THE MECHANISM OF DEGRADATION OF SIDE CHAINS OF PHYTOSTEROLS BY MICROORGANISMS

BY

CHING-SHIH CHEN

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A thesis submitted to the Graduate School of the University of Wisconsin-Madison in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Approved by Thesis Reading Committee:

Major Professor — **Date of Examination** January 10 1955

Dean, Graduate School

I wish to dedicate this to my parents and my wife Woan-ru, whose love, understanding and encouragement made the attainment of this goal possible and to whom I am forever indebted.

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THE MECHANISM OF DEGRADATION OF SIDE CHAINS OF PHYTOSTEROLS BY MICROORGANISMS

CHING-SHIH CHEN

(Under the supervision of Professor Charles J. Sih)

The degradative pathway of the phytosterols, campesterol and sitosterol by microorganisms has been shown to proceed as follows: The initial step involves hydroxylation and oxidation at C-26 to form a C-26 carboxylic acid, which is then activated via coenzyme A thioester formation. The resulting acyl-CoA derivative then undergoes dehydrogenation, mediated by a FMN-dependent oxidase. Carboxylation at C-28 results in the formation of an unsaturated dicarboxylate intermediate, which upon subsequent hydration and reverse-aldolization yields the common intermediate, 3,24-dioxo-cholest-4-en-26-oic acid. The rate of degradation of phytosterols may be greatly enhanced by the addition of sodium bicarbonate.

With the exception of the acyl-CoA synthetase, all the other enzymes required for the formation of 3.24-dioxo-cholest-4-en-26-oic acid are associated in a multienzyme complex, which includes acyl-CoA oxidase, enoyl-CoA carboxylase, hydratase, reverse-aldol lyase and ß-hydroxyacyl-CoA dehydrogenase. Both the acyl-CoA synthetase and the multienzyme complex have been purified to a nearly homogeneous state from Mycobacterium sp. NRRL-B 3805. The acyl-CoA synthetase is monomeric, with a molecular weight of 65,000. It has a restrictive substrate preference for a bulky hydrophobic ring structure. The

multienzyme complex is highly unstable and readily dissociates into subunits when the ionic strength of solution is decreased. Biotin is present in the complex, but is imbedded inside the complex and thus inaccessible to avidin.

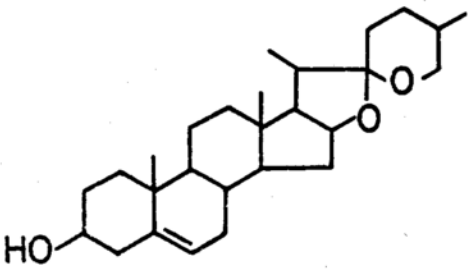
APPROVED: _____

Charles J. Sih

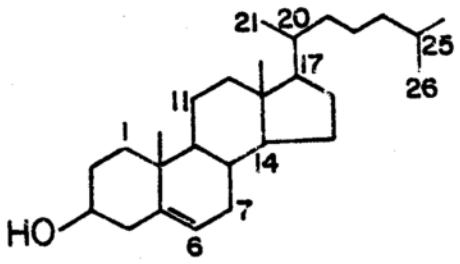
DATE: January 10, 1985

INTRODUCTION

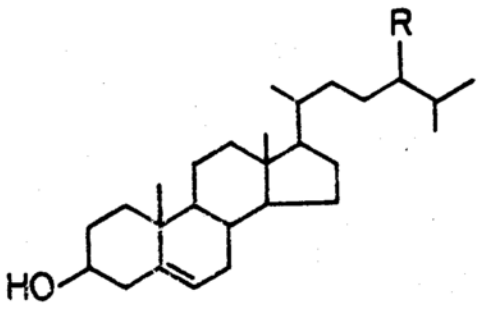
Diosgenin, a sapogenin extracted from the Mexican Barbasco root, has been the major natural raw material for the commercial production of steroid hormones. To a lesser extent, stigmasterol from soybean sterols is also used for the synthesis of corticosteroids.[1] Recently, as a result of a shortage of diosgenin, the more readily available sterols such as ß-sitosterol, campesterol, and cholesterol have attracted much attention. However, their usefulness was limited by the difficulty in selectively removing saturated aliphatic side chains while maintaining the integrity of the steroid nucleus.



Diosgenin

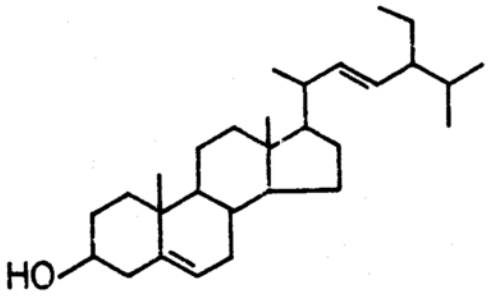


Cholesterol



$R = CH_3$ Campesterol

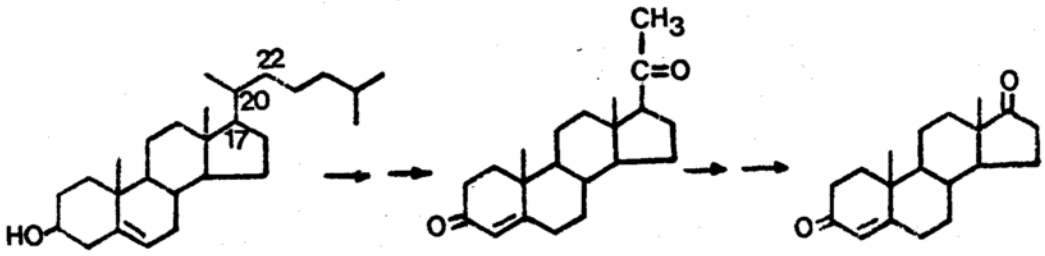
$R = C_2H_5$ Sitosterol



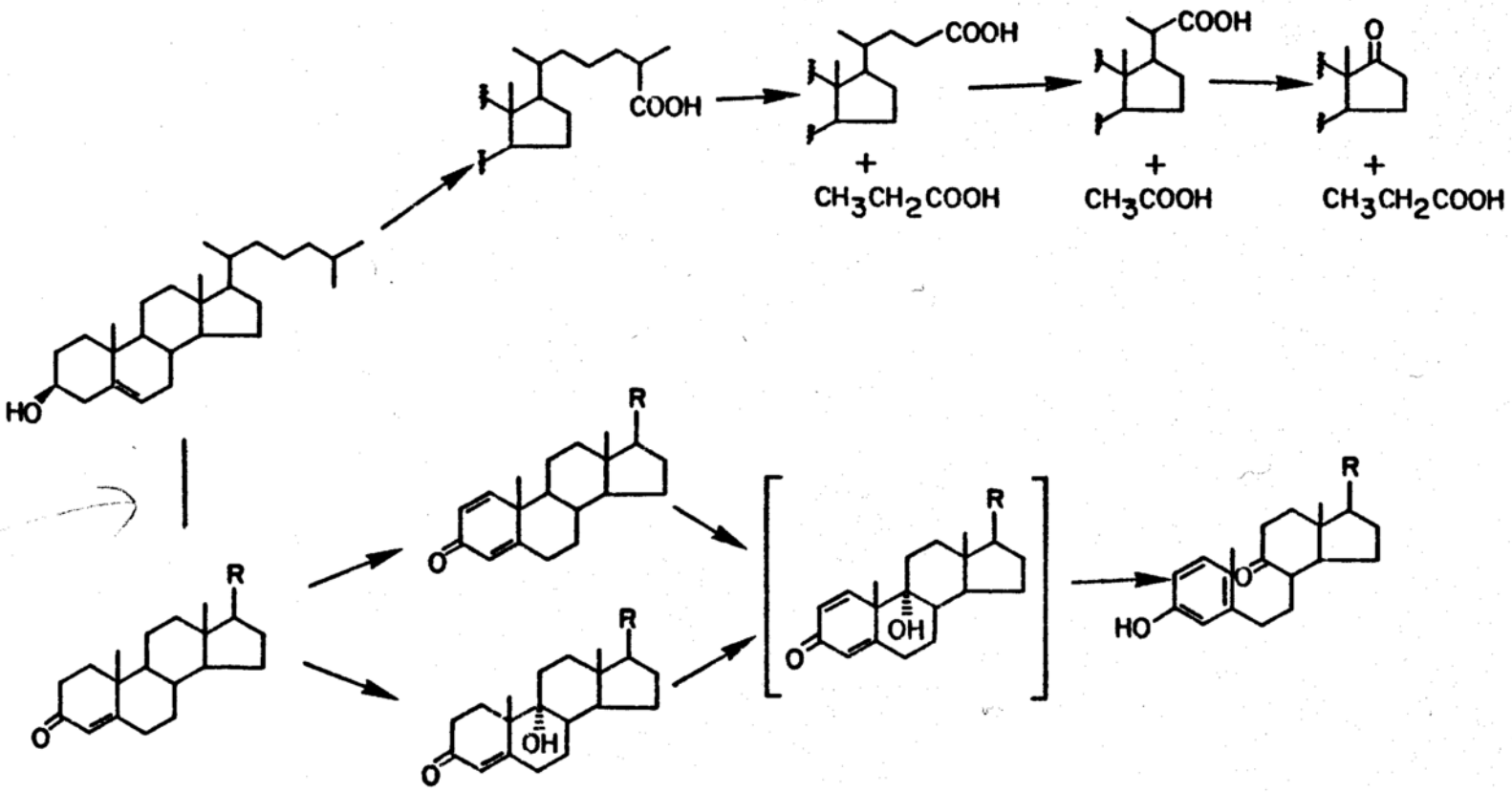
Stigmasterol

Consequently, until the development of microbiological processes they were not used as primary precursors for steroid hormone synthesis, because it has been difficult to chemically cleave efficiently the side chain of these sterols.[2] Total decomposition of cholesterol or phytosterols has been observed among a number of microorganisms of the genera Arthrobacter, Bacillus, Brevebacteria, Corynebacteria, Mycobacteria, Nocardia, and Streptomyces.[3] It was reasoned that if an organism could decompose partially the sterol by selectively cleaving the side chain without degrading the steroid nucleus, this process may have commercial application in the preparation of important steroids.

Through intensive investigations by Sih's group[4-6], the metabolic fate of cholesterol in microorganisms has been defined (Fig. 1). In contrast to mammalian systems, where 17-ketosteroids are formed via



cleavage of the C-20--C-22 bond, followed by cleavage of the C-17--C-20 bond, microbial systems degrade the side chain by a mechanism similar to the ß-oxidation of fatty acids.[7,8] After hydroxylation at C-26 and subsequent oxidation to carboxylic acid, stepwise degradation results in the formation of the C-17 keto steroid via the C-24 and C-22 carboxylic acid intermediates. The initial degradative reaction of the steroid nucleus entails the conversion of the 3ß-hydroxy-^{5} sterol to the


COOH
COOH
O
+
CH_3CH_2COOH
+
CH_3COOH
+
CH_3CH_2COOH
HO
R
O
R
OH
O
R
O
R
OH
O
HO

corresponding 3-keto-Δ^4 compound. Subsequent degradative reaction involves a 9α-hydroxylation, followed by a 1,2-dehydrogenation (or vice versa) with the formation of 3-hydroxy-9,10-secoandrost-4-ene-3,17-dione. This 9(10)-secophenol is further oxidized in a series of reactions eventually leading to carbon dioxide and water. The enzymatic reactions responsible for side chain cleavage and nucleus ring rupture do not follow a compulsory order and can proceed simultaneously and independently.

To avoid complete degradation, the enzyme involved in the initial attack on the steroid ring system, i.e., 9α-hydroxyiase or C-1(2) dehydrogenase, has to be inhibited. In principle, three different methods may be employed:

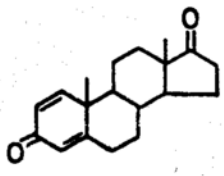
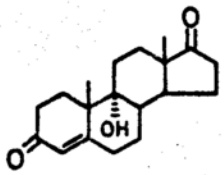
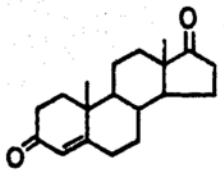
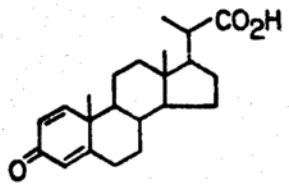
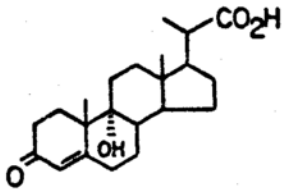
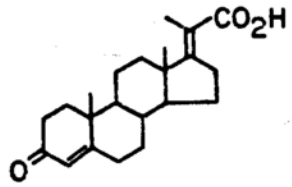
(1) Structural modification of the substrate to prevent enzymic attack of the steroid nucleus.[9,10]

(2) Fermentation of sterols in the presence of a selective enzyme inhibitor: inhibition of 9α-hydroxylation by Ni^{2+} or Co^{2+}; inactivation of 9α-hydroxylation by blocking the Fe^{2+} atom with a chelating agent such as α,α'-dipyridyl or 8-hydroxyquinoline.[11]

(3) Mutation of the microorganism to inactivate the C-1(2) dehydrogenase and/or 9α-hydroxylase.[12,13]

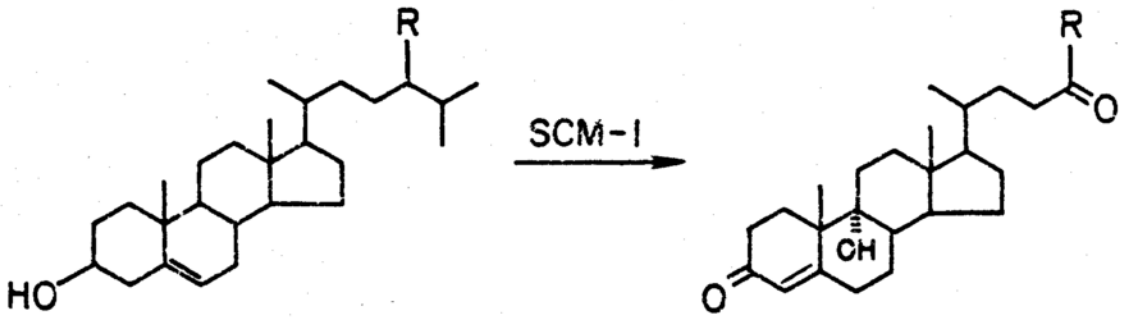
From an industrial viewpoint, it is more advantageous to use stable mutants rather than the other methods. In recent years, several economic microbiological processes have been developed to produce various important steroid intermediates[14] (Table 1).

Table 1. Accumulation of metabolites from cholesterol or phytosterol by various mutants.

Product Accumulated	Microorganism
	*Mycobacterium* sp. NRRL B-3683[12] *M. fortuitum* NRRL B-8153[18]
	*M. fortuitum* NRRL B-8119[13]
	*M.* sp. NRRL B-3805[12] *M. fortuitum* NRRL B-11045[12]
	*Nocardia corallina* IFO 3338[20]
	*M. fortuitum* NRRL B-8119[13]
	*M.* sp. NRRL B-8054[21]

However, in general, higher yields of 17-keto steroid were obtained from cholesterol, as phytosterols with branched side chains were found to be more resilient to microbial attack.[15] Apparently, the presence of the branched chain at the C-24 position interfered with the rate of ß-oxidation, as in the case of ß-substituted fatty acid. Although the mechanism of cholesterol degradation has been known for nearly two decades, very little information is available on the mechanism of degradation of the branched side chains of phytosterols. Also, the degradative enzyme systems in both cases remain unknown.

Martin and Wagner[16] reported that the enzyme system responsible for the decomposition of sitosterol was induced by either cholesterol or sitosterol, but not by long chain hydrocarbons. Knight and Wovcha[17] isolated two novel 24-oxo-intermediates, 9α-hydroxy-27-nor-4-cholestene-3,24-dione and 9α-hydroxy-26,27-dinor-4-cholestene-3,24-dione, from the incubation mixture of a mutant of Mycobacterium fortuitum (SCM-1) using a mixture of sitosterol and campesterol as substrate. These results indicated the existence of unique enzyme systems for the degradation of the branched side chain of phytosterols.



R = CH_3 Campesterol

C_2H_5 Sitosterol

Our investigation is directed to answering the following yet unresolved questions:

(1) What enzyme reactions are involved in the removal of the branched alkyl side chain at C-24?

(2) Is the enzyme system responsible for the side chain degradation of phytosterols the same as that for cholesterol?

(3) What is the regulatory mechanism of the reaction sequence?

The first portion of this study deals with the elucidation of the degradation mechanism of phytosterol using a partially purified enzyme system; whereas the second portion is concerned with the purification and characterization of the enzyme system.

PART I

MICROBIAL DEGRADATION OF PHYTOSTEROL SIDE CHAINS

EXPERIMENTAL PROCEDURES

Materials

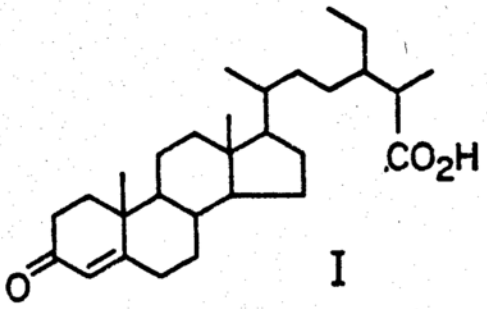
Sitosterol, cholesterol, α-toluenesulfonyl fluoride (PMSF), dithiobisnitrobenzoic acid (DTNB), phenazine methosulfate (PMS) and 40% hydrogen peroxide solution were obtained from Aldrich. Coenzyme A (CoA), adenosine triphosphate (ATP), oxidized and reduced nicotinamide adenine dinucleotide (NAD^+ and NADH), phosphoenolpyruvate, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), 4-aminoantipyrin, androst-4-ene-3,17-dione (AD), androsta-1,4-diene-3,17-dione (ADD), 2,6-dichlorophenol-indophenol (DCPIP), palmitic acid, arachidonic acid, myokinase, pyruvate kinase, lactate dehydrogenase, avidin, horseradish peroxidase, glucose oxidase and bovine serum albumin were purchased from Sigma. Coomassie blue protein assay concentrate, Affi-Gel Blue, Hydroxylapatite Bio-Gel HTP, sodium dodecyl sulfate, acrylamide, bisacrylamide, ammonium persulfate, and N,N,N',N'-tetramethylethylenediamine were purchased from Bio-Rad. DEAE Sepharose CL-6B, phenyl Sepharose CL-4B, Sephacryl S-200, Sephacryl S-300 and a molecular weight calibration kit were supplied by Pharmacia. [^{14}C]-sodium bicarbonate (specific activity 52 mCi/mmol) was obtained from New England Nuclear. Dye Matrex Gel Blue A, Diaflo ultrafilter type YM-10 and PM-10 were obtained from Amicon. Phenol and hydroxylamine hydrochloride were obtained from Mallinckrodt. 5-Cholenic acid-3ß-ol was purchased from

Steraloids Inc. All the other chemicals used were of analytical grade.

Methods

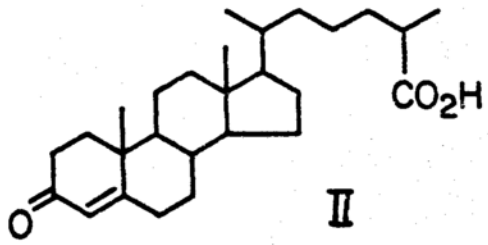
Culture condition - Mycobacterium sp. NRRL B-3805 was maintained on slants of nutrient agar, supplemented with 1% yeast extract, and stored at 4°C. A cell suspension in sterile 0.85% saline solution was used to inoculate 50 ml of medium that contained per liter: 8 g Difco nutrient broth, 5 g glycerol, 1 g yeast extract, 5 g sodium citrate and 200 mg sitosterol dissolved in 10 ml of dimethylformamide. After incubation at 25°C for 48 hr on a rotary shaker (250 rpm, 1" stroke), a 10% (v/v) inoculum was used for a second stage growth in 500 ml of the same medium. After 96 hrs, the cell suspension was concentrated by means of a Pellicon Cassette system to one-tenth of the volume, and the cells were then harvested by centrifugation at 6,000 x g. The cells were stored at -20°C without significant loss of activity.

Preparation of crude cell free extract - Cells of Mycobacterium sp. NRRL B-3805 (60 g, wet wt.) were suspended in 120 ml of 50 mM potassium phosphate buffer, pH 7.2, containing 15% (v/v) glycerol, and 0.67 mM α-toluenesulfonyl fluoride (PMSF). The cell suspension was kept at 4-8°C in an ice-salt mixture and was disrupted sonically using a Branson sonifier model S-75 (20 kc) at full power for 10 min. The cell debris was then removed by centrifugation at 10,000 x g for 20 min. The supernatant was decanted and further centrifuged at 100,000 x g for 1 hr. The clarified supernatant fraction served as the source of crude cell extract, and can be stored at -78°C



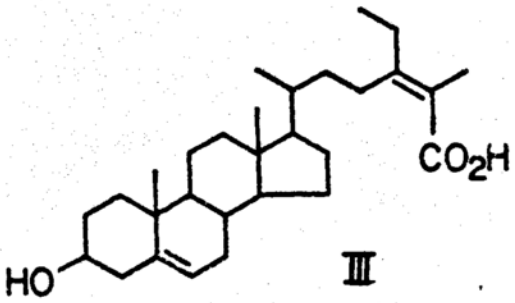
I

3-oxo-24-ethyl-cholest-4-en-26-oic acid



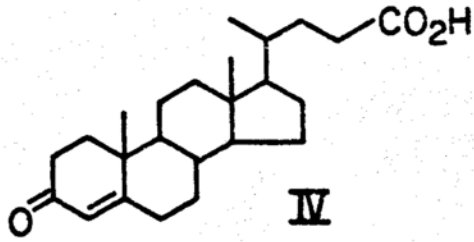
II

3-oxo-cholest-4-en-26-oic acid



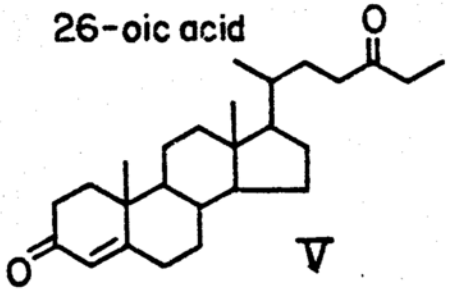
III

3-hydroxy-24-ethyl-cholest-5,24(25)-dien-26-oic acid



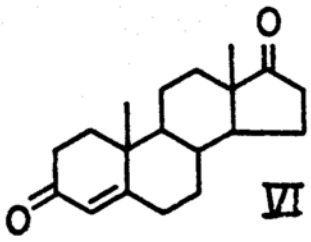
IV

3-oxo-chol-4-ene-24-oic acid



V

27-nor-cholest-4-en-3,24-dione



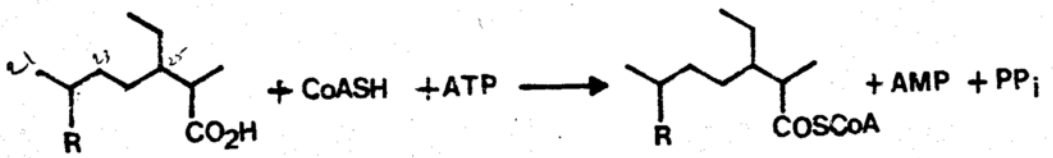
VI

androsta-4-ene-3,17-dione

indefinitely without significant loss of activity. At 4°C, the crude extract was also fairly stable in the presence of protease inhibitors, e.g., PMSF.

Enzyme assay

(A) Acyl-CoA synthetase



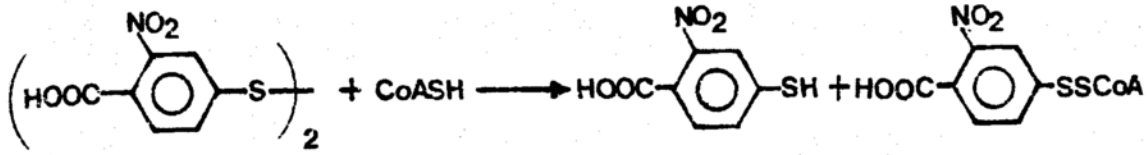
Three assay methods were employed, depending on the stages of purification:

(a) Hydroxamate-trapping method[22]

The assay system for crude enzyme contained: 1 mM of NH_2OH (pH 7.6); 5 µmol of ATP; 5 µmol of $MgCl_2$; 2 µmol of CoA and an adequate amount of enzyme in a final volume of 2 ml. The reaction was initiated by adding 1 µmol of substrate, dissolved in 50 µl of DMF, and the contents were then incubated at 30°C with shaking for 60 min. The reaction was terminated by the addition of 1 ml of 6% perchloric acid. After brief centrifugation, 1 ml of supernatant was removed and 0.1 ml of 10% $FeCl_3$ in 0.2 N HCl was added. The absorbance at 520 nm was then measured. The molar extinction coefficient of the hydroxamate was taken as 1000.

(b) Coenzyme A measurement

The amount of CoASH remaining may be measured via a chemical reaction with dithiobisnitrobenzoic acid[23] (DTNB).



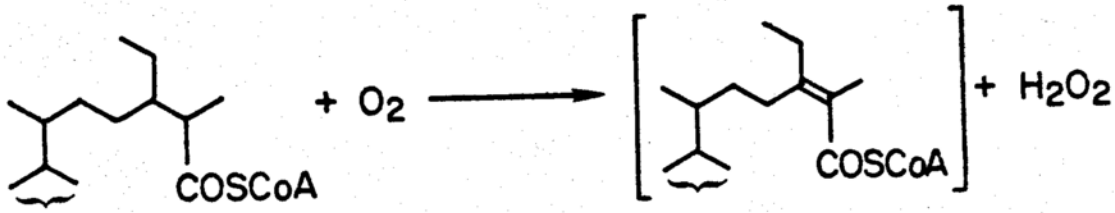
The reaction mixture contained: 0.2 µmol of CoA; 2 µmol of ATP; 2 µmol of $MgCl_2$; 10 µmol of potassium phosphate buffer, pH 7.6, and an adequate amount of enzyme, in a final volume of 1 ml. The reaction was initiated by adding 0.08 µmol of substrate, dissolved in 10 µl of DMF. After the contents were incubated at 30°C with shaking for 10 min, the reaction was terminated by adding 0.3 µmol of DTNB. The absorbance of color at 412 nm (ε = 13,600) was then measured.

(c) AMP formation from ATP[24]

The rate of AMP formation is determined by coupling the reaction of acyl-CoA synthetase with those of adenylate kinase, pyruvate kinase, and lactate dehydrogenase. The reaction mixture contained: 10 µmol of potassium phosphate buffer, pH 7.6; 2 µmol of ATP; 2 µmol of $MgCl_2$; 0.2 µmol of CoA; 0.33 µmol of NADH; 1 µmol of phosphoenolpyruvate; 5 µg of myokinase; 10 µg of pyruvate kinase; 10 µg of lactate dehydrogenase, and the purified acyl-CoA synthetase in a total volume of 1 ml. The reaction was initiated by adding 0.08 µmol of substrate, dissolved in 10 µl of DMF, and the rate of oxidation of NADH was monitored at 340 nm. For the partially purified enzyme, 0.5 mM NaF was

included to inhibit ATPase activity. In the blank, CoA was omitted. One unit of enzyme was defined as the amount of enzyme responsible for the formation of 1 nmol of product per min at 30°C.

(B) Acyl-CoA oxidase



The acyl-CoA oxidase activity was assayed by measuring the acyl-CoA dependent H_2O_2 production using a modification of the method described by Allain et al.[25]

The reaction mixture contained: 7.5 µmol potassium phosphate, pH 7.6; 0.5 µmol of CoA; 2 µmol of ATP; 2 µmol of $MgCl_2$; approximately 50 units of the partially purified acyl-CoA synthetase; 0.1 µmol of FMN, and an adequate amount of enzyme in a total volume of 0.75 ml. After preincubation at 30°C in the dark with shaking for 20 min, the reaction was initiated by the addition of 20 units of horseradish peroxidase; 20 µmol of $NaHCO_3$; 0.2 µmol of 4-aminoantipyrin; 2 µmol of phenol, and 0.1 µmol of substrate dissolved in 10 µl of DMF, in a final volume of 1.0 ml. The reaction was carried out at 30°C in the dark, and the production of H_2O_2 was measured by following the increase in absorbance at 500 nm. The molar extinction coefficient of 5,600 at pH 8.6 was used.

(C) Enoyl-CoA carboxylase

The carboxylase activity was qualitatively determined by exposing the substrate, 3-oxo-24-ethyl-cholest-4-en-26-oic acid, to the acyl-CoA synthetase and Fraction II enzymes in the presence of radioactive $NaHCO_3$ and ATP, CoA and Mg^{2+}. The nonvolatile radioactivity was measured after acidifying the reaction mixture.

The assay procedure was essentially the same as that used for acyl-CoA oxidase, except that the peroxidase-chromogen system was omitted and 1 µCi of [^{14}C]-$NaHCO_3$ was added. After incubation, the reaction mixture was acidified to pH 2 with 10% perchloric acid, and the incorporated radioactivity was measured.

(D) Thioesterase

The thioesterase activity was measured by following spectrophotometrically the increase in absorbance at 412 nm due to the reaction of liberated CoA with DTNB. A typical assay contained: 10 µmol potassium phosphate, pH 7.6; 0.03 µmol of 3-oxo-24-ethyl-cholest-4-en-26-oyl CoA; 0.1 µmol of DTNB, and an adequate amount of enzyme.

(E) Catalase[26]

Catalase activity was assayed by following spectrophotometrically the decrease in absorbance at 240 nm corresponding to the disappearance of hydrogen peroxide (ε = 43.6).

Preparation of 3-oxo-24-ethyl-cholest-4-en-26-oyl CoA - This CoA thioester could not be synthesized by conventional chemical methods,

and was prepared enzymatically from the acid and coenzyme A using a modification of the method described by Samuel et al.[27] Partially purified acyl-CoA synthetase from DEAE chromatography of the crude extract was used in the incubation. The reaction mixture consisted of: 250 µmol of potassium phosphate buffer, pH 7.6; 100 µmol of ATP; 120 µmol of $MgCl_2$; 50 µmol of CoA; 40 µmol of 3-oxo-24-ethyl-cholest-4-en-26-oic acid (in 2.5 ml of DMF) and 1000 units of enzyme in a total volume of 50 ml. After incubating at room temperature with stirring for 2 hr, the reaction was terminated by the addition of 25 ml of 5% perchloric acid. After centrifugation of the mixture at 10,000 x g for 20 min, CoA was removed from the pellet by two washes with 25 ml of 1.5% perchloric acid, and the unreacted acid was removed by extraction with ethyl acetate. The acyl CoA could be completely extracted from the pellet by a mixture of pyridine-isopropanol-water (1:1:1). After removing the solvent in vacuo, the resulting solid (43 mg, 90% yield) could be used directly without further purification. Tnin layer chromatography on Brinkman silica gel plate (0.25 mm) using n-butanol: acetic acid:water (5:2:3) as the developing solvent showed a single spot with a R_f value of 0.45.

Rf of Starting
Material ?

Protein determinations - Protein assays were performed by the Coomassie blue dye-binding method described by Bradford[28] using bovine serum albumin as a standard. Protein concentrations during purification were determined by measuring the absorbance at 280 nm and assuming that 1 OD unit = 1 mg protein/ml.

Anaerobic incubations - Incubations were performed using Thunberg tubes, and anaerobiosis was achieved by five cycles of alternative evacuation under reduced pressure and equilibration with argon. Dissolved oxygen was removed by glucose oxidase in the presence of 10 mM glucose.

Chromatographic analysis

(A) Thin layer chromatography (TLC)

TLC was performed on Brinkmann (EM) plates coated with 0.25 mm thickness of silica gel containing PF 254. The developing solvent system consisted of Skelly B-ethyl acetate-acetic acid (60:30:1). The R_f values were: 27-Norcholest-4-ene-3,24-dione (V) = 0.62; 3-oxo-24-ethyl-cholest-4-en-26-oic acid (I) = 0.53; 3-oxo-chol-4-en-24-oic acid (IV) = 0.43; and androst-4-ene-3,17-dione (VI) = 0.25.

(B) Gas-liquid chromatography

Quantitative GLC was performed on a 3 ft column of 3% ov-1 on Chromosorb W HP, 235°-265°C to determine the residual amount of sitosterol and androst-4-ene-3,17-dione in the fermentation medium. Retention times were: sitosterol = 7.5 min, cholesterol = 6 min, ADD = 2.3 min.

(C) High pressure liquid chromatography

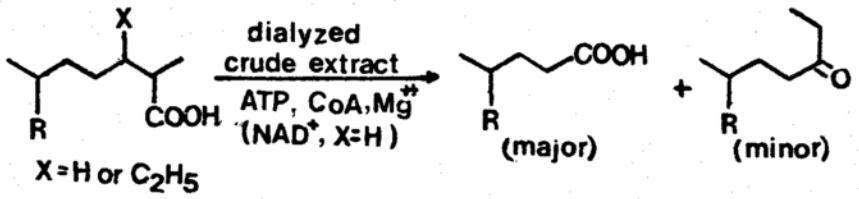
HPLC separation was effected on a Waters µPorasil (10 µ) column (0.46 I.D. x 50 cm) using Skelly B-$CHCl_3$ (2:1) as the mobile phase at a flow rate of 2 ml/min. Retention times were.

3-Oxo-24-ethyl-cholest-4-en-26-oic acid methyl ester = 9.3 min; 3-oxo-chol-4-en-24-oic acid methyl ester = 10.8 min; 27-norcholest-4-ene-3,24-dione = 11.4 min.

RESULTS

Cofactor Requirements

The dialyzed crude cell extracts in the presence of ATP, $MgCl_2$ and CoA converted 3-oxo-24-ethylcholest-4-en-26-oic acid (I) into 3-oxo-chol-4-en-24-oic acid (IV) along with a small quantity of 27-norcholest-4-ene-3,24-dione (V). Similar transformation of 3-oxo-cholest-4-en-26-oic acid (II) was noted, but NAD^+ was found to be required in this case. However, both transformations did not



proceed under anaerobic conditions, and addition of artificial electron acceptors, such as 2,6-dichlorophenol indophenol (DCPIP), or phenazine methosulfate (PMS) plus DCPIP, could not substitute for molecular oxygen (Table 2).

This observation suggested a requirement for molecular oxygen as the electron acceptor, most likely in the dehydrogenation reaction. However, because of the presence of strong catalase activity in the crude extract, no hydrogen peroxide formation could be detected at this stage.

Table 2. The inability of artificial electron acceptors to replace the oxygen requirement for enzymic conversion.

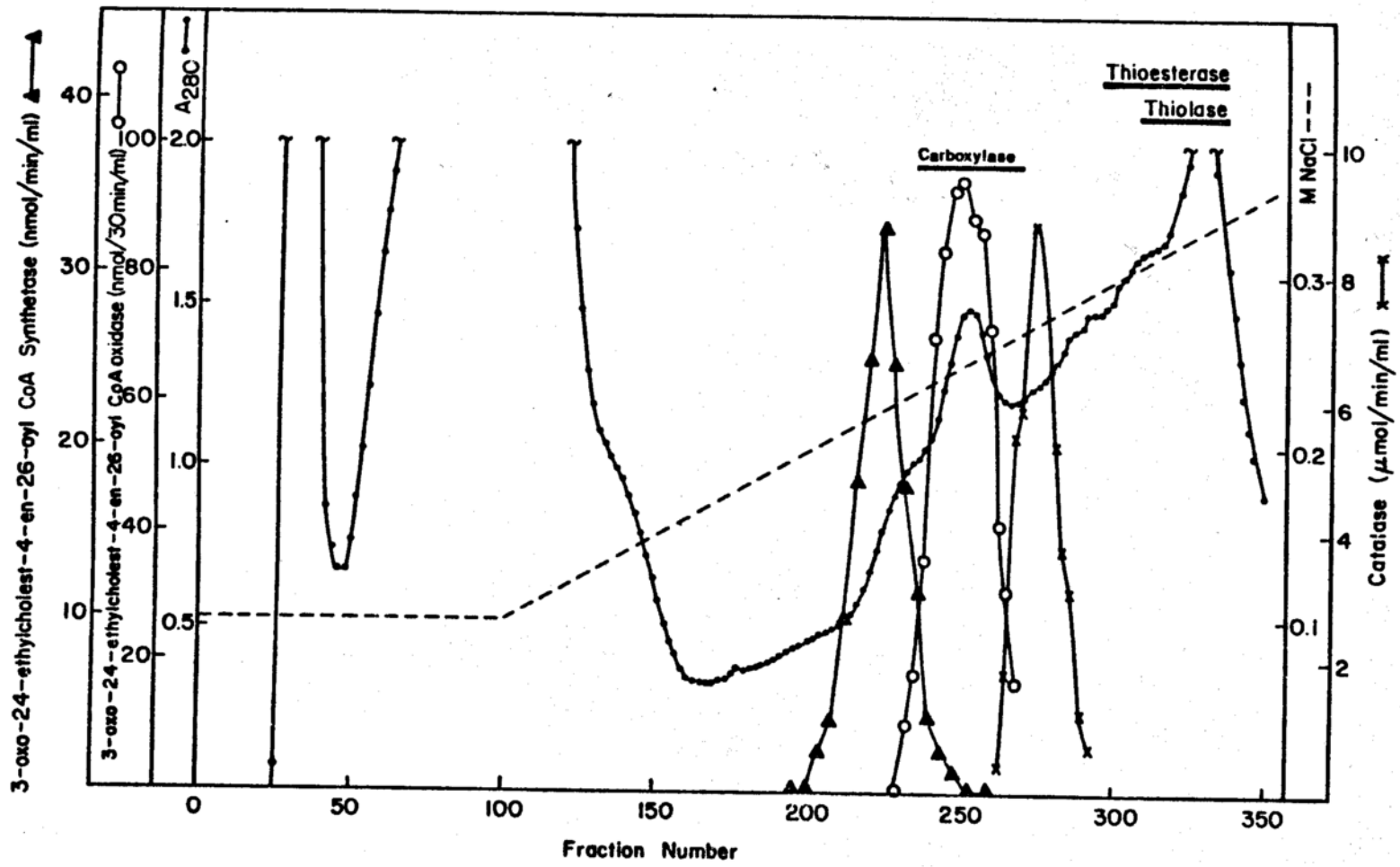
Experimental conditions		Conversion (%)	
Atmosphere	Electron acceptor added	3-Oxo-24-ethyl-cholest-4-en-26-oic acid (I)	3-oxo-cholest-4-en-26-oic acid (II)
Air	--	80	0
Air	NAD^+	80	38
Argon	--	<2	0
Argon	NAD^+	<2	0
Argon	DCPIP	<2	0
Argon	PMS	<2	0
Argon	PMS + DCPIP	<2	0

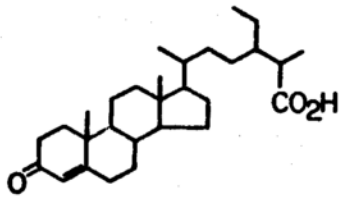
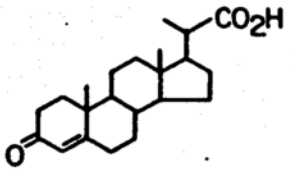
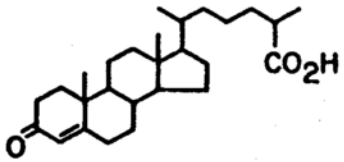
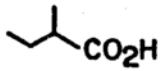
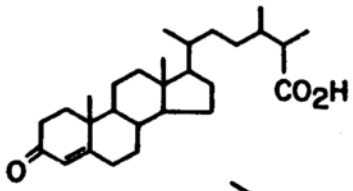
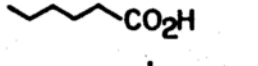
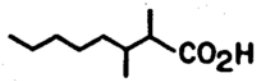
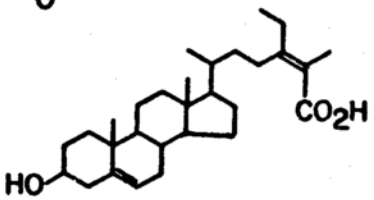
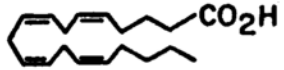
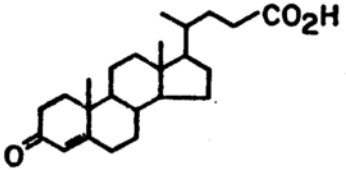
The reaction mixture contained: dialyzed crude cell free extract (30 mg of protein); 5 µmol of ATP; 5 µmol of $MgCl_2$; and 0.5 µmol of CoA in a total volume of 2 ml. Anaerobiosis was generated as described under "Experimental Procedures." The reaction was initiated by adding 0.2 µmol of 3-oxo-24-ethyl-cholest-4-en-26-oic acid (I) or 3-oxo-cholest-4-en-26-oic acid (II). The electron acceptors NAD^+, PMS and DCPIP were present in the reaction mixture at a level of 0.5 µmol. After incubation at 30°C with shaking for 1 hr, the reaction was terminated by the addition of 200 µl of 6 N HCl. The resulting mixture was extracted with equal volume of ethyl acetate twice. The organic layer was dried over Na_2SO_4 and treated with CH_2N_2. After removal of the organic solvent in vacuo, HPLC analysis of the residue was performed. Conversion was calculated based on the formation of 3-oxo-chol-4-en-24-oic acid (IV) and 27-norcholest-4-ene-3,24-dione (V).

The crude extract (1420 mg protein) was applied onto a column of DEAE-Sepharose CL-6B (5 x 21 cm, 412 ml bed volume), previously equilibrated with 50 mM potassium phosphate buffer, pH 7.2, containing 15% (v/v) glycerol, 0.1 M NaCl, and 0.67 mM PMSF. The column was first eluted with 800 ml of equilibrating buffer, and then with a 2-L linear gradient of sodium chloride from 0.1 M to 0.35 M established in the equilibrating buffer at a flow rate of 50 ml/hr, and 8 ml fractions were collected. 3-Oxo-24-ethyl-cholest-4-en-26-oyl CoA synthetase, 3-oxo-24-ethyl-cholest-4-en-26-oyl CoA oxidase and catalase activities were monitored. As shown in Fig. 3, these enzymes were well separated on a DEAE-Sepharose Cl-6B column. Active fractions of the acyl-CoA synthetase (fractions 210-234) and the acyl-CoA oxidase (fractions 240-260) were pooled separately, and were designated as Fraction I and Fraction II respectively. Moreover, strong thioesterase activity was detected in fractions 300-330.

Properties of the partially purified acyl CoA synthetase

It was found that 95% of the acyl-CoA synthetase activity of crude cell extract was recovered in the combined fractions (210-234). This enzyme exhibited rather narrow substrate specificities, being active only towards C_{24}-C_{29} steroidal acids and long chain fatty acids (Table 3). Short and medium chain fatty acids or the branched side chain analogs, such as 2,3-dimethyloctanoic acid, could not serve as substrates for the enzyme. Maximal activity was obtained with 3-oxo-cholest-4-en-26-oic acid, and the activity decreased with the presence of substituents at the C-24 position (Table 3). 3-Oxo-bisnorcholenic acid was


3-oxo-24-ethylcholest-4-en-26-oyl CoA Synthetase (nmol/min/ml)
3-oxo-24-ethylcholest-4-en-26-oyl CoA oxidase (nmol/30min/ml)
A280
M NaCl
Catalase (μmol/min/ml)
Thioesterase
Thiolase
Carboxylase
Fraction Number
0
50
100
150
200
250
300
350

Substrate	Acyl-CoA Formed (nmol/min/mg protein)	Relative Activity (%)	Substrate	Acyl-CoA Formed (nmol/min/mg protein)	Relative Activity (%)
	204	46		0	0
	448	100		0	0
	276	62		0	0
				0	0
			$CH_3(CH_2)_{14}COOH$	22	5
	75	17	$CH_3(CH_2)_{16}COOH$	26	6
				22	5
	75	16			

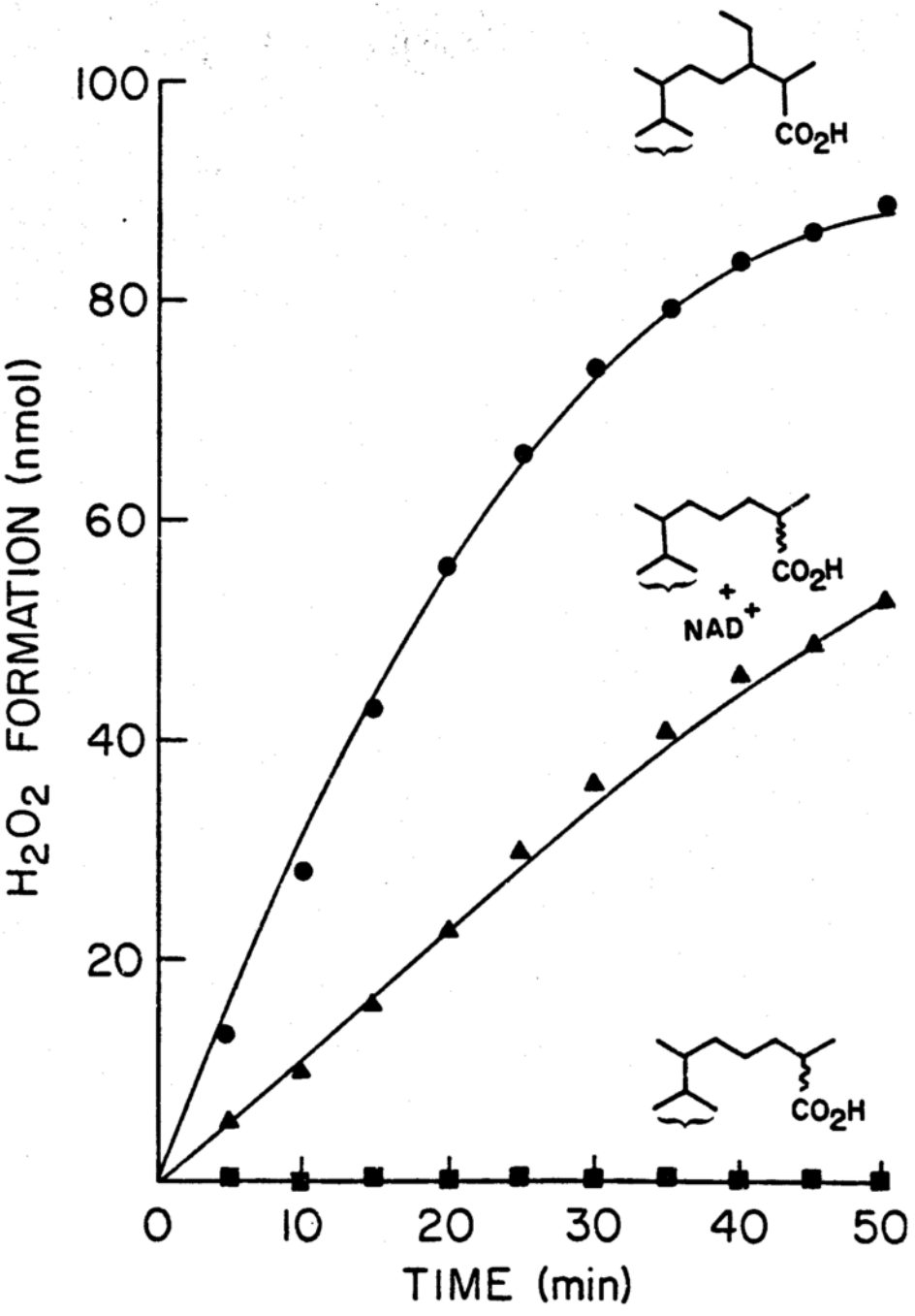
not activated by the enzyme.

The partially purified enzyme could be stored in dry ice for several months without much loss of activity. However, at 4°C, all activity was lost in one week.

Properties of the partially purified acyl-CoA oxidase (Fraction II)

After chromatography of the crude extract on DEAE-Sepharose CL-6B column, the partially purified enzyme, in the presence of acyl-CoA synthetase, CoA, ATP and $MgCl_2$, was still active towards 3-oxo-24-ethyl-cholest-4-en-26-oic acid (I) as indicated by the formation of H_2O_2 (Fig. 4). However, in the absence of NAD^+, 3-oxo-cholest-4-en-26-oic acid (II) could not serve as a substrate for the oxidase, a fact indicating that NAD^+ was required in a later step, and that its degradation was controlled in a concerted manner. The results also suggest that different mechanisms were involved in the conversion of these two compounds to the common intermediate, 3-oxo-chol-4-en-24-oic acid (IV).

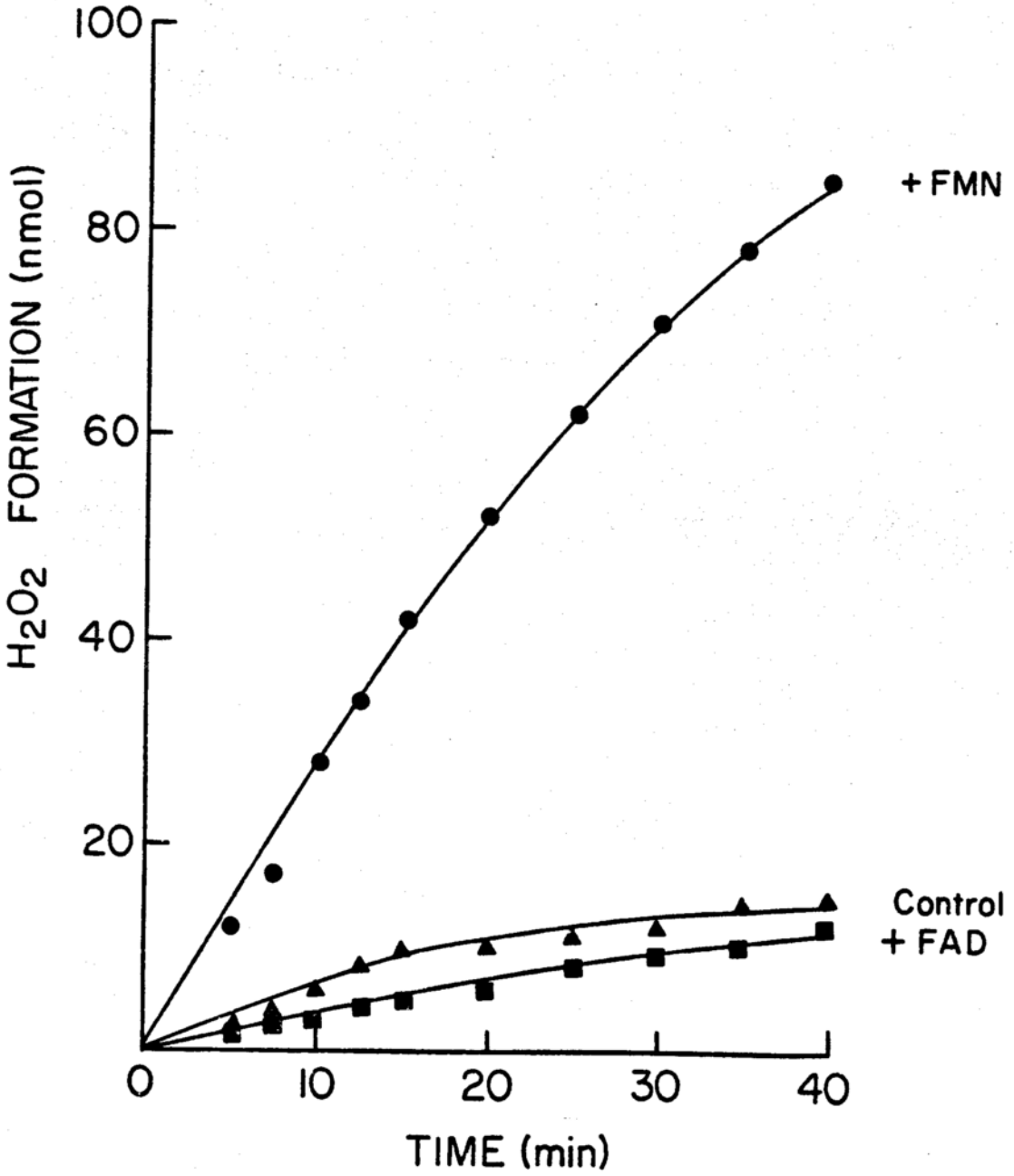
Another complication encountered periodically was the drastic loss of acyl-CoA oxidase activity when a new batch of DEAE resin was used. This loss of enzyme activity may be due to the separation of subunits from each other or the removal of noncovalently bound cofactors or metal ions, etc., as a result of the stronger binding capacity of the new resin. A series of investigations were conducted in an attempt to restore the enzyme activity. It was found that FMN stimulated the oxidase activity. To confirm that FMN was involved in the oxidase activity, KBr dialysis of the partially purified acyl-CoA oxidase was carried out to remove any noncovalently bound flavin nucleotide.[29]


100
80
60
40
20
0
10
20
30
40
50
H2O2 FORMATION (nmol)
TIME (min)
CO2H
CO2H
+
NAD+
CO2H

After exhaustive dialysis, oxidase activity was almost completely eliminated. However, the activity of the acyl CoA oxidase could be restored by preincubation with FMN, but not with FAD (Fig. 5). In contrast, the enhancement of the oxidase activity was insignificant using crude enzyme preparation, suggesting that FMN was loosely bound to the protein and that some loss of FMN from the enzyme had occurred during the purification process.

Identification of the products

3-Oxo-24-ethyl-cholest-4-en-26-oic acid (I) or 3-oxo-cholest-4-en-26-oic acid (II) were each exposed separately to a mixture consisting of Fraction I, Fraction II, ATP, CoA and $MgCl_2$. In the case of 3-oxo-cholest-4-en-26-oic acid (II), NAD^+ was also added. TLC and HPLC analysis of the ethyl acetate extract of the reaction mixture revealed two products. The chromatographic characteristics of the two unknown compounds are listed in Table 4. The less polar product (minor) exhibited chromatographic properties identical to those of 27-norcholest-4-ene-3,24-dione (V). The other product (major) was an acid which was more polar than 3-oxo-chol-4-en-24-oic acid (II), and had a maximal UV absorption at 235 nm, indicating that no other chromophore, except the Δ^4-3-oxo function, was present in the compound. The mass spectrum of its methyl ester derivative showed a molecular ion at m/e 442 (M^+) with principal fragments at 355 (M-87), 327 (M-115), 313 (M-129) and 271 (M-171) (Fig. 6), consistent with the following structural assignment.

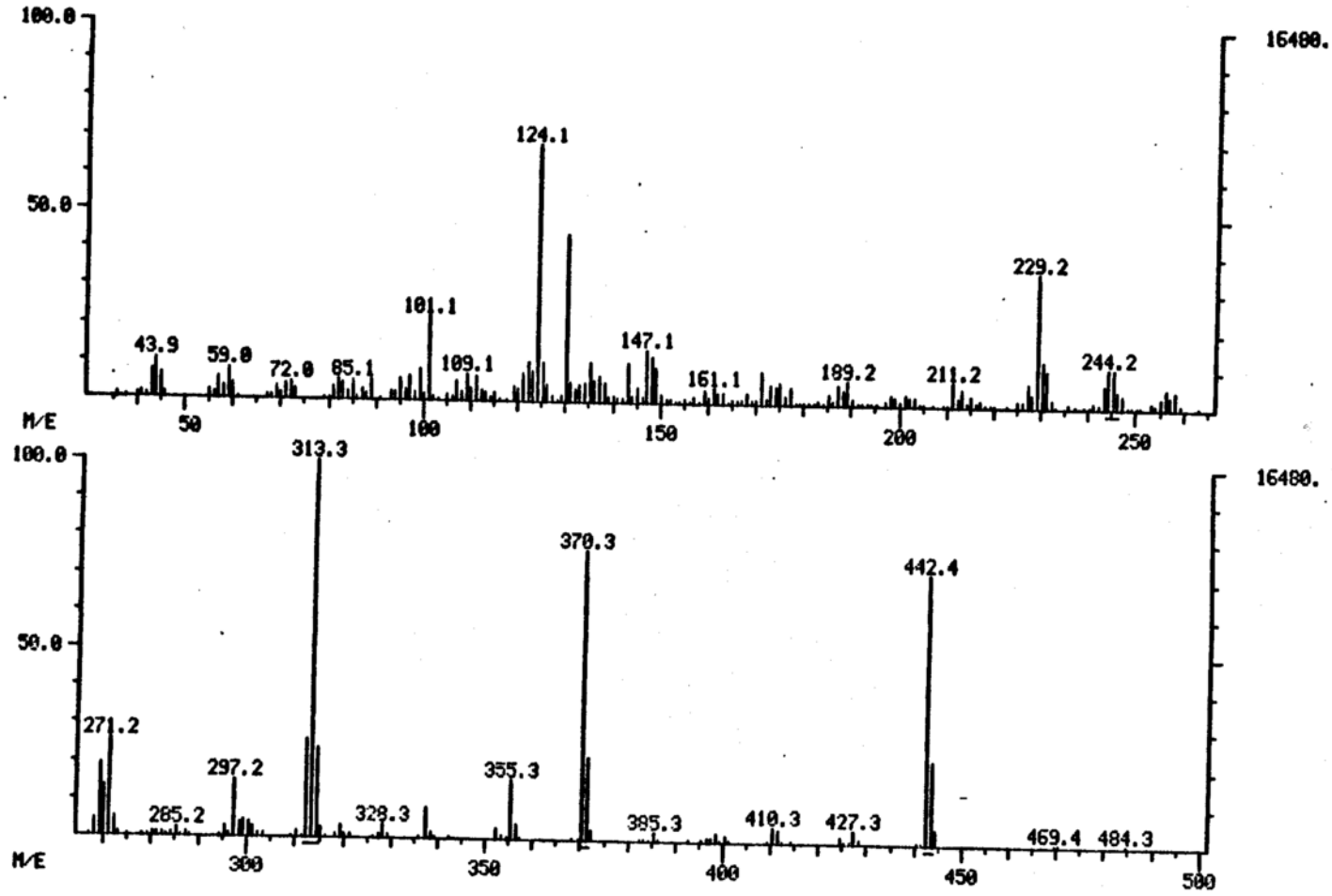

100
80
60
40
20
0
10
20
30
40
H_2O_2 FORMATION (nmol)
TIME (min)
+ FMN
Control
+ FAD

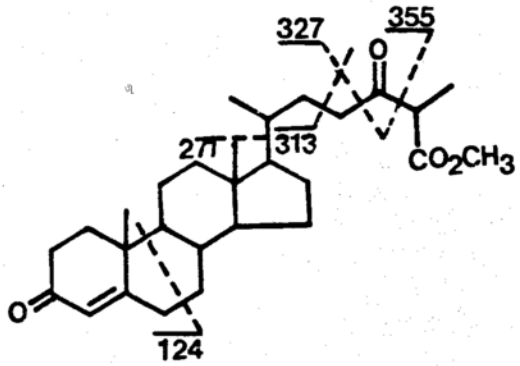
Compounds	TLC (R_f)	HPLC after treatment with CH_2N_2 (min)
3-Oxo-24-ethyl-cholest-4-en-26-oic acid (I)	0.53	9.3
3-Oxo-cholest-4-en-26-oic acid (II)	0.49	9.5
27-Norcholest-4-ene-3,24-dione (V)	0.62	11.4
3-Oxo-chol-4-en-24-oic acid (IV)	0.43	10.8
Androsta-4-ene-3,17-dione (VI)	0.25	--
Product I	0.62	11.4
Product II	0.27	14.1

MASS SPECTRUM
03/08/83 11:59:00 + 6:06
SAMPLE: CS3-SOLID PROBE,22EV,34-550AMU
CONDS.: -
GC TEMP: 178 DEG. C

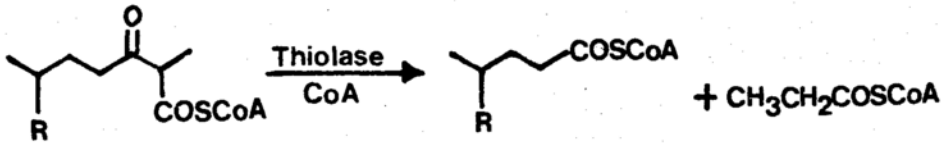
DATA: PH308CS3SP #122
CALI: B030783CALB #5

BASE M/E: 313
RIC: 215296.

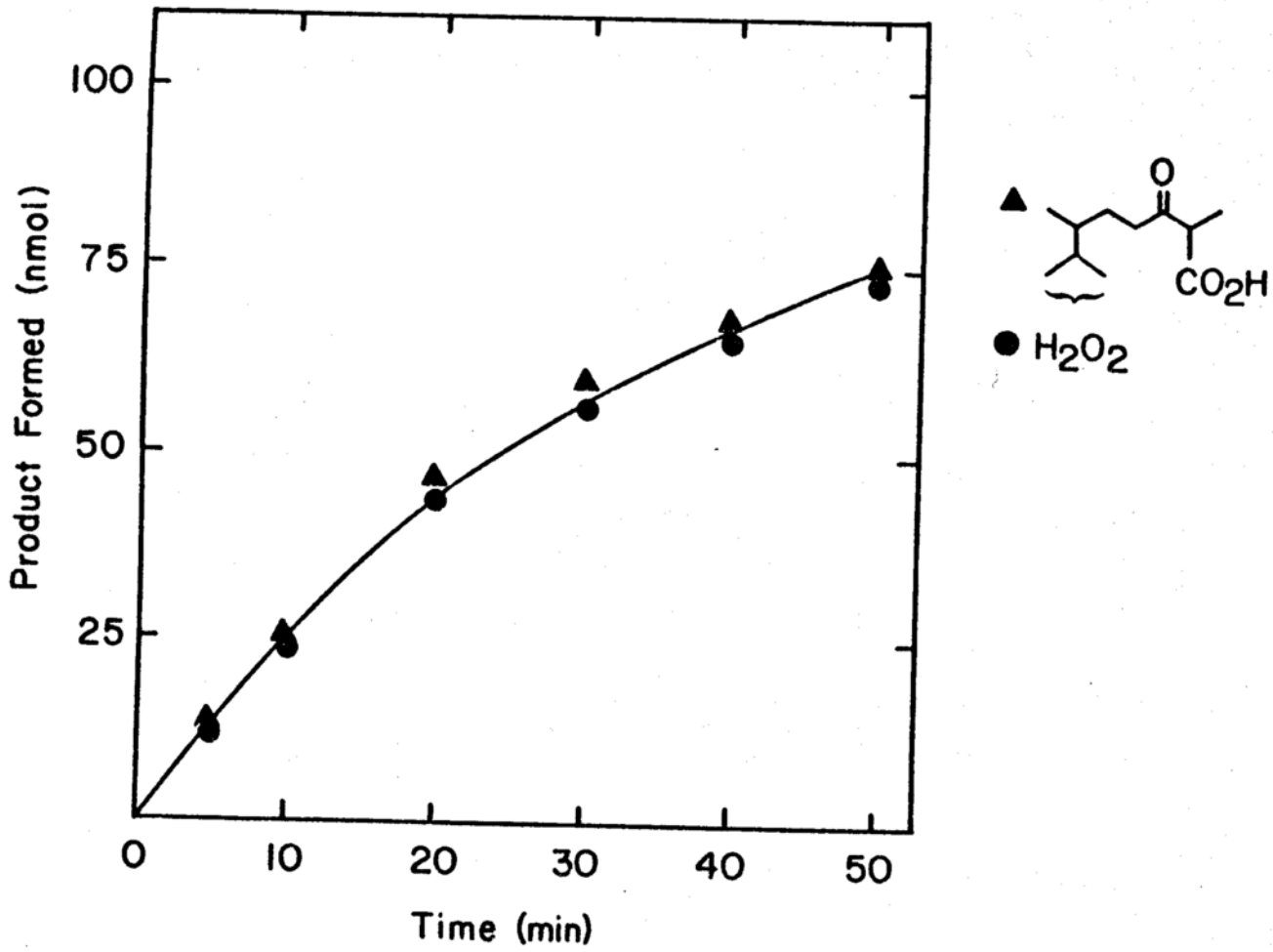


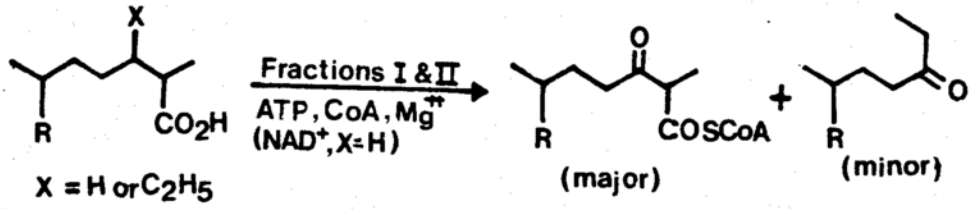


Addition of proteins, derived from fractions 310 to 330 from the DEAE column (Fig. 3), to the incubation mixture resulted in the conversion of this novel ß-keto acid into 3-oxo-chol-4-en-24-oic acid (IV), which indicated the presence of thiolase activity in these fractions (310-330).

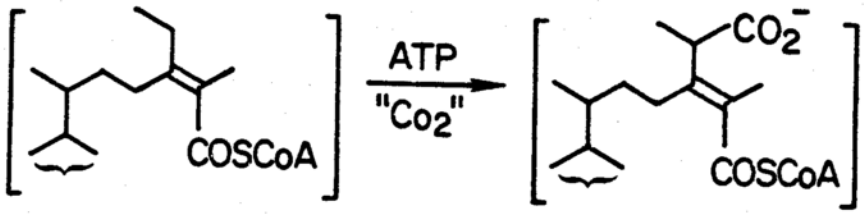


It was also noted that the amount of 27-norcholest-4-ene-3,24-dione (V) formation varied with experimental conditions, i.e., prolonged standing or extreme pH could increase its formation. Apparently, part of the ketone formed was the result of the non-enzymic decarboxylation of the ß-keto acid. These results showed that all the enzymes leading to the formation of ß-keto acid resided in Fractions I and II. The rate of formation of hydrogen peroxide coincided with the kinetics of formation of the end products (Fig. 7).


100
75
50
25
0
10
20
30
40
50
Product Formed (nmol)
Time (min)
O
CO2H
H2O2

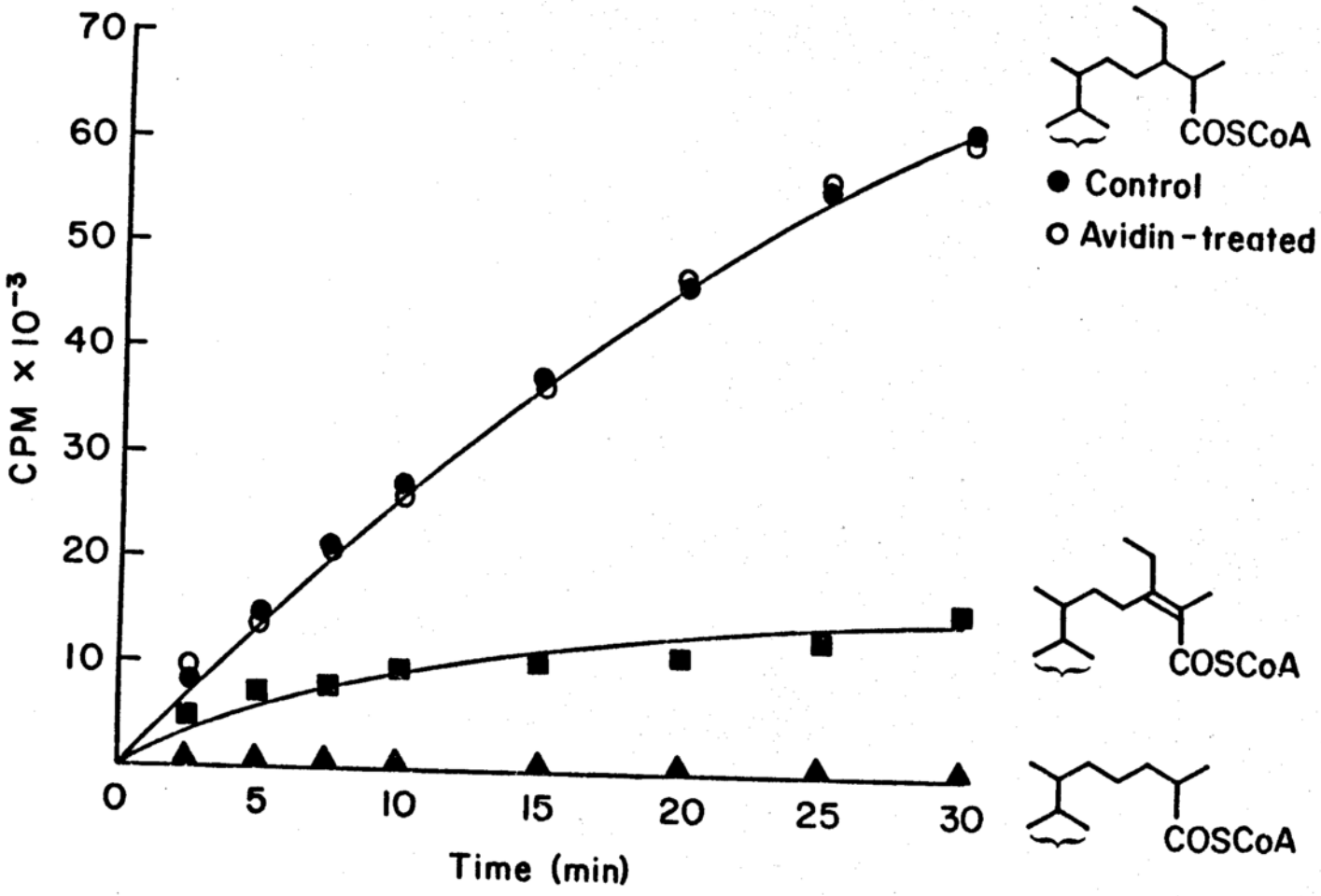


Enoyl CoA carboxylase

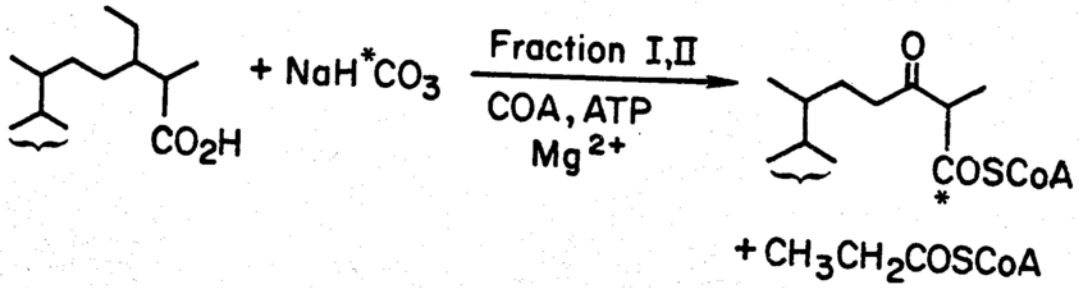


Although at this stage, the active species of carboxylation, i.e., CO_2 of HCO_3^-, was yet unknown, carboxylase assay could be carried out by measuring the incorporation of radioactive HCO_3^- into the nonvolatile molecule after acidifying the reaction mixture (Fig. 8). As shown in Fig. 3, the active fractions of carboxylase coincided with those of oxidase. It is interesting to note that the carboxylation reaction was not inhibited by avidin (Fig. 8), a known effective inhibitor of biotin-dependent enzymes. Moreover, the putative intermediate, 3-hydroxy-24-ethylcholest-5,24(25)-dien-26-oic acid (a synthetic mixture of E and Z ixomers), showed a lower incorporation rate than that of its saturated counterpart. This result may be due to the competitive inhibition by the unnatural isomer or the inaccessibility of the compound to the enzyme embedded in the enzyme complex.

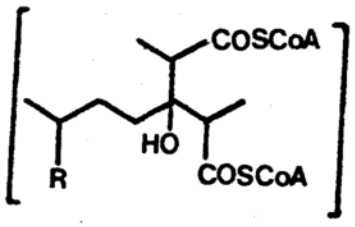
To further define the fate of the radioactive carbon after the carboxylation step, the acidified reaction mixture was extracted with


70
60
50
40
30
20
10
0
5
10
15
20
25
30
CPM x 10⁻³
Time (min)
COSCoA
Control
Avidin-treated
COSCoA
COSCoA

ethyl acetate. It was found that nearly all the nonvolatile radioactivity was extracted into the organic layer. The organic layer was treated with diazomethane, and removed <u>in vacuo</u>. Nearly all the radioactivity still remained in the residue, indicating that no radioactive propionic acid was formed (Table 5). TLC and radiochromatogram scanning analyses showed that all the radioactivity resided in the ß-keto acid (Fig. 9).



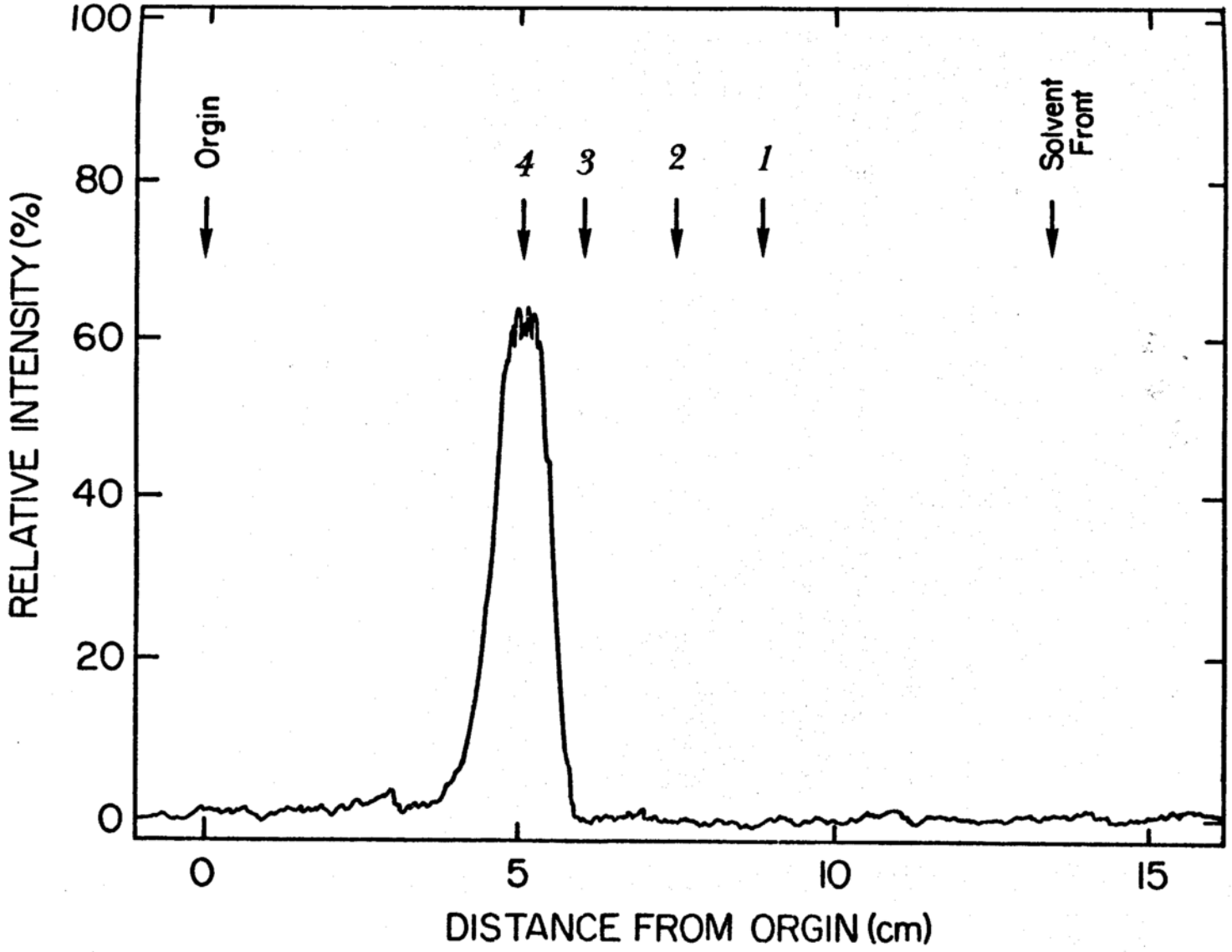
The results also reveal the ability of the enzyme system to distinguish between the two diastereotopic C_3 units, C_{25}-C_{26}-C_{27} and C_{28}-C_{29}-C_{30}, of the postulated dicarboxylate intermediate,



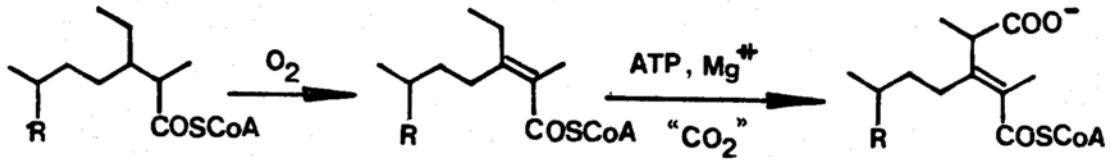
<u>Concerted regulation of the reaction sequence</u>

After the initial activation of 3-oxo-24-ethyl-cholest-4-en-26-oic-acid (I), CoA is not required in the subsequent oxidation and carboxylation steps. Hence, intermediates may accumulate if CoA is

Procedures	CPM
(1) Total radioactivity added	1.02×10^7
(2) Ethyl acetate extraction after acidifying the reaction mixture	
aqueous layer	3×10^3
organic layer	1.72×10^5
(3) CH_2N_2 treatment of the organic layer and removal of organic solvent	1.51×10^5


100
80
60
40
20
0
RELATIVE INTENSITY (%)
Orgin
4
3
2
1
Solvent Front
0
5
10
15
DISTANCE FROM ORGIN (cm)

omitted from the incubation mixture.

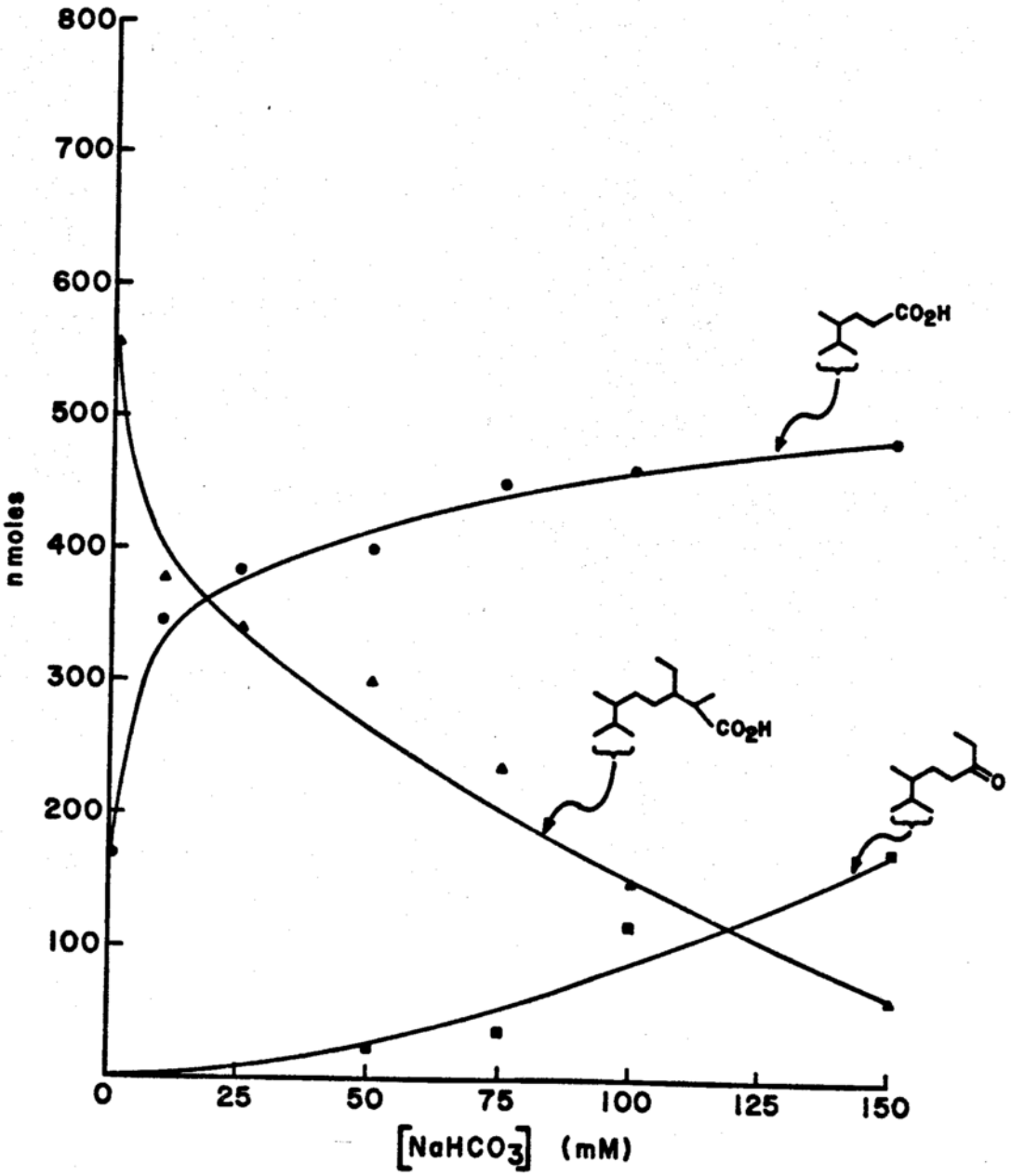


A CoA thioester of the substrate was prepared enzymatically, and exposed to the reaction mixture, but, in the absence of either CoA or ATP, no reaction occurred which strongly suggested that the degradative sequence might be controlled in a highly coordinated manner.

Enhancement of branched side chain degradation of sitosterol by sodium bicarbonate

It has been shown that bicarbonate ion is required for the degradation of the branched side chain of phytosterols. In an aqueous solution, the concentrations of bicarbonate ion and related species (CO_2 and H_2CO_3) are extremely low, <10 μM at 1 atm, and would be insufficient to fulfill the need for the enzyme system. Consequently, it is logical to supplement sodium bicarbonate in the reaction medium to enhance the degradative efficiency of phytosterols.

Fig. 10 shows the dependence of the conversion rate of 3-oxo-24-ethyl-cholest-4-en-26-oic acid on the concentration of sodium bicarbonate in the reaction medium. The rate of disappearance of the starting material increased rapidly with elevated concentrations of sodium bicarbonate, up to 150 mM.

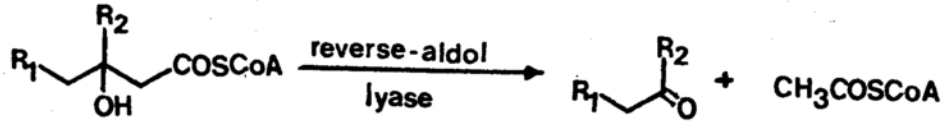

800
700
600
500
400
300
200
100
n moles
0
25
50
75
100
125
150
[NaHCO3] (mM)
CO2H
CO2H
O

In shake flask fermentation studies, sitosterol was exposed to various sterol-degrading mutants in the presence of different levels of sodium bicarbonate. In all cases, after 48 hr fermentation, 50-150% increase of bioconversion rates were observed (Table 5).

Sitosterol [$NaHCO_3$] (mM)	C_{17} Steroids (nmol)		
	AD *Mycobacterium* sp. NRRL B-3805	ADD *Mycobacterium* sp. NRRL B-3683	ADD *Mycobacterium phlei* NRRL B-15050
0	55.7	39.4	18.4
25	99.0	56.0	42.7
50	91.1	--	46.0
75	94.2	51.6	42.4
100	--	59.7	--

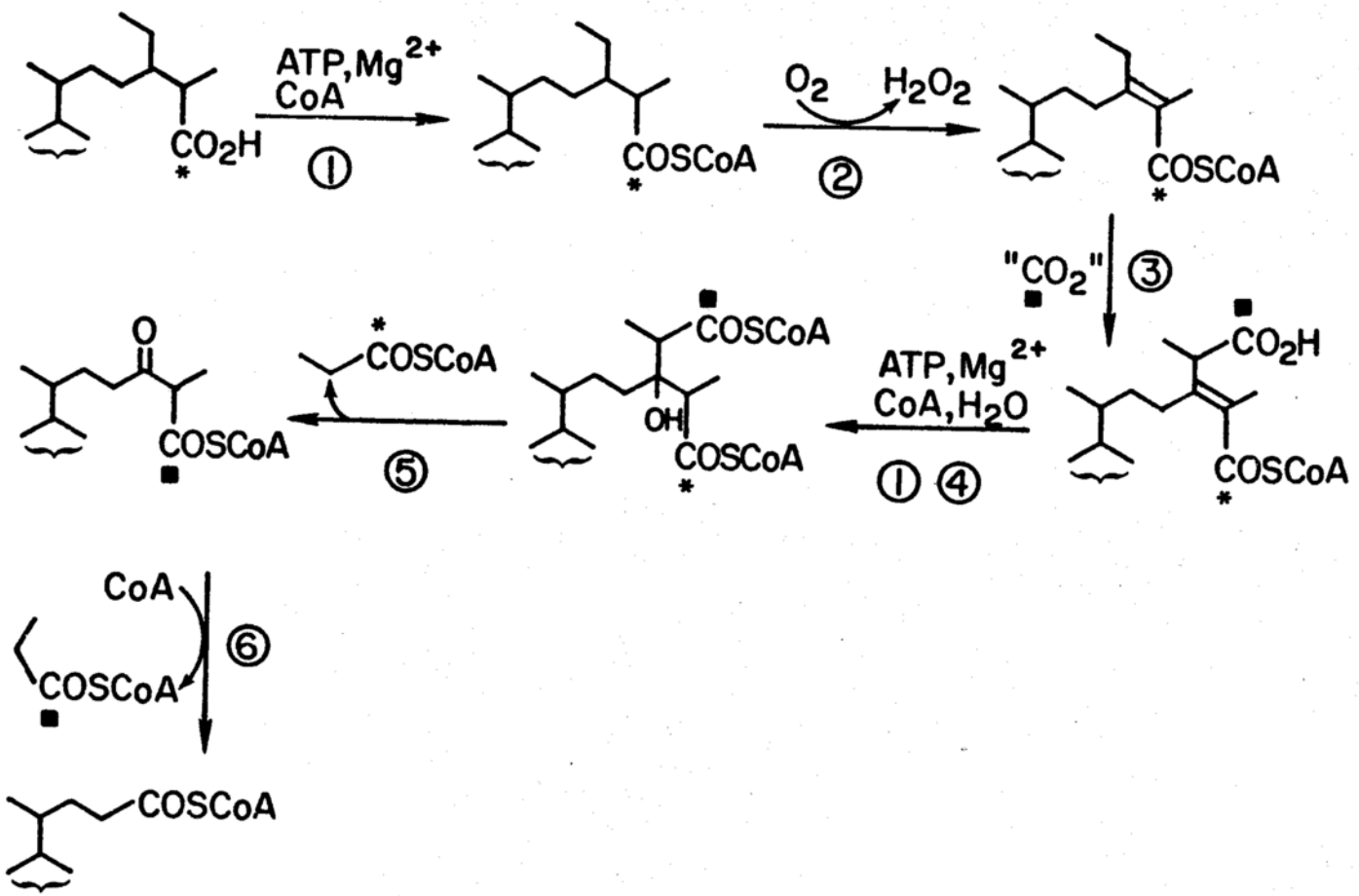
DISCUSSION

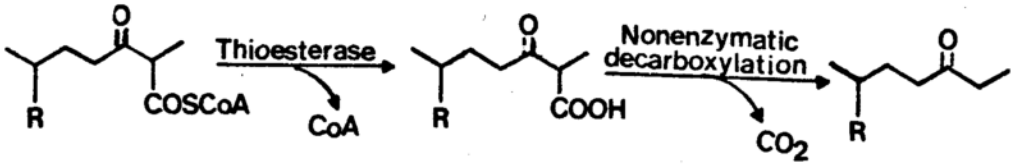
On the basis of the foregoing results, the mechanism of microbial degradation of the branched side chain of phytosterols may be envisaged as shown in Fig. 11. The carboxylic acid, derived from oxidation of the C-26 carbon, is first activated by thioester formation; subsequent dehydrogenation of the acyl-CoA derivative, mediated by an FMN-dependent oxidase, results in the formation of hydrogen peroxide. The branched side chain is first carboxylated at the C-28 position to form the putative unsaturated dicarboxylate intermediate, which is then degraded by the action of enoyl CoA hydratase and reverse-aldol lyase. Numerous enzymatic reactions have been reported where carbon-carbon fission occurs via a tertiary ß-hydroxy CoA ester. These enzymes include: malyl-CoA lyase[30,31]; citramalyl-CoA lyase[32]; 3-hydroxy-3-methylglutaryl-CoA lyase[33]; and (1-hydroxycyclohexan-1-yl)acetyl CoA lyase[34]. In all cases, the tertiary alcohol is cleaved to form acetyl CoA and the corresponding ketone.



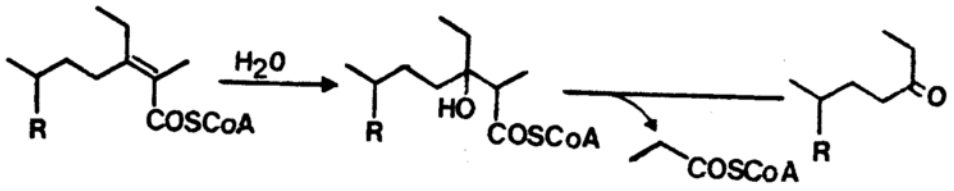
Two mechanisms have been proposed for the the formation of 3-oxo-27-norcholest-4-en-3,24-dione (V):

(1) The ketone is a product of the non-enzymatic decarboxylation of the ß-keto acid, which in turn is formed from its thioester.

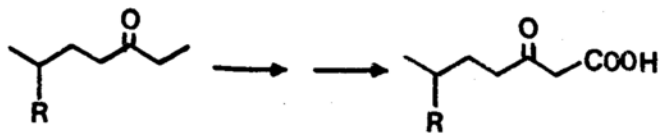

ATP, Mg2+
CoA
①
O2
H2O2
②
"CO2"
③
ATP, Mg2+
CoA, H2O
① ④
⑤
⑥
CO2H
COSCoA
OH
O
CoA



(2) The reaction sequence bypasses the carboxylation step and proceeds via direct hydration followed by reverse aldolization.

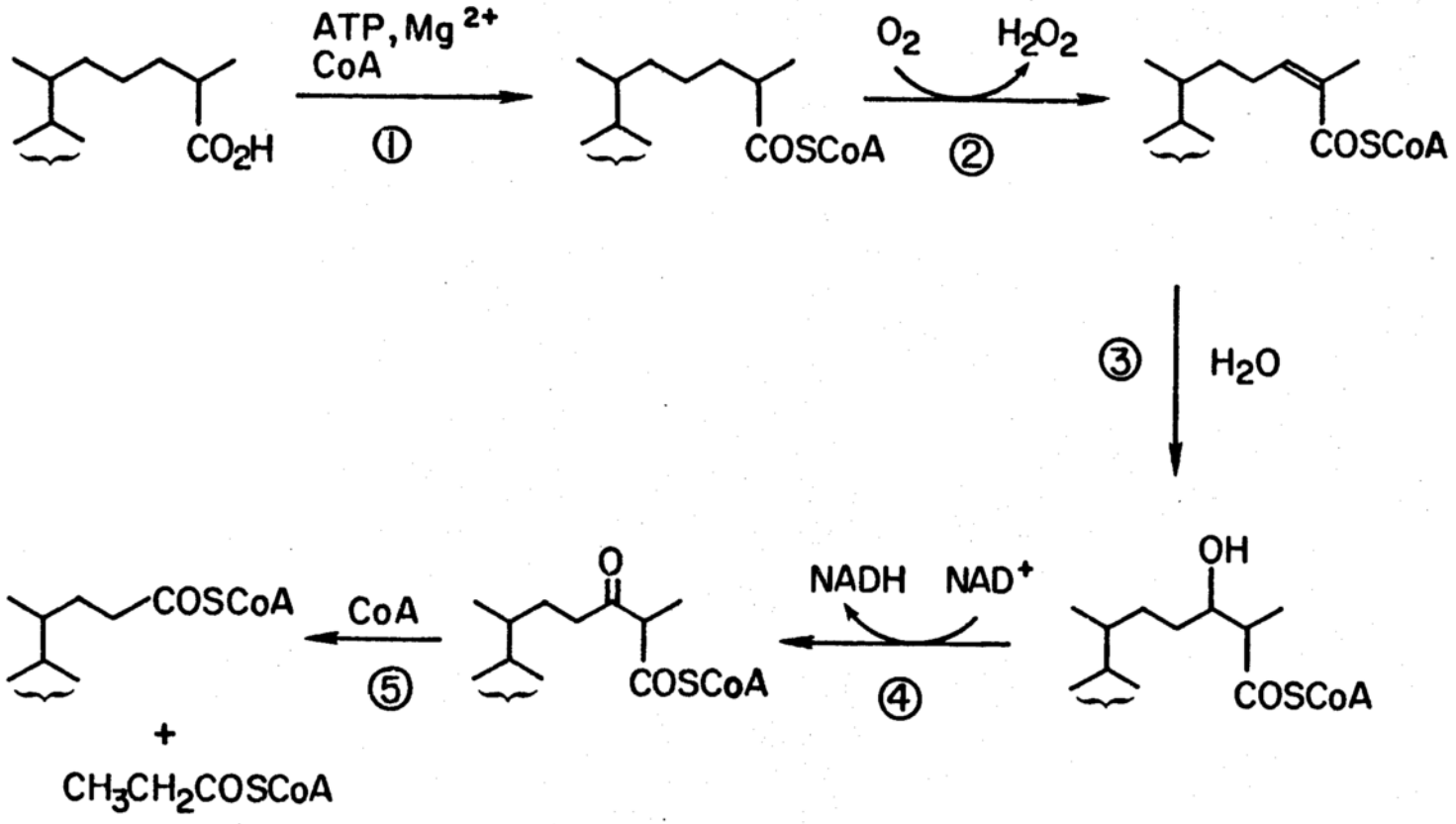


In the intact cell, the C-24 ketone (V) can be further metabolized to AD (VI), probably via terminal hydroxylation and subsequent degradation à la ß-oxidation mode. Consequently, the 24-ketone (V) will

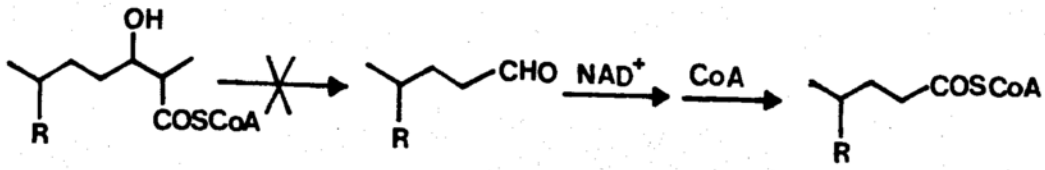


not accumulate in the medium. On the other hand, the accumulation of the 24-keto compound by the mutant[17], Mycobacterium SCM-1, is probably due to the absence of a terminal hydroxylation enzyme.

In contrast to phytosterols, degradation of the cholesterol side chain proceeds via a less complicated mechanism, similar to a ß-oxidation type of metabolism (Fig. 12). The possibility that 3-oxo-chol-4-en-24-oyl CoA might be produced via reverse-aldolytic cleavage of the C-24 hydroxy intermediate was excluded, for the same intermediate,

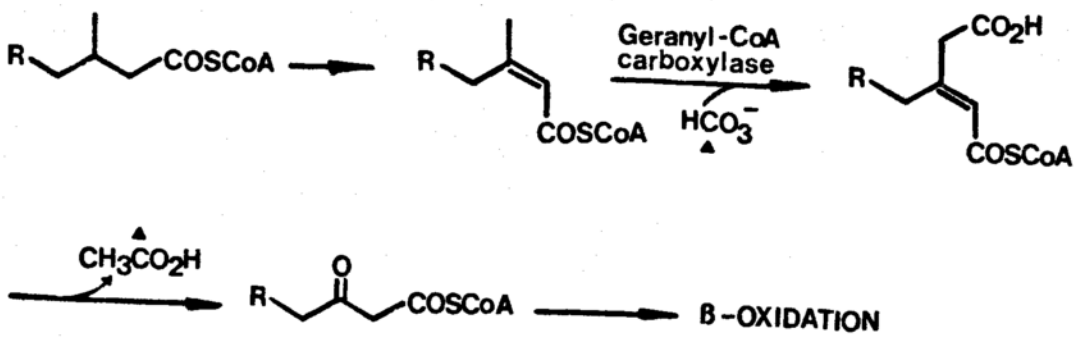

ATP, Mg 2+
CoA
①
CO2H
COSCoA
O2
H2O2
②
COSCoA
③
H2O
OH
NADH
NAD+
④
COSCoA
O
CoA
⑤
COSCoA
+
CH3CH2COSCoA

3,24-dioxo-cholest-4-en-26-oic acid, was obtained in the reaction medium.



At present, although it is still unclear whether both mechanisms share the same enzyme systems, the apparent absence of the accumulation of intermediates lends support to the contention that these enzymes function in a coordinated manner and perhaps exist in a highly ordered multienzyme complex.

The degradative mechanism of the branched side chain of phytosterols provides a unique example in biochemical metabolism. Analogous schemes have been proposed for the ß-substituted fatty acid catabolism, i.e., geranoic acid, in which a carboxylation reaction is also involved



in the removal of the branched chain. These enoyl-CoA carboxylation enzymes have been highly purified and characterized[37-41], and found to be biotin dependent and to utilize bicarbonate ion as the active

one carbon fragment. Since the biotin prothetic group acts as a "CO_2" carrier, the biotin-dependent carboxylation reaction is irreversibly inhibited by avidin, which can form an extremely tight complex with biotin.[42] Thus, the specific biotin binding property of avidin serves as a useful diagnostic tool in the characterization of biotin-dependent enzymes.[43] As seen in Fig. 8, the carboxylation of the steroid molecule was not affected by avidin, suggesting that the enzyme system may be devoid of biotin, or the prothetic group was deeply embedded in the enzyme molecule, and inaccessible to avidin. At this stage, one cannot exclude either of these possibilities.

An understanding of the degradation pathway enables us to define rate-determining steps in the overall degradation of phytosterols. Although the regulatory mechanism of the enzyme system is still unclear, the carboxylation reaction seems to be the rate limiting step, because of the low concentration of active "CO_2" species in the fermentation medium. Hence, the addition of exogenous bicarbonate ion to fermentation medium leads to a significant enhancement of the rate of phytosterol side chain degradation.

To gain further insight into the enzyme system, procedures were developed for the purification of the enzymes involved in the pathway. These investigations constitute the second part of this study.

PART II

PURIFICATION AND CHARACTERIZATION OF STEROL ACYL-CoA SYNTHETASE AND SIDE CHAIN DEGRADATION MULTIENZYME COMPLEX

EXPERIMENTAL PROCEDURES

Polyacrylamide disc gel electrophoresis

Electrophoresis under nondissociating conditions was performed according to the procedure described by Davis.[44] Gels approximately 9 cm in length were polymerized in cylindrical glass tubes with an inner diameter of 0.5 cm. The running gel consisted of 7.5% acrylamide, 0.23% bisacrylamide, 0.38 M Tris-chloride, pH 8.9, and 0.017% TEMED. When 5% polyacrylamide gels were employed, the compositions were 5% acrylamide, 0.15% bisacrylamide, 0.38 M Tris-chloride, pH 8.9, and 0.017% TEMED. Polymerization was initiated using 0.1% ammonium persulfate. A 1-cm stacking gel, composed of 3.5% acrylamide, 0.1% bisacrylamide, 0.06 M Tris-chloride, pH 6.7, and 0.017% TEMED, was formed. The electrophoresis buffer was 0.05 M Tris-glycine, pH 8.3.

Prior to electrophoresis, protein samples were mixed with one drop of glycerol and bromophenol blue tracking dye. The gels were subjected to electrophoresis at room temperature using a current of 3 mA/gel in a Pharmacia GE-2/4 electrophoresis apparatus.

Gels were stained for protein for a minimum of 2 hr in 0.25% Coomassie brilliant blue R in 10% acetic acid solution containing 50% ethanol, and destained in a solution containing 7.5% (v/v) acetic acid and 5% (v/v) methanol.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

The procedure employed was that described by Laemmli.[45] The 9-cm running gels were composed of 7.5% acrylamide, 0.23% bisacrylamide, 0.3 M Tris-chloride, pH 7.7, 0.5% SDS, and 0.033% TEMED. Polymerization was initiated using 0.1% ammonium persulfate. The 1-cm stacking gel consisted of 2.5% acrylamide, 0.15% bisacrylamide, 0.057 M Tris-HCl, pH 7.2, 0.5% SDS, 0.025% TEMED, 10% sucrose and 0.1% ammonium persulfate. The electrophoresis buffer was 0.05 M Tris-glycine, pH 8.3. The protein samples were denatured by incubation for 3 min at 100°C in the presence of 1% SDS, 5% mercaptoethanol, 50% glycerol and bromophenol blue tracking dye. Electrophoresis was carried out in a Pharmacia GE 2/4 at room temperature using a voltage of 60 volts until the sample had stacked, at which time the voltage was increased to 120 volts until completion. Gels were stained overnight in 0.25% Coomassie brilliant blue R in 10% acetic acid, 50% ethanol, and destained in 7.5% acetic acid.

The known proteins used as molecular weight standards were ribonuclease (13,700), chymotrypsinogen (25,000), ovalbumin (43,000), catalase (60,000 x 4), and serum albumin (67,000).

Determination of molecular weight by chromatography on Sephacryl S-200[46]

Protein samples were applied onto a 328 ml (2.5 x 67 cm) column of Sephacryl S-200 and eluted with 50 mM potassium phosphate buffer, pH 7.6, containing 0.1 M NaCl at a flow rate of 15 ml/hr. Fractions

of 3 ml were collected. Protein was monitored at 280 nm. The void volume of the column was determined with blue dextran (M.W. 2,000,000). A standard curve was constructed from the elution volume of the following known proteins: catalase (232,000), aldolase (158,000), bovine serum albumin (67,000), ovalbumin (43,000), and chymotrypsinogen (25,000). The molecular weight of the enzyme was determined from the plot of K_{av} vs. log of the molecular weight of standard proteins.

Protein determinations

Protein determinations were performed using a Coomassie blue dye-binding method (Bio-Rad protein assay) using BSA as a standard. Protein concentrations during purification were determined by measuring the absorbance at 280 nm and assuming 1 OD unit = 1 mg protein/ml.

Determination of the active species of "CO_2" utilized by the enoyl-CoA carboxylase

The method employed for identification was that of Cooper et al.[47,48], which is based upon the observation that this equilibration

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

requires more than 60 sec to be attained at a temperature below 15°C when the initial reactants are CO_2 and H_2O. Consequently, if HCO_3^- is the active species and [^{14}C]-HCO_3^- is the source of ^{14}C, the incorporation of ^{14}C is rapid during the first two minutes as compared to that using CO_2 as the source of ^{14}C.

The complete reaction mixture contained the following in a final volume of 2 ml: 10 mM potassium phosphate buffer, pH 7.6, containing 0.2 M NaCl; 1 µmol of CoA; 10 µmol of ATP; 10 µmol of $MgCl_2$; 50 µg of the purified acyl-CoA synthetase and 500 µg of the purified sterol side chain degradation multienzyme complex, which was preincubated with 200 µM FMN. After adding 0.2 µmol of 3-oxo-24-ethyl-cholest-4-en-26-oic acid, the resulting mixture was incubated at 10°C with shaking for 10 min, and 0.4 µmol of [^{14}C]-$NaHCO_3$ or 0.4 µmol of [^{14}C]-$NaHCO_3$ plus 0.4 µmol of HCl was added (the specific activity of [^{14}C]-$NaHCO_3$ was 55 µCi per µmol). An aliquot of the reaction mixture (100 µl) was withdrawn every 20 sec intervals, and added to 200 µl of 1 N $HClO_4$ to remove any unreacted $NaHCO_3$. The remaining radioactivity was measured.

Identification of biotin

The procedure used was a modification of the ABC (Avidin-Biotin-Peroxidase Complex) immunoenzymatic method described by Hsu et al.[49,50]. The technique involves an application of avidin followed by the addition of biotin-peroxidase complex. Avidin serves as a link between biotin-containing protein and biotin-peroxidase complex.

Protein samples at various dilutions were prepared, and were dispensed 200 µl/well into wells (96) on a Dynatech Immulon microtiter plate. The coating solution in the wells was incubated for at least 2 hr at room temperature, and the plate was washed three times with Tris-HCl buffer, pH 9.0, using the plate washer. Any residual liquid was pounded out on an absorbent towel. The same procedure was repeated using 200 µl of BSA solution (100 µg/ml), 200 µl of a mixture of BSA and

avidin (1 mg of BSA + 0.1 mg of avidin/ml) and 200 µl of biotin-peroxidase solution (10 µg/ml) on the same plate. The final reaction was carried out by adding 200 µl of 50 mM Tris buffer, pH 7.2, containing 0.05% o-phenylenediamine and 0.01% hydrogen peroxide. After incubating the solution at room temperature for 1 hr, the reaction was terminated by adding 100 µl of 1 N HCl. Absorbance at 412 nm was then measured on a spectrophotometer plate reader.

In control experiments, pyruvate carboxylase and bovine serum albumin served as positive and negative controls.

RESULTS

I. Purification and characterization of acyl coenzyme A synthetase

All enzyme purification procedures described herein were carried out at 0-4°C unless otherwise mentioned. All buffers routinely used contained 15% (v/v) glycerol to stabilize the activity of enzyme.

Purification Procedures

Step 1. Preparation of crude cell extract.

The Mycobacterium sp. NRRL B-3805 cells (180 g wet wt., suspended in 300 ml of 50 mM potassium phosphate buffer, pH 7.6) were sonicated and ultracentrifuged at 100,000 x g as described in page 12. The clarified supernatant served as the source of crude cell extract.

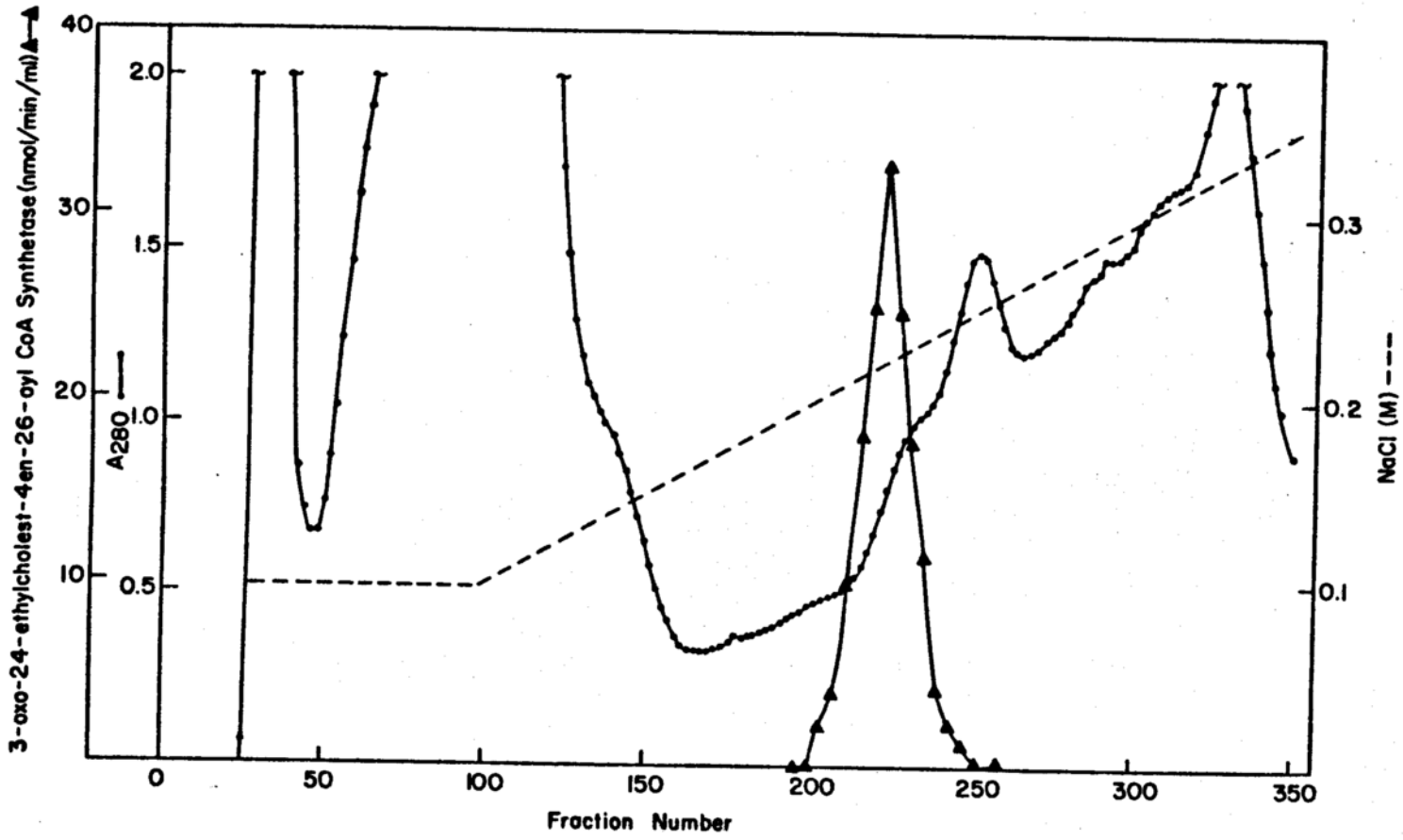
Step 2. DEAE-Sepharose CL-6B chromatography.

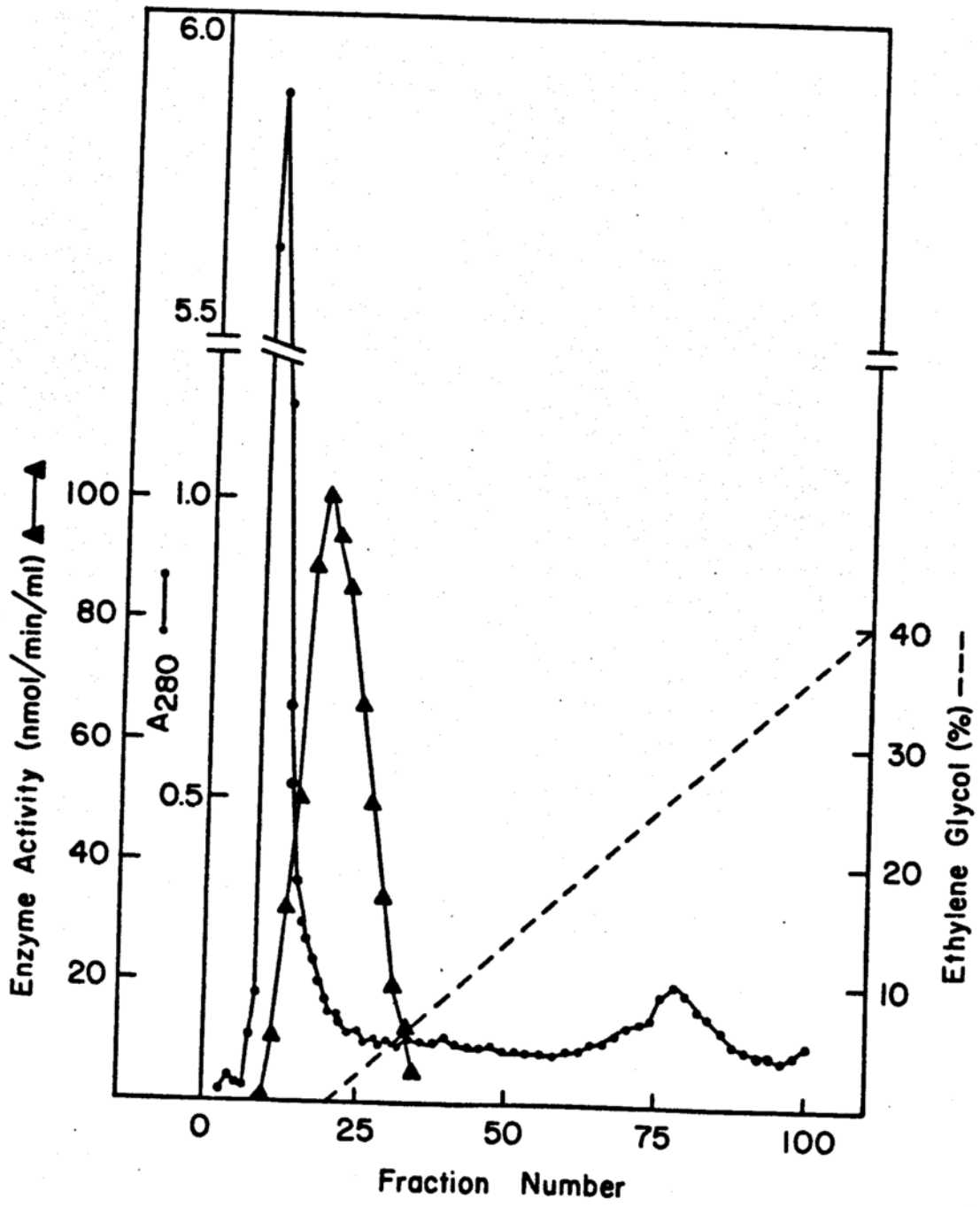
The crude cell extract was divided into three equal aliquots.

Each 100 ml aliquot (1420 mg of protein) was applied onto a DEAE-Sepharose CL-6B column (5 x 21 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.6) containing 0.67 mM PMSF. The column was first washed with 800 ml of the equilibrating buffer containing 0.1 M NaCl, and then eluted with a linear gradient (2 L) consisting of 0.1 to 0.35 M NaCl. Fractions 210 to 234 from each of the three columns, which contained the acyl-CoA synthetase activity, were pooled as shown in Fig. 1. After concentrating the pooled fractions by ultrafiltration on a YM-10 membrane to approximately 150 ml, the proteins were recovered by ammonium sulfate precipitation (60% saturation) and centrifugation at 10,000 x g. The precipitate was redissolved in 15 ml of 10 mM potassium phosphate buffer (pH 7.6), containing 0.67 mM PMSF.

Step 3. Hydrophobic interaction chromatography.

The resulting enzyme solution was applied, directly without dialysis, onto a column of phenyl-Sepharose CL-4B (2 x 10 cm), previously equilibrated with 10 mM potassium phosphate, pH 7.6. The column was washed with 100 ml of the equilibrating buffer, and then eluted with 500 ml of a linear 0-40% gradient of ethylene glycol established in the same buffer. Fractions 15 to 31, which contained the acyl-CoA synthetase, were pooled as shown in Fig.2. The pooled fractions were concentrated by ultrafiltration using a YM-10 membrane to 10 ml, and then subjected to dialysis against 10 mM potassium phosphate, pH 7.6 for 16 hr with two buffer changes.


3-oxo-24-ethylcholest-4en-26-oyl CoA Synthetase (nmol/min/ml)
A280
NaCl (M)
Fraction Number
0
50
100
150
200
250
300
350
10
20
30
40
0.5
1.0
1.5
2.0
0.1
0.2
0.3


6.0
5.5
1.0
0.5
100
80
60
40
20
A_{280}
Enzyme Activity (nmol/min/ml)
40
30
20
10
Ethylene Glycol (%)
0
25
50
75
100
Fraction Number

Step 4. Affi-Gel Blue chromatography.

The dialyzed enzyme solution (42 mg in 10 ml) was applied onto an Affi-Gel Blue column (2 x 8 cm), previously equilibrated with 10 mM potassium phosphate buffer, pH 7.6. The column was washed with the same buffer until the absorbance at 280 nm returned to the base line. The protein was then eluted with 400 ml of the equilibrating buffer with a linear gradient consisting of 0-0.4 M NaCl. As shown in Fig. 3, the acyl-CoA synthetase was eluted as a single peak in fractions 55-95, and the specific activity of the enzyme remained constant across the entire elution peak, which indicated that the enzyme was highly purified. The fractions were combined and concentrated by ultrafiltration. The affinity chromatography resulted in a 5-fold enhancement of specific activity.

A summary of the purification steps and the results are outlined in Table 1. The final enzyme preparation has a specific activity of 1678 nmol of 3-oxo-24-ethylcholest-4-en-26-oyl CoA formed/min/mg of protein. Throughout the enzyme purification procedure, enzymatic activity was monitored using 3-oxo-cholest-4-en-26-oic acid (II), 3-oxo-24-ethylcholest-4-en-26-oic acid (I) and palmitic acid, and the ratio of respective activity remained constant (2.2:1:0.1). The overall purification was 253 fold, with a recovery of 34% of the total activity. The purified enzyme could be stored in dry ice for several months without appreciable loss of activity.

Enzyme Activity (nmol/min/ml)
A280
MKCl
Specific Activity (nmol/min/mg)
Fraction Number

.... = PROTEIN ?

	Total activity units[a]	Total protein mg	Specific activity units/mg protein	Purification -fold	Yield %	Ratio of activities[b]
Crude extract	30070	4248	7.0	--	--	---
DEAE-Sepharose CL-6B	28560	140	204	29	95	2.19:1:0.11
Phenyl-Sepharose CL-4B	19026	42.0	453	65	63	2.24:1:0.11
Affi-Gel Blue	10116	5.72	1768	253	34	2.21:1:0.11

✓Homogeneity

The purity of the purified acyl-CoA synthetase was examined electrophoretically in both polyacrylamide gel and sodium dodecyl sulfate-polyacrylamide gel. Both electrophoreses showed a single protein band with a negligible amount of impurity (Fig. 4).

✓Molecular weight

The native molecular weight of the purified acyl-CoA synthetase was determined to be approximately 67,000 by gel filtration on Sephacryl S-200, as shown in Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a single protein band with an apparent molecular weight of 65,000 (Fig. 6). These results indicate that the acyl-CoA synthetase exists as a monomer, and no apparent aggregation phenomenon was observed.

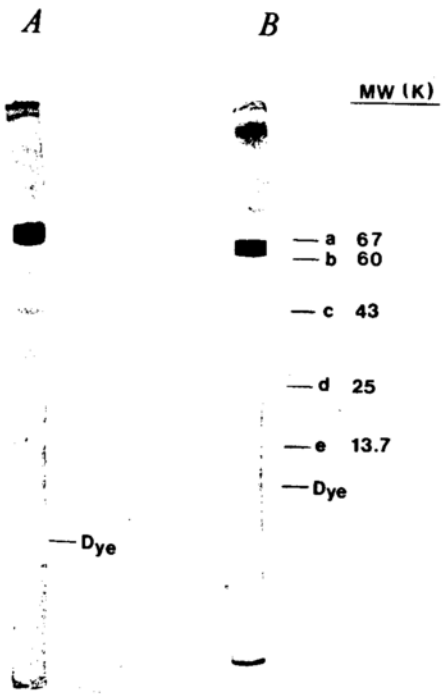
Catalytic properties

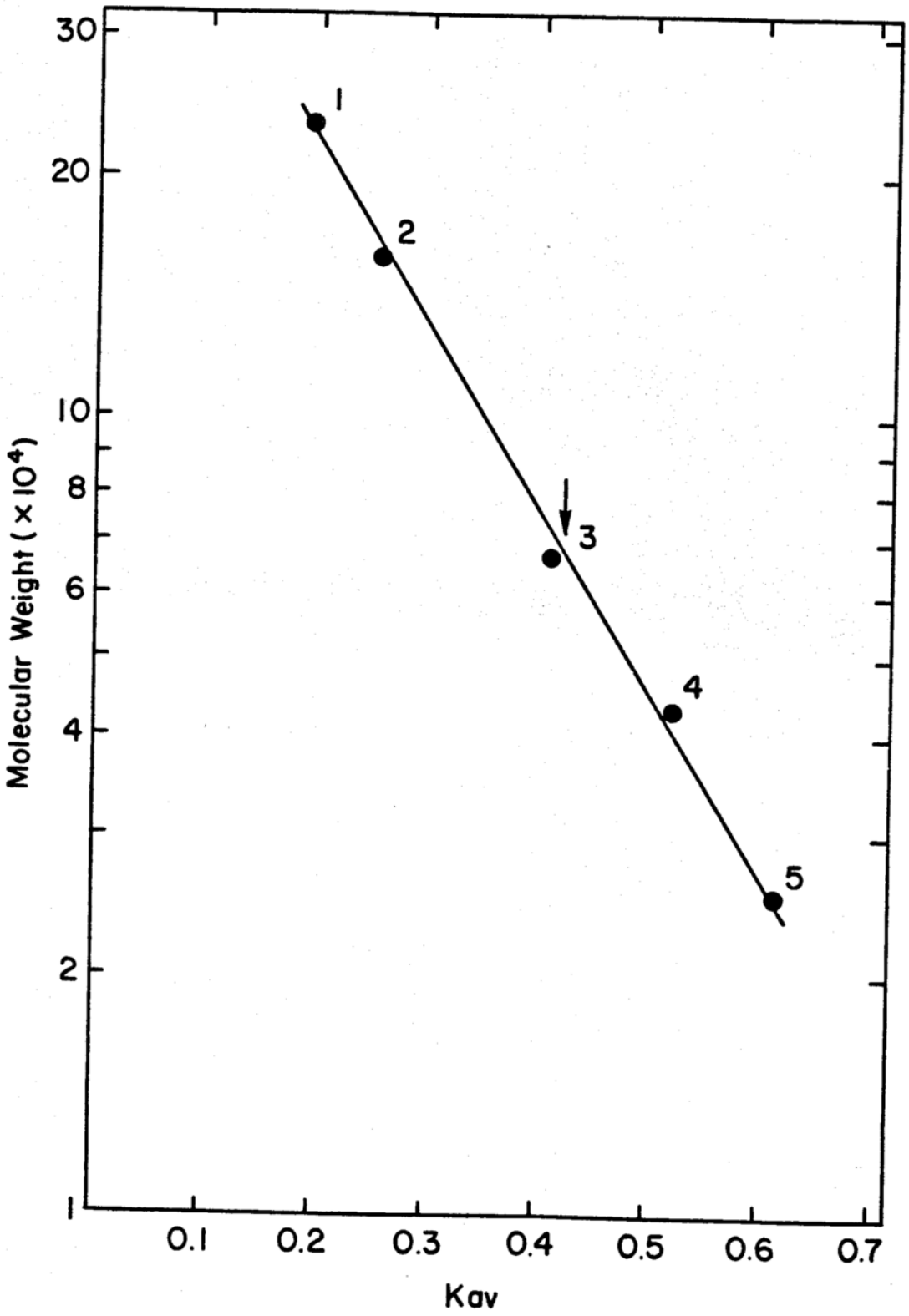
pH optimum

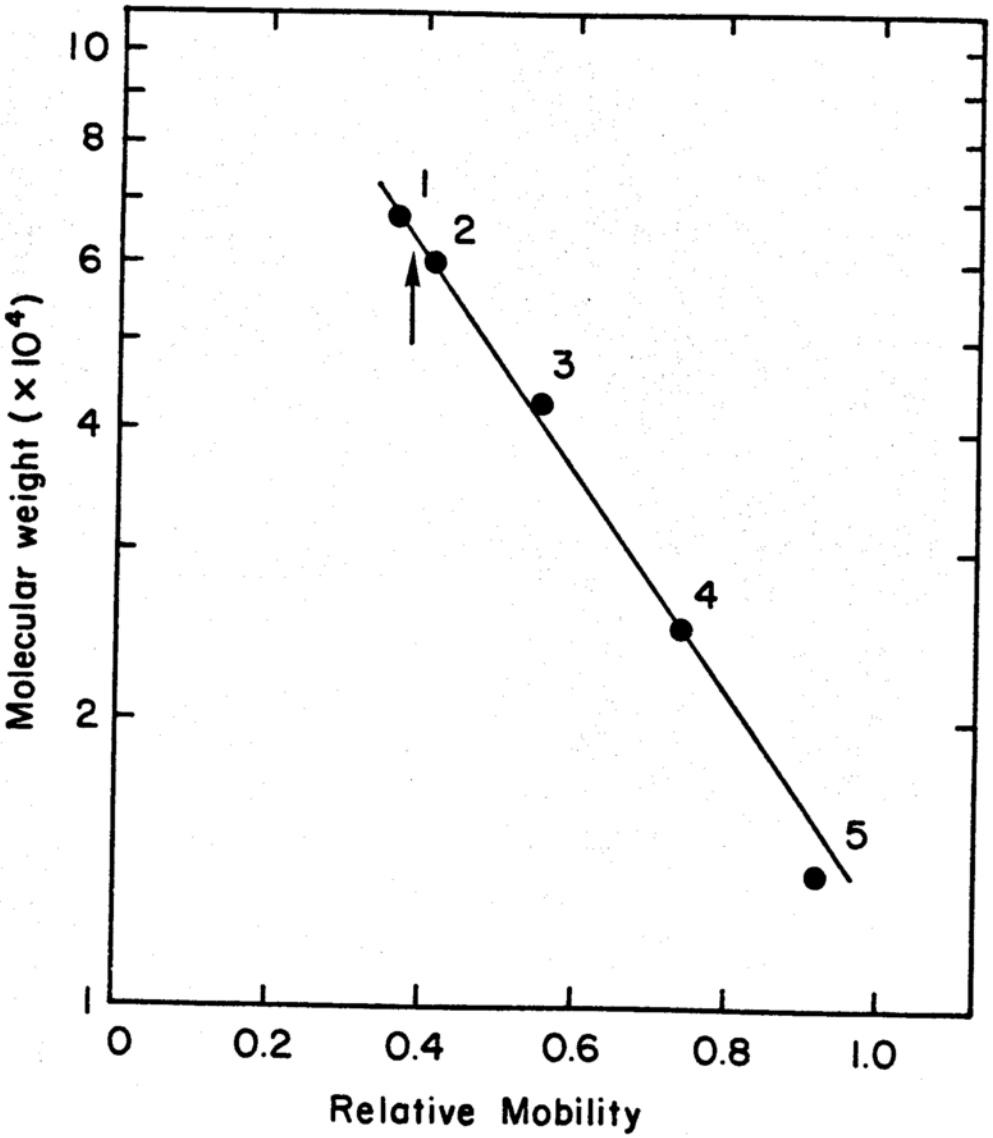
Fig. 7 shows a bell-shaped pH profile for the purified enzyme, with a pH optimum around 7.6.

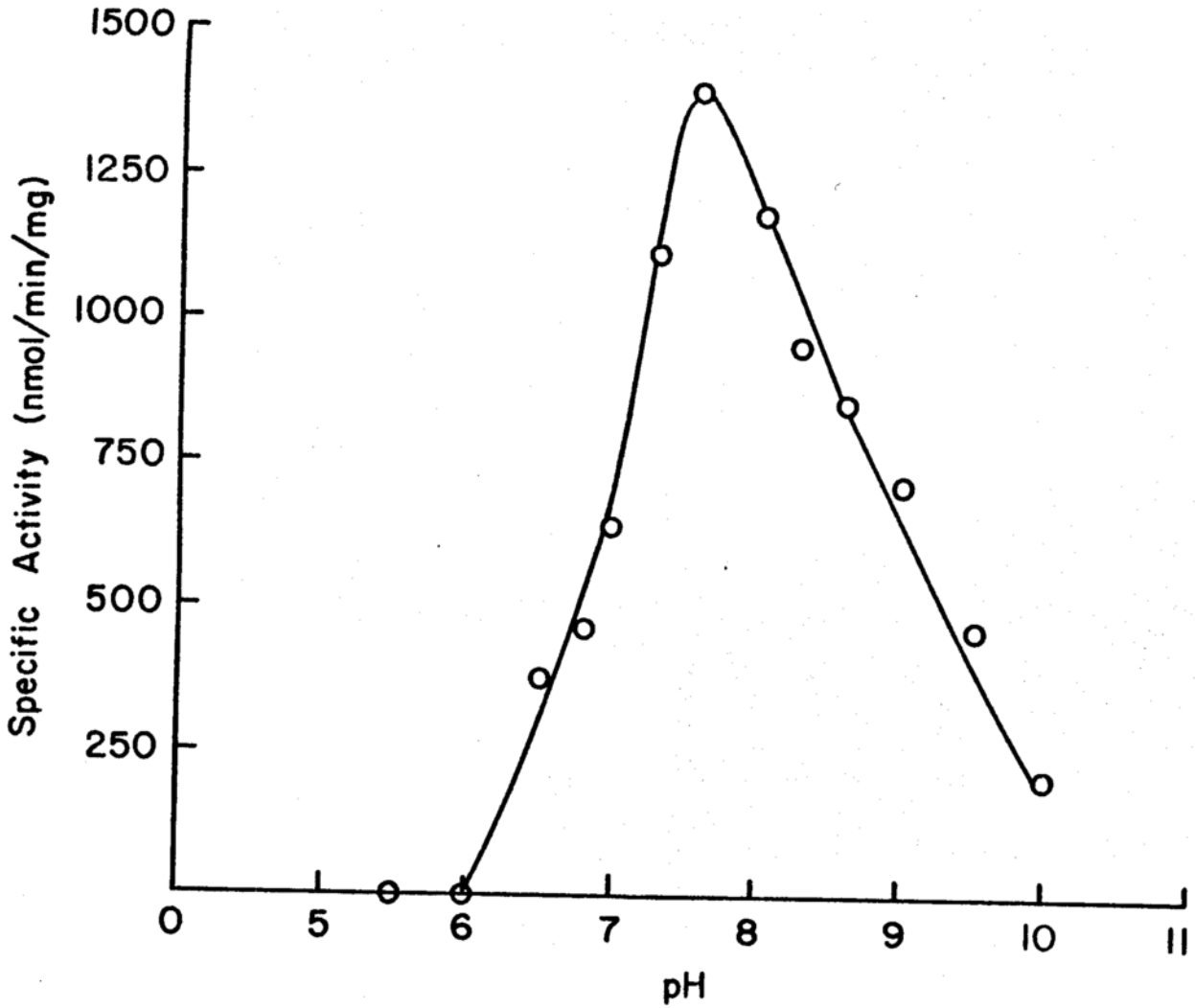
Time course and the effect of enzyme concentration on the acyl-CoA synthetase reaction

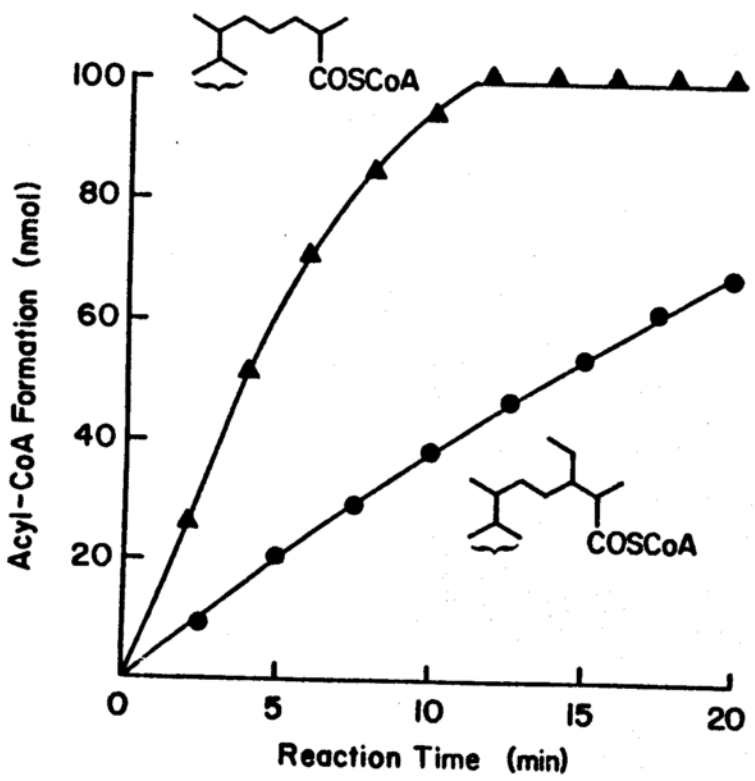
Acyl-CoA formation as a function of enzyme concentration and time is shown in Fig. 8. For both substrates, the reaction rate is a linear function of enzyme concentration up to 4 µg/ml, and the initial reaction rate remains constant for 5 minutes.

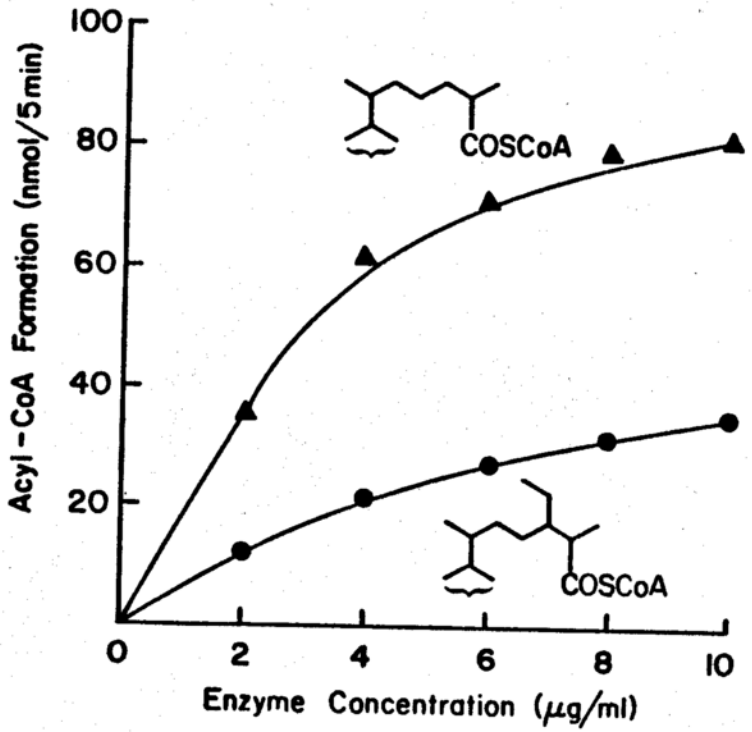

A
B
MW (K)
a 67
b 60
c 43
d 25
e 13.7
Dye
Dye


30
20
10
8
6
4
2
1
Molecular Weight (x 10⁴)
1
2
3
4
5
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Kav


10
8
6
4
2
1
Molecular weight (x 10⁴)
1
2
3
4
5
0
0.2
0.4
0.6
0.8
1.0
Relative Mobility


Specific Activity (nmol/min/mg)
1500
1250
1000
750
500
250
0
5
6
7
8
9
10
11
pH


COSCoA
COSCoA
Acyl-CoA Formation (nmol)
100
80
60
40
20
0
5
10
15
20
Reaction Time (min)


COSCoA
COSCoA
Acyl-CoA Formation (nmol/5min)
100
80
60
40
20
0
2
4
6
8
10
Enzyme Concentration (μg/ml)

Substrate specificity

The results in Table 2 show the relationship of the enzymatic kinetic parameters with the substrate structure. The purified enzyme could effectively catalyze the activation only of substrates having a bulky, hydrophobic structure. The highest specificity constant (k_{cat}/K) and maximal rate (V_{max}) were obtained with 3-oxo-cholest-4-en-26-oic acid (II). In general, the structure of the aliphatic side chain had little effect on the K_m values, but critically affected the maximal velocity. The shortening of side chain length by merely two carbons dramatically diminished the maximal activity more than ten fold, and further reduction in the chain resulted in a complete loss of activity. Palmitic acid could also serve as a substrate, but with poor affinity and low reaction rate. A short chain fatty acid and a branched side chain analog of phytosterol were completely inactive.

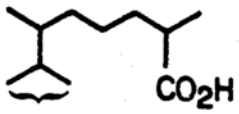
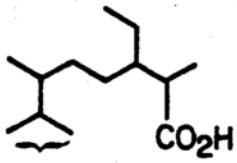
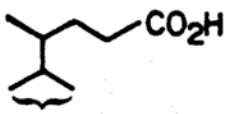
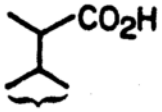
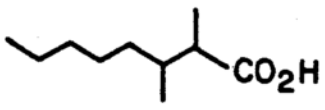
II. Purification and characterization of sterol side chain degradation multienzyme complex

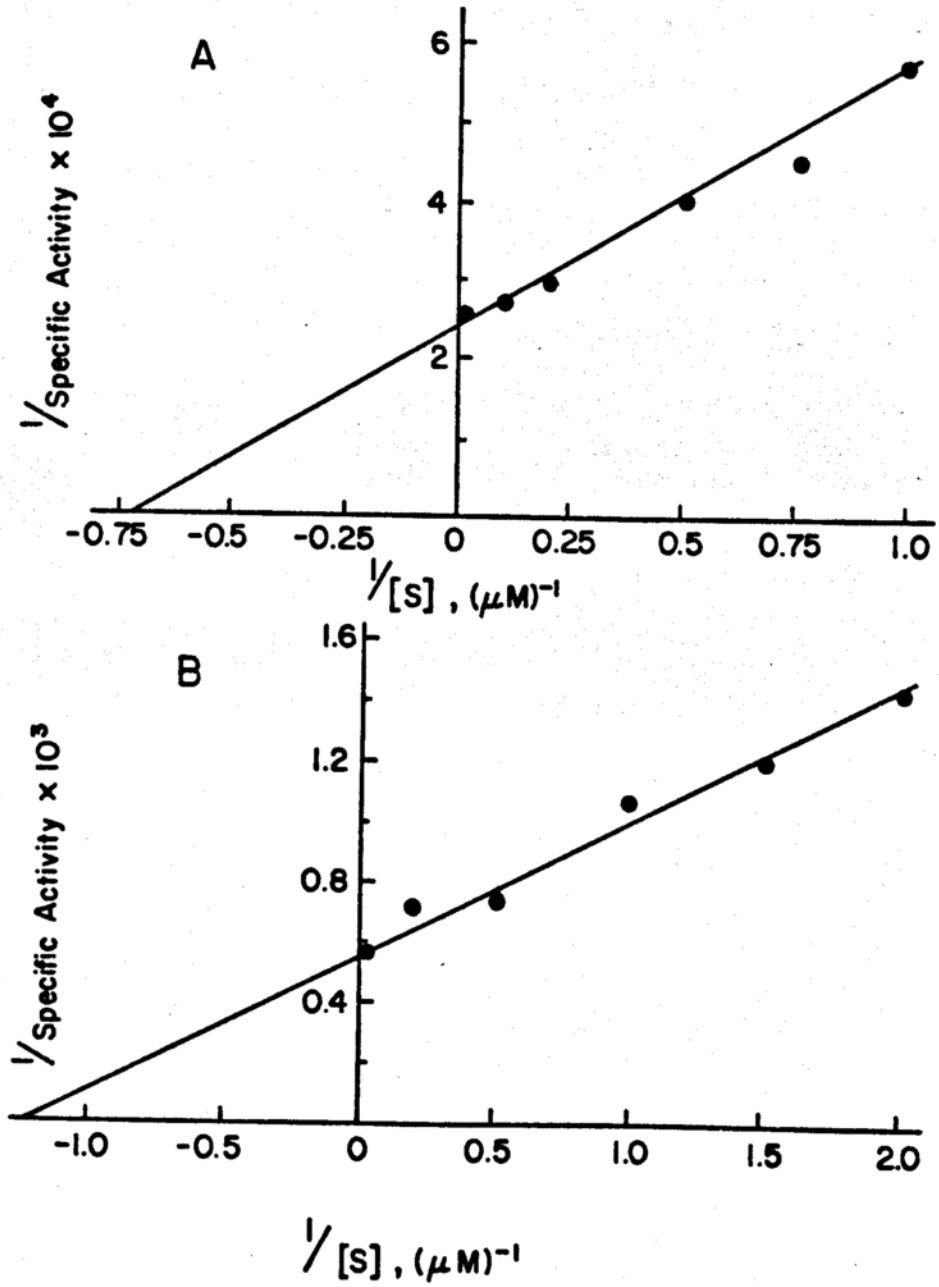
Purification procedures

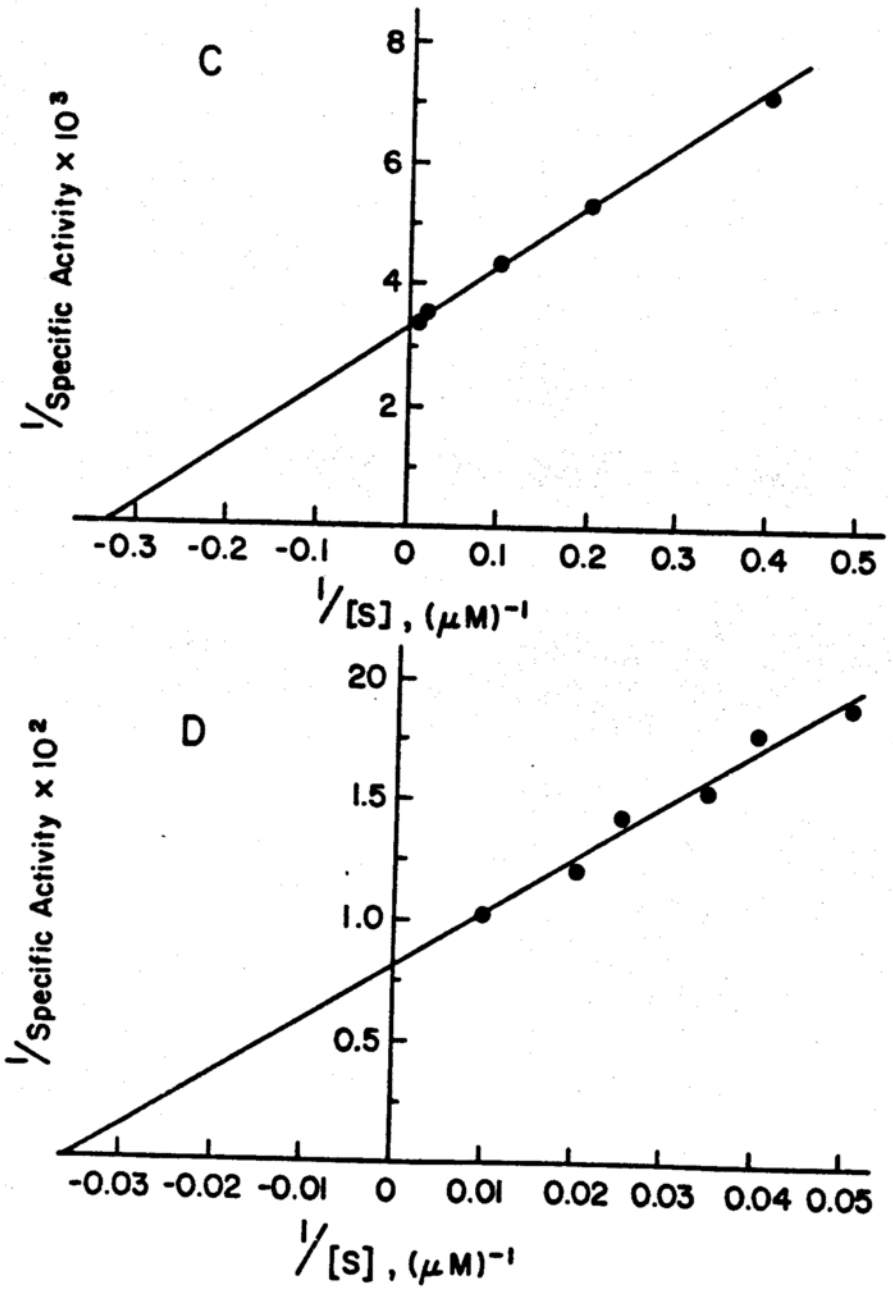
All procedures described herein were carried out at 0-4°C unless otherwise mentioned. Buffers contained 15% glycerol (v/v) to stabilize the enzyme.

Step 1. Preparation of crude cell extract.

The Mycobacterium sp. NRRL B-3805 cells (60 g wet wt, suspended in 120 ml of 50 mM potassium phosphate buffer, pH 7.6) were sonicated and ultracentrifuged at 100,000 x g, as described on page 12. The

SUBSTRATE	Km	Vmax
	μM	nmol/min/mg protein
	1.4	4166
	0.8	1770
	3.2	313
$CH_3(CH_2)_{14}CO_2H$	27.8	127
	No Reaction	
$CH_3(CH_2)_4CO_2H$	No Reaction	
	No Reaction	


A
6
4
2
1/Specific Activity x 10⁴
-0.75
-0.5
-0.25
0
0.25
0.5
0.75
1.0
1/[S], (μM)⁻¹
B
1.6
1.2
0.8
0.4
1/Specific Activity x 10³
-1.0
-0.5
0
0.5
1.0
1.5
2.0
1/[S], (μM)⁻¹


C
8
6
4
2
1/Specific Activity x 10³
-0.3 -0.2 -0.1 0 0.1 0.2 0.3 0.4 0.5
1/[S], (μM)⁻¹
D
20
1.5
1.0
0.5
1/Specific Activity x 10²
-0.03 -0.02 -0.01 0 0.01 0.02 0.03 0.04 0.05
1/[S], (μM)⁻¹

clarified supernatant served as crude cell extract.

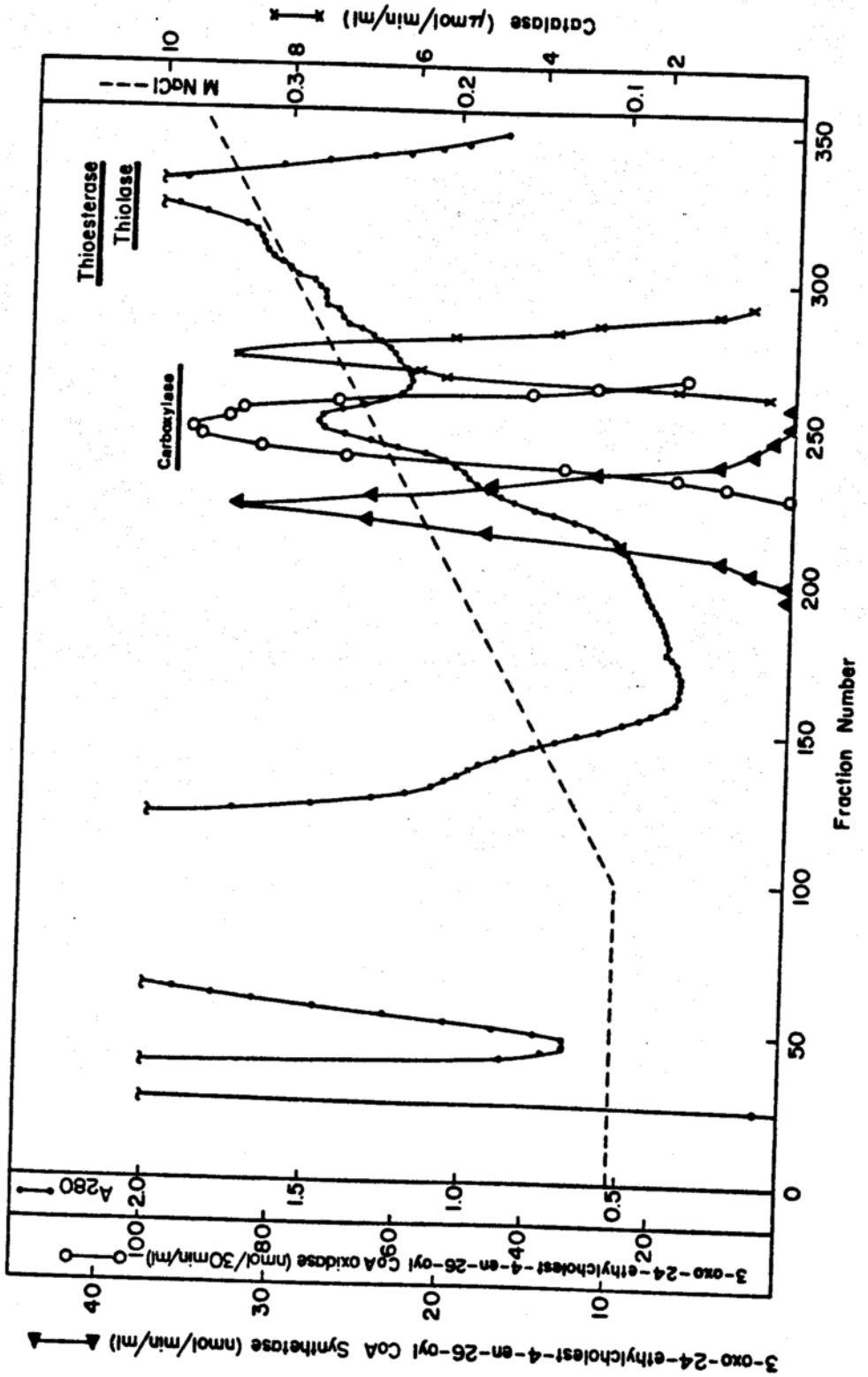
Step 2. DEAE-Sepharose CL-6B chromatography.

The chromatographic procedure was essentially the same as that described previously. Fractions 240 to 260, which contained the acyl-CoA oxidase activity, were collected as shown in Fig. 10. The pooled fractions were concentrated by ultrafiltration using a YM-10 membrane to approximately 20 ml, and only about 30% of the total activity was recovered. At this stage, the enzyme was fairly stable at 4°C in the presence of the protease inhibitor, PMSF, and could be stored at -78°C for several months without significant loss of oxidase activity.

However, further attempts to purify the enzyme by various conventional chromatographies, e.g., hydrophobic interaction, hydroxylapatite, Affi-Gel Blue or even gel filtration, always resulted in a dramatic loss of oxidase activity, a loss not due to the removal of noncovalently bound cofactor, e.g., flavin nucleotide. Because of the instability of the enzyme, much effort was expended to establish a rapid and efficient purification step. Numerous affinity chromatographic resins were screened, e.g., FMN-agarose, CoA-agarose and various dye-ligand-linked agarose, but only Matrex Gel Blue A gave a satisfactory purification.

Step 3. Matrex Gel Blue A chromatography.

The concentrated enzyme solution was applied onto a column of Matrex Gel Blue A (2 x 10 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.6, containing 0.2 M NaCl and 0.67 mM PMSF.

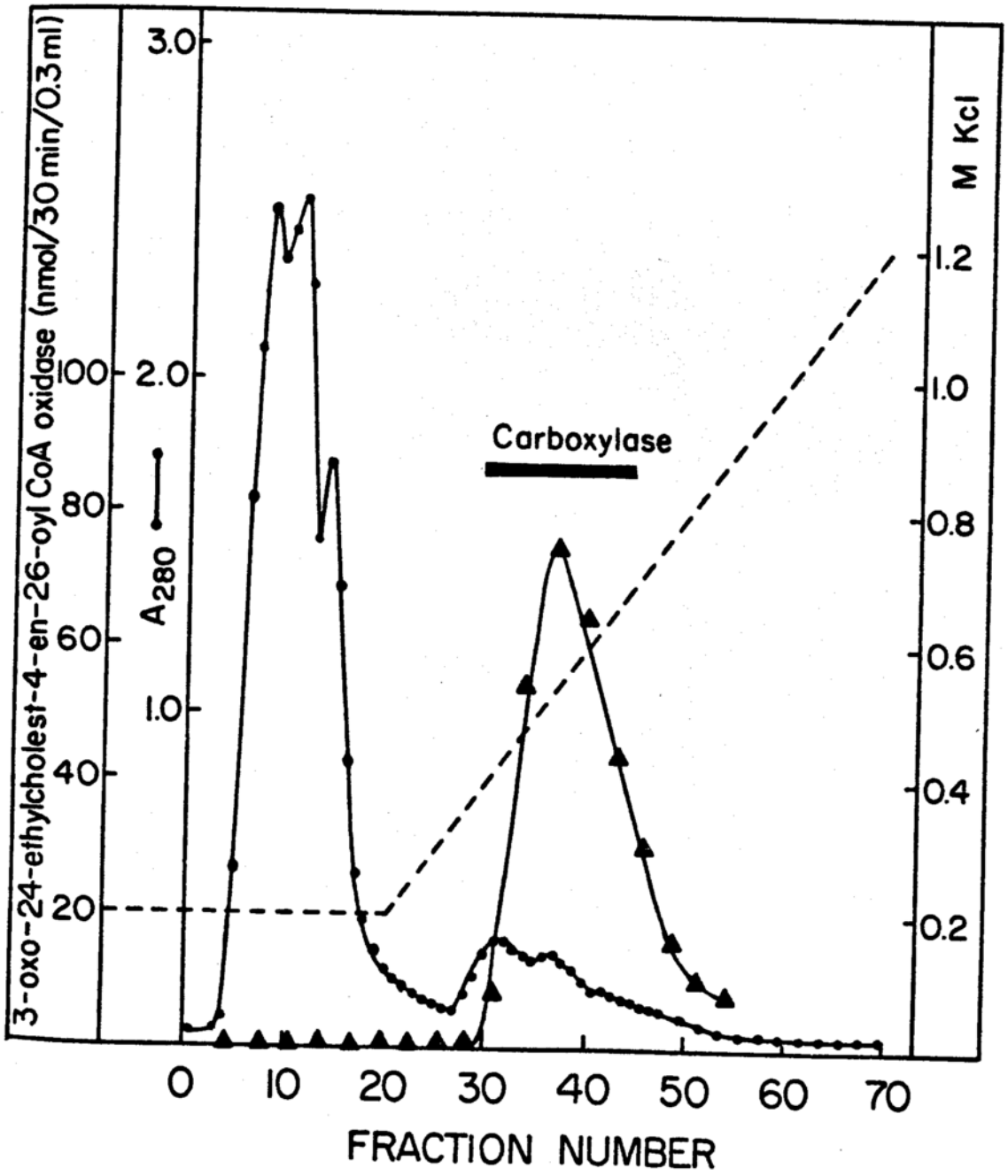

3-oxo-24-ethylcholest-4-en-26-oyl CoA Synthetase (nmol/min/ml)
3-oxo-24-ethylcholest-4-en-26-oyl CoA oxidase (nmol/30min/ml)
A280
Catalase (μmol/min/ml)
M NaCl
Thioesterase
Thiolase
Carboxylase
Fraction Number

Prior to further elution, the enzyme solution was kept in the gel for 1 hr. The column was first washed with the equilibrating buffer until the absorbance at 280 nm returned to the base line, and then eluted with a 400 ml linear gradient of NaCl from 0.2 to 1.2 M in 10 mM potassium phosphate buffer, pH 7.6 (Fig. 11). Fractions of 5.8 ml were collected. The protein in each fraction was assayed for oxidase activity, and subjected to 5% polyacrylamide disc gel electrophoresis to examine the purity. Fractions 35-41 contained nearly homogeneous, oxidase-active protein, and were pooled separately from other oxidase-active but impure fractions (31-34 and 42-48). The pooled enzyme solution was concentrated to approximately 400 µg per ml, divided into several portions, and stored in dry ice. This affinity chromatographic step resulted in a 9-fold purification with 35 percent recovery of oxidase activity.

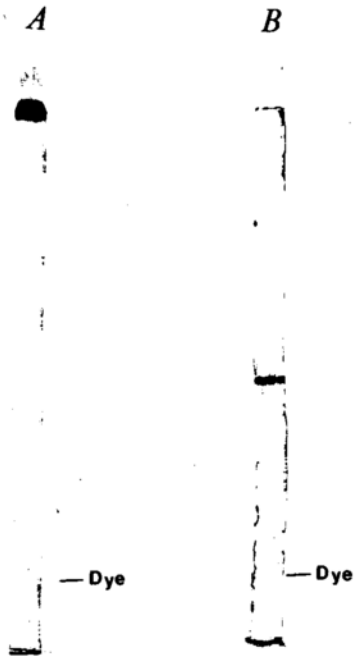
A summary of the purification steps and the results are outlined in Table 3. The overall purification was 47 fold with a recovery of 10.7 percent of total oxidase activity.

Homogeneity

Both 5% and 7.5% polyacrylamide disc gel electrophoreses were performed. As shown in Fig. 12, in 7.5% gel electrophoresis, the protein band stayed at the top of the running gel, and the 5% gel electrophoresis exhibited a single protein band with trace amounts of impurity.


3-oxo-24-ethylcholest-4-en-26-oyl CoA oxidase (nmol/30min/0.3ml)
100
80
60
40
20
3.0
2.0
1.0
A280
M KCl
1.2
1.0
0.8
0.6
0.4
0.2
Carboxylase
0
10
20
30
40
50
60
70
FRACTION NUMBER

	Total activity units[a]	Total protein mg	Specific activity units/mg protein	Purification -fold	Yield %
Crude extract	49100[b]	1420	35	--	--
DEAE-Sepharose CL-6B	15120	82	185	5.3	30.8
Matrex-Gel Blue A	5256	3.2	1643	47	10.7


A
B
Dye
Dye

Stability

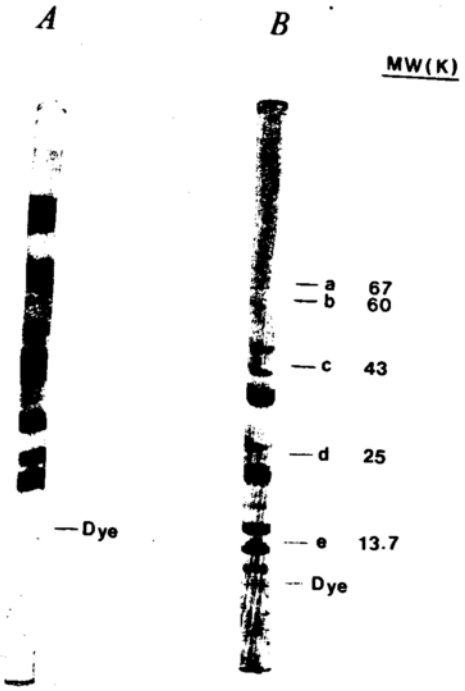
The enzyme exhibited high instability at 4°C, and could be stored in dry ice only in the presence of high ionic strength for a longer period of time without much loss of activity. Frequent thawing and freezing should be avoided. As shown in the electrophoretic pattern (Fig. 13), dialysis of the enzyme led to the complete dissociation of the protein into numerous subunits. These dissociated subunits could not be renatured by increasing ionic strength or adding reducing agents such as dithiothreitol.

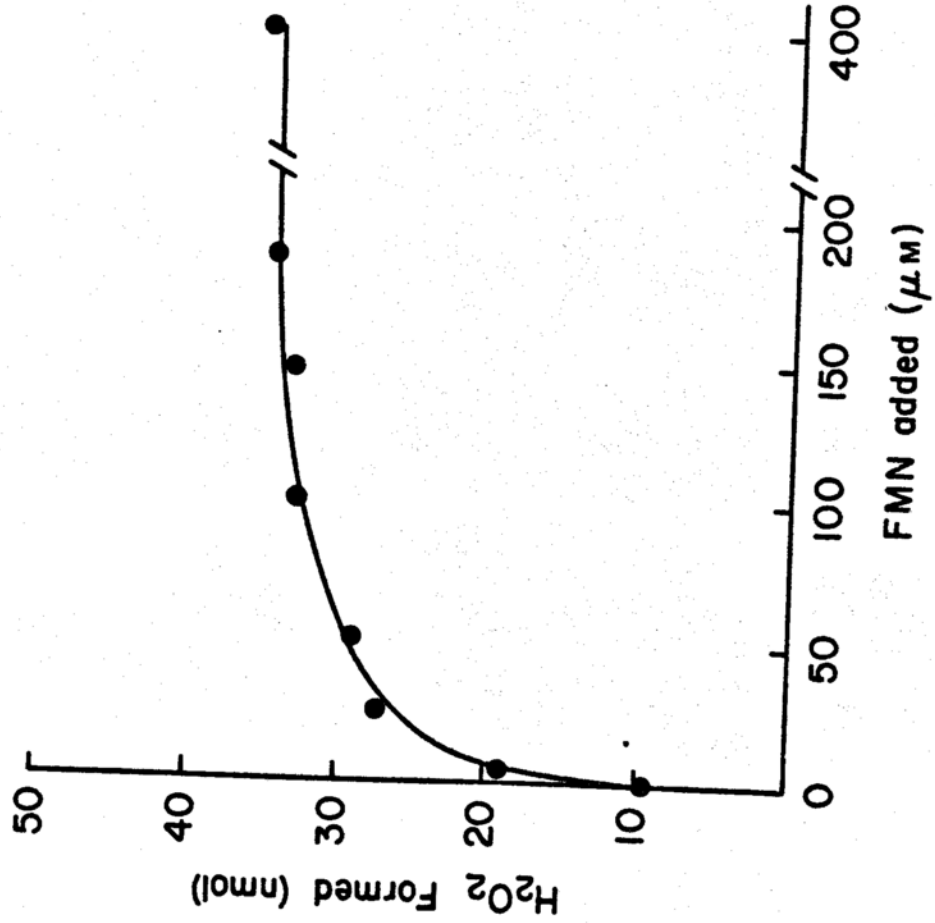
Molecular weight

Because of the instability of the enzyme complex, no conventional methods for the determination of native molecular weight, e.g., gel filtration or sedimentation equilibrium, could be employed. However, the inability of the enzyme complex to migrate in 7.5% gel indicated that the molecular weight of the complex exceeded 250,000. According to SDS gel electrophoresis, the complex contained at least 6 different subunits, with molecular weights of 46,000, 41,000, 35,000, 24,000, 16,500 and 11,000.

Exogenous cofactor requirement

After passing the enzyme solution through the affinity column, the oxidase activity decreased, indicating partial loss of the loosely bound cofactor. Preincubation of the enzyme with 50 μM FMN regenerated 90% of the original activity (Fig. 14).


A
B
MW(K)
a 67
b 60
c 43
d 25
e 13.7
Dye
Dye

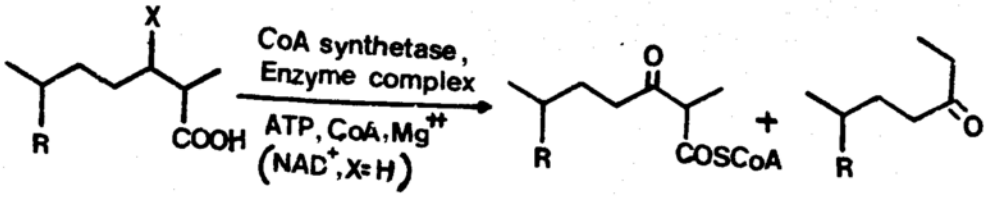

H_2O_2 Formed (nmol)
50
40
30
20
10
0
50
100
150
200
400
FMN added (μM)

Substrate specificity

Both 3-oxo-cholest-4-en-26-oyl CoA or 3-oxo-24-ethyl-cholest-4-en-26-oyl CoA served as substrates for the oxidase (Fig. 15). The lower activity of the former compound may be due to the presence of an isomer epimeric at C-25, which may reduce the effective substrate concentration or it may competitively inhibit the reaction.

Product identification

Exposure of 3-oxo-24-ethyl-cholest-4-en-26-oic acid (I) to the enzyme complex, which was preincubated with FMN, in the presence of acyl-CoA synthetase, ATP, CoA and $MgCl_2$ gave a mixture of 3,24-dioxo-cholest-4-en-26-oic acid and 27-nor-cholest-4-en-3,24-dione at a ratio of approximately 2 to 1. In contrast, when 3-oxo-cholest-4-en-26-oic acid (II) was used as a substrate in the presence of NAD^+, the ß-keto acid was predominantly produced, with small amounts of the 24-keto compound.

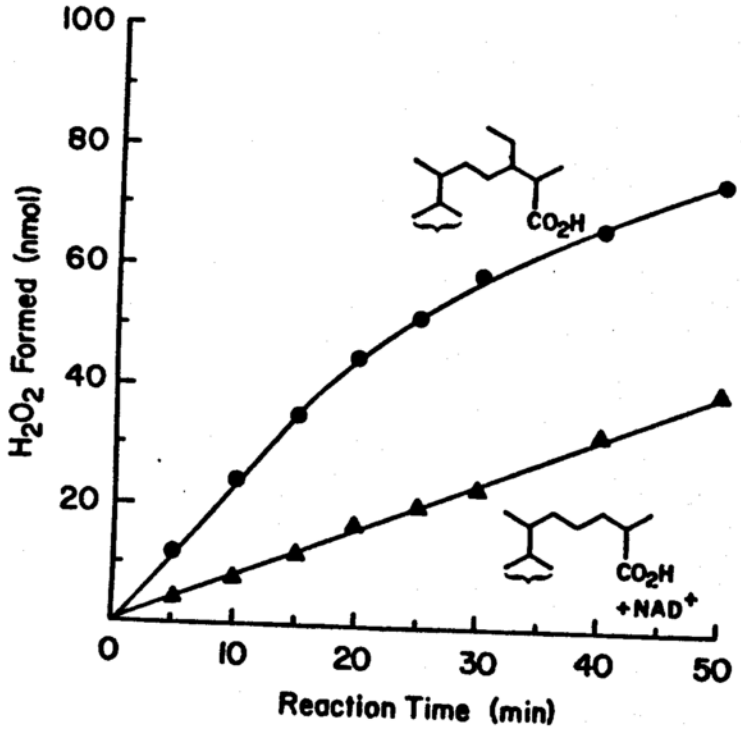


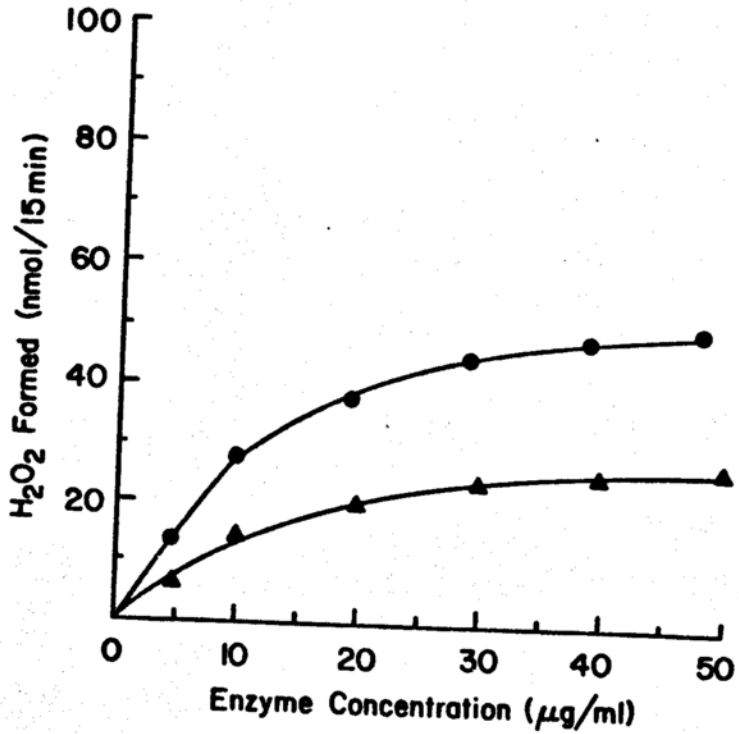
X = H 10 : 1

X = C_2H_5 2 : 1

Absorption spectrum of purified enzyme

The absorption spectrum of purified enzyme (Fig. 16) showed no distinctive peaks in the visible region. Although the enzyme contained flavin nucleotide, no characteristic peaks of flavin were observed,


100
80
60
40
20
0
10
20
30
40
50
H_2O_2 Formed (nmol)
Reaction Time (min)
CO_2H
CO_2H
+NAD^+


100
80
60
40
20
0
10
20
30
40
50
H_2O_2 Formed (nmol/15 min)
Enzyme Concentration (μg/ml)

probably because of its low content.

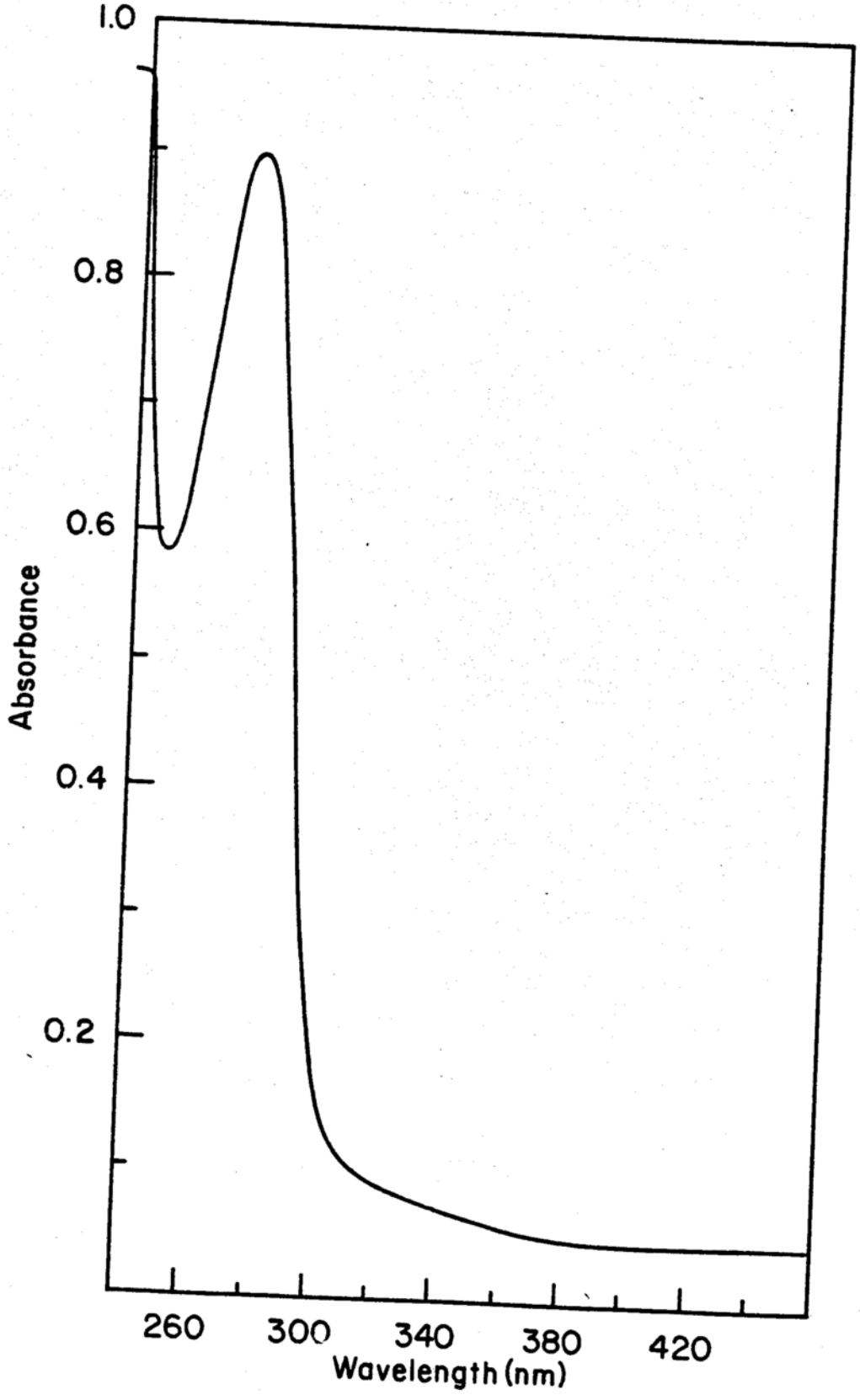
Identification of biotin

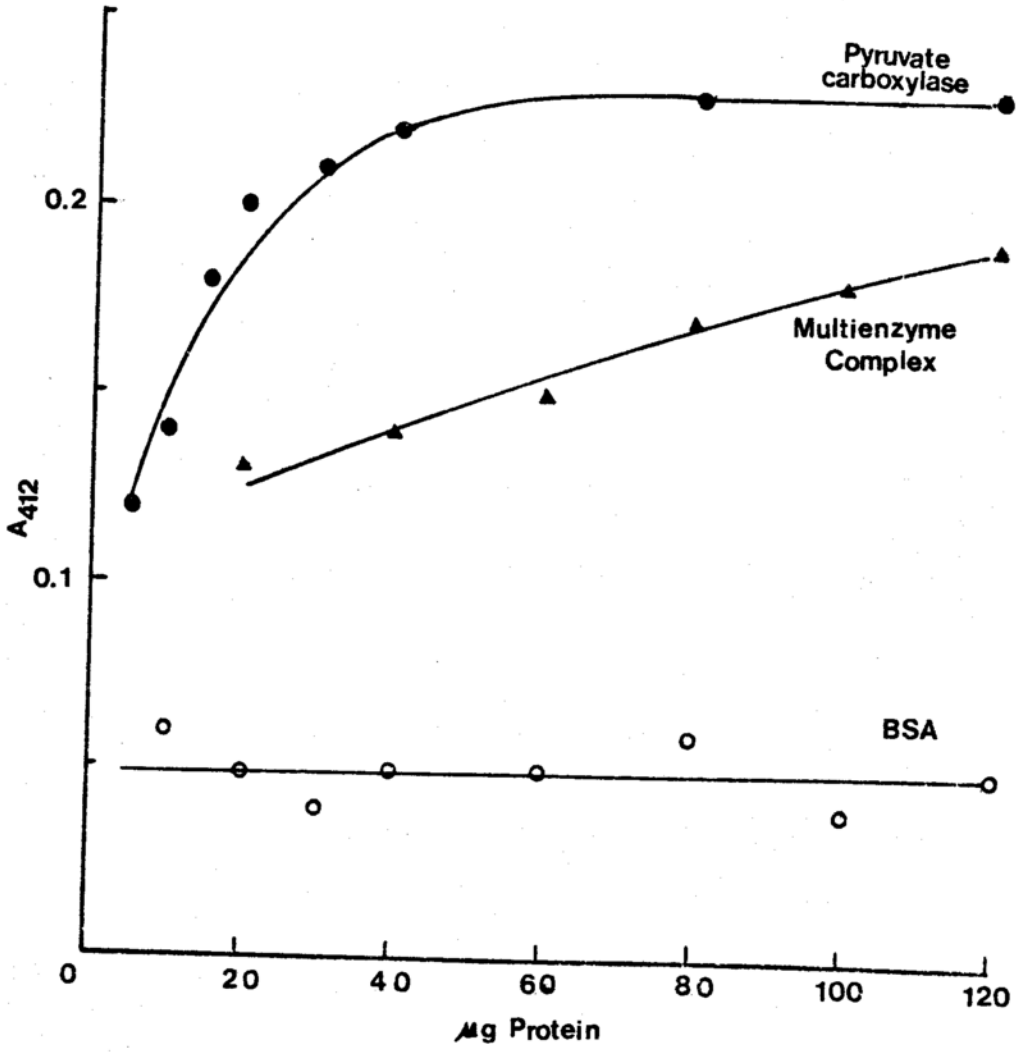
Both PEP carboxylase and the multienzyme complex assays showed a protein concentration-dependent increase of peroxidase activity, indicating the presence of biotin in the multienzyme complex (Fig. 17). On the other hand, BSA did not cause an increase of peroxidase activity.

DISCUSSION

The sterol acyl-CoA synthetase and side chain degradation multienzyme complex from Mycobacterium sp. NRRL B-3805 have been been highly purified to a nearly homogeneous state as analyzed by polyacrylamide gel electrophoresis. The purification of these two enzymes represents the first preparation of a highly purified enzyme system which accounts for the partial cleavage of sterol side chains. The most important element leading to the successful purifications is the availability of the natural intermediate, 3-oxo-24-ethyl-cholest-4-en-26-oic acid (I), which enabled us to bypass the terminal hydroxylation and oxidation steps.

The acyl-CoA synthetase is a fairly stable enzyme, and can be handled without any problems. Rapid purification was achieved in three steps with a 253-fold purification. The enzyme exhibited a distinctive substrate preference, in which a bulky hydrophobic ring structure is required. Another interesting feature is that only a monomeric


1.0
0.8
0.6
0.4
0.2
Absorbance
260
300
340
380
420
Wavelength (nm)


Pyruvate carboxylase
Multienzyme Complex
BSA
0.2
0.1
A_{412}
0
20
40
60
80
100
120
μg Protein

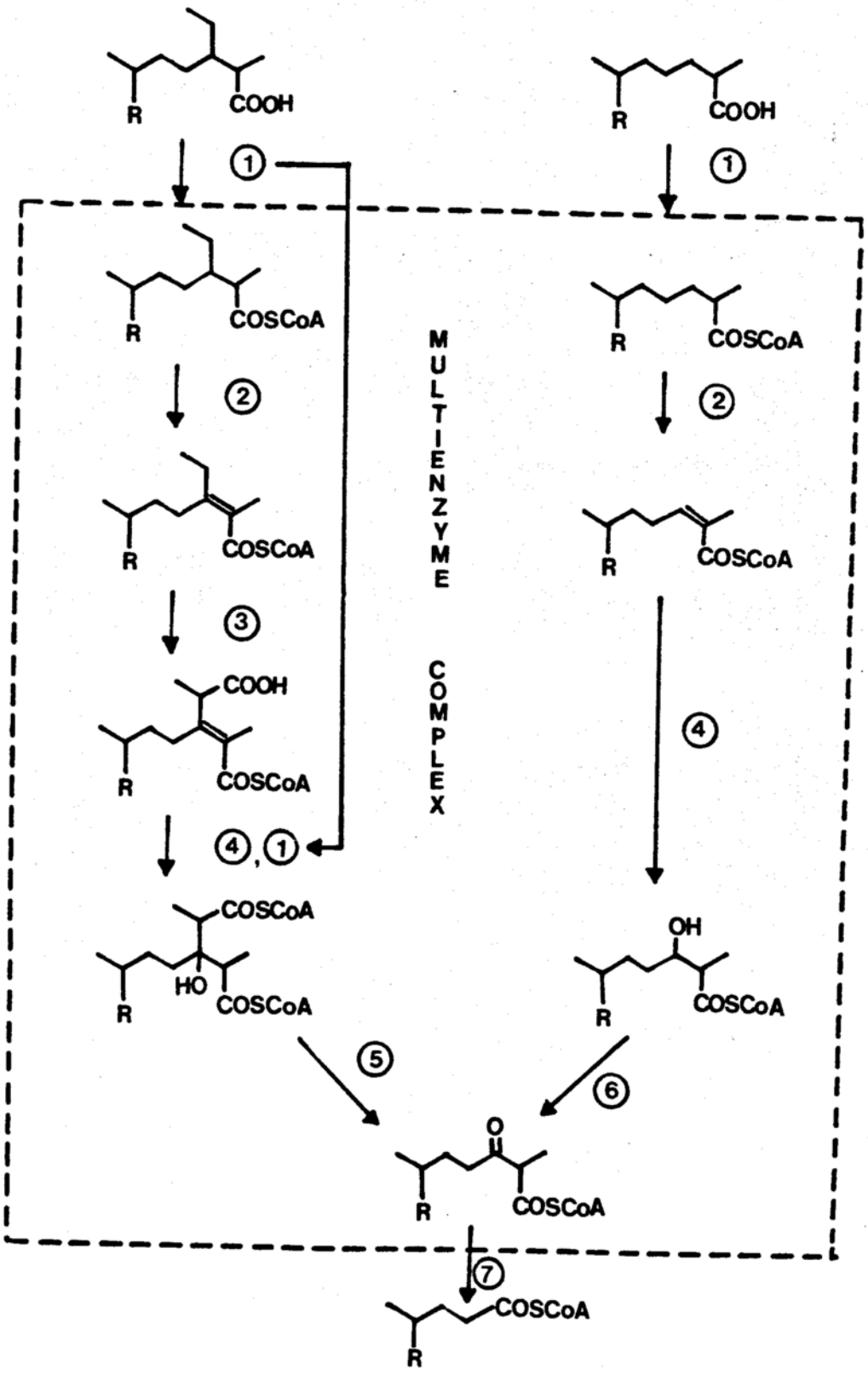
form of the enzyme molecule exists, whereas most other acyl-CoA synthetases are in aggregated forms.[51,52] However, the CoA synthetase might associate weakly to the multienzyme complex, and may be part of the complex in vivo.

On the other hand, considerable difficulties were encountered during the purification of the multienzyme complex. Numerous types of chromatographic procedures, e.g., hydrophobic interaction, gel filtration, chromatofocusing or hydroxylapatite, failed to give satisfactory recovery of enzyme activity. The key step leading to the successful purification was the affinity chromatography coupled to a DEAE-Sepharose CL-6B column. Matrex-Gel Blue A was selected from several affinity chromatographic resins. Prior to sample application, the column had to be equilibrated with 0.2 M KCl to reduce the interaction between the enzyme complex and the resin. Otherwise, the complex could not be eluted from the column even with a salt gradient up to 1.5 M KCl. Moreover, Affi-Gel Blue could not substitute for the Matrex-Gel Blue A resin because the binding of the enzyme complex to the former gel is too tight.

Polyacrylamide disc gel electrophoresis of the dialyzed preparation of the purified multienzyme complex showed the dissociation of the enzyme into numerous subunits upon dialysis, disclosing the extreme instability of the complex. The multienzyme complex easily dissociated when the ionic strength of the medium decreased. This suggested that the major forces contributing to association were due to hydrophobic interaction. This is consistent with the observation that the complex is more stable in high ionic strength buffer. The instability of the

complex prohibited the determination of the molecular weight of the native complex by conventional methods, e.g., gel filtration and sedimentation equilibrium. SDS-gel electrophoresis of the complex showed the molecular weight of subunits to range from 11,000 to 46,000. Since different proteins did not stain equally on SDS gel, the molar ratio of these subunits could not be exactly determined.

All the enzymes responsible for the partial breakdown of both cholesterol and phytosterol side chain were shown to be present in the complex. Presumably, these include acyl-CoA oxidase, enoyl-CoA carboxylase, reverse-aldol lyase and ß-hydroxyacyl-CoA dehydrogenase, which are required in the formation of the key C_{24} intermediate from both CoA esters of 3-oxo-24-ethyl-cholest-4-en-26-oic acid (I) and 3-oxo-cholest-4-en-26-oic acid (II) (Fig. 18). Since the relevant substrates are not available, the relative activities and substrate specificities of the component enzymes could not be determined. More 27-nor-cholest-4-en-3,24-dione (V) accumulated during the incubation of 3-oxo-24-ethyl-cholest-4-en-26-oic acid (I) with the purified enzyme complex than when 3-oxo-cholest-4-en-26-oic acid (II) was used as the substrate. This cannot be attributed to nonenzymatic decarboxylation of the ß-keto acid. Therefore, it may have been a result of unequal inactivation of the component enzymes during the purification procedure. The residual enoyl-CoA carboxylase activity did not coincide with that of oxidase, and some dehydrogenated intermediate may directly undergo the hydration and reverse-aldol reactions without carboxylation to form the 24-keto compound. The advantage of having an integrated complex is the greater catalytic efficiency, due to the channeling

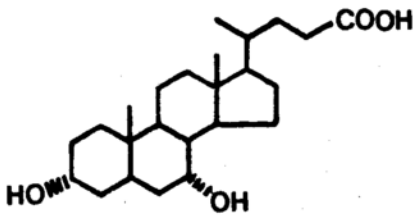

R
COOH
①
COSCoA
②
③
④, ①
④
OH
HO
⑤
⑥
O
⑦
MULTIENZYME COMPLEX

of the intermediate from one active site to the next directly without equilibrating with the medium. On the other hand, the complex functions in a highly coordinated manner, so that no intermediate will accumulate. At this stage, the structural organization of the enzyme complex still remains unknown.

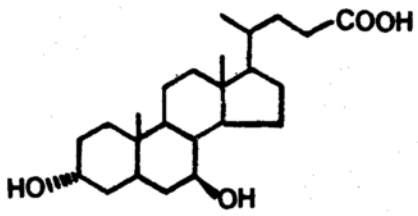
The addition of FMN, but not of FAD, caused an enhancement of oxidase activity and total degradative activity of the purified enzyme, which suggested that the enzyme complex contained loosely bound FMN or an analog as the cofactor. Several acyl-CoA oxidases for fatty acids have been found from various sources[53-55], and in all cases, tightly bound FAD was found. However, one common observation was noted among these oxidases: the enzyme's requirement for oxygen cannot be replaced by adding artificial electron acceptors, e.g, PMS or DCPIP, etc.

Numerous efforts have been made to determine the active species of the carboxylation reaction, i.e., CO_2 or HCO_3^-. However, the incorporation rate of radioactivity was too low in the short time incubation experiment at low temperature to provide any useful information. Hence, attention was shifted to the determination of the presence of biotin in the enzyme. Our result shows the existence of biotin, but this prothetic group is apparently imbedded inside the complex and inaccessible to avidin. Since it is well known that carboxylases which have biotin as their prothetic groups use bicarbonate ions as the active species, it is reasonable to assume that bicarbonate ions may also be the active species for the enzyme.

In considering the scheme for the microbial degradation of sterol side chains, it can be dissected into three stages, i.e., C_{29} (or C_{27}) $\longrightarrow$ C_{24} $\longrightarrow$ C_{22} $\longrightarrow$ C_{17}. It is believed that each stage is controlled by a distinctive enzyme system. In theory, at each stage some intermediates may accumulate if a proper mutant is selected or a suitable inhibitor is used. These intermediates are useful starting materials for the syntheses of various types of steroid drugs. Undoubtedly, an understanding of these enzyme systems will allow one to design suitable experiments in achieving this objective. For example, much progress has been made in developing novel steroid chemotherapeutic agents, such as chenodeoxycholic acid[56] (CDC) and ursodeoxycholic acid[47] (UDC).



CDC



UDC

They are effective agents in the dissolution of cholesterol gallstones, and have become a viable alternative to the surgical removal of the gallbladder. However, one problem encountered in producing these compounds on a large scale is the cost of the starting material, i.e., deoxycholic acid and cholic acid.[58] On the other hand, if the C_{24}-acid can be produced in large quantities from either cholesterol or phytosterol, it will be a welcome substitute for cholic acid.

Because of the uncertainty of the supply of diosgenin and the ready accessibility of cholesterol and especially of beta-sitosterol from "tall oil" and soybeans, it is likely that beta-sitosterol will become the primary source of raw material for steroid hormone synthesis in the future especially since C_{19} steroids can be converted into C_{21} steroids chemically.

By defining the intermediates, reaction sequence and the regulatory mechanism of the overall side chain cleavage process it should now be possible to further enhance the efficiencies of these transformations. Furthermore, mutants may then be selected for the accumulation of various types of oxygenated side chain intermediates, suitable for further elaboration into other useful steroidal compounds.

REFERENCES

1. Weichert, R. (1970) Angew. Chem. Int. Ed., 9, 321-332.
2. Fieser, L., and Fieser, M. (1959) "Steroids," Reinhold, New York.
3. Snider, B. B., Corcoran, R. J., and Breslow, R. (1975) J. Am. Chem. Soc., 97, 6580-6581.
4. Charney, W., and Herzog, H. L. (1967) "Microbial Transformation of Steroids," Academic Press, New York.
5. Sih, C. J. (1980) Roussel Prize Lecture, pp. 35-43.
6. Schoemer, U., and Martin, C. K. A. (1979) Adv. Appl. Microbiol., 22, 25-58.
7. Sih, C. J., Tai, H. H., and Tsong, Y. Y. (1967) J. Am. Chem. Soc., 89, 1957-1958.
8. Sih, C. J., Tai, H. H., Tsong, Y. Y., Lee, S. S., and Coombe, R. G. (1968) Biochemistry, 7, 808-818.
9. Sih, C. J., and Wang, K. C. (1965) J. Am. Chem. Soc., 87, 1387-1388.
10. Sih, C. J., Wang, K. C., and Tai, H. H. (1968) Biochemistry, 7, 796-807.
11. Nagasawa, M., Watanabe, N., Hashiba, H., Murakami, M., Bae, M., Tamura, G., and Arima, K. (1970) Agric. Biol. Chem., 34, 838-844.
12. Marsheck, W. J., Kraychy, S., and Muir, R. D. (1972) Appl. Microbiol., 23, 72-77.
13. Wovcha, M. G., Antosz, F. J., Knight, J. C., Kominek, L. A., and Pyke, T. R. (1978) Biochim. Biophys. Acta, 531, 308-321.

14. Kieslich, K. (1980) "Steroid conversions" in Economic Microbiology, Vol. 5, Academic Press, New York, pp. 370-465.

15. Nagasawa, M., Watanabe, N., Hashiba, H., Tamura, G., and Arima, K. (1970) Agric. Biol. Chem., 34, 798-800.

16. Martin, C. K. A., and Wagner, F. (1976) Eur. J. Appl. Microbiol., 2, 243-255.

17. Knight, J. C., and Wovcha, M. G. (1980) Steroids, 36, 723-730.

18. Wovcha, M. G., and Biggs, C. B. (1978) German Patent 2,746,383.

19. Wovcha, M. G., and Biggs, C. B. (1981) U.S. Patent 4,293,644.

20. Arima, K., Nakamatsu, T., and Beppu, T. (1978) Agric. Biol. Chem., 42, 411-416.

21. Jiu, J., and Marsheck, W. R. (1971) U.S. Patent 3,994,933.

22. Kornberg, A., and Pricer, W. E. (1953) J. Biol. Chem., 204, 329-343.

23. Alpers, D. H., Appel, S. H., and Tomkins, G. M. (1965) J. Biol. Chem., 240, 10-13.

24. Spector, T., Miller, R. L., Fyfe, J. A., and Krenisky, T. A. (1974) Biochim. Biophys. Acta, 370, 585-591.

25. Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W., and Fu, C. (1974) Clin. Chem., 20, 470-475.

26. Roggenkamp, R., Sahm, H., and Wagner, F. (1974) FEBS Lett., 41, 283-287.

27. Samuel, D., Estroumza, J., and Ailhaud, G. (1970) Eur. J. Biochem., 12, 576-582.

28. Bradford, M. (1976) Anal. Biochem., 72, 248-254.

29. Massey, V., and Curti, B. (1966) J. Biol. Chem., 241, 3417-.

30. Hacking, A. J., and Quayle, J. R. (1974) Biochem. J., 139, 399-405.

31. Hersh, L. B. (1973) J. Biol. Chem., 248, 7295-7303.

32. Cooper, R. A., and Kornberg, H. L. (1964) Biochem. J., 91, 82-91.

33. Stegink, L. D. and Coon, M. J. (1968) J. Biol. Chem., 243, 5272-5279.

34. Ougham, H. J., and Trudgill, P. W. (1982) J. Bacteriol., 14=50, 1172-1182.

35. Pirnik, M. P. (1977) Crit. Rev. Microbiol., 5, 413-422.

36. Fall, R. R., Brown, J. L., and Schaffer, T. L. (1979) Appl. Environ. Microbiol., 38, 715-722.

37. Hector, M. L., and Fall, R. R. (1976) Biochemistry, 15, 3465-3472.

38. Fall, R. R., and Hector, M. L. (1977) Biochemistry, 16, 4000-4005.

39. Schiele, U., and Lynen, F. (1981) Methods Enzymol., 71, 781-791.

40. Fall, R. R. (1981) ibid., 791-799.

41. Rainwater, D. L., and Kolattukudy, P. E. (1982) J. Bacteriol., 151, 905-911.

42. Moss, J., and Lane, M. D. (1971) Adv. Enzymol., 35, 321-442.

43. Green, N. M. (1964) Biochem. J., 90, 564-568.

44. Davis, B. J. (1964) Ann. N. Y. Acad. Sci., 121, 404-427.

45. Laemmli, U. K. (1970) Nature, 227, 680-685.

46. Andrews, P. (1964) Biochem. J., 96, 595-606.

47. Cooper, T. G., Tchen, T. T., Wood, H. G., and Benedict, C. R. (1968) J. Biol. Chem., 243, 3857-3863.

48. Cooper, T. G., Filmer, D., Wishnick, M., and Lane, M. D. (1969) J. Biol. Chem., 244, 1081-1083.

49. Hsu, S. M., Raine, L., and Fanger, H. (1981) J. Histochem. Cytochem., 29, 577-580.

50. Hsu, S. M., Raine, L., and Fanger, H. (1981) Am. J. Clin. Pathol., 75, 734-738.

51. Philip, D. P., and Parson, P. (1979) J. Biol. Chem., 254, 10776-10784.

52. Kameda, K., and Nunn, W. D. (1981) J. Biol. Chem., 256, 5702-5707.

53. Stokes, G. B., and Stumpf, P. K. (1976) Arch. Biochem. Biophys., 176, 591-603.

54. Hryb, D. J., and Hogg, J. F. (1979) Biochem. Biophys. Res. Commun., 87, 1200-1206.

55. Shimizu, S., Yasui, K., Tani, Y., and Yamada, H. (1979) Biochem. Biophys. Res. Commun., 91, 108-113.

56. Thistle, J. L., Hofmann, A. F., Paulina, Y. S., Yu, B. A., and Ott, B. (1977) Digestive Diseases, 22, 1-6.

57. Salen, G., Colalillo, A., Verga, D., Bagan, E., Tint, G. S., and Shefer, S. (1980) Gastroenterology, 78, 1412-1418.

58. Sutherland, J. D., Macdonald, I. A., and Forrest, T. P. (1982) Prep. Biochem., 12, 307-321.

APPENDIX

APPLICATION OF BIOCHEMICAL SYSTEMS TO ORGANIC SYNTHESIS

Esterases

1. Chen, C.S.; Fujimoto, Y.; Sih, C.J. (1981) "Bifunctional Chiral Synthons via Microbiological Methods. 1. Optical Active 2,4-Dimethylglutaric Acid Monomethyl Esters." J. Am. Chem. Soc. 103, 3580-3582.
2. Chen, C.S.; Fujimoto, Y.; Girdaukas, G.; Sih, C.J. (1982) "Quantitative Analyses of Biochemical Kinetic Resolutions of Enantiomers." J. Am. Chem. Soc. 104, 7294-7299.
3. Wang, Y.F.; Chen, C.S.; Girdaukas, G.; Sih, C.J. (1984) "Bifunctional Chiral Synthons via Biochemical Methods. 3. Optical Purity Enhancement in Enzymic Asymmetric Catalysis." J. Am. Chem. Soc. 106, 3695-3696.

Oxidoreductases

1. Chen, C.S.; Zhou, B.N.; Shieh, W.R.; Vanmiddlesworth, F., Goplan, A.S.; Sih, C.J. (1983) "Stereochemical Control of Yeast Reductions. 2. Quantitative Treatment of the kinetics of Competing Enzyme Systems for a Single Substrate." Bioorg. Chem. 12, 98-117.

2. Sih, C.J.; Chen, C.S. (1984) "Microbial Asymmetric Catalysis — Enantioselective Reduction of Ketones." Angew. Chem. Int. Ed. Engl. 23, 570-578.