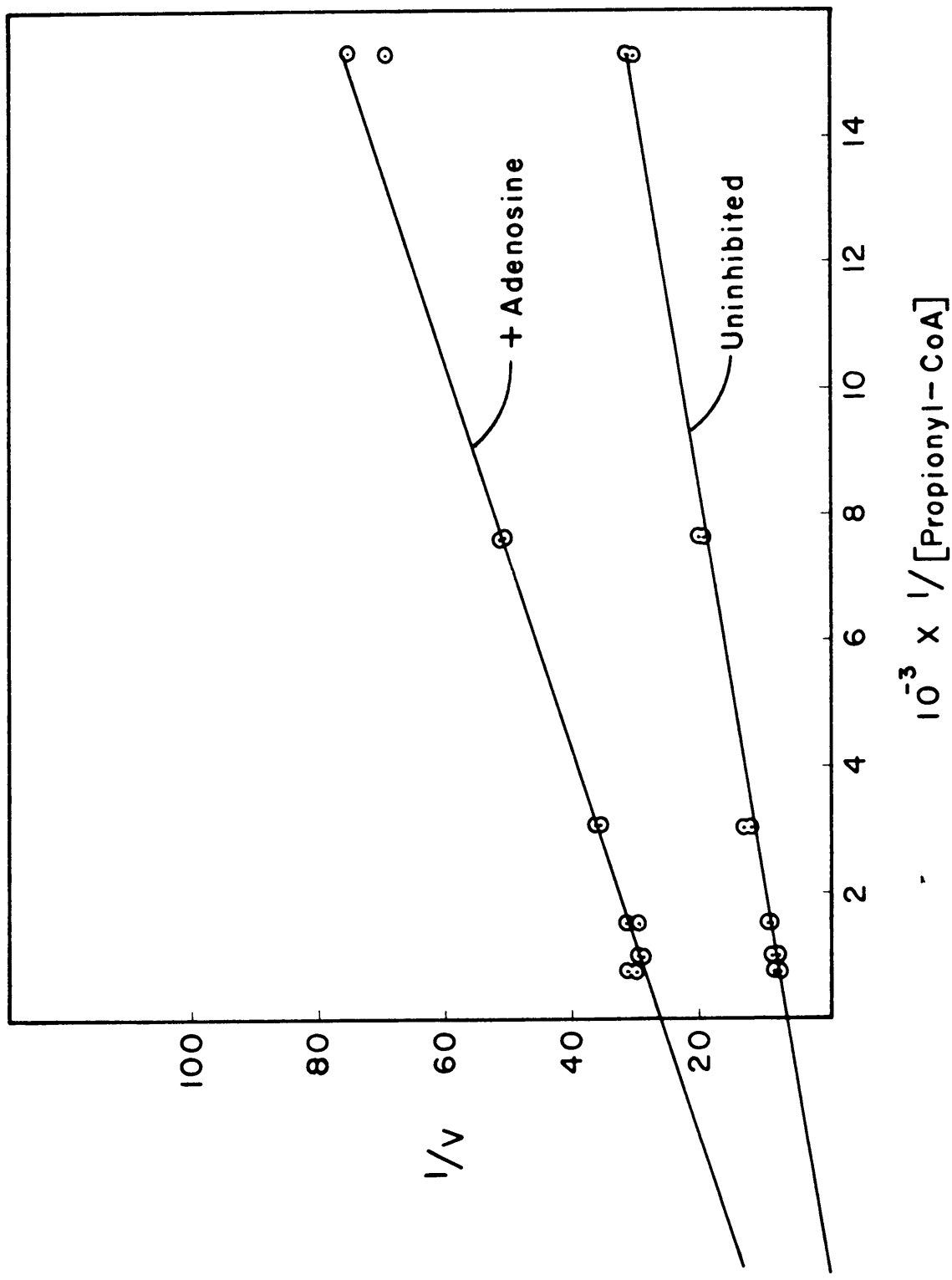

+ Adenine
Uninhibited
1/v
60
50
40
30
20
10
2
4
6
8
10
12
14
10^{-3} x 1/[Propionyl-CoA]

FIGURE XI

LINEWEAVER-BURKE PLOTS FOR PROPIONYL-CoA IN THE PRESENCE AND ABSENCE OF 5×10^{-3} M ADENOSINE

V is expressed as micromoles of propionyl-CoA-dependent bicarbonate fixation per hour under standard propionyl-CoA carboxylase assay conditions, and propionyl-CoA is expressed in molar concentration.


100
80
60
40
20
1/v
2
4
6
8
10
12
14
10^{-3} x 1/[Propionyl-CoA]
+ Adenosine
Uninhibited

the Km for propionyl-CoA remains unexplained, but it does seem clear that propionyl-CoA is unable to prevent adenine or adenosine inhibition. It is tempting to speculate that these inhibitors are acting only at the ATP site since both adenine (not shown) and adenosine (Figure XII) show mixed competitive inhibition possibly with a strong competitive element with respect to ATP.

4) Propionyl-pantetheine

As shown in Figure XIII, propionyl-pantetheine in the absence of GSH appears to be a non-competitive inhibitor of propionyl-CoA carboxylase. Since this compound is carboxylated by the enzyme, some competitive element was anticipated. However, in view of the low levels used in relation to its Km (Km = 2.4×10^{-2} M, Ref. 25) it is possible that the competitive element is too small to be observed under these conditions. The non-competitive effect may be ascribed to steric hinderance by binding at a site near the propionyl-CoA site. The prevention of propionyl-pantetheine inhibition by GSH (Figure XIV) may indicate that enzyme-propionyl-pantetheine association involves an enzyme sulfhydryl. Thus, saturation of propionyl-pantetheine with sulfhydryl would prevent binding to enzyme.

FIGURE XII

LINEWEAVER-BURKE PLOTS FOR ATP IN THE PRESENCE AND ABSENCE OF 5×10^{-3} M ADENOSINE

V is expressed as micromoles of propionyl-CoA-dependent bicarbonate fixation per hour under standard propionyl-CoA carboxylase assay conditions and in the presence of 2.7×10^{-3} M $MgCl_2$. ATP is expressed in molar concentration.

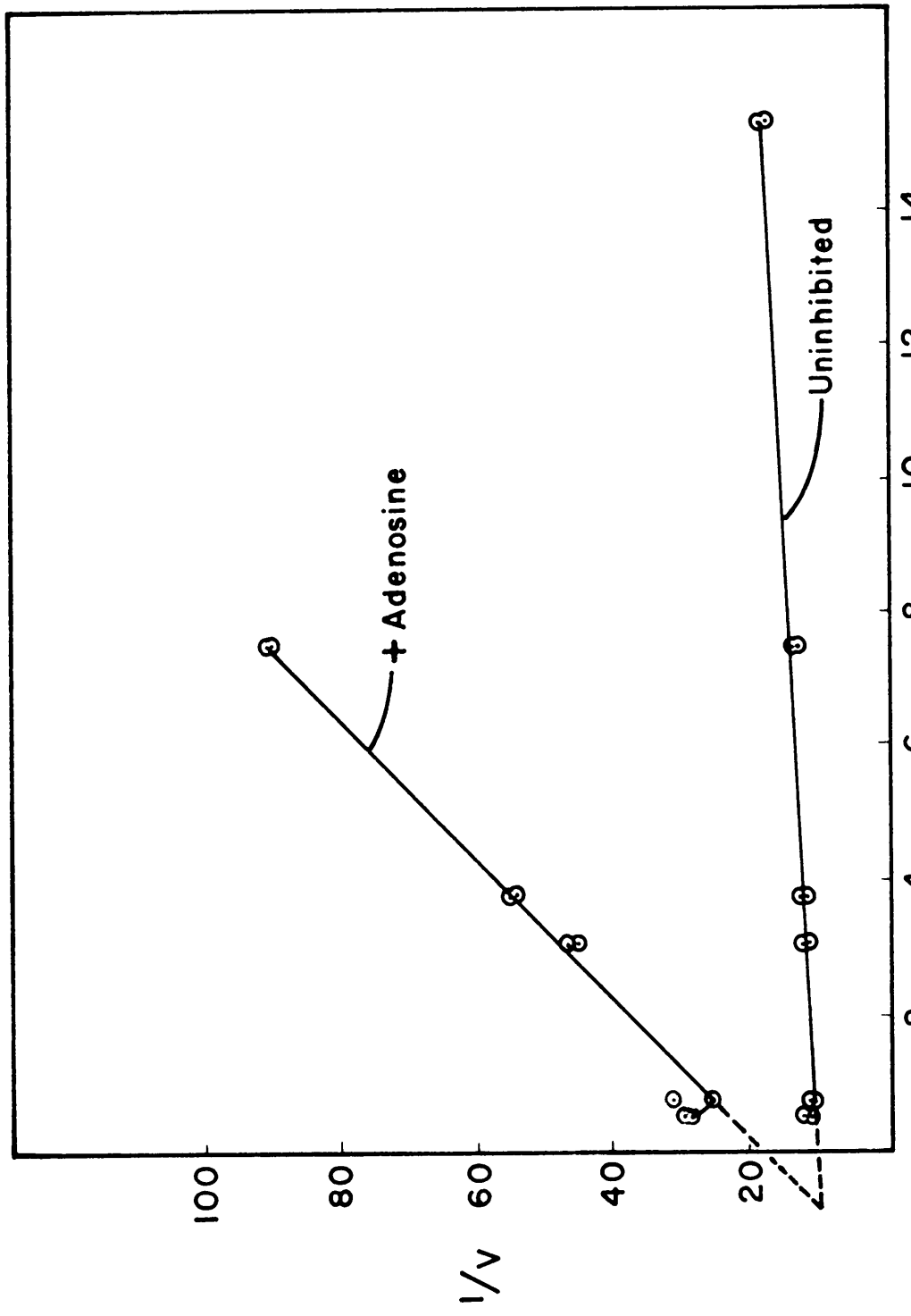

+ Adenosine
Uninhibited
1/v
100
80
60
40
20
2
4
6
8
10
12
14
10^{-3} x 1/[ATP]

FIGURE XIII

LINEWEAVER-BURKE PLOTS FOR PROPIONYL-CoA
IN THE ABSENCE OF GSH AND IN THE PRESENCE
AND ABSENCE OF 4.6×10^{-3} M PROPIONYL-PANTETHEINE

V is expressed as micromoles of propionyl-CoA-dependent bicarbonate fixation per hour under standard propionyl-CoA carboxylase assay conditions, and propionyl-CoA is expressed in molar concentration.

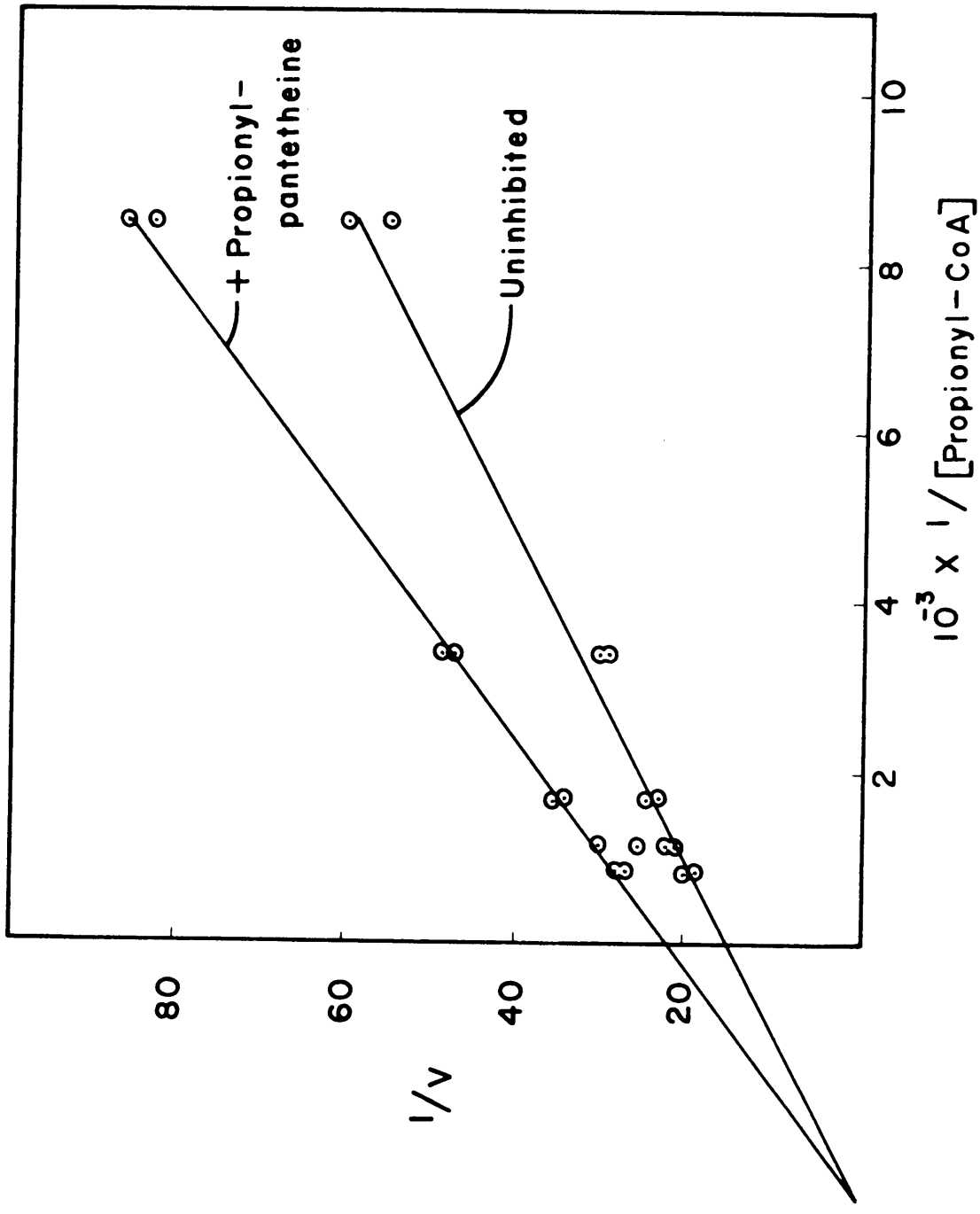
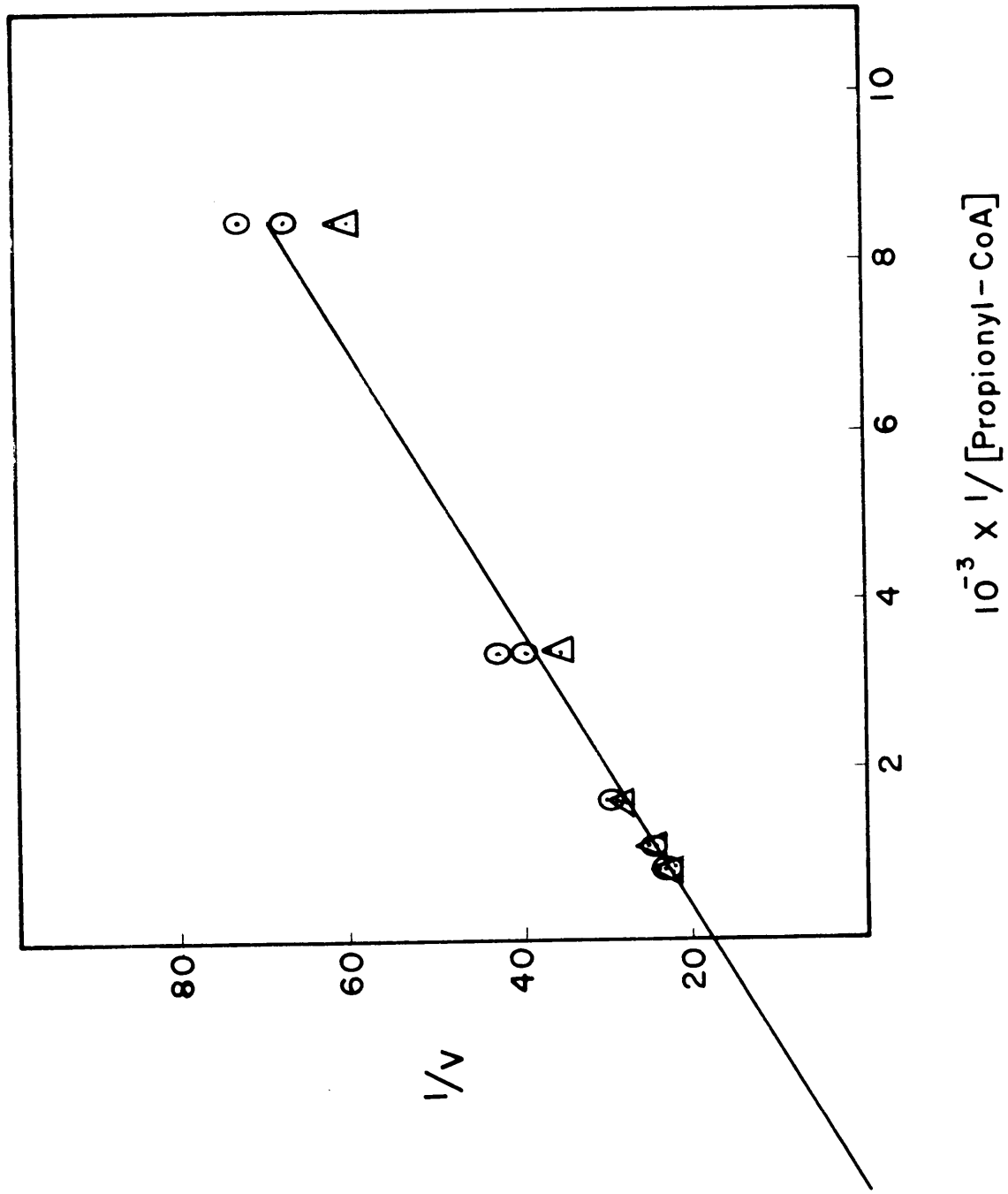

+ Propionyl-
pantetheine
Uninhibited
1/v
80
60
40
20
2
4
6
8
10
10^{-3} x 1/[Propionyl-CoA]

FIGURE XIV

LINEWEAVER-BURKE PLOTS FOR PROPIONYL-CoA IN THE PRESENCE OF GSH AND IN THE PRESENCE AND ABSENCE OF 4.6×10^{-3} M PROPIONYL-PANTETHEINE

V is expressed as micromoles of propionyl-CoA-dependent bicarbonate fixation per hour under standard propionyl-CoA carboxylase assay conditions, and propionyl-CoA is expressed in molar concentration.

Triangles denote the presence and circles the absence of propionyl-pantetheine.


80
60
40
20
1/v
2
4
6
8
10
10^{-3} x 1/[Propionyl-CoA]

VI. DISCUSSION

Reaction Mechanism

The fact that tritium exchange into the α-carbon of propionyl-CoA was not observed (Table I, treatments A and B) under the conditions employed would not in any case distinguish between an SE_1 or an SE_2 mechanism for the carboxylation of propionyl-CoA, since stereospecific SE_1 reaction mechanisms not involving the participation of solvent water can be postulated. However, the lack of tritium exchange would tend to rule out any SE_1 mechanism which involves a resonance-stabilized full carbanion or an unstabilized carbanion asymmetrically solvated by water rather than by enzyme. Such an interpretation of the tritium exchange experiments presented here is dependent on proof that, under the conditions employed to permit exchange, propionyl-CoA was not only bound to the uncarboxylated enzyme but also properly oriented with respect to the catalytic site. Such proof was not obtained in subsequent experiments. Although propionyl-CoA and propionyl-pantetheine protection against PCMB may be taken as evidence that propionyl-CoA is bound to the uncarboxylated enzyme, no evidence has been seen that the necessary highly specific orientation takes place under these conditions. Furthermore, the increased sensitivity of the enzyme to PCMB (Table IV) in the presence of ATP (which would allow formation of enzyme-CO_2) heightens the possibility that, although bound, the propionyl-CoA may not be properly oriented on the uncarboxylated enzyme and that further orientation and α-carbon activation must await a conformational change concomitant with the formation of enzyme-CO_2.

As mentioned previously, the quantitative recovery of tritium incorporated in the reverse reaction upon recarboxylation (Table I, treatment D) is further evidence of the absolute stereospecificity of the reaction. If either the forward or the reverse reaction, or both, were not stereospecific with respect to the α-carbon, only 50% of the enzymatically incorporated tritium could have been recovered since the reaction was being forced to completion in both cases and thus each molecule could pass through the system only once.

The Active Site of Propionyl-CoA Carboxylase

The probability that enzyme sulfhydryl forms part of the active site of propionyl-CoA carboxylase is increased by the observation (Tables IV and V) that propionyl-CoA and propionyl-pantetheine protect the enzyme against the action of the sulfhydryl reagent PCMB. However, these effects can be attributed to mutual steric hinderance between substrate and PCMB. Propionyl-pantetheine may be binding to sulfhydryls both at and near the propionyl-CoA site since it is both carboxylated and a non-competitive inhibitor. If so, PCMB might inhibit native enzyme by binding only near, not at this site, and the site itself may be susceptible to PCMB attack only after formation of enzyme-CO_2. If this is the case, the number of active sulfhydryls could be compared with the number of active biotins in a given amount of enzyme by titrating with PCMB in the presence and absence of ATP using C^{14}-bicarbonate, and comparing the molar difference with the moles of enzyme-$C^{14}O_2$ formed determined from a separate aliquot.

Inhibition of the enzyme by TNBS suggests the involvement of amino groups at the active site of the enzyme. Here again, the possibility of non-specific steric hinderance by this reagent certainly exists.

Functional Groups of Propionyl-CoA and ATP

Information has been obtained concerning the participation of some functional groups of the CoA moiety of the substrate in enzyme-substrate interactions. The competitive nature of inhibition by 3'-AMP, in contrast to that shown by either 2'- or 5'-phospho-adenosine, together with a Ki approximating that of coenzyme A is indicative that the 3'-phosphate is of great importance in the binding, if not the orientation, of propionyl-CoA. It would also appear that the 3'-AMP portion of the molecule was ineffective in protecting against PCMB, while propionyl-pantetheine did protect. This is taken as an indication that sulfhydryl groups of the enzyme may be involved in an orientative rather than binding interaction with the acyl-pantoic acid portion of the substrate molecule, but not with the 3',5'-ADP portion. The comparison of Km's and Vm's for the three substrate propionyl-thioesters, and the non-carboxylation of propionyl-N-acetylcysteamine again suggests the requirement of the 3'-phosphate for full activity and also implicates the pantoic acid moiety rather than the entire pantetheine chain.

The association between enzyme and propionyl-CoA does not appear to involve covalent bonding of the type acyl-S-enzyme since the enzyme does not act as a propionyl- or methylmalonyl-transacylase. This association seems to be electrostatic in nature, and the enzyme-propionyl-CoA

complex is readily dissociable as judged by the failure to isolate enzyme-propionyl-CoA complex by gel filtration methods.

The nearly equivalent inhibitions obtained with the nucleoside triphosphates, none of which will replace the ATP requirement of the enzyme[3], suggests the involvement of the 5'-phosphates in binding ATP. While aliphatic amino compounds such as β-alanine and L-tryptophan are not inhibitory, aromatic amino compounds such as adenine, adenosine and (with the reservation previously noted) aniline seem to be strongly inhibitory. Adenosine shows a strong tendency toward competitive inhibition (Figure XII), and Kaziro et al have found ADP to be competitive with ATP. This suggests the involvement of the adenine amino group in the binding of either ATP or propionyl-CoA, or both. If this is the case, the lack of inhibition by cytosine and weak inhibition by CTP and GTP might be due to electrostatic repulsions between the acidic hydroxyls of these three compounds and the acidic group on the enzyme molecule to which the aromatic amine must bind, or to the reduction of the bacisity of the amino group by resonance effects following ionization of a substituent hydroxyl.

More definitive kinetic studies of the inhibitions reported here might result from studying their effects on transcarboxylations catalyzed by propionyl-CoA carboxylase such as the ethylmalonyl-CoA--propionyl-CoA transcarboxylation. Tritium exchange experiments carried out with this system would also be of interest.

Proposed Mechanism of α-Carbon Activation

Of the possible mechanisms for α-carbon activation enumerated in the introduction to the "Experimental" section, a concerted mechanism of the SE_2 type seems most reasonable since it does not involve the stabilization of a highly reactive carbanion intermediate. Such a reaction could be facilitated by partial abstraction of one of the α-hydrogens by a nitrogen base on the enzyme surface, thus stabilizing a weak negative charge at the α-carbon rendering it susceptible to electrophillic attack. It is conceivable that such a nitrogen base could be accessible to an α-hydrogen only when both hydrogens are above (or below, but not both) the rest of the propionyl chain with respect to the enzyme surface. Such limited accessibility resulting from the surface conformation of the enzyme would lead to the stereospecificity observed.

VII. SUMMARY

Propionyl-CoA carboxylase has been purified to a state of near homogeniety, and some of its enzymatic properties relating to substrate binding and mechanism of action have been studied. The enzyme was not found to catalyze the incorporation of solvent tritium at the α-carbon of propionyl-CoA in the absence of ATP. Absolute stereospecificity was observed with regard to which α-hydrogen is replaced during the addition of CO_2.

Investigation of the characteristics and interrelations of enzyme inhibition by propionyl-pantetheine, coenzyme A, adenosine-3'-monophosphate, adenosine-2'-monophosphate, adenosine-5'-monophosphate, and chloromercuribenzoate have led to the following conclusions about the nature of enzyme-substrate interactions. First, propionyl-CoA is bound to the enzyme surface largely through the agency of the 3'-phosphate group. Second, some portion of the pantoic acid moiety of CoA (possibly the hydroxyl group) is involved in a more orientative than binding interaction with the enzyme. Third, the adenine amino group, characteristic of both ATP and propionyl-CoA, has been implicated as essential for ATP activity, but not necessarily for CoA activity.

The enzyme-propionyl-CoA complex does not appear to be of the type acyl-S-enzyme, but rather seems to result from electrostatic interaction of enzyme and substrate. Inhibition of the enzyme by chloromercuribenzoate and 2,4,6-trinitrobenzenesulfonic acid seems to implicate enzyme sulfhydryl and amino groups respectively in such an interaction.

Some evidence has been seen that a conformational change may accompany the formation of enzyme-CO_2. Such a conformational change may be necessary before propionyl-CoA can be properly oriented with respect to the catalytic site of the enzyme, but is not required for binding of this substrate.

Possible mechanisms for the activation of the α-carbon of propionyl-CoA toward electrophillic attack have been discussed. An SE_2-type mechanism involving incomplete α-hydrogen abstraction by a nitrogen base on the enzyme surface is considered most probable and is discussed.

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STUDIES ON THE MECHANISM OF ACTION OF PROPIONYL-CoA CARBOXYLASE

C. S. Hegre

An Abstract

Propionyl-CoA carboxylase has been purified to a state of near homogeniety, and some of its enzymatic properties relating to substrate binding and mechanism of action have been studied. The enzyme was not found to catalyze the incorporation of solvent tritium at the α-carbon of propionyl-CoA in the absence of ATP. Absolute stereospecificity was observed with regard to which α-hydrogen is replaced during the addition of CO_2.

Investigation of the characteristics and interrelations of enzyme inhibition by propionyl-pantetheine, coenzyme A, adenosine-3'-monophosphate, adenosine-2'-monophosphate, adenosine-5'-monophosphate, and chloromercuribenzoate have led to the following conclusions about the nature of enzyme-substrate interactions. First, propionyl-CoA is bound to the enzyme surface largely through the agency of the 3'-phosphate group. Second, some portion of the pantoic acid moiety of CoA (possibly the hydroxyl group) is involved in a more orientative than binding interaction with the enzyme. Third, the adenine amino group, characteristic of both ATP and propionyl-CoA, has been implicated as essential for ATP activity, but not necessarily for CoA activity.

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