STUDIES ON THE ISOLATION AND PURIFICATION OF ACETYL CoA SYNTHETASE FROM MITOCHONDRIA OF LACTATING BOVINE MAMMARY GLAND

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY
SHAHIDA QURESHI

1971

This is to certify that the

thesis entitled

STUDIES ON THE ISOLATION AND PURIFICATION
OF ACETYL CoA SYNTHETASE FROM
MITOCHONDRIA OF LACTATING
BOVINE MAMMARY GLAND

presented by

Shahida Qureshi

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry and
Dairy Science

Robert M. Cook

Major professor

Date November, 1971 William A. Black

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ABSTRACT

STUDIES ON THE ISOLATION AND PURIFICATION OF ACETYL CoA SYNTHETASE FROM MITOCHONDRIA OF LACTATING BOVINE MAMMARY GLAND

By

Shahida Qureshi

This work was undertaken to develop a method for purifying acetyl-CoA synthetase from lactating bovine mammary gland mitochondria. The different purification methods studied were ammonium sulphate fractionation, adsorption on alumina C_γ gel, chromatography on TEAE cellulose using $KHCO_3$ or Tris-HCl buffers, DEAE cellulose, carboxymethyl cellulose, Sephadex G-100 and G-200, Bio-gel P-100 and P-200, ultracentrifugation in sucrose density gradient, adsorption chromatography on calcium phosphate gel, and polyacrylamide gel electrophoresis.

The method that yielded an enzyme preparation more than 90 per cent pure was ammonium sulphate fractionation followed by chromatography on DEAE cellulose (DE-23), followed by chromatography on DEAE cellulose (DE-52), and finally chromatography on calcium phosphate gel using potassium phosphate buffers. The enzyme was concentrated,

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transferred to Tris-HCl buffer, and stored at -60° C. Substrate specificity studies showed the enzyme to be most active on acrylate followed by acetate, propionate, and maleate. Michaelis-Menten constants for the various cofactors and substrate were 6.51×10^{-4} M, 2.92×10^{-4} M, 2.24×10^{-4} M, and 6.1×10^{-4} M for Mg, CoA, ATP, and acetate, respectively. The molecular weight of the enzyme is 63,000. AMP at high levels inhibited enzyme activity. Acetyl-CoA synthetase, from bovine mammary gland mitochondria, exhibits a strong tendency to aggregate. This was shown by gel filtration, sedimentation equilibrium, and gel electrophoresis studies. The enzyme is not active in the aggregated form. The presence of salt in the enzyme preparation tends to prevent aggregation. The exact nature of this phenomenon is not understood clearly at present.

STUDIES ON THE ISOLATION AND PURIFICATION OF ACETYL CoA SYNTHETASE FROM MITOCHONDRIA OF LACTATING BOVINE MAMMARY GLAND

By

Shahida Qureshi

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY

Departments of Biochemistry and Dairy Science

1971

To my parents,

Zakia Khatoon and Abdul Shakoor Quraishi,
whose many sacrifices have so meaningfully
enriched my life,

and my husband,

Wahid Hosain, whose patience, understanding
and encouragement made this work possible.

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I would like to take this opportunity to extend my heart felt gratitude to Dr. Robert M. Cook, my academic advisor, without whose encouragement, guidance, and patient understanding this work would not have been possible. I also appreciate the kindness and understanding he showed for the difficulties a foreign student may encounter. Thanks are due also to Dr. William W. Wells for graciously consenting to be my academic advisor in the Department of Biochemistry.

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I am indebted to my brother, Aslam, for drawing some of the figures included in this thesis.

Last, but not least, many thanks are due to my husband, Wahid, and my brother, Asad Quraishi, for being so patient, understanding, and helpful during the course of this study.

VITAE

The author, Shahida Qureshi, was born in Alwar, India on March 16, 1940. In 1947 her family migrated to Karachi, Pakistan. She graduated from Government Girls High School in 1954. She spent two years in Central Government College for Women and then transferred to D. J. Government Science College where she graduated in 1958, with Zoology and Chemistry as majors. She obtained an M.Sc. in Chemistry (organic) from the University of Karachi in 1960. She then joined the Pakistan Council of Scientific and Industrial Research as a research assistant. She came to the United States of America on August 10, 1965, and joined the Agricultural Biochemistry and Soils Department of the University of Idaho as a graduate student. In 1966 she transferred to Michigan State University and joined the Biochemistry and Dairy Science Departments to work for her doctorate.

On December 20, 1969, she married Wahid Hosain Qureshi.

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ATP	adenosine triphosphate
GTP	guanosine triphosphate
CTP	cytidine triphosphate
ITP	inosine triphosphate
UTP	uridine triphosphate
AMP	adenosine monophosphate
CoA	Coenzyme A
K_m	Michaelis-Menten constant
GSH	glutathione
2-ME	2-mercaptoethanol
DEAE	diethylaminoethyl
TEAE	triethylaminoethyl
BSA	bovine serum albumin
TCA	trichloroacetic acid
NADH	nicotinamide adenine dinucleotide, reduced form
EDTA	ethylene diamine tetraacetic acid
Tris	tris (hydroxymethyl) amino methane
$P-P_i$	pyrophosphate

CHAPTER I

INTRODUCTION

In ruminant animals the microbial population in the rument ferments carbohydrates to acetate, propionate, butyrate, carbon dioxide, and methane. Acetate is produced in the largest amount and is the principal source of energy in the ruminant. In fed ruminants the concentration of acetate in whole blood is usually 1 to 2 mM. Acetate not only is a major source of energy in ruminants, but it plays a major role in lipogenesis, comparable to the one played by glucose in nonruminants. The activity of ATP-citrate lyase is negligible in ruminants. Therefore, glucose can only supply limited amounts of acetyl-CoA for fatty acid synthesis.

Before being utilized by the cell acetate must be activated (covalently linked to the thiol group of coenzyme A). This reaction is catalyzed by the thiokinase, acetyl-CoA synthetase {acetate: CoA ligase (AMP) (6.2.1.1)}.

$$CH_3\text{-}COOH + ATP + CoA\text{-}SH \overset{Mg^{++}}{\rightleftharpoons} CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}SCoA + AMP + P\text{-}P_i$$

Acetyl CoA synthetase is widely distributed in plants, animals, and microorganisms. Kinases such as thiokinases are generally considered to be nonequilibrium enzymes and, therefore, they can function in metabolic control processes. It is proposed that the thiokinase, acetyl-CoA synthetase, is an important rate limiting step in acetate metabolism in ruminants. In order to fully elucidate the role acetyl-CoA synthetase plays in control, the enzyme needs to be purified so that its physical and catalytic properties can be determined.

The purpose of this work is to study methods for purifying acetyl-CoA synthetase from lactating bovine mammary gland mitochondria.

The lactating mammary gland was chosen as a source of the enzyme because of the high rate of acetate utilization for both oxidative and synthetic reactions and because milk synthesis is of major economic importance.

CHAPTER II

LITERATURE REVIEW

Acetyl-CoA Synthetase

General

The enzyme acetyl-CoA synthetase belongs to the general class of enzymes [acid: CoA ligase (AMP) (6.2.1)] which catalyze the activation of a free fatty acid to its corresponding thioester according to the following general reaction:

$$\text{Fatty acid} + \text{ATP} + \text{CoA-SH} \overset{Mg^{++}}{\rightleftharpoons} \text{Fatty acyl-CoA} + \text{AMP} + \text{P-P}_i$$

Acetyl-CoA synthetase is widely distributed in nature. It has been partially purified from many sources like yeast, bacteria, higher plants, mammalian organs, and pigeon liver.

The activation of acetate was reported for the first time by Nachmansohn and Machado (1943). They prepared the activating system from rat brain homogenate and used it for acetylating choline.

Lipmann and Tuttle (1945) showed that when ATP and acetate were incubated with fresh pigeon liver extracts and hydroxylamine added in low concentrations, an appreciable amount of acethydroxamate accumulated in the reaction mixture.

Using the same enzyme source Stern and Ochoa (1950) observed citrate synthesis from acetate, ATP, and oxaloacetate. The reaction required Mg^{++} and CoA and was thought to proceed through two steps:

(1) ATP + acetate $\longrightarrow$ "active acetate"

(2) "active acetate $\longrightarrow$ citrate

+ oxaloacetate

Lynen et al. (1951) identified "active acetate" as acetyl-CoA, thus elucidating the function of CoA as an acyl carrier.

The enzyme was partially purified, for the first time from beef heart and pig heart mitochondria (Hele, 1954; Beinert et al., 1953). About the same time, partial purification of acetyl-CoA synthetase was also achieved from spinach leaf mitochondria (Millerd et al., 1954) and later on from yeast (Berg, 1956). It was finally crystallized from beef heart mitochondria with 9% recovery and 64 fold purification (Webster, 1965). The enzyme was also studied in rabbit heart myocardium (Severin, 1967), rat liver (Aas, 1968), potato tuber (Huang et al., 1970), and yeast (DeVincenzi, 1970).

The purified enzyme showed instability after dilution or dialysis and lost activity if kept at 4°C. (Hele, 1954; Webster, 1963). It was also reported to be heat labile (Huang et al., 1970). Crude extracts of spinach leaf enzyme required GSH but the purified enzyme did not (Millerd et al., 1954). The beef heart enzyme was found to be more stable in the presence of 2-mercapto-ethanol (Webster, 1965). The rabbit heart enzyme was reported to have 5 sulfhydryl groups which were not required for enzyme activity (Severin, 1967).

The formation of acetyl-CoA from acetate, ATP, and CoA was reversible; if $P-P_i$ was added acetyl-CoA disappeared (Hele, 1954; Beinert et al., 1953). The enzyme activated acetate, propionate, and acrylate (Hele, 1954; Webster, 1963; Aas, 1968). Other acids like butyrate, fluoroacetate, succinate, acetoacetate, formate, malonate, glycine, cyanoacetate, oxaloacetate, and glycollate were not activated (Webster, 1963; Hele, 1954; Huang et al., 1970). The spinach enzyme did not activate propionate, but butyrate, succinate, valerate, and caproate were activated at slow rates.

The Michaelis-Menten constants, for different substrates, for the enzyme from various sources are given in Table 1. The absolute requirements of Mg^{++} was established by Berg (1956). In addition to that a

Table 1

Properties of Acetyl-CoA Synthetase

Source	Reference	K_m (M)					
		Acetate	Propionate	Acrylate	CoA	ATP	$MgCl_2$
Beef heart mito.	Hele, 1954	1.42×10^{-3}	5.0×10^{-3}	2.9×10^{-3}			
Beef heart mito.	Webster, 1963, 1965	8.00×10^{-4}	1.1×10^{-2}	1.1×10^{-2}	4.00×10^{-4}	9.00×10^{-4}	1.40×10^{-3}
Rat liver	Aas et al., 1968	4.40×10^{-3}	4.0×10^{-3}				
Beef heart mito.	Farrar, 1970	6.50×10^{-4}			2.10×10^{-4}	7.10×10^{-4}	(Mg ATP)
Yeast	DeVincenzi, 1970						
aerobic enzyme		3.00×10^{-4}				1.00×10^{-3}	
anaerobic enzyme		3.00×10^{-3}				1.00×10^{-2}	
Potato	Huang et al., 1970						
A-1		1.67×10^{-5}			5.00×10^{-5}	3.57×10^{-5}	3.45×10^{-4}
A-2		2.00×10^{-5}			2.90×10^{-6}	3.85×10^{-5}	2.50×10^{-4}
A-3		2.00×10^{-5}			1.67×10^{-5}	3.85×10^{-5}	3.45×10^{-4}
A-4		5.00×10^{-5}			1.67×10^{-5}	3.85×10^{-5}	3.45×10^{-4}
A-5		2.20×10^{-5}			1.67×10^{-5}	3.50×10^{-5}	4.00×10^{-3}

second divalent cation and a monovalent cation were also needed. Tris, NH_4^+ and K^+ were stimulating while Na^+ and Li^+ were inhibitory (Webster, 1965).

Mechanism

The mechanism of acetyl-CoA formation from acetate, ATP, and CoA, in the presence of Mg^{++} was studied by Berg (1956), using the yeast enzyme. The reaction was diphasic; the initial step was the formation of acetyl-AMP, which then reacted with CoA to form acetyl-CoA.

(1) acetate + ATP + CoA-SH $\rightleftharpoons$ acetyl-AMP + $P\text{-}P_i$

(2) acetyl-AMP + CoA $\rightleftharpoons$ acetyl-CoA + AMP

Mg^{++} was required for the first partial reaction. Acetyl-AMP was chemically synthesized which, upon incubation with the enzyme, $P\text{-}P_i$ and Mg^{++}, gave rise to ATP and acetate. AMP and acetyl-CoA were formed when acetyl-AMP was incubated with CoA. The attempt to isolate acetyl-AMP from the reaction mixture was not successful and, therefore, the intermediate was considered to be tightly bound to the enzyme. Webster (1962) did, however, isolate acetyl-^{14}C-AMP from the reaction mixture containing ^{14}C-labelled acetate, ATP, $MgCl_2$, and an excess of acetyl-CoA synthetase.

Berg (1956) showed that the yeast enzyme followed a Bi Uni Uni Bi Ping Pong mechanism. One of the five different forms of the potato enzyme was reported to follow an

Iso Bi Uni Uni Bi Ping Pong mechanism and at least two catalyzed the enzymatic activation of acetate by ordered Ter Ter mechanism (Huang et al., 1970). To quote the authors, "A single reaction is catalyzed by an enzyme which exists in different forms and each form of the enzyme catalyzes the reaction by a different mechanism."

Farrar (1970) carried out kinetic studies on acetyl-CoA synthetase isolated and partially purified from beef heart mitochondria. The data were consistent with a Bi Uni Uni Bi Ping Pong mechanism as proposed earlier by Berg (1956). According to this mechanism acetate and ATP were added to the enzyme first. Pyrophosphate was released. Next CoA was added, and finally AMP and acetyl-CoA were released.

Molecular Weight

The molecular weight first reported for the partially purified acetyl-CoA synthetase from beef heart mitochondria was between 40,000 and 80,000 (Hele, 1954). Webster (1963) reported a sedimentation constant of 4.45S which corresponded to a molecular weight of 70,000. However, the sedimentation constant reported for the freshly purified crystalline enzyme was 3.5S which agreed well with the value, 3.86S, for the rabbit heart myocardium enzyme (Severin, 1967). The molecular weight calculated from the sedimentation equilibrium experiment was 30,570 at the meniscus and 55,740 at the bottom of

the cell. The weight average molecular weight was calculated to be 35,790 while the z-average molecular weight was 71,000. The molecular weight calculated by the binding studies of acetyl-AMP to the enzyme was between the range of 31,000-34,000, assuming a 1:1 stoichiometry between enzyme and acetyl-AMP (Webster, 1962).

The other molecular weight values reported for acetyl-CoA synthetase were 59,500 for the potato enzyme (Huang et al., 1970) and 130,000 for the yeast enzyme (DeVincenzi, 1970).

Multiple Forms

Multiple forms of acetyl-CoA synthetase were reported in yeast (DeVincenzi, 1970) and potato tuber (Huang, 1970). The enzyme activity was reported to be associated with mitochondria both in yeast and potato tuber as well as with yeast microsomes. Three different forms of acetyl-CoA synthetase were reported in yeast. Two forms were associated with the microsomes of the standing and 24-hour-old aerobic culture while the third was localized in the mitochondria of 48-hour-old culture. The three forms had the same molecular weight, electrophoretic mobility, pH optimum and were inhibited by Na^+ to the same extent. They differed, however, in their catalytic properties, namely, K_m values, for acetate and ATP, and catalytic behavior toward propionate.

The potato enzyme existed in five different forms. They had identical molecular weight, pH optimum, and were specific for acetate. They had similar K_m values for ATP and Mg^{++} but different values for acetate and CoA.

Intracellular Localization

Acetyl-CoA synthetase activity was found in the cytosol of adipose and mammary gland tissue of ruminants (Hanson et al., 1967). The enzyme was localized predominantly in the mitochondria in lung and liver, while in kidney it was equally divided between mitochondria and cytosol. In heart and mammary gland two-thirds of the enzyme activity was in the cytosol and one-third in the mitochondria (Quraishi et al., 1971).

Aas and Bremer (1968) have reported the distribution of acetyl-CoA synthetase in rat liver. They found that 51% of the total activity was associated with the mitochondria and about 22% was found in the particle free supernatent.

Intramitochondrial Localization

To investigate the submitochondrial localization of these enzymes, the mitochondria were disrupted and the enzyme activity determined in relation to marker enzymes (Aas and Bremer, 1968). L-glutamate dehydrogenase is for the matrix, D-β-hydroxybutyrate dehydrogenase and

carnitine-palmitoyl transferase for the inner membrane, and long-chain acyl-CoA synthetase for the outer membrane. Acetyl-CoA synthetase activity was localized in the matrix of the mitochondria.

Medium-Chain Fatty Acid acyl-CoA Synthetase

Acyl-CoA synthetase (ATP dependent) (EC.6.2.1.2) catalyzes the activation of medium-chain (C_4-C_{12}) fatty acids and does not activate acetate. It was isolated and partially purified for the first time from beef liver mitochondria (Mahler et al., 1953). The enzyme was also isolated from hog liver (Jenck et al., 1957), beef heart mitochondria (Webster, 1965), ox liver particles (Bar-tana et al., 1968), and from beef liver (Graham et al., 1969). The enzyme required a divalent metal ion for maximal activity but no monovalent cation was required (Webster, 1965). AMP and ADP inhibited the reaction. The enzyme was heat unstable and was sensitive to extremes of pH (Mahler et al., 1953). The enzyme prepared from ox liver acetone powder separated into two enzymatically active protein peaks upon chromatography on Bio-Gel and DEAE-Sephadex columns (Bar-tana et al., 1968). One of the enzymes catalyzed the reaction by "Bi Uni Uni Bi Ping Pong" mechanism, as suggested by Berg (1956) for acetyl-CoA synthetase, while the other followed an ordered Ter Ter mechanism. The octanoyl-CoA synthetase from beef liver

also followed a Bi Uni Uni Bi Ping Pong mechanism (Graham et al., 1969).

The medium-chain fatty acyl-CoA synthetase is localized in the matrix of the mitochondria (Aas et al., 1968).

Long-Chain Fatty Acid acyl-CoA Synthetase

Kornberg and Pricer (1953) were first to demonstrate the long-chain fatty acyl-CoA synthetase (acid: CoA ligase (AMP), EC 6.2.1.3; trivial name: Palmitoyl-CoA synthetase) in guinea-pig and rat liver microsomes as well as the cytosol. The preparation was active on fatty acids ranging from C_5 to C_{22}. Acetate, propionate, and butyrate were not activated. Maximum enzyme activity was observed using C_{12}. The enzymatic activation followed the identical over-all equation as for acetate or medium-chain fatty acid activation.

Farstad et al. (1966) presented evidence for the bimodal distribution of palmitoyl-CoA synthetase, i.e., both in mitochondria and microsomes of rat liver. Approximately 70% of the total palmitoyl-CoA synthetase activity is found in microsomes and approximately 30% in the mitochondria. The enzyme is firmly bound to membranes both in microsomes and in mitochondria.

Allman et al. (1966) have shown that the enzyme is located in the outer membrane of beef liver mitochondria.

Lippel and Beattie (1970) also investigated the submitochondrial localization of palmitoyl-CoA synthetase. The activity was found mostly to reside in the outer mitochondrial membrane and some in the inner membrane, the ratio between the two being (O.M./I.M.) 26. Skrede and Bremer (1970) confirmed the above findings of Lippel and Beattie (1970).

GTP-Dependent Fatty Acid Synthetase

Rossi and Gibson (1964) were first to demonstrate the GTP-specific thiokinase from beef liver mitochondria. The enzyme catalyzes the activation of fatty acids according to the following reaction:

$$GTP + R.COOH + CoA\text{-}SH \rightleftharpoons R\text{-}CO\text{-}S\text{-}CoA + GDP + P_i$$

Fatty acids of chain length C_4 to C_{12} were active substrates for the GTP-kinase system. CTP, UTP, and ATP were inactive, but ITP replaced GTP.

The same enzyme was reported by Galzigna et al. (1966) from rat liver mitochondria. This enzyme catalyzed the activation of both short- and long-chain fatty acids. They found that the GTP-dependent activation was sensitive to P_i and F^-. The difference between the substrate specificity of the beef liver enzyme and that of rat liver was shown to be due to the use of organic solvent in isolating the beef liver enzyme. It

was shown by Sartorelli (1967) that lecithin was bound to the GTP-specific enzyme and its removal with organic solvents affected the enzyme activity as well as its substrate specificity. The enzyme was further purified by Galzigna et al. (1967). They reported one band on polyacrylamide gel electrophroesis. The molecular weight was estimated to be around 20,000 and the sedimentation coefficient was 1.0S.

Rossi et al. (1970) identified 4'-phosphopantetheine as the cofactor which was required for enzyme activity. The cofactor was bound to the apoenzyme in a molecular ratio of 1:1 by weak secondary bonds. The GTP-dependent fatty acid synthetase is located in the outer membrane of beef liver mitochondria (Allman et al., 1966).

Methods Used to Purify acyl-CoA Synthetases

Several different methods have been used to partially purify acyl-CoA synthetases. Fractional precipitation by protamine was used for the yeast enzyme (Berg, 1956), ammonium sulphate precipitation in the case of heart and liver enzymes (Mahler et al., 1953; Hele, 1954) and various column chromatography techniques (Webster, 1963 and 1965; Severin et al., 1967; Huang et al., 1970; Farrar, 1970). The acid pH step, adsorption by calcium phosphate gel, and hydroxylapatite have also

been used to purify both ATP-dependent and GTP-dependent acyl-CoA synthetases (Galzigna et al., 1967; Huang et al., 1970).

The most extensively purified enzyme among the acyl-CoA synthetases is acetyl-CoA synthetase, which was isolated and purified from bovine heart mitochondria (Webster, 1965). The various steps used to purify this enzyme were: ammonium sulphate precipitation, adsorption by alumina C_γ-gel, chromatography using Sephadex and TEAE-cellulose columns, and finally crystallization. That the enzyme protein was not homogeneous was evident by the type of data obtained by the sedimentation equilibrium experiment. The enzyme showed four protein bands upon gel electrophoresis (personal communication).

Farrar (1970) has purified acetyl-CoA synthetase from beef heart mitochondria using a different procedure. After the initial ammonium sulphate precipitation steps the enzyme was taken up in 0.02 M $KHCO_3$, $NiCl_2$, and acetyl-adenylate added to it before the RNA-pH precipitation step. Yeast RNA was added to the enzyme and the pH of the solution lowered to 4.7, and kept there for 3 minutes. The cloudy solution was then centrifuged and the precipitate taken up in $KHCO_3$ and the pH raised to 6.2. Insoluble material was removed by centrifugation. After two more steps of ammonium sulphate precipitation the protein was chromatographed on TEAE-collulose column using a linear KCl gradient

(0 to 0.25 M KCl) in $KHCO_3$. The eluted enzyme was precipitated by ammonium sulphate. The purification procedure resulted in 41.6 fold purification of the enzyme with 2.3% yield. The specific activity of the purified enzyme was, however, less than one-third of the value reported by Webster (1965) for both the crystalline enzyme and that from the Sephadex column. Gel electrophoresis of the purified enzyme revealed several protein bands (personal communication).

The potato enzyme (Huang et al., 1970) has been partially purified by ammonium sulphate precipitation, DEAE-cellulose, and hydroxylapatite column chromatography. However, the enzyme was far from being a homogeneous protein; to quote the authors, "a homogeneous potato acetyl-CoA synthetase is at present unavailable." Also, the attempts to purify the long-chain fatty acyl-CoA synthetase have not been successful (Farstad, 1968).

It is evident that none of the enzyme preparations of acyl-CoA synthetase, from various sources, have been purified to homogeneity. Therefore, the present knowledge about their physical properties is somewhat speculative, and, hence, further studies on purification are needed.

CHAPTER III

EXPERIMENTAL PROCEDURE

Materials and Methods

Reagents

All chemicals were purchased from commercial sources. Acetyl-CoA, CoA, ATP, Tris (Trizma base), and other nucleotides were purchased from Sigma Chemical Company, St. Louis, Missouri. TEAE-cellulose was purchased from Brown Company, Berlin, New Hampshire. DEAE-celluloses (DE-23 and DE-52) were purchased from Whatmann, W and R Balston Ltd, England. Ovalbumin, Sephadex gels, and Sephadex columns were purchased from Pharmacia Fine Chemical Inc., Piscataway, New Jersey. Bio-gels and cellex-CM were obtained from Bio-Rad Laboratories, Richmond, California. Calcium phosphate gel was prepared according to the method of Miller et al. (1965). Chemicals for gel electrophoresis were purchased from Canal Industrial Corporation, Rockville, Maryland. 2-Mercaptoethanol was from Eastman Organic Chemicals, Rochester, New York. Ammonium sulphate used throughout the experiment was a special enzyme research grade from

General Biochemicals, Chagrin Falls, Ohio. Ampholine, Carrier Ampholytes was from LKB-Produkter AB, Bromma, Sweden. The dialysis tubing was purchased from Union Carbide Corporation, New York, New York.

Spectrophotometery

The Coleman Junior Spectrophotometer, the Beckman DB-G Spectrophotometer, and the Gilford Model 2400 Spectrophotometer equipped with Gilford automatic sample changer were used for spectrophotometric measurements.

Enzyme Assay

Acetyl-coenzyme A synthetase activity was measured by the acetate-dependent disappearance of the free sulfhydryl group of coenzyme A as described by Mahler, Wakil, and Bock (1953). In a total volume of 0.20 ml, the complete reaction mixture contained 5 μmoles of K-acetate, 1.1 μmoles of ATP, 1.5 μmoles of $MgCl_2$, 0.17 μmoles of CoA-SH, 16 μmoles of Tris (hydroxymethyl) amino methane hydrochloride buffer. From 25 to 4 μg of enzyme protein were used. Blank tubes did not contain acetate.

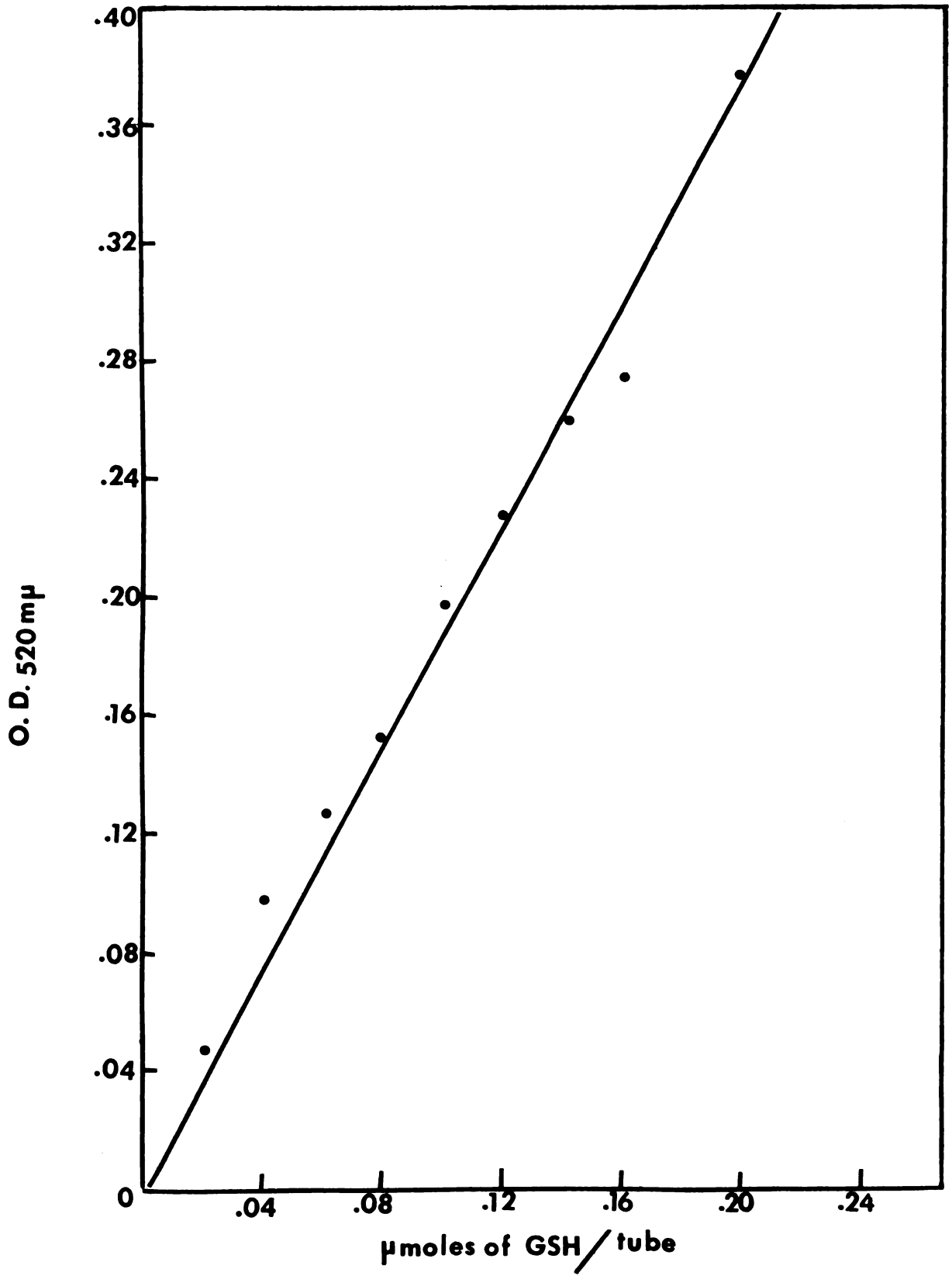
All tubes were preincubated for one minute at 37°. After the enzyme addition the incubation period was three minutes at 37°. In some assays the incubation period was for 10 minutes. The reaction was terminated by the addition of 2.8 ml of the nitroprusside color reagent

prepared by the method of Grunert and Phillips (1951). The optical density was read after 30 seconds at 520 mμ. The difference in optical density between the blank and the complete reaction mixture is the measure of enzyme activity. Enzyme concentration is adjusted to give a difference in optical density between 0.075 and 0.250. Within this range ΔOD_{520} is proportional to enzyme concentration. Under the assay conditions a difference of 0.185 in optical density corresponds to the disappearance of 0.10 μm of CoA. Glutathione was used as the reference standard (Figure 1). One unit of enzyme activity is defined as the amount which catalyzes the disappearance of 1 μmole of coenzyme-A per hour under standard assay conditions. The ΔO.D. was converted to units by multiplying by a factor of 10.81 for a 3-minute assay, and by 3.243 for a 10-minute assay. Specific activity is expressed in units of enzyme activity per mg of protein.

Protein Determination

Protein was measured by the Lowry (1951) method using bovine serum albumin (BSA) as a reference standard. The purified enzyme protein was measured by the absorbance at 280 and 260 μm according to the method of Warburg and Christian (1941).

FIGURE 1.--Glutathione standard curve.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Sucrose Density Centrifugation

The molecular weight of acetyl-CoA synthetase was determined by the method of Martin and Ames (1961) using a Beckman Model L2 65B preparative ultracentrifuge. Linear sucrose gradient of from 5 to 20 per cent sucrose containing 0.05 M Tris-HCl (pH 7.5) in a volume of 4.4 ml was prepared in cellulose nitrate tubes. 43.8 µg of enzyme protein in 0.1 ml of the same buffer were applied to the top of the gradient and centrifuged at 50,000 rpm (246,000 xg) for 10 hours at 4 C° in a Beckman SW-56 rotor. Ovalbumin was used as a marker. At the end of the run fractions were collected by puncturing the bottom of the tube. Eight drops per fraction were collected. Ovalbumin was measured by the absorption at 210 mµ using BSA as the standard (Tombs, 1959). The fractions from the tube containing the enzyme were assayed for activity.

The above technique was also employed to determine the sedimentation coefficient of acetyl-CoA synthetase using E. Coli t-RNA as a marker. This centrifugation was carried out in a Beckman SW-39 rotor at 35,000 rpm for 28 hours. t-RNA was determined by measuring the absorption at 260 mµ.

Electrophoresis

Polyacrylamide Disc gel (5.5% acrylamide) electrophoresis was performed by the method of Davis (1964).

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The acrylamide and bisacrylamide were recrystallized from chloroform and acetone respectively, according to the method of Loening (1957). The 5.5% gel was prepared as follows: one part solution A [IN HCL, 48 ml; Tris, 36.3 g; Temed (N, N, N^1, N^1 - tetra methyl ethylene diamine), 0.23 ml; water to 100 ml] was mixed with two parts of solution C [Acrylamide, 22.2 g, BIS (N, N^1 - methylenebisacrylamide), 0.30 g; H_2O to make 100 ml], four parts H_2O and one part of solution E [Riboflavin, 4 mg/100 ml]. One end of each tube (5 mm ID x 75 mm) was sealed using parafilm. 1.6 ml of the above solution were transferred to each tube. The gel solution polymerized within 25-40 minutes under fluorescent light.

The protein sample was made denser by adding a few crystals of sucrose. In addition, 5 µl of 0.05% bromophenol blue dye were also added to the sample. The buffer used was 0.025 M Tris-HCL - 0.20 M Glycine, pH 8.3. All gel tubes were pre-electrophoresed for 15 minutes before the sample addition. The electrophoresis was carried out with a current of 6 ma/tube for 30' at 4° C. After electrophoresis the gel columns were stained for proteins either with 0.5% Amido-black in 7% acetic acid for 1 hour and then destained electrophoretically, or with coomassie blue according to the method of Chrambach et al. (1967). The gels were kept in 10% TCA for 15 minutes and then put in the staining solution [0.4 g of

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coomassie blue dye in 100 ml of 20% methanol and 10% TCA] for 12 hours. The gels were then rinsed with 33% methanol and 10% TCA for 6 hours and finally transferred to 10% TCA for 10-12 hours.

To locate the enzyme activity the gel columns that were not stained for protein were sliced into 2 mm segments and each segment was placed in 0.1 ml of 0.2 M Tris-HCl buffer, pH 8.6. The extract was assayed for enzyme activity the next day.

Ultracentrifugation

The sedimentation equilibrium experiment was performed using a Spinco Model E analytical ultracentrifuge equipped with mechanical speed control and Rayleigh Interference optics. Double sector cells with an An-D rotor were used. The enzyme protein from a TEAE-cellulose column was diluted to 0.5 mg/ml with 0.2 M Tris-HCl buffer, pH 8.6. After dialysis against 100 volumes of the same buffer for 5 hours, 0.11 ml (50 µg protein) of the sample were transferred to the cell. The centrifugation was carried out at 20,410 rpm at 4° C. for 18 hours, according to Yphantis (1964). Protein was determined by 280/260 ratio.

Iso-Electric Point Determination

The isoelectric focusing experiment was carried out to achieve further purification of acetyl-CoA

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synthetase isolated from the TEAE cellulose column, and to determine the Isoelectric point of the enzyme. For this purpose 110 ml column was used at a temperature of 4° C. The experiment was carried out according to the procedure outlined in the LKB brochure.[1] The anode solution was prepared by diluting 0.1 ml of H_2SO_4 to 10 ml with water. The cathode solution was 0.4 ml of ethylenediamine and 12 g of sucrose dissolved in 14 ml of water. The anode was located at the top and the cathode at the bottom. Twenty-four tubes were prepared by mixing different amounts of the dense and less dense solution. Each tube contained 4.6 ml. The dense solution was prepared by dissolving 1.9 ml of 40% ampholyte, pH 3 to 10, and 28 g of sucrose in 42 ml of water. The less dense solution was prepared by diluting 0.6 ml of the ampholyte to 60 ml of water. Two mg of enzyme protein from the TEAE-cellulose fraction were added to tube #11. The sucrose density column was then prepared by carefully layering these tubes. The experiment was carried out for 42 hours using a potential of 300 volts. At the end of the experiment 2 ml fractions were collected. Aliquots of the fractions were analyzed for acetyl-CoA synthetase activity. Also, the pH of every fifth fraction was determined. In one experiment the ampholytes were

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[1]LKB Instruments Inc., 12221 Parklawn Drive, Rockville, Maryland 20852.

removed, from each fraction by dialysis, and protein was determined by 280/260 ratio.

Fractionation of Bovine Mammary Gland

The mammary gland tissue used in these studies was obtained from five different lactating Holstein cows. The cows were fed normal rations and were slaughtered at peak lactation. Acetyl-CoA synthetase is known to have highest activity at peak lactation. The activity diminishes considerably when the cows dry up. The mammary gland tissue from cows 329 and 330 was used in the early studies. These cows were fed a high grain and low roughage ration. Cow 330 was also fed magnesium oxide. The second enzyme preparation was from cow 444 and cow 445. Cow 445 was fed hay and concentrates while 444 was fed only concentrates. The tissue used in the final studies was obtained from cow 1063. This cow was fed a normal ration of corn silage, alfalfa hay, and grain.

All of the fractionation steps were conducted at 4° C. The enzyme isolation procedure used through #5 ammonium sulphate fraction was that described by Webster (1965).

Isolation of Mitochondria

Mammary gland tissue was immediately chilled in ice after slaughter. After removal of fat and connective

tissue, the tissue was cut into thin long strips and ground in a meat grinder. One kg of the tissue was then homogenized in 2 liters of 0.13 M KCl, adjusted to pH 8 with KOH, using a one-gallon Waring blender at high speed for 20 seconds and then at low speed for 20 seconds. The homogenate was then centrifuged in an MSE six-liter centrifuge at 1200 xg for 15 minutes. The 1200 xg supernatant was filtered through several layers of cheese cloth and centrifuged in a Sorvall RC-2B at 20,200 xg for 25 minutes. The top fluffy layer was discarded and the mitochondrial pellet was taken up in 0.13 M KCl [1 gm (wet weight) per 7 ml of KCl] and briefly homogenized. The resulting suspension was frozen in plastic bottles at -20° C. Before freezing an aliquot of the 20,200 g pellet was characterized for intactness, observed by NADH uptake, phosphorylating activity and purity (KCN sensitive) using a polarograph equipped with a clark oxygen electrode. The preparations showed good phosphorylating activity and oxygen uptake was inhibited by KCN. There was slight electron transport when NADH was the substrate. This data is taken as evidence that 20,200 xg pellet is respiring mitochondria.

Ammonium Sulfate Fractionation

The mitochondria were thawed rapidly by swirling in a water bath at 40° C. After each thaw the pH was adjusted to 8 with 1 N NH_4OH. This process was repeated

three times. After the last thaw the mitochondrial suspension was centrifuged at 20,200 xg for 45 minutes. The supernatant was adjusted to pH 8 and to 0.1 M in 2-mercaptoethanol. To the fraction thus obtained, the mitochondrial extract, 21 gms of ammonium sulphate per 100 ml were added slowly and with stirring. The solution was adjusted to pH 8 with IN NH_4OH, stirred for one hour and then centrifuged at 20,200 xg for 10 minutes. An additional 23.5 gms of ammonium sulphate were added to each 100 ml of the supernatant. The precipitate obtained was recentrifuged to remove excess ammonium sulphate, and was then taken up in 0.02 M $KHCO_3$ and stored at -60° C. (Figure 2).

TEAE-Cellulose Chromatography

TEAE-cellulose was washed according to Whatman's procedure[1] and then equilibrated in 0.05 M Tris-HCl buffer, pH 7.8. In some cases 0.005 M Tris-HCl buffer was used. (Also in the initial studies 0.02 M $KHCO_3$ buffer was used.) The column dimensions were 1.7 cm x 42 cm. The column was washed overnight with the buffer at a flow rate of 30 ml/hr.

About 200 mg of ammonium sulphate precipitate were dialyzed against 0.05 M Tris-HCl buffer, pH 7.8. The

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[1] Advanced Ion-exchange Celluloses Laboratory Manual (H. Reeve Angel and Co. Ltd., 14 New Bridge, London, England.)

FIGURE 2.--Fractionation of bovine mammary gland tissue.

Mammary gland tissue was homogenized in
0.13 M KCl (1 g tissue/2 ml KCl) and the
homogenate

Add 23 g $(NH_4)_2SO_4$ (100 ml

Mammary gland tissue was homogenized in 0.13 M KCl (1 g tissue/2 ml KCl) and the homogenate was centrifuged:

1200 x g - 10 min

Sup. | PPT

20,200 x g - 25 min

PPT (mito) | Sup.

Suspend in 0.13 M KCl

Freeze and thaw 3 times

Centrifuge at

20,200 x g - 45 min

Sup. (mito Ext.) | PPT

F-3 Mito Ext.

Add 22.5 g $(NH_4)_2SO_4$/100 ml

20,200 x g - 10 min

F-4 Sup. | PPT

Add 23 g $(NH_4)_2SO_4$/100 ml

20,200 x g - 10 min

F-5 PPT | Sup.

Taken up in $KHCO_3$

C_γ-gel step

F-6 Sup. | PPT

Added 45.5 g $(NH_4)_2SO_4$

Centrifuged

F-7 PPT | Sup.

Liquid $(NH_4)_2SO_4$ step

F-8 Sup. | PPT

Add 18.0 g $(NH_4)SO_4$/100 ml

Centrifuge

F-9 PPT | Sup.

p

t

e

w

g

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K

a

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D
Ch

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protein sample was added to the column and washed with the starting buffer until the optical density of the effluent buffer read below 0.1 at 280 mμ. The protein was then eluted from the column using a linear KCl gradient of 0.0 to 0.6 M in 0.05 M Tris-HCl buffer, in a total volume of 600 ml. The enzyme eluted at a KCl concentration of 0.26 M. Fractions having specific activity of about 80 or above were combined and concentrated using the Dia-flo cell. The concentrated protein was stored at -60° C.

DEAE-Cellulose Chromatography

The DEAE-cellulose was washed[2] as described in the Whatmann brochure and suspended as a thick slurry in 0.005 M Tris-HCl buffer, 3 mM in 2-ME, pH 7.5. The column dimensions were 1.7 cm x 42 cm. The column was equilibrated overnight with the same buffer, at a flow rate of 15-20 ml/hr. The ammonium sulphate precipitate was dialyzed for 30 minutes, diluted to 10 mg of protein/ml, and added to the column. The column was then washed with 140 ml of buffer and then eluted using a linear KCl gradient. Six ml fractions of eluate were

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[2] Ibid.

collected. The tubes with the highest enzyme activity were pooled and concentrated in a Dia-flow cell. The concentrated enzyme protein was stored at -60° C.

Carboxymethyl-Cellulose Chromatography

Carboxymethyl-cellulose (Cellex-CM) was prepared by washing first with 0.5 M NaOH - 0.5 M NaCl then with 1 N HCl and finally with water until free of acid. The cellulose was then suspended in 0.01 M potassium phosphate buffer, pH 7.5, which was used throughout the experiment. The column dimensions were 0.9 x 26 cm. The column was equilibrated overnight with the buffer at a flow rate of 6 ml/hr. About 50 mg of ammonium sulphate precipitate were dialyzed against the buffer. This was diluted to give 5 mg/ml of enzyme protein and then added to the column. The column was eluted with 0.01 M potassium buffer, pH 7.5. Three ml fractions of the eluate were collected.

CHAPTER IV

RESULTS

Mammary gland tissue from five lactating holstein cows were used for these studies. When experiments on purification had exhausted the supply of mitochondria, another cow was selected for slaughter and a fresh supply of mitochondria was prepared. In the initial studies attempts were made to purify acetyl-CoA synthetase according to the procedure described by Webster (1965) for purification of acetyl-CoA synthetase from bovine heart mitochondria. The procedure involved after the ammonium sulphate fractionation steps final purification by column chromatography using TEAE cellulose and $KHCO_3$ buffers. This procedure did not give extensive purification using mitochondria prepared from Cow 330 (Table 2). Column chromatography using TEAE cellulose gave less than a two-fold increase in specific activity over the #9 ammonium sulphate precipitate (Table 2). The #9 precipitate was only slightly more active than the #5 precipitate. The enzyme eluted in two peaks when

TABLE 2.--Purification of acetyl-CoA synthetase
(Cow 330)

Mammary gland weight = 3.8 kg
Wet weight of mitochondria = 376 gm

Fraction	Volume ml	Total Protein mg	Specific Activity µmole/hr/ mg	Total Units	Recovery %
#3 (Mito Ext.)	2,160	11,880	4.9	58,212	100
#4 (Sup.)	2,300	5,060	6.9	34,914	60
#5 (PPT)	128	3,161	18.3	57,846	99
#7 (PPT)	150	2,250	19.3	43,425	74
#9 (PPT)	24	1,404	27.3	38,329	66

chromatographed on TEAE cellulose (Figure 3). When the tubes with peak enzyme activity were combined and rechromatographed, the enzyme eluted in three separate peaks (Figure 4).

In an attempt to separate the enzyme protein from the non-enzyme protein a column was run in which a KCl gradient was not used. The enzyme protein eluted with the major protein peak (Figure 5). These studies exhausted the supply of the #9 precipitate prepared from Cow 330 (Table 2). Mitochondria were prepared from Cow 329 and the enzyme was purified by ammonium sulphate fractionation through the #7 precipitate (Table 3). The #9 precipitate was not prepared because previous data showed that a major increase in specific activity was not obtained. Further purification of the #7 precipitate was attempted using Bio-gel P-100 (Figure 6) or Bio-gel P-200 (Figure 7), or Sephadex G-100 (Figure 8) or Carboxymethyl cellulose (Figure 9). None of these column chromatography techniques using various buffers gave a significant purification.

Another preparation of mitochondria was obtained from Cow 444 (Table 4). The effect of Tris-HCl concentration on enzyme activity is shown in Figure 10. Optimum enzyme activity was obtained over a Tris-HCl concentration range of 0.04 M to 0.2 M. Potassium chloride did not increase the activity of the enzyme (Figure 11).

FIGURE 3.--Chromatography of acetyl-CoA synthetase on TEAE cellulose using $KHCO_3$ buffers and a KCl gradient.

The buffer used throughout this procedure was 0.02 M $KHCO_3$ - 0.5 mM EDTA - 3 mM 2-ME, pH 8. 500 mg of protein (13,500 units of enzyme) were diluted, dialyzed, and added to the column. The protein was eluted by washing the column with a linear KCl gradient of 0.05 M to 1.4 M in 440 ml of the buffer. The two peaks of enzyme activity were combined and chromatographed (Figure 4).

(———), protein mg/ml; (-.-.-), specific activity.

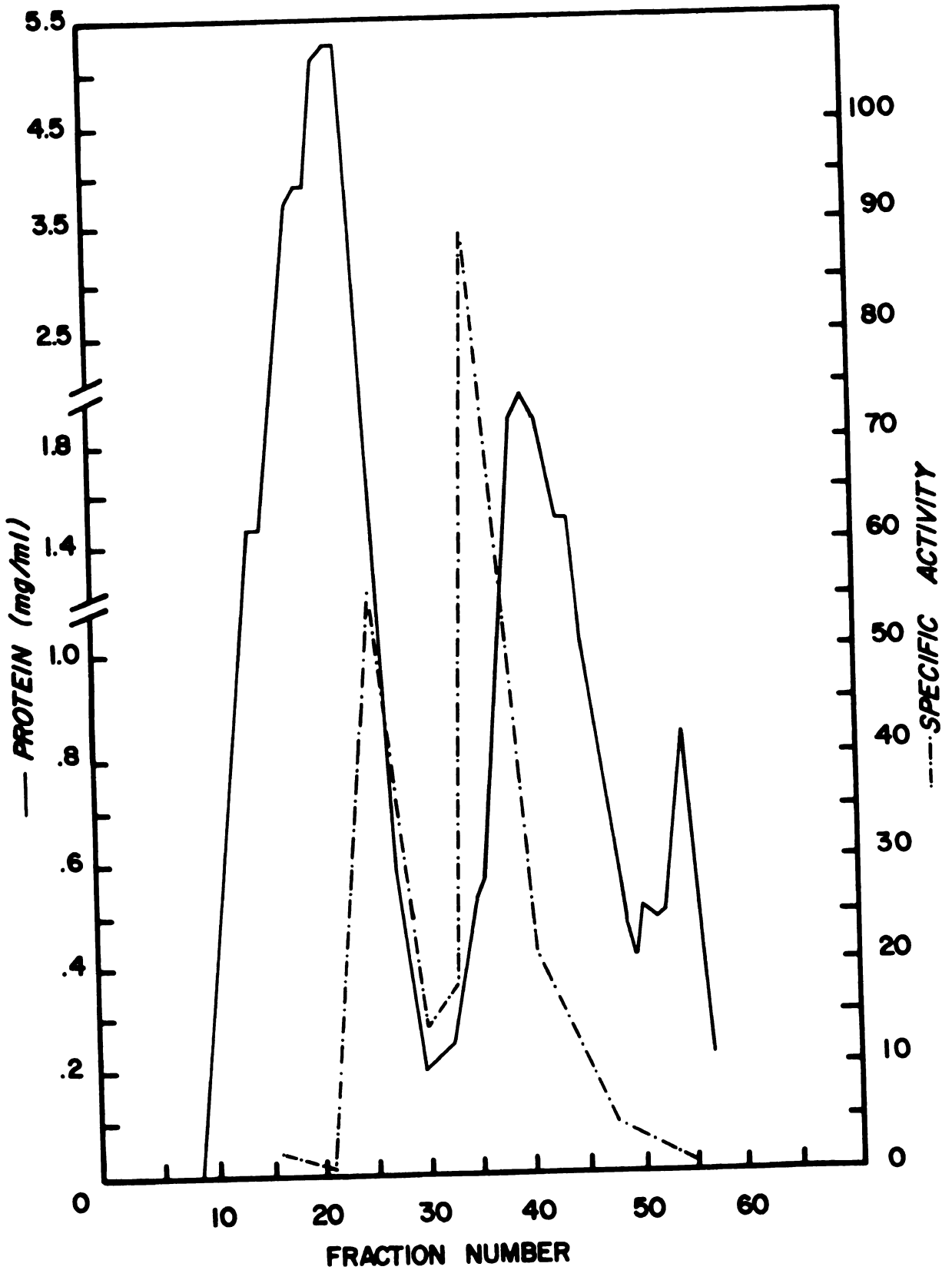

5.5
4.5
3.5
2.5
1.8
1.4
1.0
.8
.6
.4
.2
0
— PROTEIN (mg/ml)
100
90
80
70
60
50
40
30
20
10
0
---- SPECIFIC ACTIVITY
0
10
20
30
40
50
60
FRACTION NUMBER

FIGURE 4.--Rechromatography of acetyl-CoA synthetase on TEAE-cellulose using $KHCO_3$ buffers and a KCl gradient.

The combined enzyme fractions (Figure 3, 120 mg protein) were rechromatographed. A linear KCl gradient of 0.05 M to 0.5 M was used in a total volume of 420 ml.

(———), protein mg/ml; (-.-.-), specific activity.

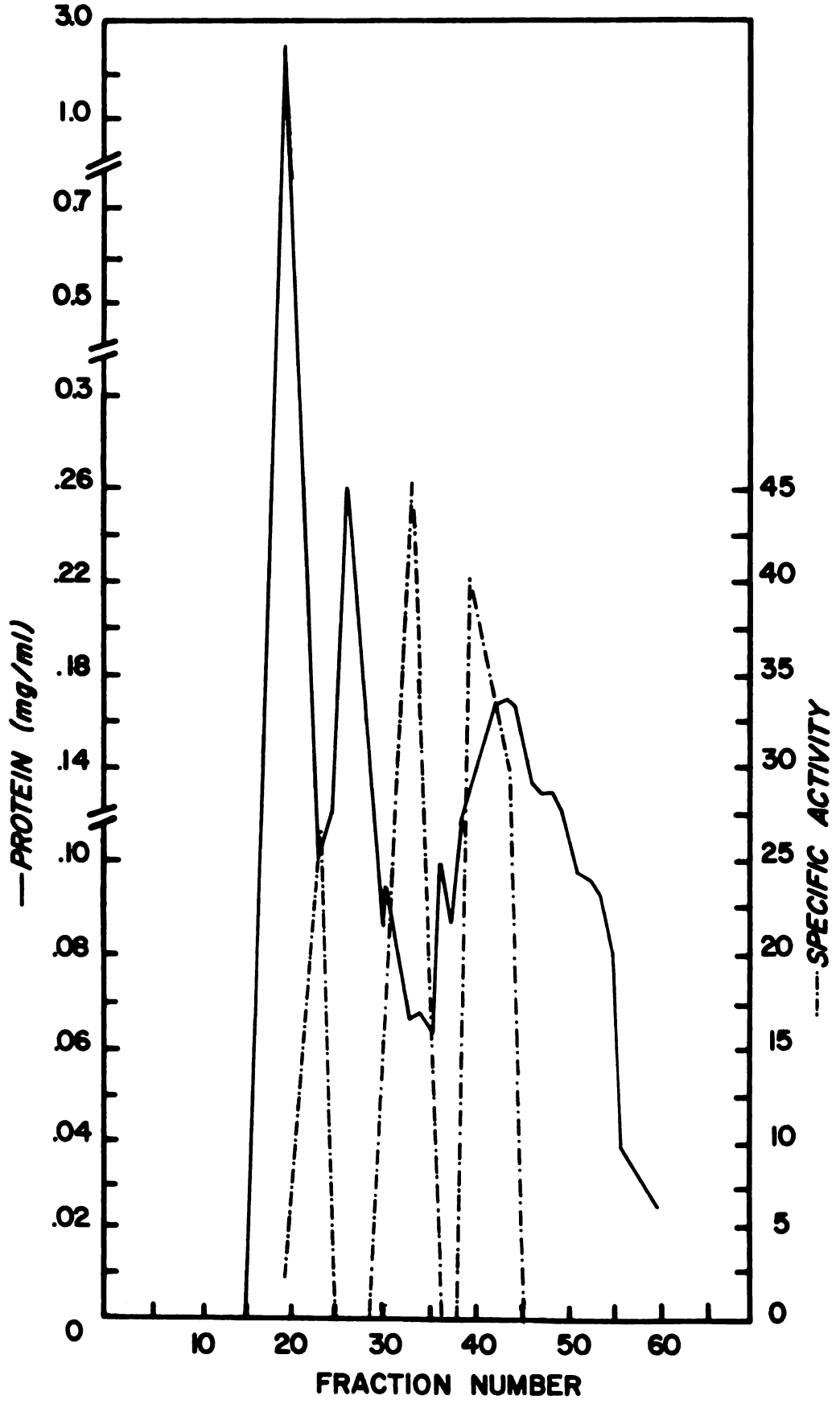

3.0
1.0
0.7
0.5
0.3
.26
.22
.18
.14
.10
.08
.06
.04
.02
0
45
40
35
30
25
20
15
10
5
0
10
20
30
40
50
60
FRACTION NUMBER
—PROTEIN (mg/ml)
----SPECIFIC ACTIVITY

FIGURE 5.--Chromatography of acetyl-CoA synthetase on TEAE cellulose using $KHCO_3$ buffers without a KCl gradient.

About 500 mg of enzyme protein were added to the column. Elution was carried out using 0.02 M $KHCO_3$ - 0.5 mM EDTA - 3 mM 2-ME buffer, pH 8. A KCl gradient was not used.

(———), protein mg/ml; (-.-.-), specific activity.

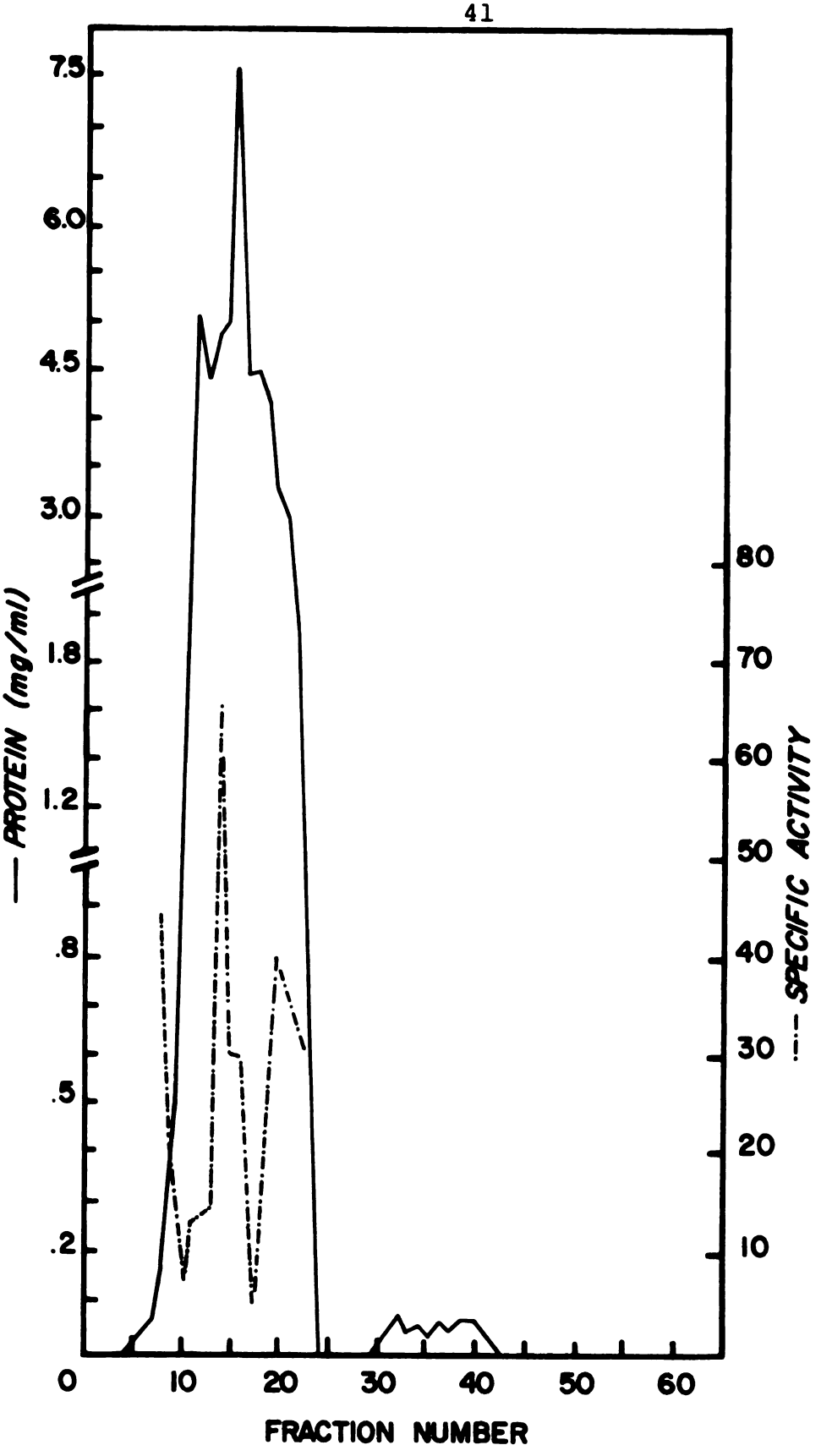

7.5
6.0
4.5
3.0
1.8
1.2
.8
.5
.2
— PROTEIN (mg/ml)
80
70
60
50
40
30
20
10
-·- SPECIFIC ACTIVITY
0
10
20
30
40
50
60
FRACTION NUMBER

TABLE 3.--Purification of acetyl-CoA synthetase
(Cow 329)

Mammary gland weight = 4 kg
Wet weight of mitochondria = 147 gm

Fraction	Volume ml	Total Protein mg	Specific Activity µmole/hr/ mg	Total Units	Recovery %
#3 (Mito Ext.)	752	2,857	5	14,285	100
#4 (Sup.)	810	1,701	5.5	9,355	65.4
#5 (PPT)	100	920	15.1	13,892	97.2
#7 (PPT)	12.5	487.5	17.1	8,336	58.3

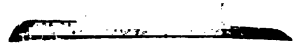


FIGURE 6.--Chromatography of acetyl-CoA synthetase on Bio-Gel P-100.

The buffer used was 0.05 M $KHCO_3$ - 0.005 M Tris-HCl, pH 7.6. The column dimensions were 2.5 x 34 cm.

(———), protein mg/ml; (----), units/ml.

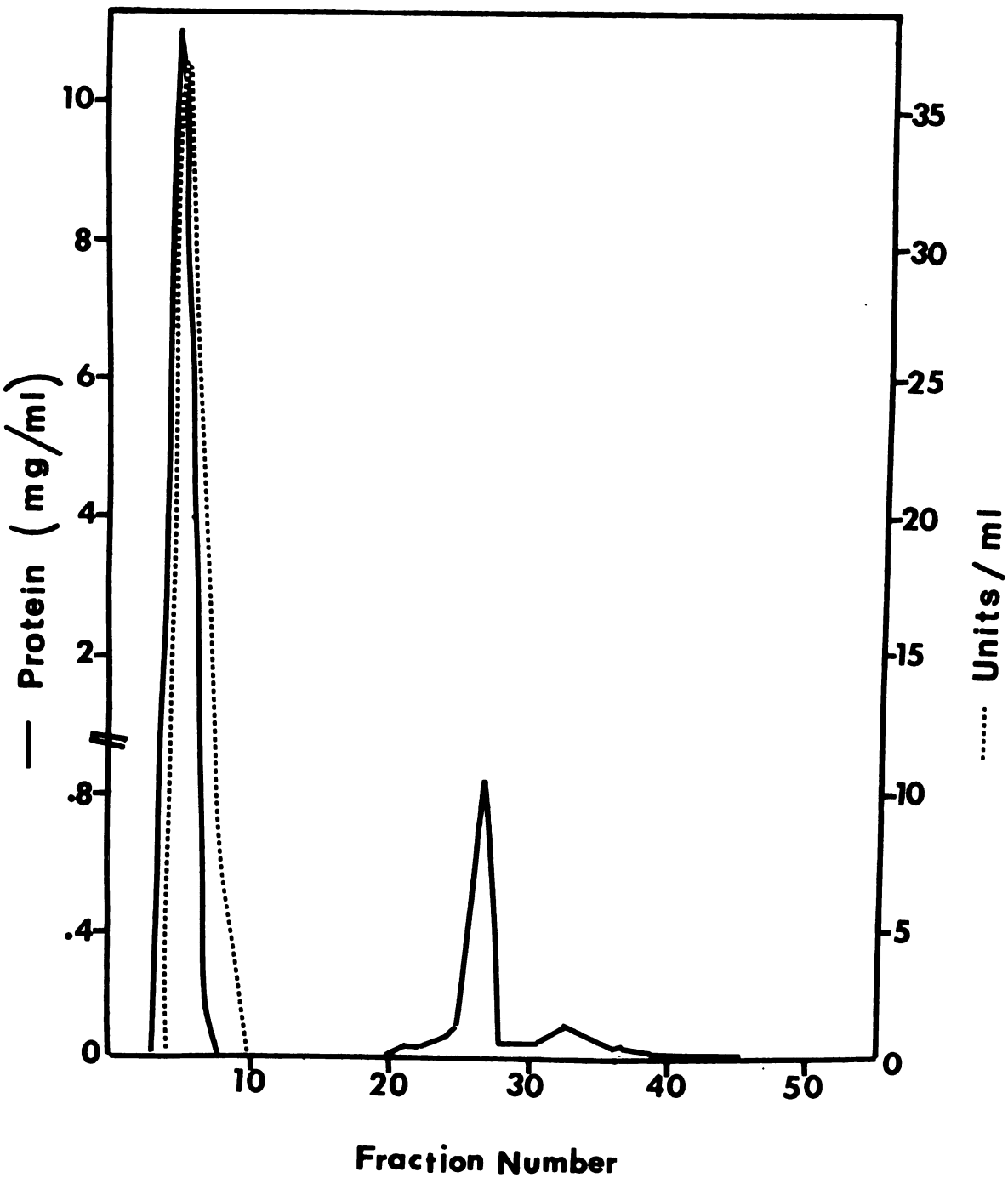

10
8
6
4
2
.8
.4
0
— Protein (mg/ml)
35
30
25
20
15
10
5
0
..... Units / ml
10
20
30
40
50
Fraction Number

FIGURE 7.--Chromatography of acetyl-CoA synthetase on Bio-Gel P-200.

The column dimensions were 1.8 x 70 cm. The buffer is described before (Figure 6).

(———), protein mg/ml; (----), units/ml.

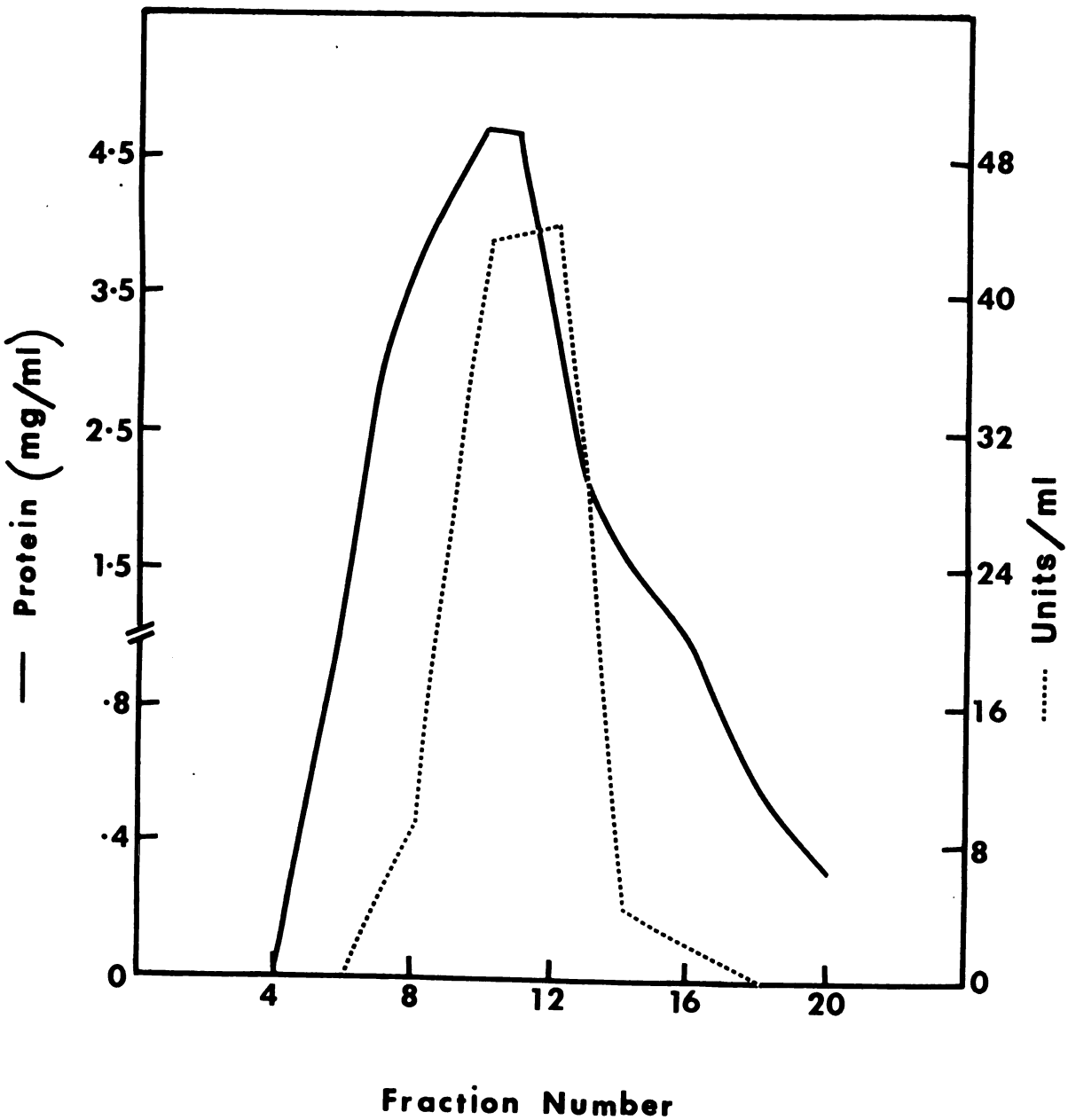

— Protein (mg/ml)
4·5
3·5
2·5
1·5
·8
·4
0
48
40
32
24
16
8
0
4
8
12
16
20
Units/ml
Fraction Number

FIGURE 8.--Chromatography of acetyl-CoA synthetase on Sephadex G-100.

The column dimensions were 2.5 x 37 cm. The buffer used was 0.02 M $KHCO_3$, pH 8.

(———), protein mg/ml; (----), units/ml.

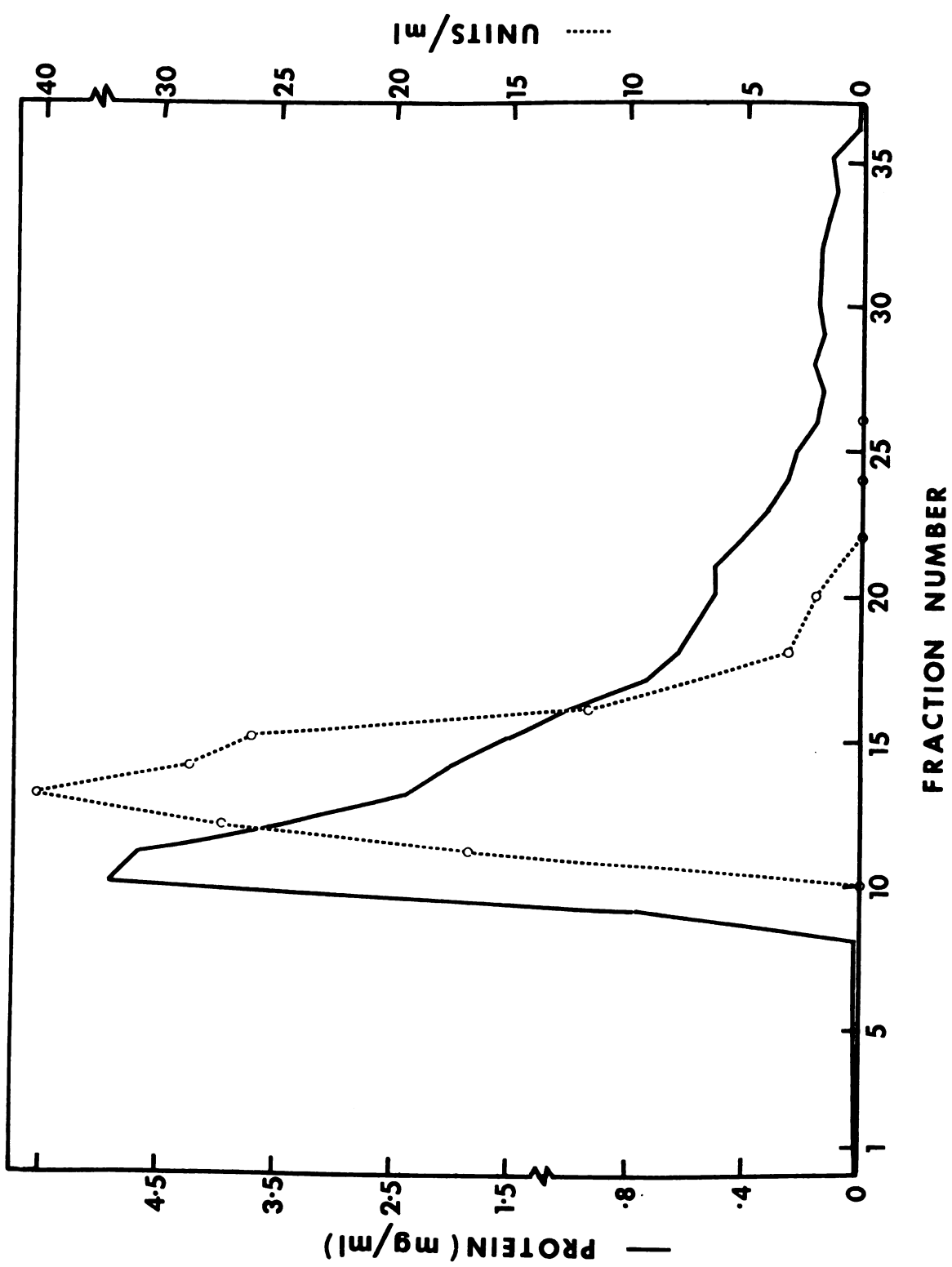

UNITS/ml
PROTEIN (mg/ml)
FRACTION NUMBER
40
30
25
20
15
10
5
0
4·5
3·5
2·5
1·5
·8
·4
0
1
5
10
15
20
25
30
35

FIGURE 9.--Chromatography of acetyl-CoA synthetase on carboxymethyl cellulose.

The column dimensions were 0.9 x 26 cm. 0.01 M potassium phosphate buffer, pH 7.5 was used to eluate the column.

(———), protein mg/ml; (-----), units/ml.

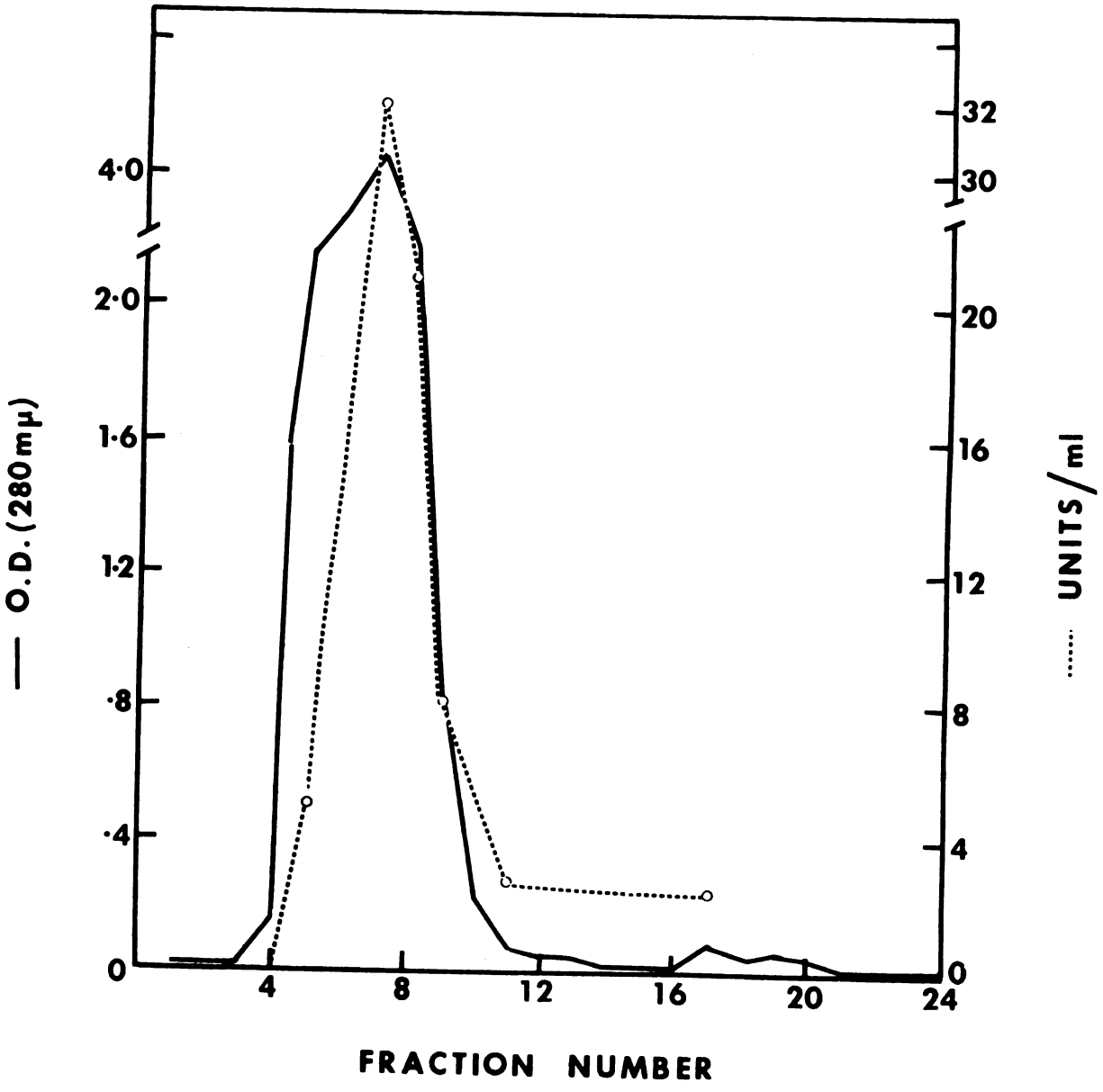

— O.D. (280mμ)
4·0
2·0
1·6
1·2
·8
·4
0
32
30
20
16
12
8
4
0
UNITS/ml
4
8
12
16
20
24
FRACTION NUMBER

TABLE 4.--Purification of acetyl-CoA synthetase
(Cow 444)

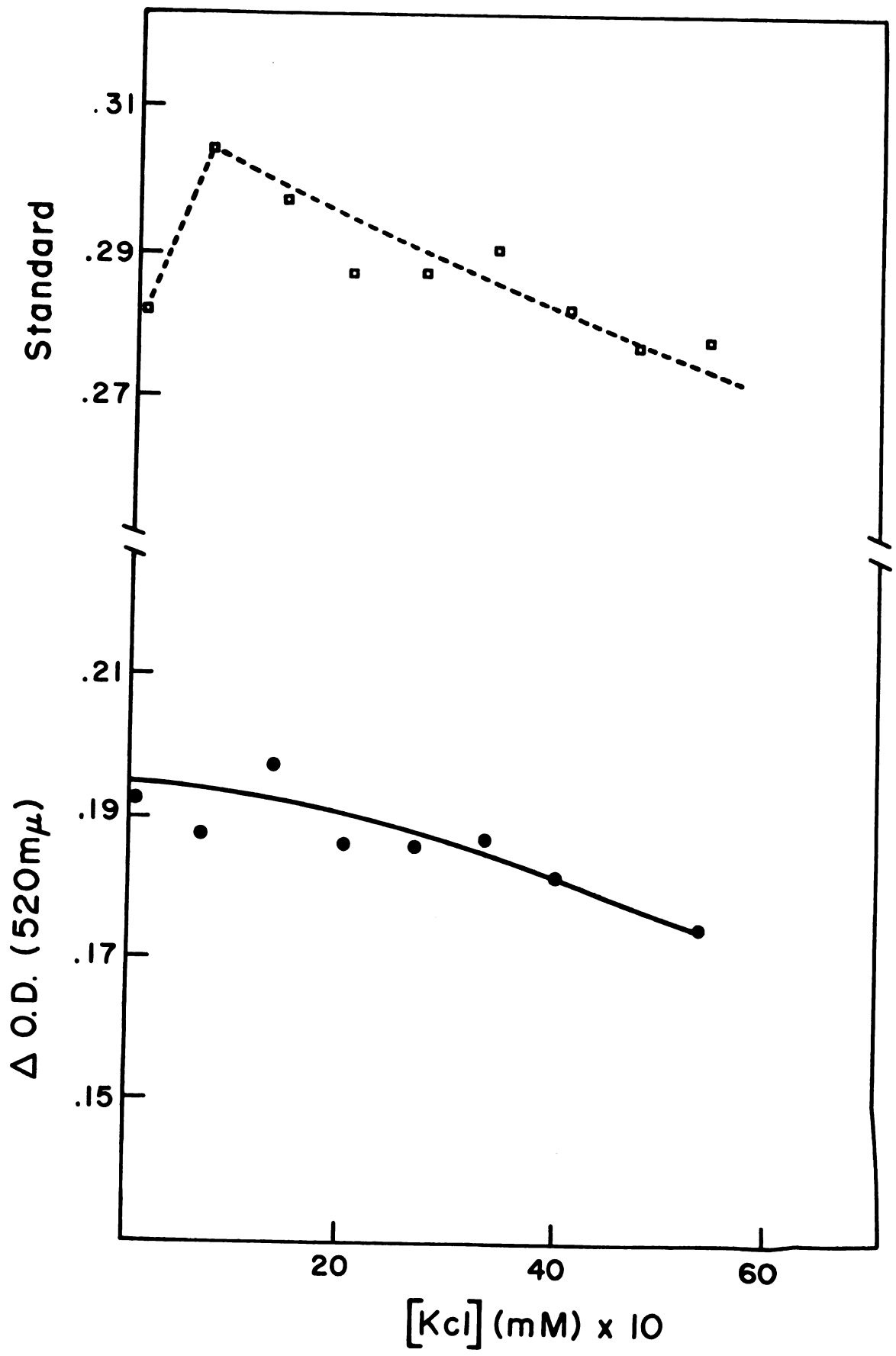
Mammary gland weight = 1.8 kg
Wet weight of mitochondria = 156 g

Fraction	Volume ml	Total Protein mg	Specific Activity µmole/hr/mg	Total Units	Recovery %
#3 (Mito Ext.)	1,180	3,799	4.1	15,575	100
#4 (Sup.)	1,370	1,575	-	-	-
#5 (PPT)	14	627	8.8	5,517	35
TEAE-cellulose (Columns I-IV)	24	64	72.6	4,650	30
TEAE-cellulose (Column V)	19.2	27	158.0	4,270	27

FIGURE 10.--Effect of Tris-HCl concentration on acetyl-CoA synthetase activity.

O.D.(520mμ)
.40
.35
.30
.20
.16
.12
.08
.04
0
8
16
24
[TRIS-HCL](mM)x10⁻¹
— ΔO.D.(520mμ)

FIGURE 11.--Effect of KCl concentration on acetyl-CoA synthetase activity.


Standard
.31
.29
.27
.21
.19
.17
.15
Δ O.D. (520mμ)
20
40
60
[Kcl] (mM) x 10

Studies using KF as an inhibitor of ATPase indicated that this enzyme was not interfering with the enzyme assay (Figure 12). A major purification using TEAE cellulose was achieved when Tris-HCl buffer was used instead of $KHCO_3$ buffer. In contrast with the results in Figure 5, the enzyme protein separated from the major protein peak when a Tris-HCl buffer was used in the absence of a KCl gradient (Figure 13). The enzyme isolated from four such columns was combined, concentrated, and rechromatographed on TEAE cellulose (Figure 14).

Since a major purification of the enzyme was achieved (Figure 14, Table 4), further studies on the purity of the preparation were carried out. One protein band was observed upon polyacrylamide gel electrophoresis using 10 μg of protein and amido black dye. However, sedimentation equilibrium studies indicated the presence of at least two molecular species, one with a molecular weight of 49,000 and one with a molecular weight of 84,500 (Figure 15). Polyacrylamide gel electrophoresis using 100 μg of protein showed the presence of four protein bands (Figure 16). Enzyme activity was found in the third protein band (Figure 16).

Using sucrose density gradient centrifugation, the sedimentation coefficient was estimated to be 4.5 S and the molecular weight was estimated to be 62,000 (Figure 17). This did not correspond to any of the values obtained from the sedimentation equilibrium study.

FIGURE 12.--Effect of KF on acetyl-CoA synthetase activity.

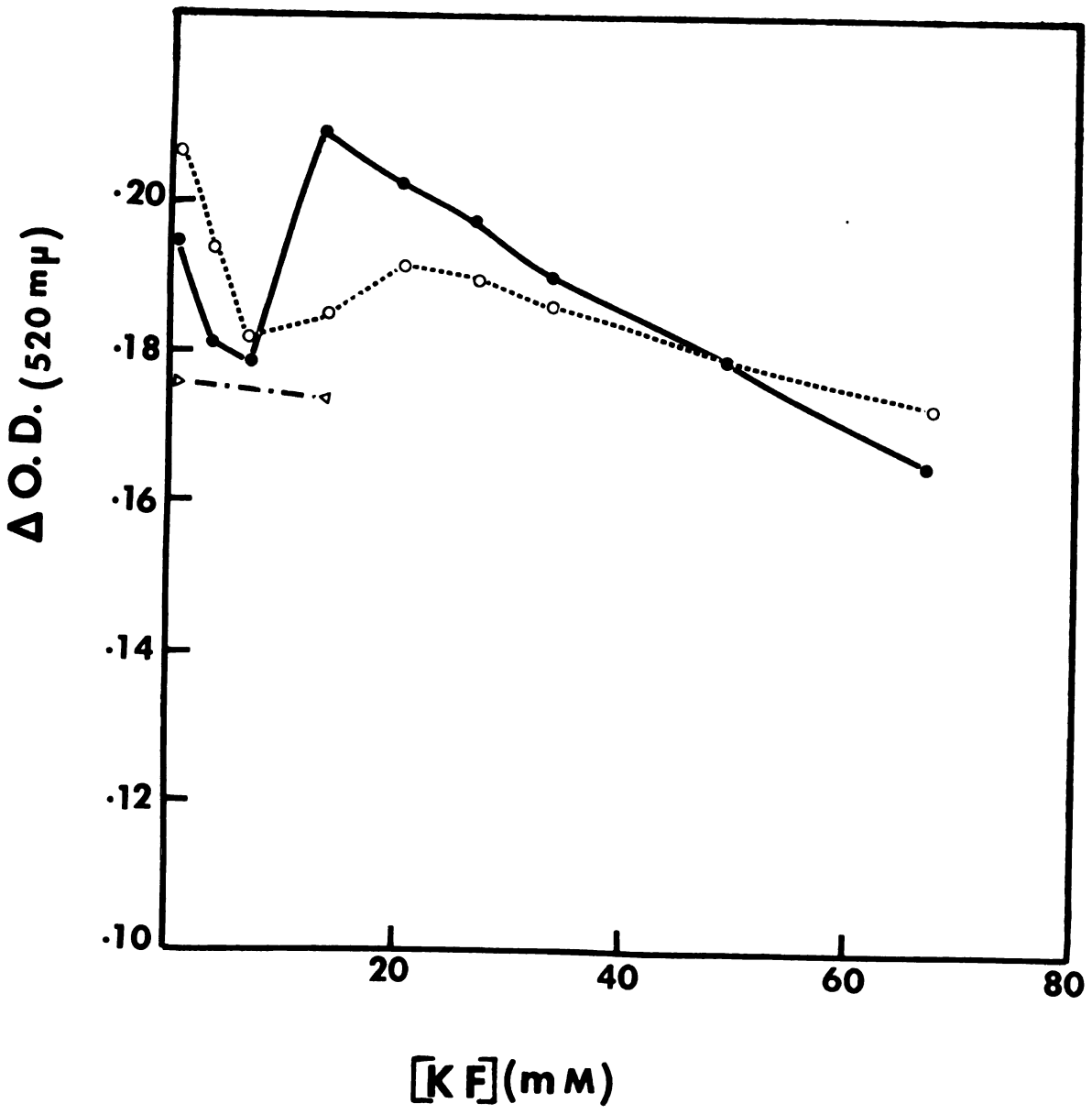

ΔO.D. (520 mμ)
·20
·18
·16
·14
·12
·10
20
40
60
80
[K F] (m M)

FIGURE 13.--Chromatography of acetyl-CoA synthetase on TEAE cellulose using Tris buffers and a KCl gradient.

The eluting buffer was 0.005 M Tris-HCl, pH 7.0. A linear KCl gradient of 0.01 M to 0.6 M was used in a total volume of 600 ml. The gradient was started after tube 70.

(———), protein mg/ml; (----), units/ml.

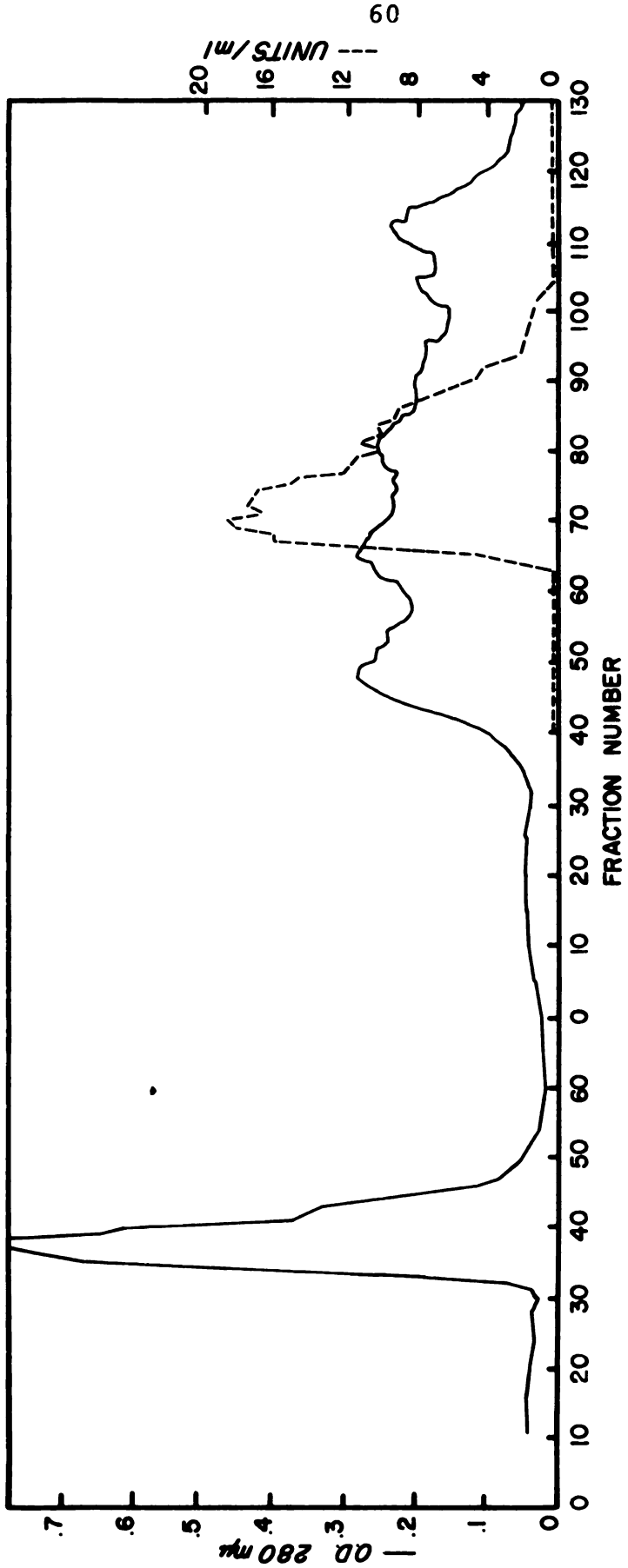

--- UNITS/ml
20
16
12
8
4
0
— O.D. 280 mμ
.7
.6
.5
.4
.3
.2
.1
0
0 10 20 30 40 50 60 0 10 20 30 40 50 60 70 80 90 100 110 120 130
FRACTION NUMBER

FIGURE 14.--Rechromatography of acetyl-CoA synthetase on TEAE cellulose using Tris buffers and a KCl gradient.

The column dimensions were 0.9 x 20 cm. The linear KCl gradient used was 0.1 M to 0.6 M in a total volume of 100 ml. 3 ml fractions of the eluate were collected. The gradient was started at tube 1.

(———), protein mg/ml; (----), units/ml.

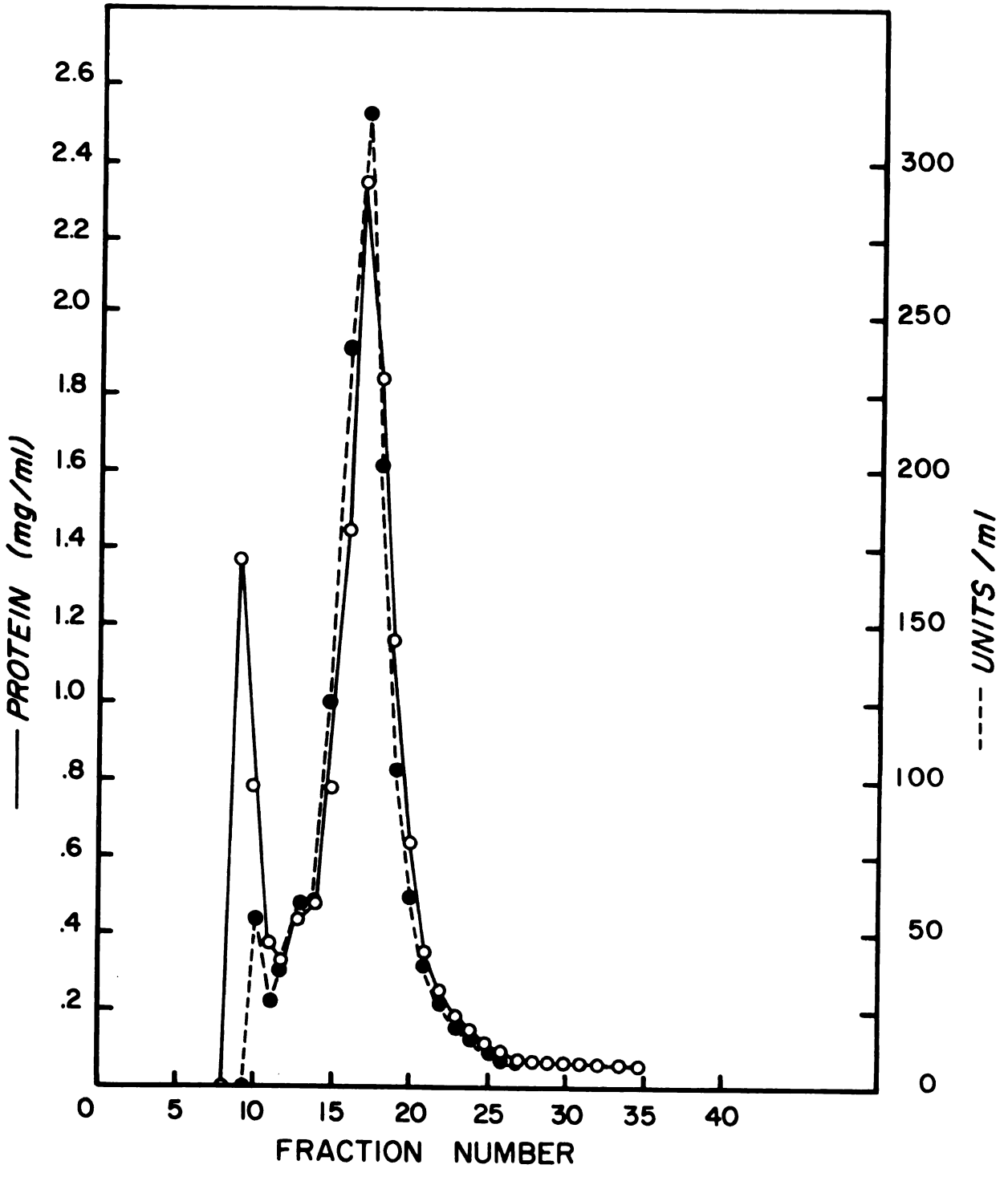

2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
PROTEIN (mg/ml)
300
250
200
150
100
50
0
UNITS/ml
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

FIGURE 15.--Sedimentation equilibrium study of acetyl-CoA synthetase.

Equilibrium was reached in 18 hours, at 24,410 rpm at 4° C. A value of 0.76 for partial specific volume was assumed. The details are described in the Experimental Procedure.

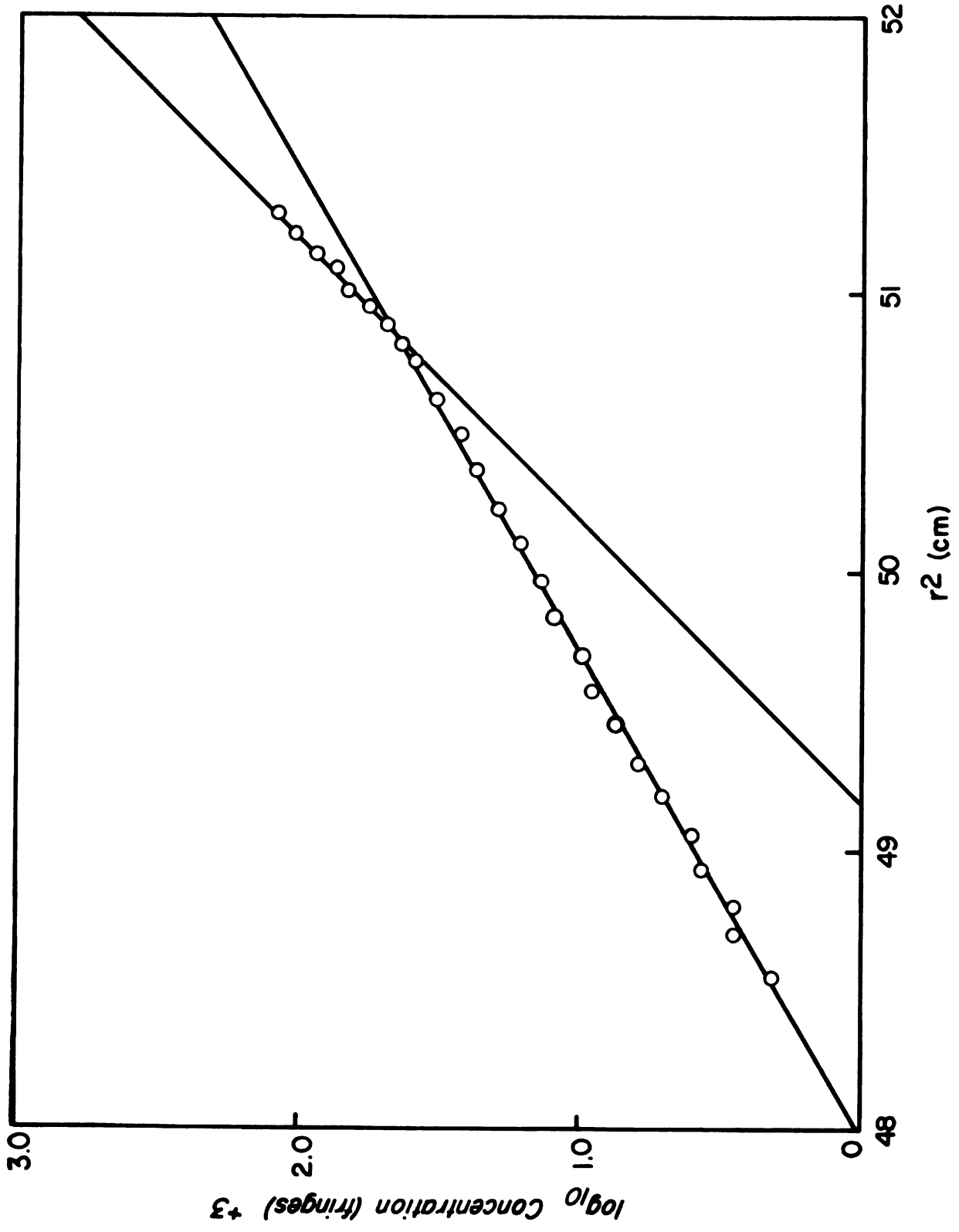
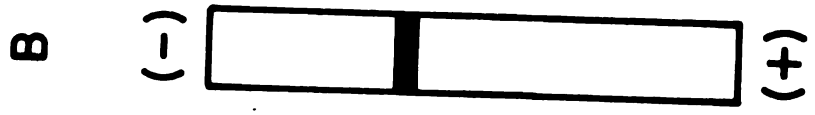

3.0
2.0
1.0
0
48
49
50
51
52
r2 (cm)
log10 Concentration (fringes) +3

FIGURE 16.--Schematic presentation of polyacrylamide gel electrophoresis of acetyl-CoA synthetase.

The buffer used was 0.05 M Tris-HCl, pH 9. The run was carried out for 2 hours. The details are described in the Experimental Procedure.


B
(-)
(+)

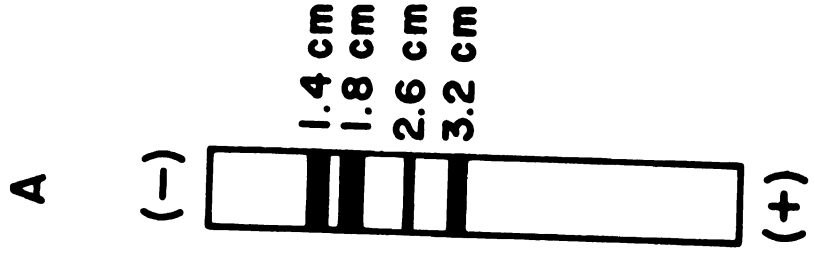
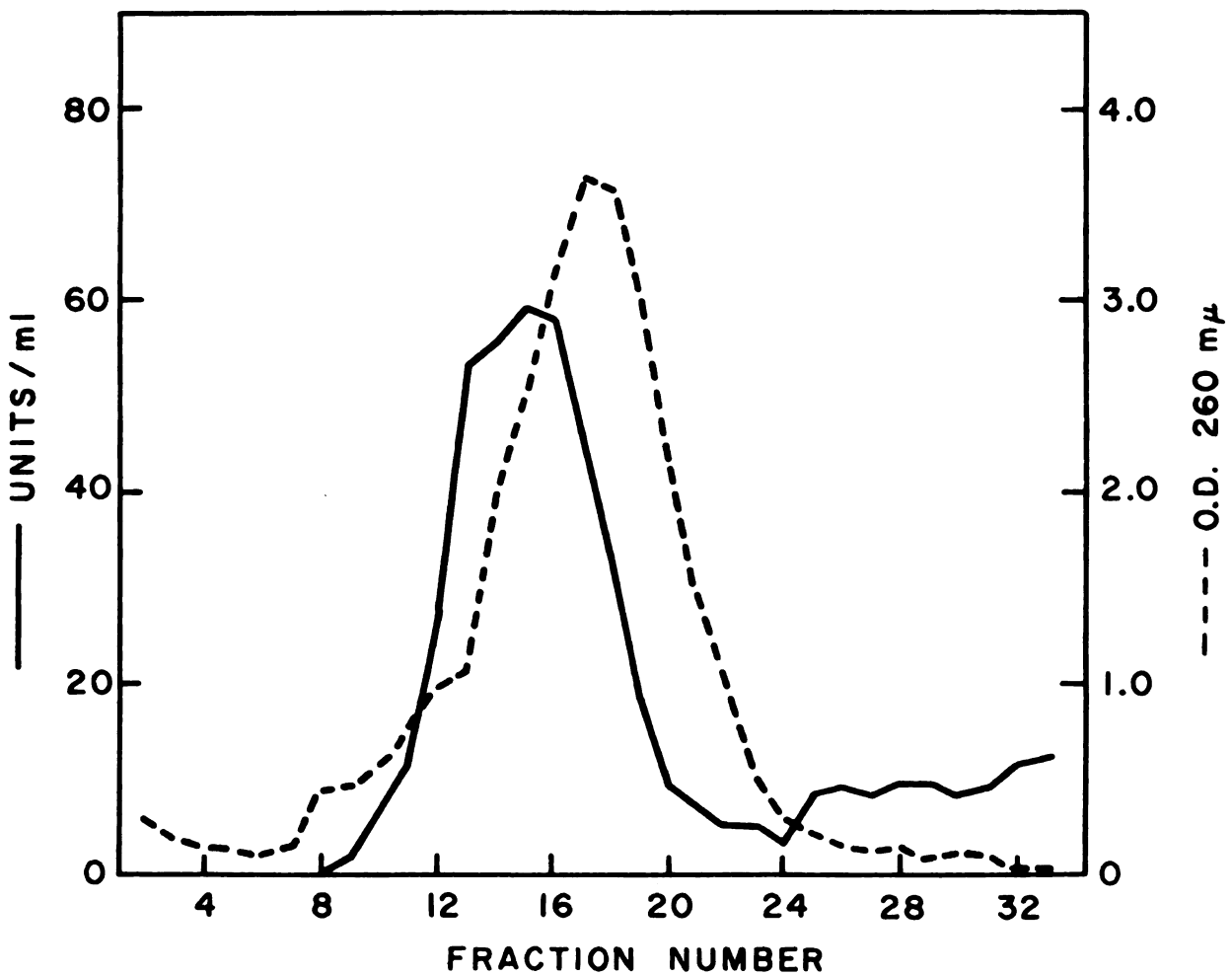

A
(-)
1.4 cm
1.8 cm
2.6 cm
3.2 cm
(+)

FIGURE 17.--Sucrose density gradient centrifugation of acetyl-CoA synthetase at 35,000 rpm for 28 hours at 4° C. and using a 5-20% sucrose gradient.


80
60
40
20
0
4.0
3.0
2.0
1.0
0
4
8
12
16
20
24
28
32
FRACTION NUMBER
UNITS/ml
O.D. 260 mμ

Although the data in Figure 14 suggested that the enzyme may be pure, the other studies showed that a high molecular weight species may be present. Consequently an attempt was made to separate the high molecular weight protein using Sephadex G-200. The buffer contained 0.1 M KCl to prevent aggregation. However, the protein emerged from the column in the high molecular weight fraction and the specific activity decreased from 158 to 53 (Figure 18).

Finally, isoelectric focusing in a sucrose density gradient was studied as a method of further purification of the enzyme (Figure 19). This technique was not successful because the enzyme precipitated at its isoelectric point (pH 5.7) and lost considerable activity.

Mitochondria were prepared from Cow 445 (Table 5). Extensive purification was achieved after chromatography on TEAE cellulose (Figure 20) by using adsorption chromatography on calcium phosphate gel (Figure 21). The enzyme from the calcium phosphate gel columns was used in studies to determine the optimum conditions for polyacrylamide gel electrophoresis and subsequent measurement of enzyme activity in the protein bands.

The final preparation of mitochondria was from Cow 1063. The enzyme was purified as in previous studies (Table 6) except that the #5 ammonium sulphate precipitate was chromatographed on a DE-23 cellulose column. The laboratory supply of TEAE cellulose was exhausted after

FIGURE 18.--Chromatography of acetyl-CoA synthetase on Sephadex G-200.

The column dimensions were 1.2 x 16 cm. The buffer used was 0.005 M Tris-HCl - 0.1 M KCl, pH 7.0. 5 mg of protein were added to the column. 1 ml fractions of the eluate were collected. The void volume was 7 ml.

(———), O.D. at 280 μm; (····), O.D. at 215 μm; (----), units/ml.

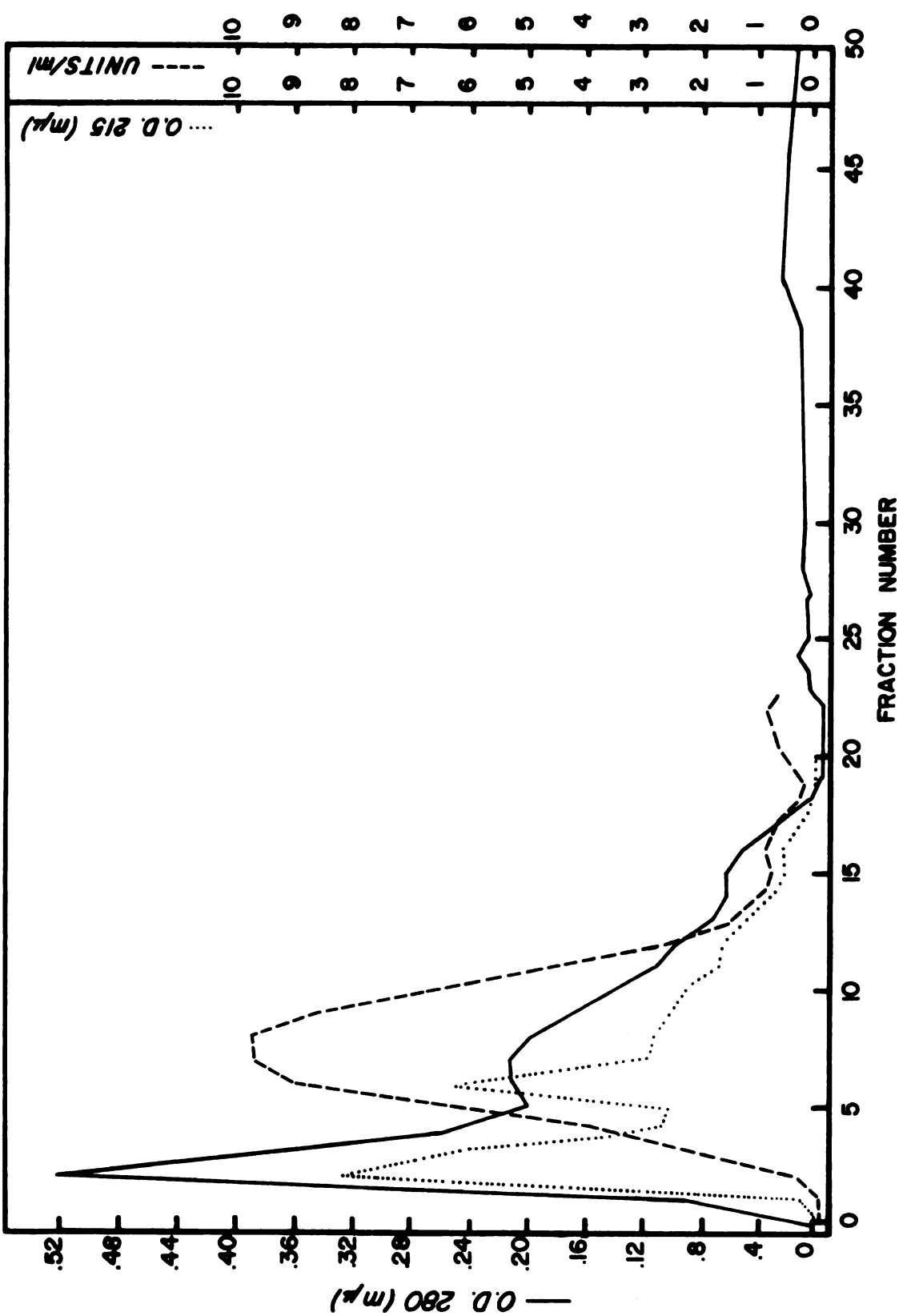

— O.D. 280 (mμ)
.... O.D. 215 (mμ)
---- UNITS/ml
FRACTION NUMBER
0
.4
.8
.12
.16
.20
.24
.28
.32
.36
.40
.44
.48
.52
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
25
30
35
40
45
50

FIGURE 19.--Iso-electric focusing of acetyl-CoA synthetase.

The details are described in the Experimental Procedure.

(———), protein mg/ml; (----), units/ml; (O-O-O), pH.

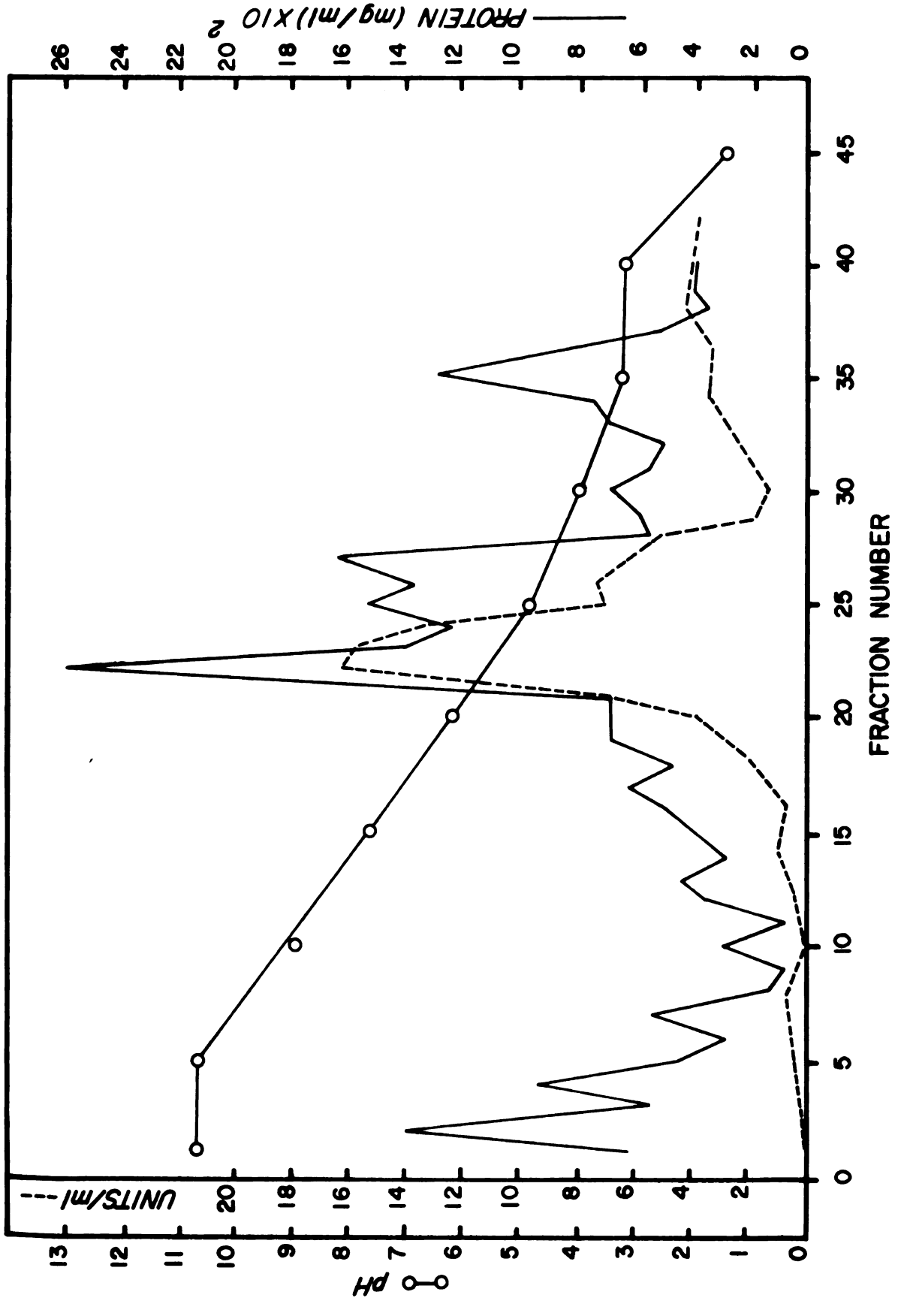

PROTEIN (mg/ml) X10 ²
UNITS/ml
pH
FRACTION NUMBER

TABLE 5.--Purification of acetyl-CoA synthetase
(Cow 445)

Mammary gland weight = 1.14 kg
Wet weight of mitochondria = 97.8 g

Fraction	Volume ml	Total Protein mg	Specific Activity µmole/hr/mg	Total Units	Recovery %
#3 (Mito Ext.)	690	1,725	8.8	15,180	100
#4 (Sup.)	780	748	17	12,729	84
#5 (PPT)	16	614	21	12,900	85
TEAE-cellulose	87	61	156	9,560	63
TEAE-cellulose (purified)	2.4	15	156	2,340	100
Calcium phosphate gel	30	0.86	346	297	1.27

FIGURE 20.--Chromatography of acetyl-CoA synthetase on TEAE cellulose using Tris buffer and a KCl gradient.

The column was prepared as described in the Experimental Procedure.

(———), protein mg/ml; (----), units/ml.

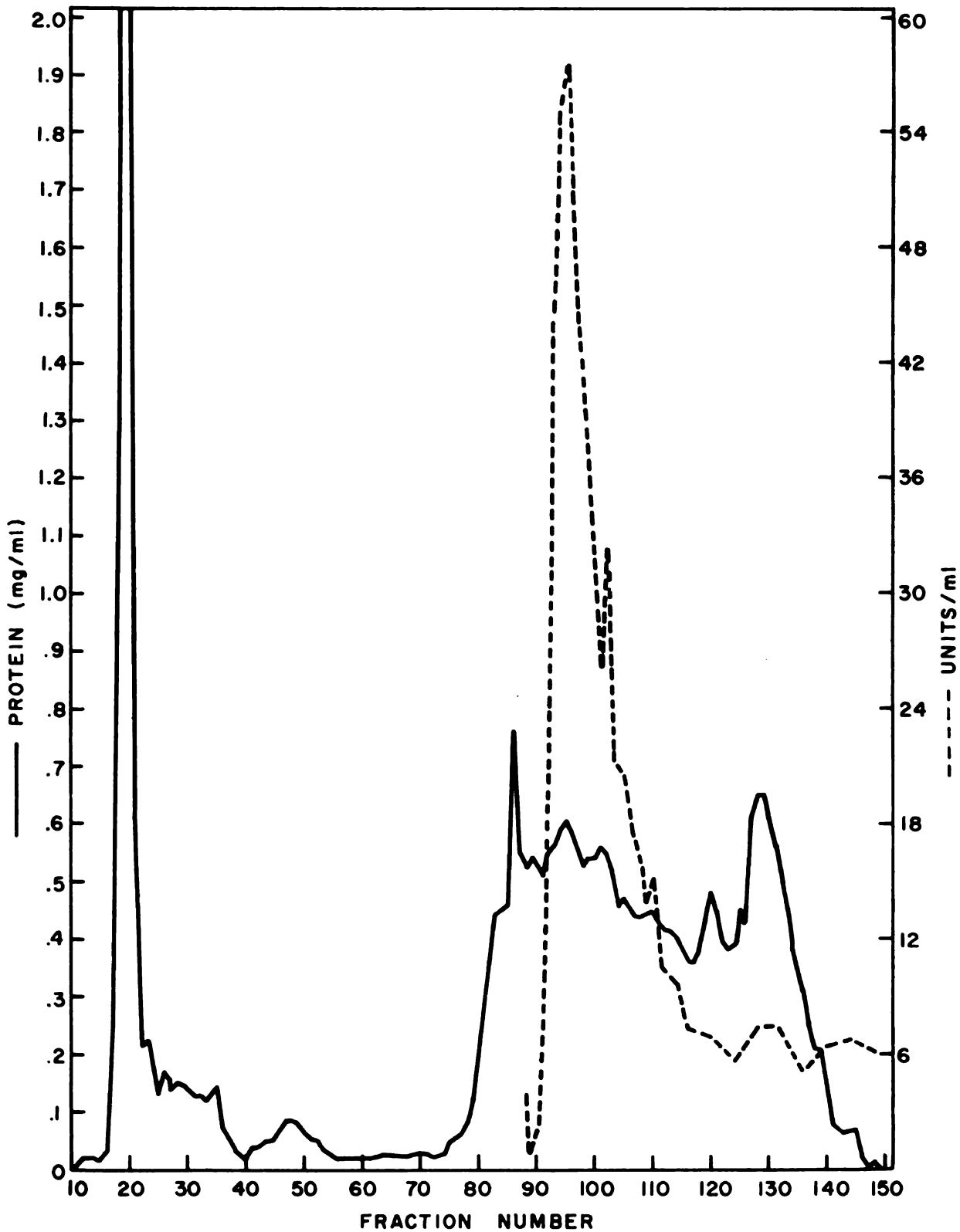

PROTEIN (mg/ml)
UNITS/ml
FRACTION NUMBER
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
60
54
48
42
36
30
24
18
12
6
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150

FIGURE 21.--Chromatography of acetyl-CoA synthetase on calcium phosphate gel (enzyme prepared from mitochondria of Cow 445).

The column dimensions were 3 x 4 cm. A stepwise gradient of increasing concentration of potassium phosphate buffer, pH 7, was used to elute the protein. The eluate was collected in 2.8 ml fractions.

(———), protein mg/ml; (----), units/ml.

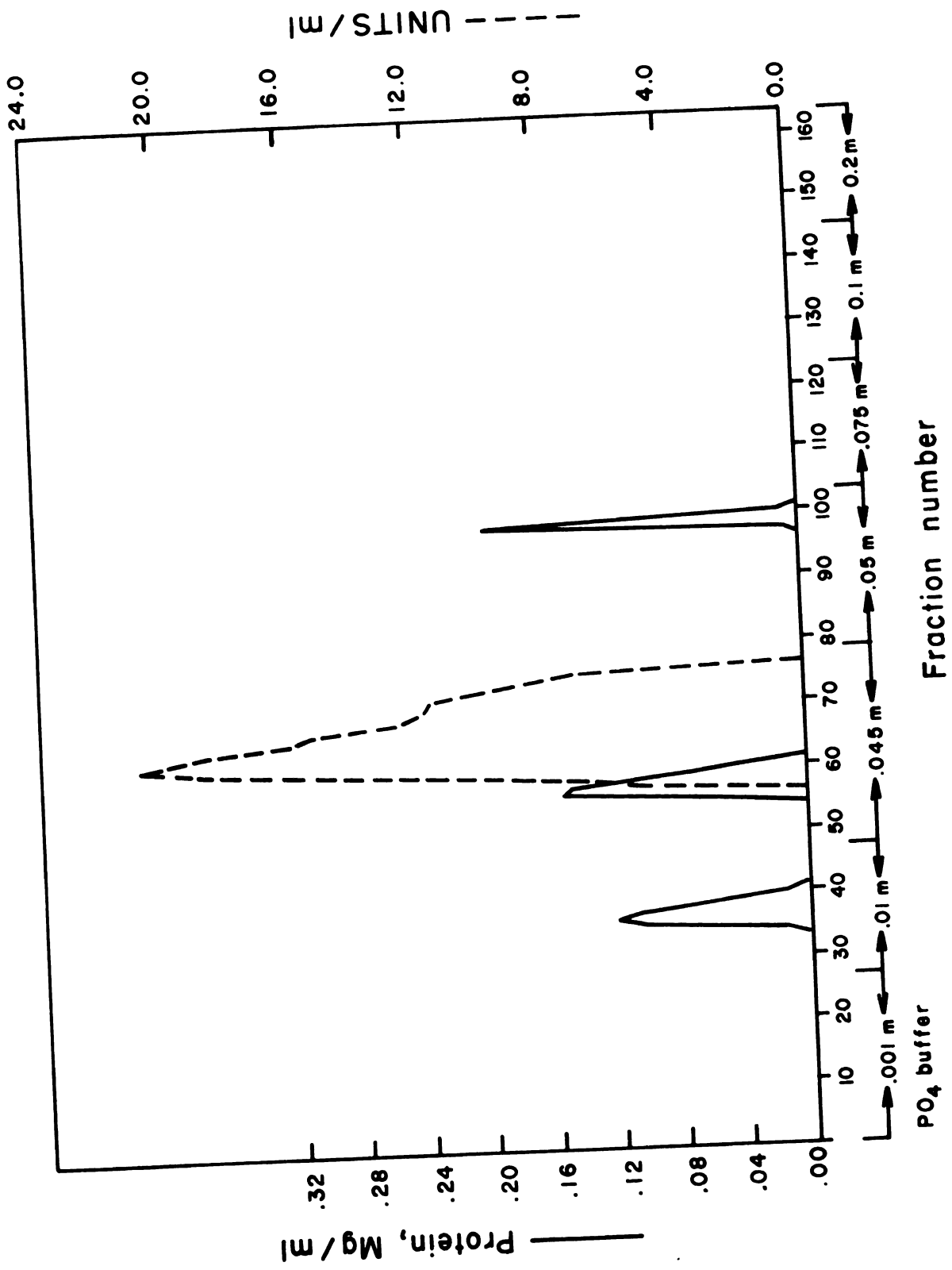

---- UNITS/ml
24.0
20.0
16.0
12.0
8.0
4.0
0.0
0.2 m
0.1 m
.075 m
.05 m
.045 m
.01 m
.001 m
PO4 buffer
Fraction number
Protein, Mg/ml
.32
.28
.24
.20
.16
.12
.08
.04
.00

TABLE 6.--Purification of acetyl-CoA synthetase
(Cow 1063)

Mammary gland weight = 6.15 kg
Wet weight of mitochondria = 457 g

Fraction	Volume ml	Total Protein mg	Specific Activity µmole/hr/mg	Total Units	Recovery %
#3 (Mito Ext.)	4,090	9,587	6.7	64,232	100
#4 (Sup.)	4,530	4,953	-	-	-
#5 (PPT)	107	3,040	21.8	66,272	103
#5 (PPT) (purified)	32	852	21.8	18,573	100
DE-23 cellulose	46	64.4	104.9	6,750	36.4
DE-52 cellulose	18	27.6	111.0	2,930	15.8
Calcium phosphate gel:					
First peak	34	3.6	-	-	-
Second peak	36	3.09	360	1,115	6.0

purification of the enzyme from Cow 445. Purification of the #5 precipitate could not be achieved with new lots of TEAE cellulose from other sources. Approximately 2 g of the #5 precipitate (Table 6) were consumed in this attempt. However, a preparation of DEAE cellulose (DE-23) was found to give good resolution of the enzyme (Figure 22).

The enzyme from the DE-23 column was rechromatographed on DE-52 column (Figure 23). DE-52 is a microgranular material that Huang and Stumpf (1970) found would give good resolution of acetyl-CoA synthetase isozymes from potato. Further resolution was not achieved. However, when the enzyme from the DE-52 column was diluted with 0.001 M potassium phosphate buffer and chromatographed on calcium phosphate gel, extensive purification was achieved (Figure 24). The enzyme eluted in two peaks. The fractions containing enzyme activity from the first peak were combined and rechromatographed on calcium phosphate gel to determine whether or not the enzyme would eluate at the same place. The protein did appear at the same place. However, the enzyme activity was lost.

Acetyl-CoA synthetase activity in the second peak (Figure 24) appeared to fall on the protein curve. The specific activity varied from 200 to 424, but most of the tubes had a specific activity of 325 to 400. The

FIGURE 22.--Chromatography of acetyl-CoA synthetase on De-23 cellulose.

The column was washed first with 140 ml of 3 mM 2-ME - 0.005 M Tris-HCl buffer, pH 7.5; then with 160 ml of 3 mM 2-ME - 0.01 M Tris-HCl buffer, pH 7.5. The activity was eluted with 600 ml of a linear KCl gradient of 0 to 0.6 M in 3 mM 2-ME - 0.01 M Tris-HCl buffer, pH 7.5.

(———), protein mg/ml; (----), units/ml.

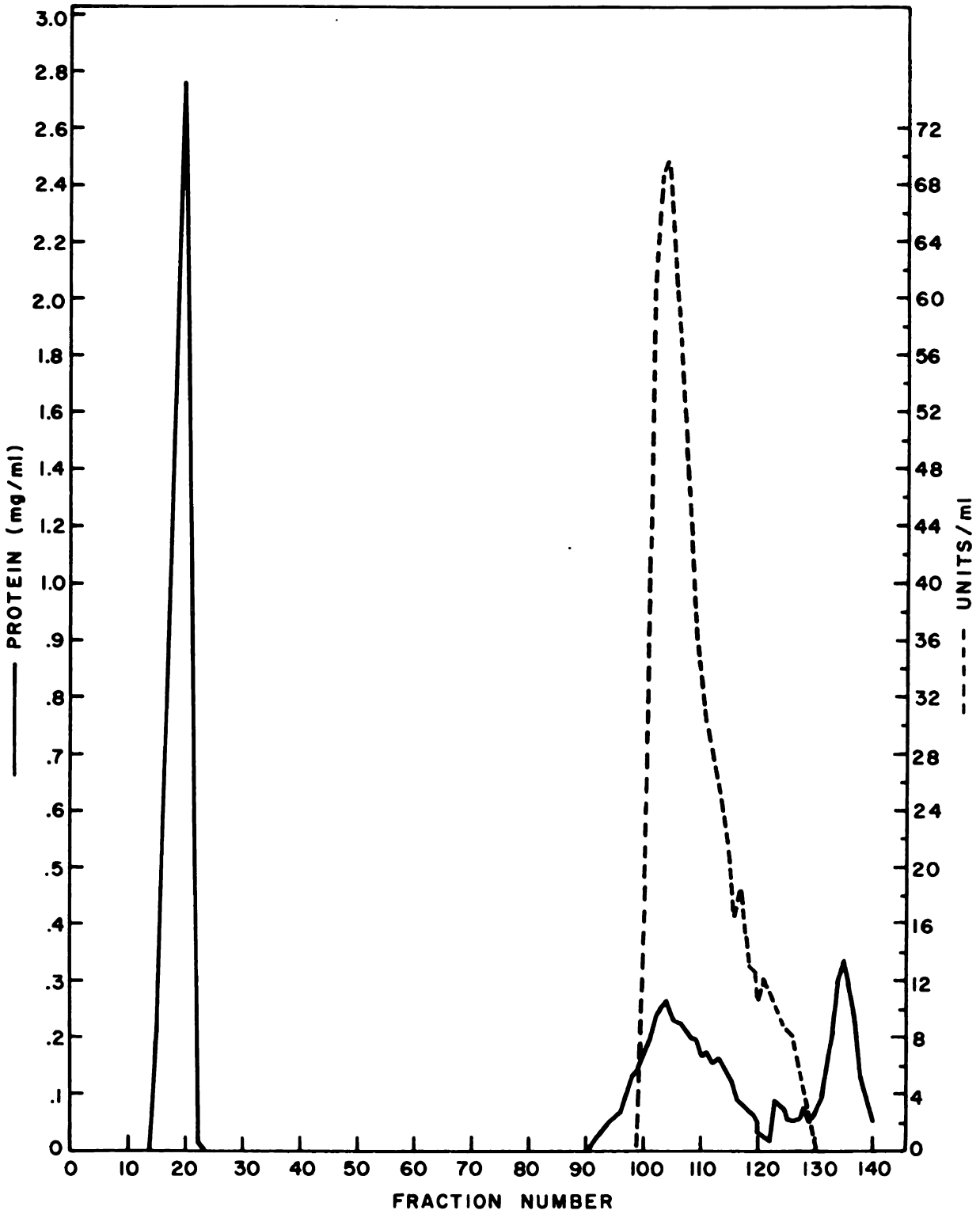

PROTEIN (mg/ml)
UNITS/ml
FRACTION NUMBER
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
72
68
64
60
56
52
48
44
40
36
32
28
24
20
16
12
8
4
0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140

FIGURE 23.--Rechromatography of acetyl-CoA synthetase on DE-52 cellulose.

The column was washed first with 75 ml of 0.005 M Tris-HCl - 3 mM 2-ME buffer, pH 7.5. The activity was eluted with 400 ml of a linear KCl gradient of 0.15 M to 0.4 M in, 3 mM 2-ME - 0.01 M Tris-HCl buffer, pH 7.5.

(———), protein mg/ml; (----), units/ml.

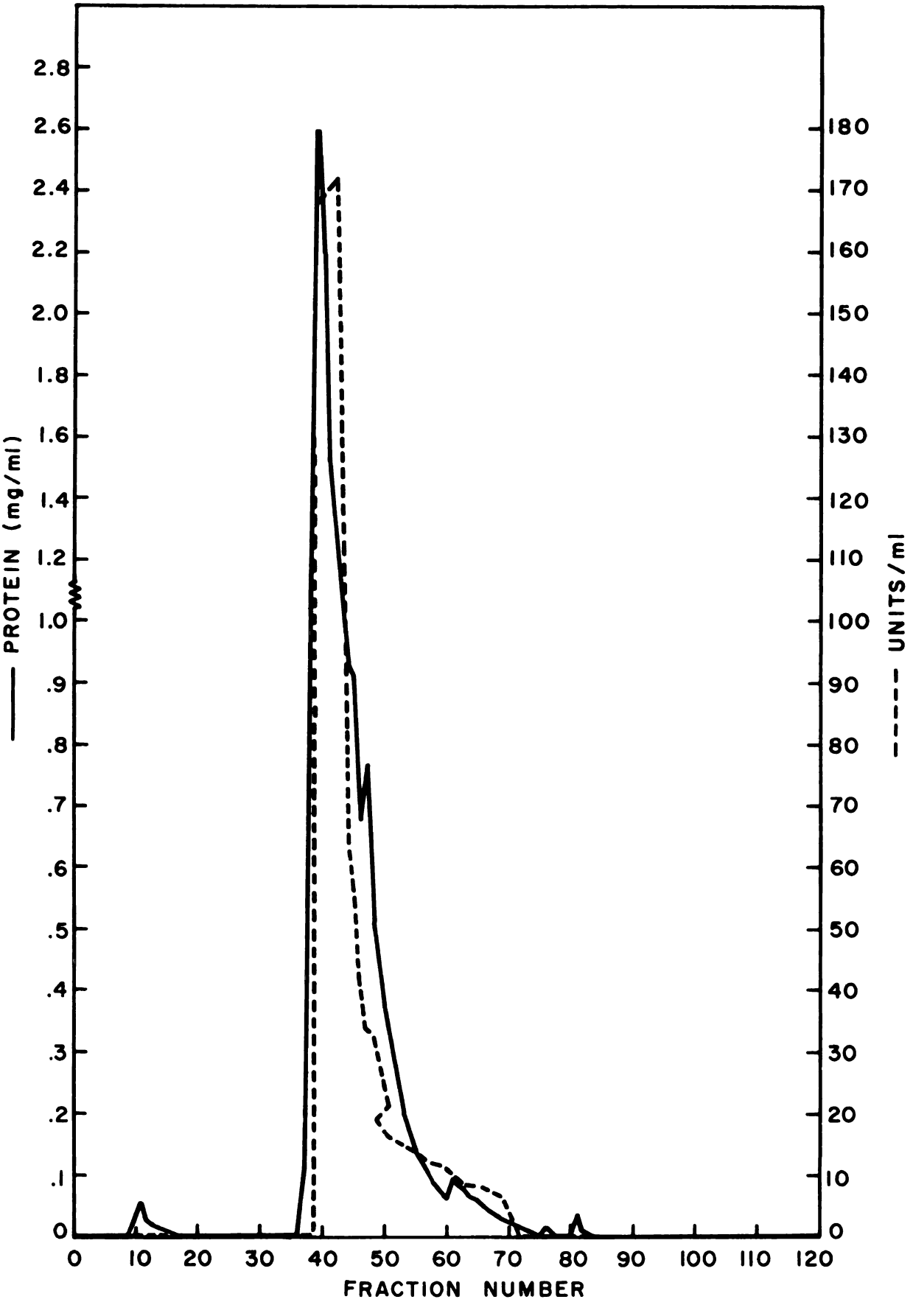
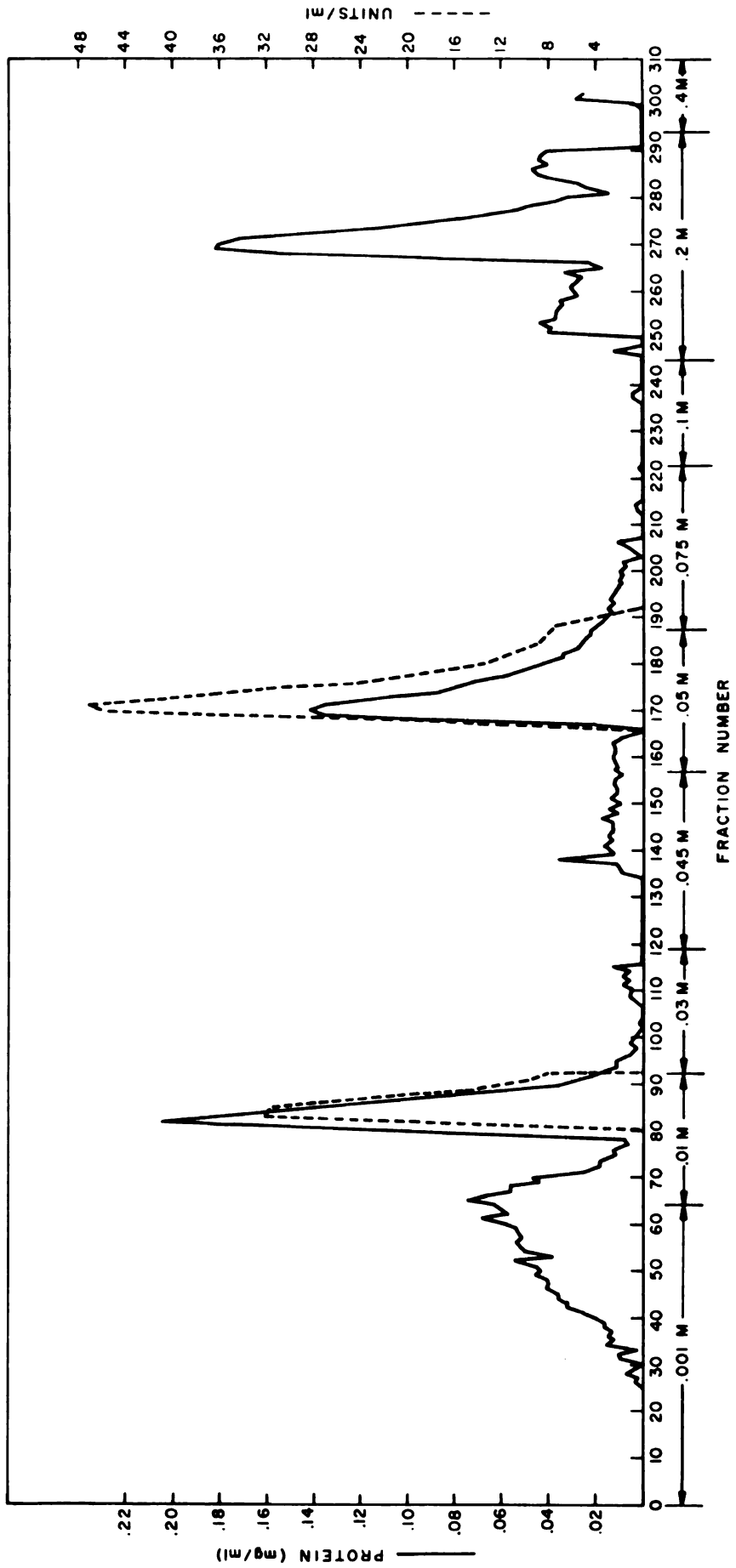

PROTEIN (mg/ml)
UNITS/ml
FRACTION NUMBER
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180

FIGURE 24.--Chromatography of acetyl-CoA synthetase on calcium phosphate gel (enzyme prepared from mitochondria of Cow 1063).

The column was washed with stepwise gradient of increasing concentration of potassium phosphate buffer, pH 7.

(———), protein mg/ml; (----), units/ml.


UNITS/ml
PROTEIN (mg/ml)
FRACTION NUMBER
.001 M
.01 M
.03 M
.045 M
.05 M
.075 M
.1 M
.2 M
.4 M

specific activity when plotted gave almost a straight line. These data indicated that the enzyme protein was pure. The enzyme from this second peak was concentrated, divided into small aliquots, and frozen at -60° C.

The molecular weight of the enzyme was determined to be 63,000 using sucrose density gradient centrifugation (Martin and Ames, 1961) (Figure 25). This value agreed with the one obtained for the enzyme from Cow 444 (Figure 17). Substrate specificity studies showed that the enzyme was most active with acrylate followed by acetate, propionate and maleate (Table 7). The enzyme was not active with C_4 to C_8 straight chain fatty acids.

Enzyme activity was linear with time and protein concentration (Figures 26 and 27). The effect of pH on enzyme activity is shown in Figure 28. Michaelis-Menten constants for Mg, CoA, ATP, and acetate are presented in Figures 29, 30, 31, and 32, respectively. These values agreed with those reported by others (Table 1). In contrast with the heart and potato enzymes (Farrar, 1970; Huang et al., 1970) relatively high levels of AMP were required for enzyme inhibition (Figure 33).

Of major significance were the characterization studies using polyacrylamide gel electrophoresis. When stained with coomassie blue dye, there were seven protein bands (Figure 34). It appeared that more than 90% of the protein was in the fourth band. This fourth protein band,

FIGURE 25.--Sucrose density gradient centrifugation of acetyl-CoA synthetase (using ovalbumin as a reference).

The centrifugation was carried out at 50,000 rpm for 10 hours at 4° C. using a 5-20% sucrose gradient. (Details of the experiments are given in the Experimental Procedure.)

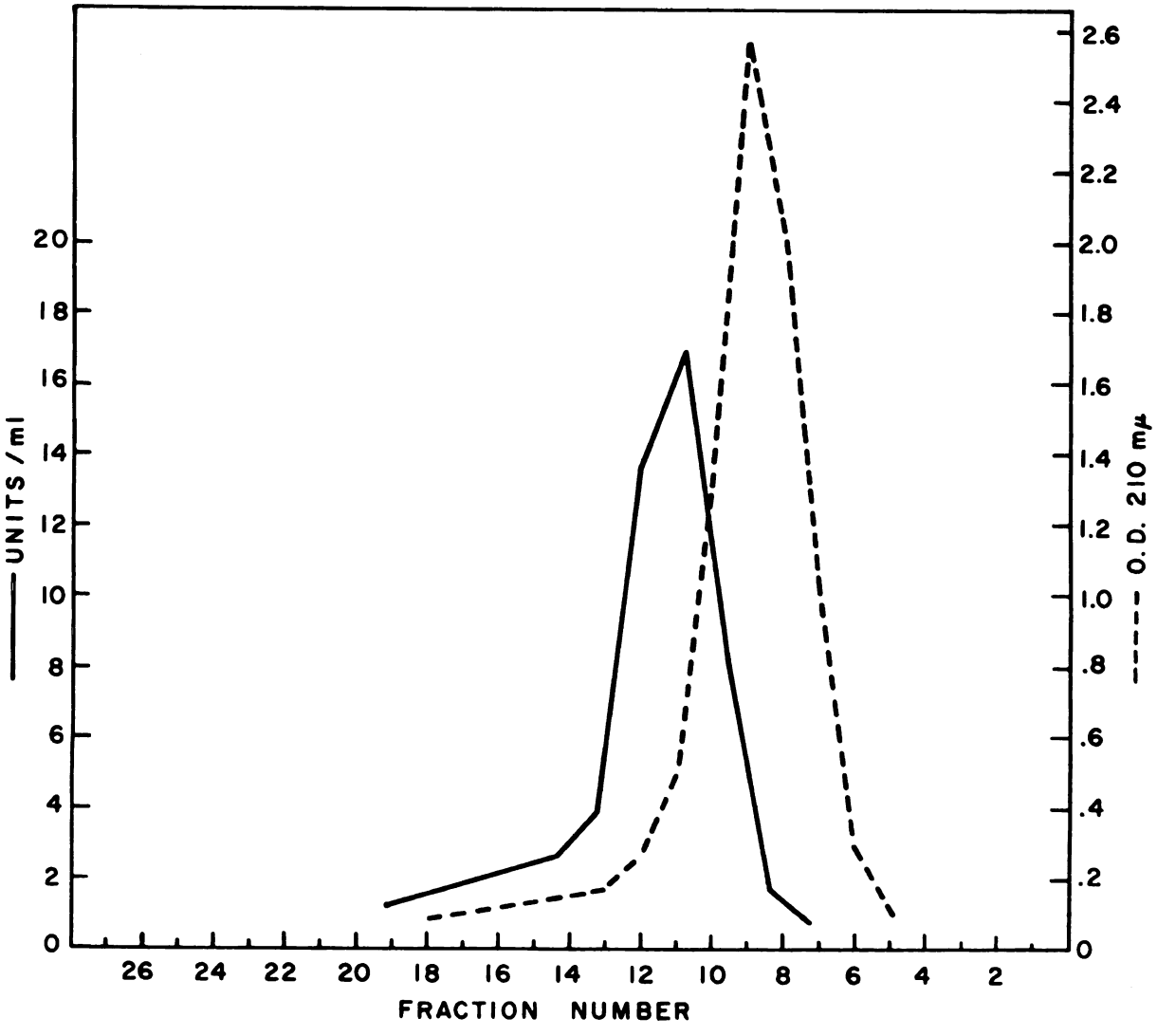

UNITS / ml
O.D. 210 mμ
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
18
20
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
26
24
22
20
18
16
14
12
10
8
6
4
2

TABLE 7.--Substrate specificity of acetyl-CoA synthetase

Substrates Tested	Relative Activity
Acetate	100
Propionate	65
Butyrate	0
Valerate	0
Hexanoate	0
Heptanoate	0
Octanoate	0
Acrylate	151
Maleate	25
Crotonate	0

FIGURE 26.--Effect of time on the linearity of the acetyl-CoA synthetase reaction.

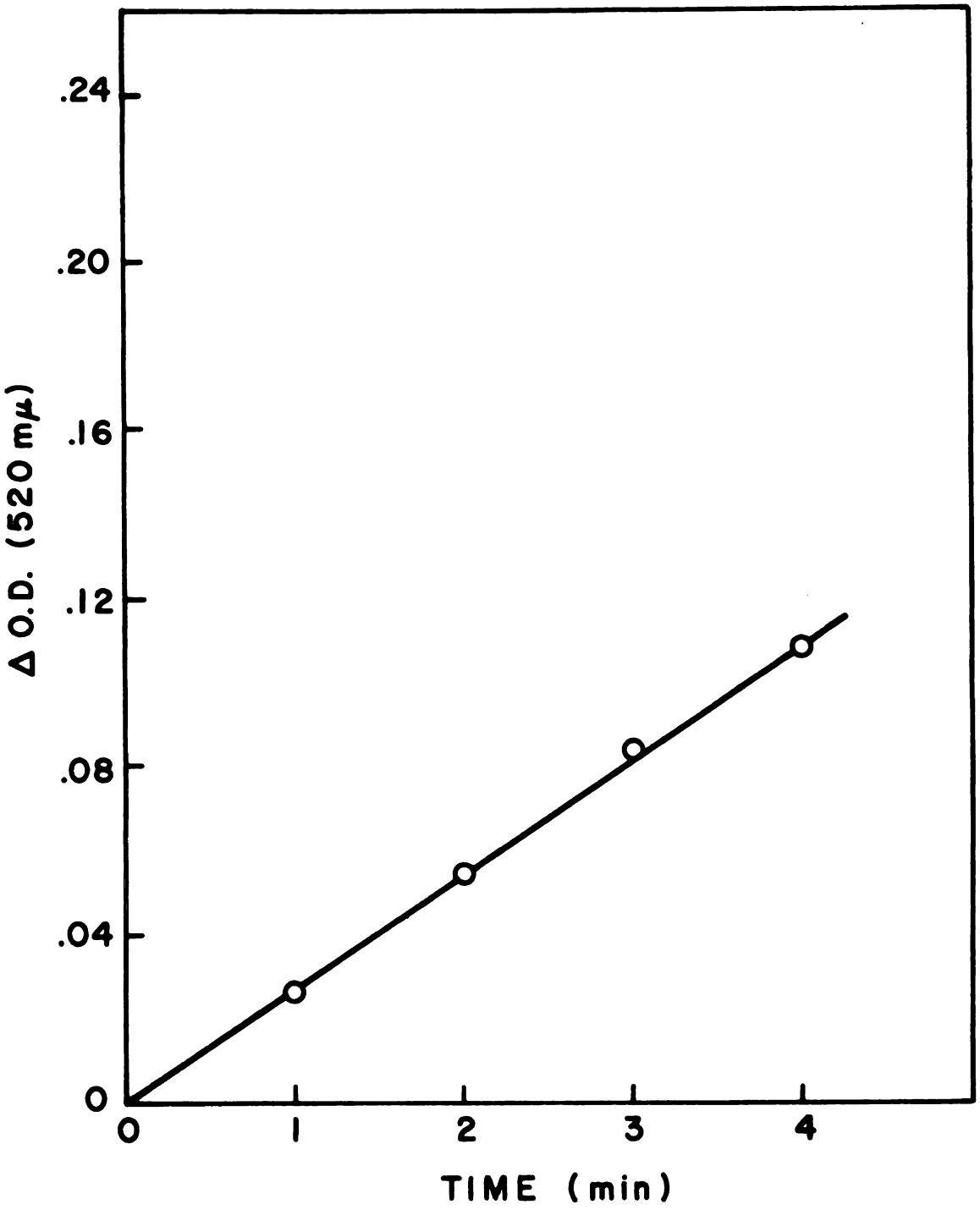

.24
.20
.16
.12
.08
.04
0
Δ O.D. (520 mμ)
0
1
2
3
4
TIME (min)

FIGURE 27.--Effect of protein concentration on the linearity of the acetyl-CoA synthetase reaction.

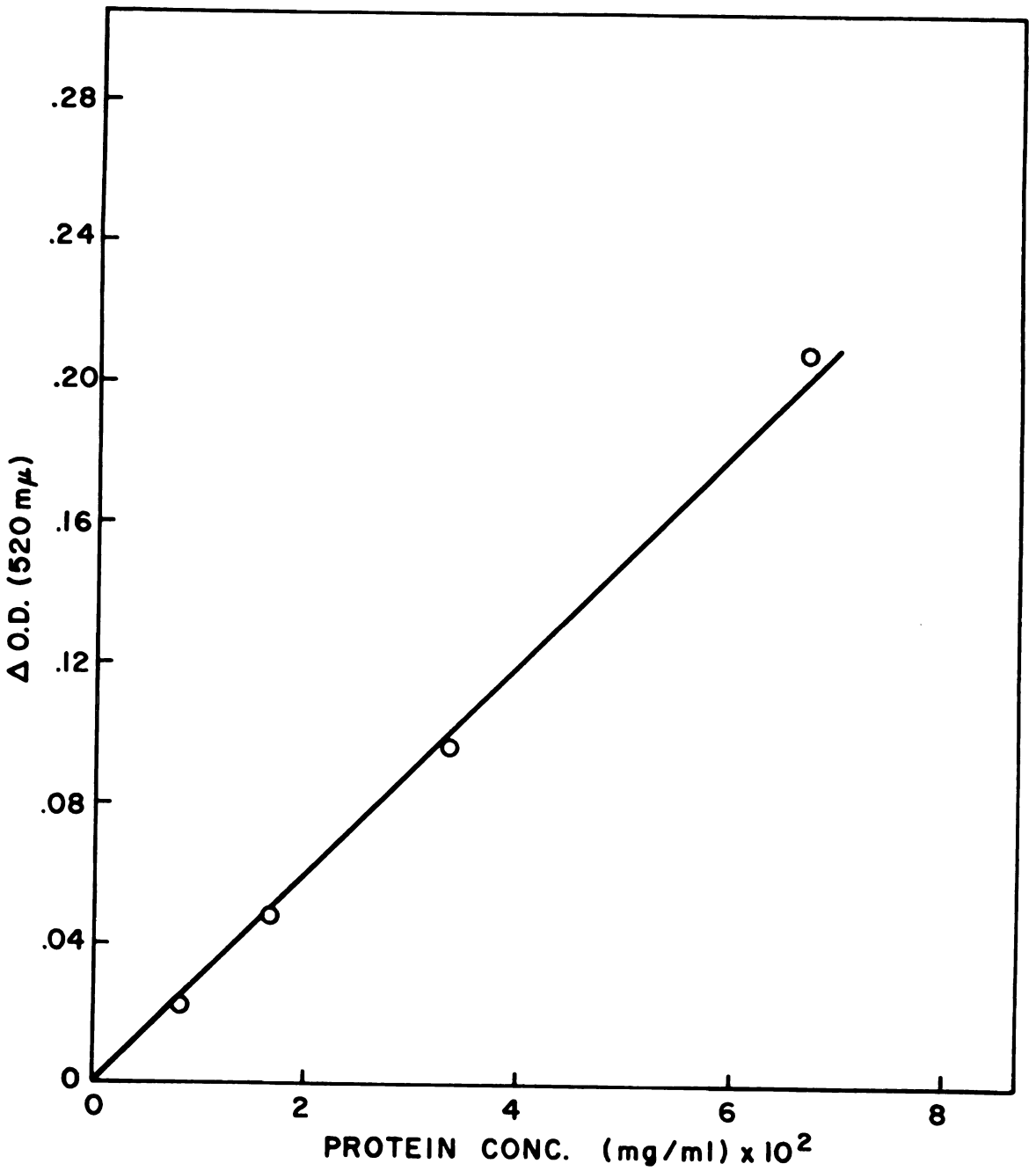

.28
.24
.20
.16
.12
.08
.04
0
Δ O.D. (520 mμ)
0
2
4
6
8
PROTEIN CONC. (mg/ml) x 10²

FIGURE 28.--Effect of pH on acetyl-CoA synthetase activity.

⊙—⊙—⊙ Tris-HCl buffer

Δ—Δ—Δ Potassium phosphate buffer

□—□—□ Glycine buffer

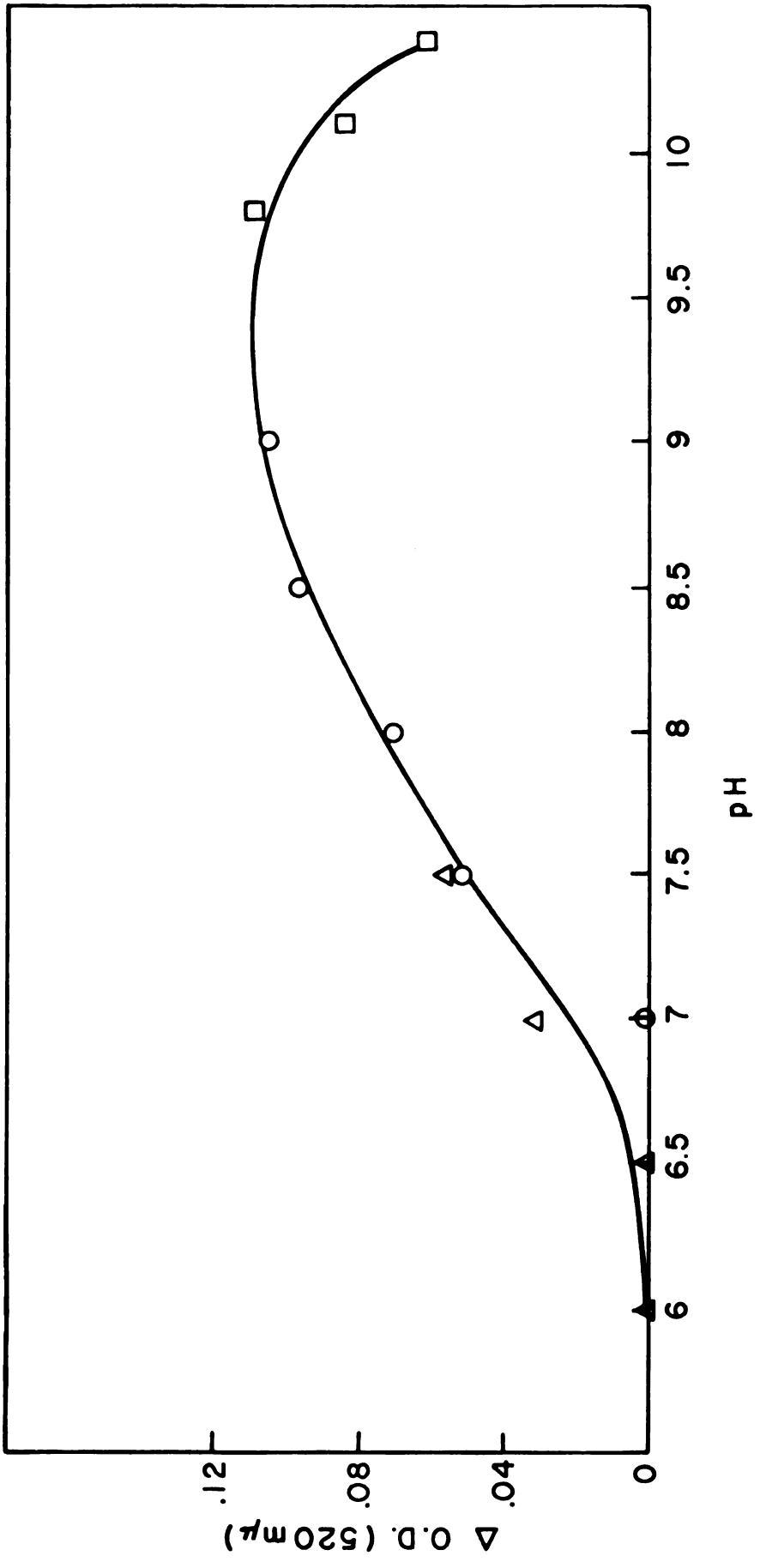

Δ O.D. (520mμ)
0
.04
.08
.12
6
6.5
7
7.5
8
8.5
9
9.5
10
pH

FIGURE 29.--Effect of Mg concentration on acetyl-CoA synthetase activity. The inset is the Lineweaver-Burk plot of the same data.

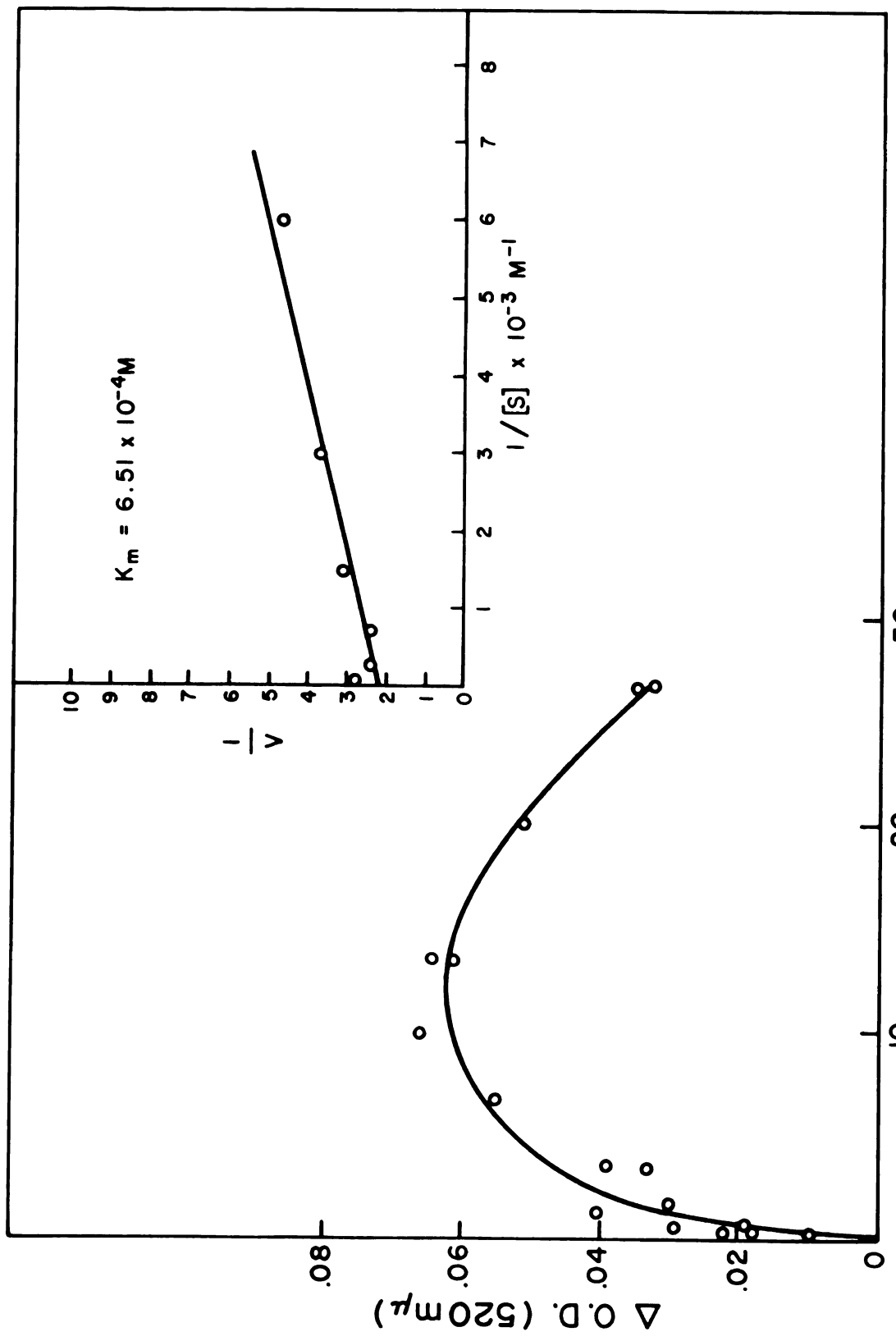

Δ O.D. (520mμ)
[MgCl2] (mM)
1/v
1/[S] x 10-3 M-1
Km = 6.51 x 10-4M

FIGURE 30.--Effect of CoA concentration on acetyl-CoA synthetase activity. The inset is the Lineweaver-Burk plot of the same data.

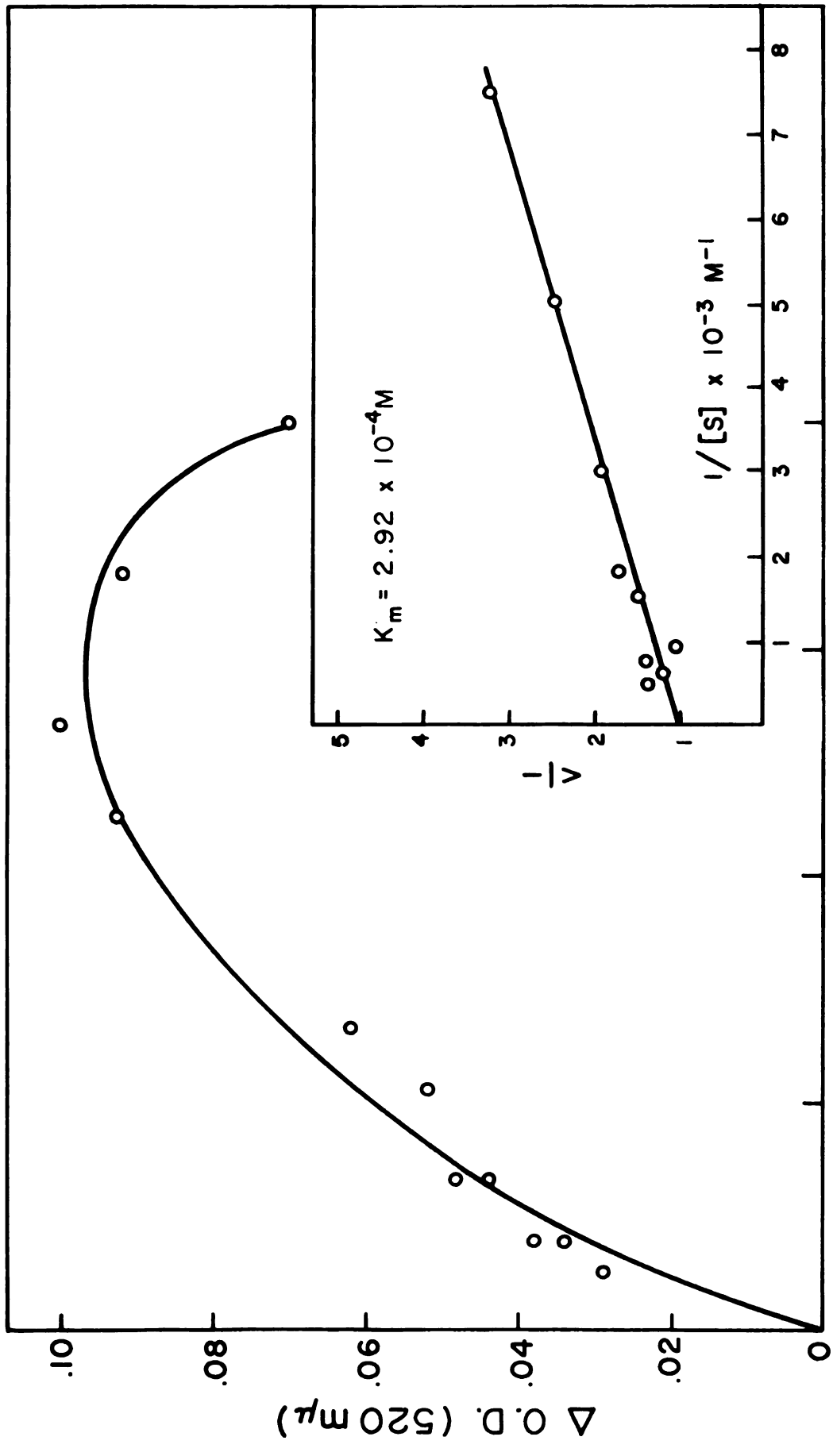

Δ O.D. (520 mμ)
.10
.08
.06
.04
.02
0
0.5
1
1.5
2
[CoA] (mM)
$\frac{1}{v}$
5
4
3
2
1
$K_m = 2.92 \times 10^{-4} M$
1/[S] x 10^{-3} M^{-1}
1
2
3
4
5
6
7
8

FIGURE 31.--Effect of ATP concentration on acetyl-CoA synthetase activity. The inset is the Lineweaver-Burk plot of the same data.

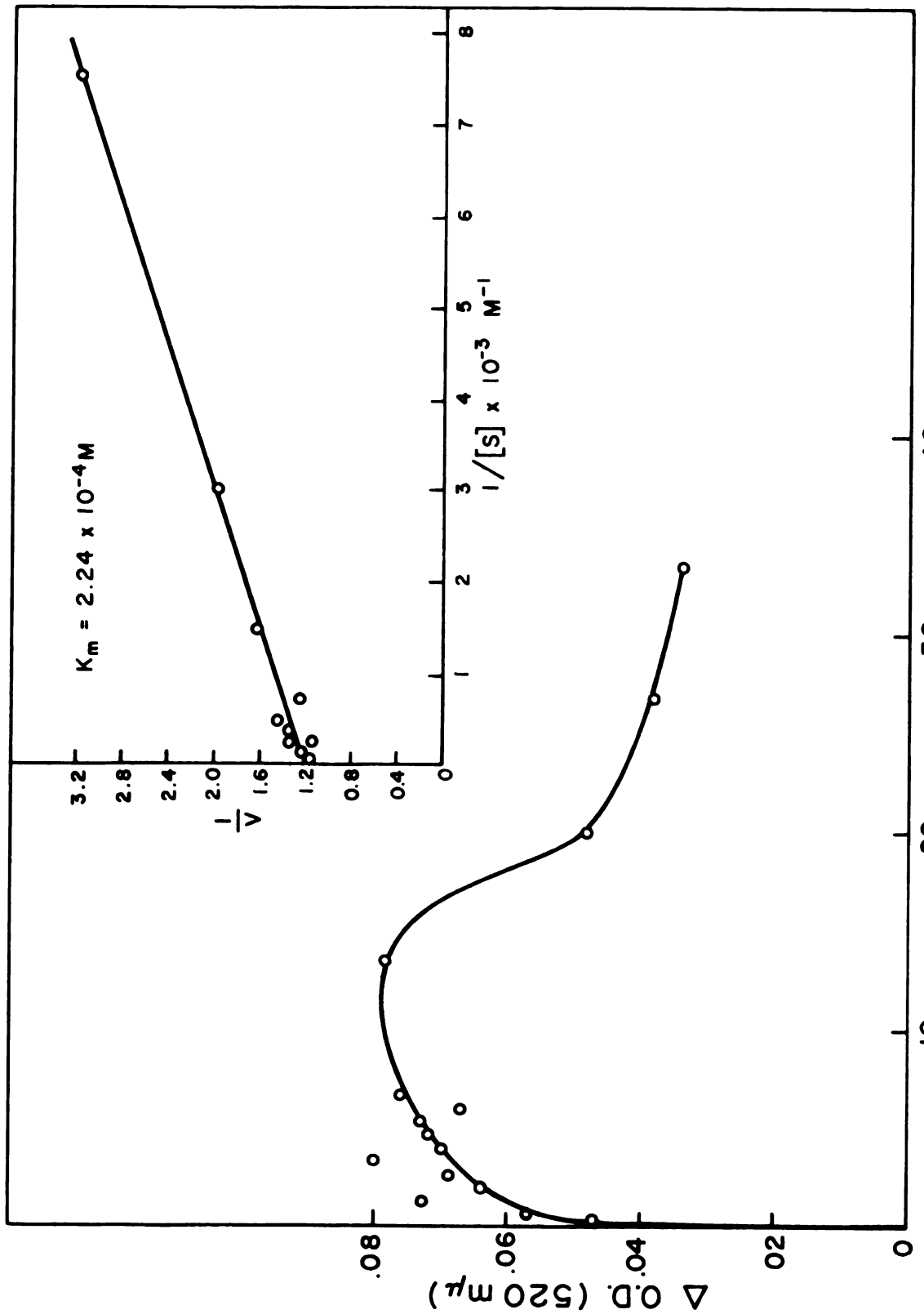

Δ O.D. (520 mμ)
[ATP] (mM)
$K_m = 2.24 \times 10^{-4} M$
$\frac{1}{V}$
$1/[S] \times 10^{-3}\ M^{-1}$

FIGURE 32.--Effect of acetate concentration on acetyl-CoA synthetase activity.

The inset is the Lineweaver-Burk plot of the same data.

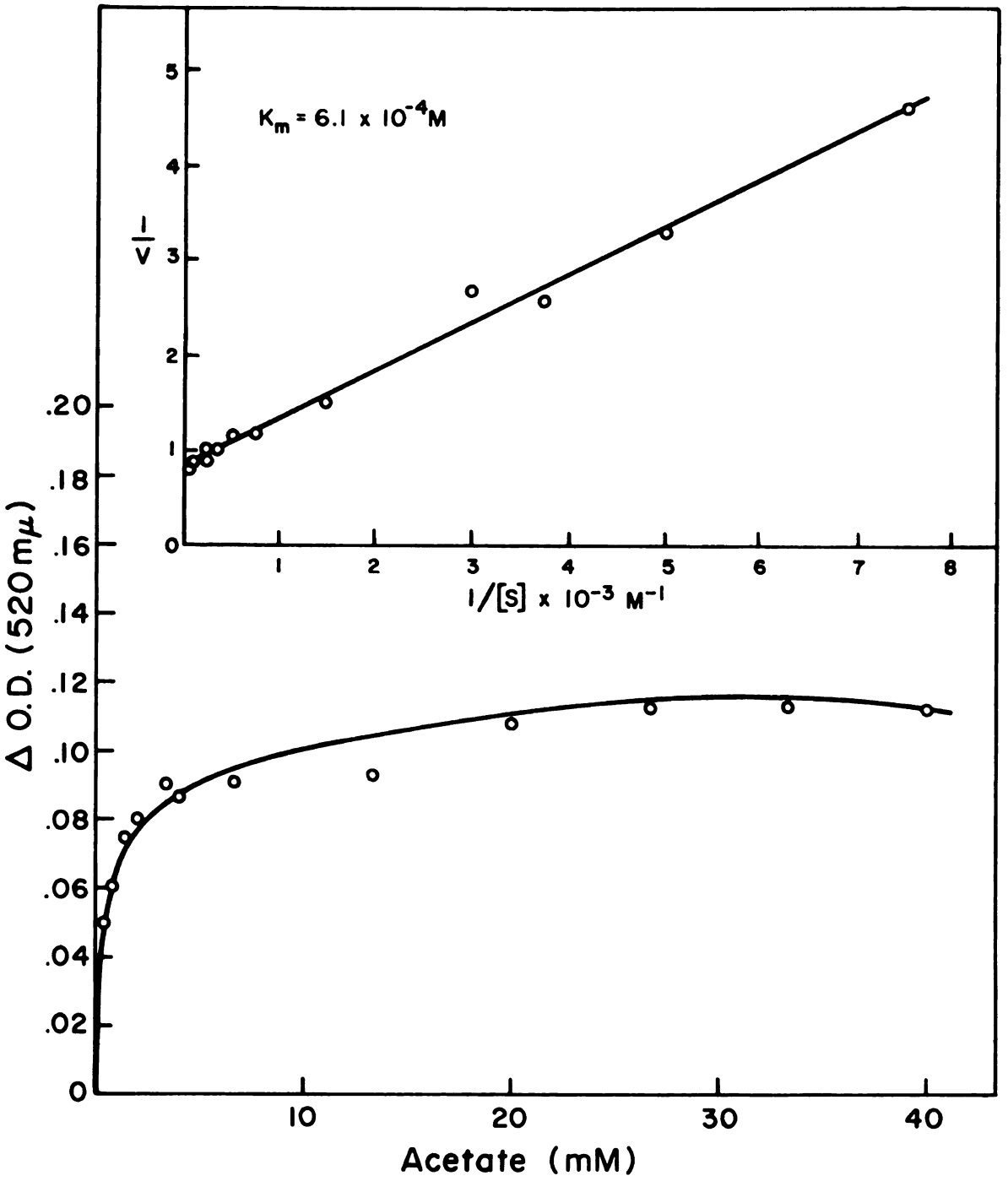

$K_m = 6.1 \times 10^{-4} M$
$\frac{1}{V}$
$1/[S] \times 10^{-3}\ M^{-1}$
Δ O.D. (520mμ)
Acetate (mM)

FIGURE 33.--Effect of AMP concentration on acetyl-CoA synthetase activity.

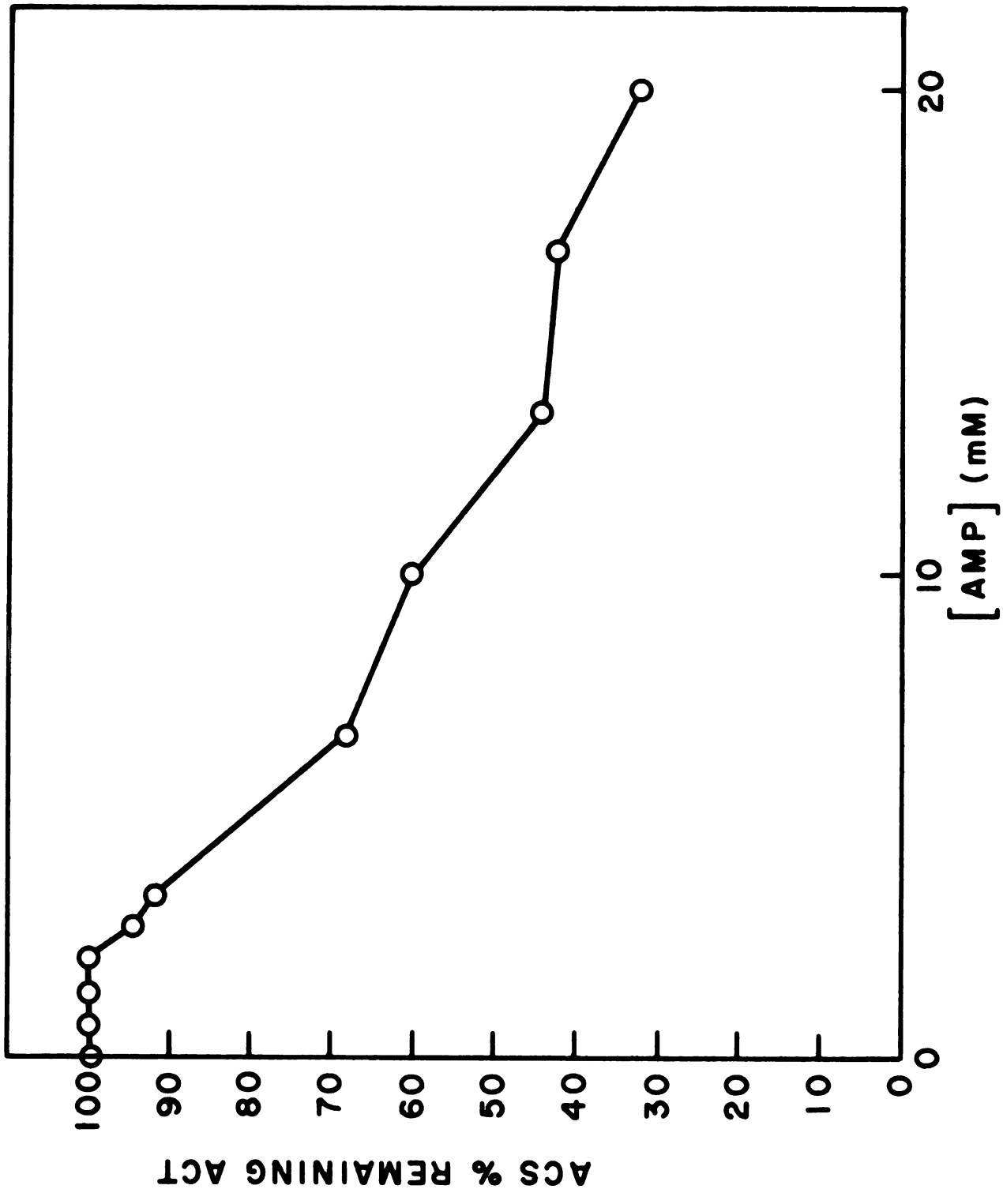

ACS % REMAINING ACT
100
90
80
70
60
50
40
30
20
10
0
0
10
20
[AMP] (mM)

FIGURE 34.--Schematic presentation of polyacrylamide gel electrophoresis of purified acetyl-CoA synthetase.

A pH 8.3 buffer system (Davis, 1969) was used. 20 µg of protein were layered on top of the gel. The run was carried out for 30' using 6 ma/gel at 4° C. Gel A shows protein bands and Gel B shows enzyme activity.

B

A

along with two other bands, contained acetyl-CoA synthetase activity. The enzyme activity from the first peak of the calcium phosphate gel column (Figure 24) was lost during rechromatography. Consequently, the nature of the proteins that were present in this enzyme peak is not known.

CHAPTER V

DISCUSSION

The data indicate that acetyl-CoA synthetase exists in multiple molecular forms. The exact nature of these forms is not understood at present. Since it is now known that many enzymes do exist in more than one molecular form, it is not surprising to find multiple forms of acetyl-CoA synthetase in bovine mammary gland. If a single enzyme occurs in different forms in a single organism and each form catalyses the same reaction, then isozymes of the enzyme exist. The term isozyme, then, can be defined as those enzymes that exist in more than one structural form in the same species. This term was coined for the first time by Markert and Møller (1959) to designate the multiple molecular forms of lactic dehydrogenase and other enzymes found within a single organism. Isozymes are of different kinds, homopolymeric, heteropolymeric, conformational, hybrid, conjugated, and others. On the other hand certain enzyme proteins occur

in different aggregate forms of a monomer. In such a case either the aggregated or the monomer form could be enzymatically active.

It is not entirely clear whether the multiple molecular forms of acetyl-CoA synthetase are aggregates of a monomeric unit or whether isozymes exist. Acetyl-CoA synthetase activity did not separate from other proteins but emerged within the void volume when chromatographed on Sephadex G-100 column (Figure 8). The enzyme behaved similarly when Bio-gel P-100 (Figure 6) and Bio-gel P-200 (Figure 7) columns were used. These results suggest aggregation of the enzyme under the experimental conditions.

Sedimentation equilibrium studies of the enzyme purified from a TEAE Cellulose column (Figure 13, Figure 14) can be interpreted in three different ways (Figure 15). First that the enzyme may exist in a monomer-dimer equilibrium. The lower molecular weight value of 49,000 obtained for acetyl-CoA synthetase could represent the monomer form of the enzyme while the higher value of 85,000 could represent the dimer form or else some average value of the two species present. However, the molecular weight value of the enzyme from sucrose density gradient centrifugation is estimated to be 62,000 (Figure 17). The minimum molecular weight reported for acetyl-CoA synthetase

from bovine heart mitochondria is 31,000 to 34,000 (Webster, 1965). If it is assumed that the enzymes from both tissues have similar molecular weights then the mammary gland enzyme could possibly have been aggregated to a dimer form under the conditions of the experiment to give a value of 62,000.

The second possibility suggested by the sedimentation equilibrium studies of the mammary gland enzyme is that a high molecular weight protein may be present in the enzyme preparation. To test this possibility the enzyme was chromatographed on Sephadex G-200 to separate a high molecular weight impurity from acetyl-CoA synthetase (Figure 18). However, the enzyme lost two-thirds of the activity. An inactive protein peak eluted in the void volume and the enzyme right after the void volume. This can be explained either on the basis that Sephadex denatures the enzyme as has been reported for many enzymes or else that the first inactive protein peak that emerged from the column represents an aggregate form of the enzyme which is not active. The concentration of KCl in the buffer was 0.1 M and perhaps not enough to break up the aggregation. The fractions of the eluate from the Sephadex G-200 column were combined but original enzyme activity was not restored.

Non-specific aggregation of acetyl-CoA synthetase is another possible interpretation of the sedimentation

equilibrium studies. There is not enough evidence, at present, to determine the exact nature of aggregation exhibited by acetyl-CoA synthetase.

Disc gel electrophoresis studies of the enzyme purified from Cow 444 mammary gland tissue also supported the hypothesis that the enzyme acetyl-CoA synthetase exists in multiple molecular forms. Staining of the gel with amido black dye showed four protein bands (Figure 16). The enzyme activity was found associated with the third band, next to the fastest moving band. The two slower moving protein bands which represented about 70% of the total protein applied had no enzyme activity. These two protein bands were also not well separated indicating some kind of equilibrium between the proteins. These protein bands possibly could represent aggregate forms of the enzyme which are not active.

The hypothesis that acetyl-CoA synthetase has a strong tendency to aggregate, the state in which it is not active, is further supported by the fact that the enzyme is very stable in the presence of ammonium sulphate and can be stored frozen for a long period of time without losing activity. The presence of the salt is known to prevent aggregation. This might explain why the enzyme remains stable longer in the presence of salt. Also, the activity of the enzyme is found to increase

when assayed in a more dilute solution, a condition where the proteins are known to exist in a non-aggregate form.

When more enzyme was isolated and purified from the mammary gland tissue of cows 445 and 1063, the purification was carried a step beyond chromatography on TEAE cellulose. Adsorption chromatography using calcium phosphate gel gave more than a three-fold purification (Table 6). Acetyl-CoA synthetase activity which emerged as a single peak from the DE-52 cellulose column (Figure 23) separated into several protein peaks emerging at different ionic strengths of phosphate buffer. The enzyme activity was found to be associated with two protein peaks (Figure 24). The proteins, comprising the other inactive peaks, should have very similar overall charges since they emerged as a single peak from the DE-52 cellulose column. It is not known whether they represent impure foreign proteins or inactive forms of acetyl-CoA synthetase. Aggregation of the active enzyme to form an inactive aggregate is possible. This kind of aggregate, however, would also be expected to separate upon centrifugation in a sucrose density gradient. The two sucrose density gradient centrifugation experiments carried out using the enzyme from a TEAE cellulose column (Figure 17) and from a calcium phosphate gel column (second enzyme activity peak, Figure 25) showed only

one activity peak. It can be argued, however, that the conditions were not right for the different forms of the enzyme to separate.

Although much of the data suggest that acetyl-CoA synthetase aggregates to yield multiple molecular forms, it is possible that isozymes exist. The enzyme purified using calcium phosphate gel could be separated into seven protein bands using gel electrophoresis. Enzyme activity was found to be associated with three of these protein bands (Figure 34). This data suggest the existence of acetyl-CoA synthetase as isozymes. The earlier report that acetyl-CoA synthetase exists as isozymes in potato tuber strengthens this interpretation (Huang and Stumpf, 1970). Five isozymes of acetyl-CoA synthetase were reported which were separated by initial chromatography on DEAE cellulose and then further purified by adsorption chromatography on hydroxylapatite. Each isozyme catalyzed the activation of acetate and exhibited similar kinetic properties. Unpublished studies in this laboratory indicate that acetyl-CoA synthetase in bovine fetal tissues preferentially activates propionate rather than acetate, a situation in contrast to the adult enzyme. This observation can also be explained on the basis of isozymes. That is, isozymes exist in fetal tissues that preferentially activate propionate, whereas an isozyme in adult tissue predominate that preferentially activates acetate.

The nature of the multiple molecular forms of lactic dehydrogenase, glutamate dehydrogenase, adenosine deaminase, carbonic anhydrase, and acetyl-CoA carboxylase has been studied extensively. Of these enzymes the nature of the multiple forms of acetyl-CoA synthetase most closely resembles that of glutamate dehydrogenase and acetyl-CoA carboxylase. The most extensively studied example of an isozyme is that of lactic dehydrogenase from mammalian tissues. This enzyme exists as a tetramer and is made up of two different types of subunits, M and H kind, which are produced by two different genes. The different combination of these two subunits results in the production of five isozymes. A single subunit is not found to be enzymatically active (Markert, 1968).

A single polypeptide chain may also polymerize to yield a series of isozymes of different polymer size, like glutamate dehydrogenase (Bitensky et al., 1965).

$$\text{Polymer} \rightleftharpoons \text{monomer x} \rightleftharpoons \text{monomer y}$$

Monomer x is the form of glutamate dehydrogenase which catalyzes the reaction. At high protein concentration the monomer aggregates to form the higher molecular weight polymer while certain small allosteric modifiers influence the formation of y monomer causing the disaggregation of the polymeric form of the enzyme.

A single polypeptide chain can also fold in different conformations giving rise to isozymes called "conformors," like adenosine deaminase (Murphy et al., 1969) and carbonic anhydrase (Edsall, 1968). These conformors have the same catalytic properties but can be separated by electrophoresis or ion-exchange chromatography.

Acetyl-CoA carboxylase provides an example of the phenomenon where the same enzyme can exist in different forms of aggregation (Lane, 1969). Acetyl-CoA carboxylase exists as an unbranched, filamentous structure with a sedimentation coefficient of 50-68 S and molecular weight of several million. Lane had carried out extensive studies with avian liver and bovine adipose tissue acetyl-CoA carboxylases. Both exhibit aggregation phenomenon. The polymeric form of each carboxylase can be dissociated into protomers of the same molecular weight 410,000 and sedimentation coefficient, 13 to 14 S. Each protomer, in its turn, is composed of four non-identical subunits each of about 100,000 molecular weight. The catalytic properties of acetyl-CoA carboxylase are intimately associated with its state of aggregation. The enzyme is active in the aggregated, polymeric form while the dissociated subunits have no activity. The rat liver acetyl-CoA carboxylase exhibits similar phenomenon (Numa et al., 1966). Tricarboxylic acids like

citrate and isocitrate activate acetyl-CoA carboxylase by shifting the equilibrium toward the formation of the polymeric form.

Acetyl-CoA synthetase from the mitochondria of lactating mammary gland may exhibit an aggregation phenomenon similar to that observed for acetyl-CoA carboxylase, except that as the extent of aggregation increases enzyme activity decreases. In this sense, acetyl-CoA synthetase is more like glutamate dehydrogenase where the polymeric form is not active but the monomer form is active. Similarly there is a possibility that some small molecules might act as an allosteric modifier of acetyl-CoA synthetase and play an important role in maintaining the proper equilibrium between the different molecular forms of the enzyme.

In summary, acetyl-CoA synthetase prepared as described herein represents the most highly purified form of the enzyme ever obtained from any mammalian tissue. The enzyme exists in multiple molecular forms. This work provides a sound basis for continued study of the various properties of the enzyme and the role it plays in control of cell metabolism.

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