PARTIAL PURIFICATION OF 3-HYDROXY-3-METHYL-GLUTARYL COENZYME A REDUCTASE FROM TURKEY LIVER AND ITS ISOLATION FROM SPINACIA OLERACEA CHLOROPLASTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

HMG-CoA	3-Hydroxy-3-Methylglutaryl Coenzyme A
MVA	Mevalonic Acid
MVAP	5-Phosphomevalonic Acid
MVAPP	5-Pyrophosphomevalonic Acid
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
IPP	Isopentenylpyrophosphate
DMAPP	Dimethylallylpyrophosphate
NADP	Nicotinamide Adenine Dinucleotide Phosphate
EDTA	Ethylene Diamine Tetraacetic Acid
MES	2-(N-Morpholine)ethane Sulfonic Acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid

CHAPTER I

INTRODUCTION

It has been known that 3-hydroxy-3-methyl glutaryl coenzyme A reductase is the key enzyme for controlling cholesterol synthesis. This enzyme catalyzes the conversion of HMG-CoA to mevalonic acid. Cholesterol in excessive amounts can be lethal as in cholesterolemia, arteriosclerosis and coronary artery disease.

Experiments with mammalian tissue have shown that this enzyme is regulated by diets, hormones, and other factors and exhibits a diurnal rhythm. Also studies on solubilization techniques and purification have been done.

Little research has been done on this important enzyme in turkey liver and in plants. The objectives of this study were as follows: 1) to solubilize HMG-CoA reductase from turkey liver microsomes, 2) to study some partially purified HMG-CoA reductase from turkey liver microsomes and, 3) to establish the presence of HMG-CoA reductase activity in spinach chloroplast.

CHAPTER II

LITERATURE REVIEW

A. Cholesterol Biosynthesis

The study *in vivo* of Schoenheimer and Breusch in 1933 (1) provided the first evidence of cholesterol synthesis, absorption and destruction in an intact animal. In 1933, Rittenberg and Schoenheimer (2) showed that a rapid increase in isotopically labelled cholesterol was observed when deuterium (D_2O) was fed to animals for long periods of time. Siperstein (3) has reviewed the synthesis of cholesterol from acetate. Bucher and McGarrahan (4) concluded that the biosynthesis of cholesterol from acetate *in vitro* requires both microsomes and soluble cell constituents and *in vivo* over 90% of newly formed cholesterol is in the microsomal fraction. The synthesis of radioactive mevalonic acid synthesized from 1-^{14}C acetate in rat liver homogenates was first demonstrated by Knauss, Porter and Wasson (5).

The work of Block, Lynen, Cornforth and Popjak (6) led to the elucidation of the biosynthetic pathway of cholesterol from acetate. The first step of the pathway is the conversion of HMG-CoA to mevalonate. HMG-CoA reductase converts HMG-CoA to mevalonic acid. The enzyme was first found in mammalian microsomes by Bucher, Overath and Lynen (7). This enzyme has also been found in yeast

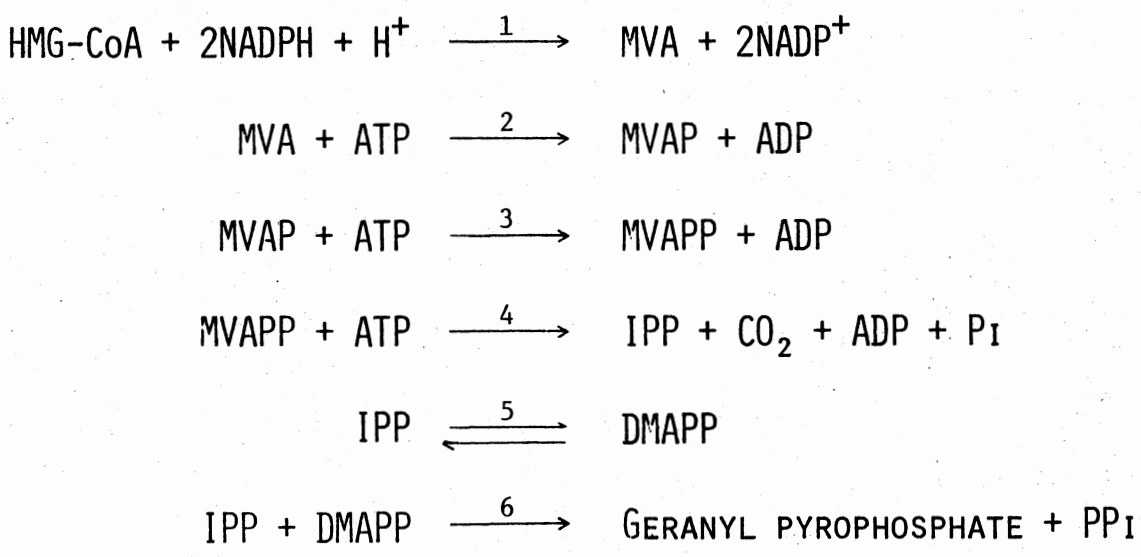
(8, 9). Mevalonate is phosphorylated to 5-phosphomevalonate, 5-phosphomevalonate is phosphorylated to 5-pyrophosphomevalonate, and 5-pyrophosphomevalonate is decarboxylated to isopentenyl pyrophosphate. An isomerization, with loss of 4-5 ^{3}H from mevalonate (2-R^{3}H of isopentenyl pyrophosphate), was observed in the formation of dimethylallyl pyrophosphate. A head to tail condensation of isopentenyl pyrophosphate and dimethylallyl pyrophosphate results in the formation of the acyclic monoterpene pyrophosphate (Figure 1).

B. Regulation of Cholesterol Biosynthesis

There is a significant amount of evidence to support the belief that the rate of cholesterol synthesis from acetate is regulated at the site of action of HMG-CoA reductase. This regulation is accomplished by many factors such as 1) cholesterol feeding, 2) fasting, 3) compounds present in bile, 4) hormones, 5) x-rays, 6) time of day, 7) and cyclic rhythm (10).

1. Regulation by Cholesterol Feeding and Fasting

In fasted rats, the hepatic HMG-CoA reductase activity was depressed dramatically when compared to hepatic HMG-CoA reductase activity in rats injected with triton WR 1339; this was first observed by Bucher et al. (7). Siperstein and Fagan (11) used a gas liquid chromatography to compare the amount of MVA formed and the amount of β-hydroxy-β-methyl glutaric acid formed in rats fed cholesterol; from these experiments they concluded that the control step was the reduction of HMG-CoA to MVA. The first direct evidence for reduction



1 HMG-CoA REDUCTASE

2 MEVALONATE KINASE

3 PHOSPHOMEVALONATE KINASE

4 PYROPHOSPHOMEVALONATE DECARBOXYLASE

5 ISOPENTENYL PYROPHOSPHATE ISOMERASE

6 GERANYL PYROPHOSPHATE SYNTHETASE

Figure 1. The Pathway From Mevalonic Acid To Acyclic Monoterpene Pyrophosphate

of HMG-CoA reductase activity in microsomal preparations and solubilized fractions by cholesterol feeding and fasting in rats was reported by Linn (12). Experimentation on cholesterol fed rats by Shapiro and Rodwell (13) suggested that cholesterol affects the rate of sterol synthesis by changing the amount of enzyme, not the activity.

Dietschy and Siperstein (14) studied the effect of cholesterol feeding and fasting on sterol synthesis in seventeen tissues of the rat. They observed a difference between the biosynthetic pathway for sterols in hepatic tissue and the biosynthetic pathway in extrahepatic tissues. Only the liver responds to exogenous cholesterol and fasting. In turkey liver and kidney, HMG-CoA reductase activity was suppressed with a 1% cholesterol diet (15).

2. Regulation by Dietary Fats

Feeding shorter chain fatty acids to rats cause a greater lowering in HMG-CoA reductase activity than to feeding longer chain fatty acids. A higher degree of unsaturation also causes the lower activity (16).

3. Regulation by Factors Present in Bile and Hormone

Bile contains many substances including bile acids, phospholipids and cholesterol. Hamprecht and Lynen (7) demonstrated that cholesterol synthesis is suppressed by feeding bile acids to rats. However, these bile acids play no direct role in the control of sterol synthesis by the liver; in fact, suppression of cholesterol synthesis actually resulted from the change in the enterolymphatic circulation of endogenous cholesterol (18).

There is evidence to show that hormones are involved in the maintenance of a diurnal rhythm of reductase activity and overall regulation of cholesterol synthesis. These include epinephrin, norepinephrin, glucocorticoid, insulin, glycagon and triiodothyronine (11). Both insulin and thyroid hormone increased HMG-CoA reductase activity (19). Physiological doses of insulin and glucagon first stimulated and then inhibited the cholesterol synthesis from labelled acetate in rat liver in vivo. This group also reported that an influence of insulin on cholesterogenesis in rat liver is controversial (20). Guder et al. (21) demonstrated the dependence of HMG-CoA reductase upon the thyroid state of the animal and the stimulation of this enzyme by thyroid hormone given in vivo. Glucagon, which is an antagonist of the action of insulin and triiodothyroxine, blocked the increase in activity initiated by these hormones. Hydrocortisone prevented both hormonally-induced and diurnal increase in reductase activity (11). Epinephrine and norepinephrine stimulated reductase activity, but steroid sex hormones, like estrogens, progesterone and androgen, suppressed reductase activity (22, 23).

A diurnal rhythm in HMG-CoA reductase was first noted in mouse liver by Kandutsch and Saucier (24), and shortly after that was found in rat liver (17, 25), rat intestine (26), certain rat and mouse hepatoma (27), and in turkey liver and kidney (15). The work on the mechanism of diurnal variation of hepatic HMG-CoA reductase activity suggested that the diurnal variation in enzyme is at least due to a change in rate of synthesis (28). Rat liver microsomal HMG-CoA reductase exhibited a cyclic rhythm with a peak activity at midnight (13) but it is the opposite of HMG-CoA reductase activity in turkey

liver which showed the highest peak at noon (15). This is directly related to the feeding habits of the animal. Turkeys eat the bulk of their meal before noon.

Of the enzyme catalyzing the conversion of acetate to squalene, only one other enzyme in the cholesterol metabolic pathway, cholesterol 7-α-hydroxylase was known to have a diurnal rhythm. This enzyme catalyzes the first reaction in the catabolism of cholesterol to bile acids (10). The effect of fasting and refeeding of fat-free diets on HMG-CoA reductase showed a significant diurnal variation (29).

The developmental pattern of rat hepatic HMG-CoA reductase showed a sharp rise immediately prior to birth and exhibit an equally precipitous postnatal decline. The activity remained constant close to normal adult levels for the first 8 days, then declined and remained low prior to weaning. By the third day after weaning, reductase activity increased by three-fold and did not decline to the adult value until the second week after weaning (10). One observation on the neo-natal developmental pattern of HMG-CoA reductase from the liver of turkey poults showed maximum activity at 18 days old (15). The changes of HMG-CoA reductase in rat brain during development was first increased between 8 and 12 days after birth followed by a fall until 18 days (30).

C. Enzymic Reduction of HMG-CoA to Mevalonate

The enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (mevalonate:NADP oxidoreductase[acylating CoA] E.C. 1.1.1.34) which catalyzes the reduction of 3-hydroxy-3-methyl-glutaryl-CoA to

mevalonic acid is considered to be the rate-limiting factor in the biosynthesis of cholesterol from acetate. Guder et al. (23) fractionated microsomal suspension into smooth membrane and rough membrane by a sucrose gradient technique and suggested that the localization of this enzyme was in the rough membrane of endoplasmic reticulum. Shapiro and Rodwell upon using a discontinuous sucrose gradient confirmed Guder's results that the rough membrane contained over 95% of HMG-CoA reductase (13). By using continuous sucrose gradients Goldfarb (13) indicated that over 80% of the activity was found in the fraction of smooth membranes composed of smooth endoplasmic reticulum, golgi apparatus and plasma membrane. Mitropoulous' (32) experiments suggested that HMG-CoA reductase was in the membrane of endoplasmic reticulum, very similar to those in which cholesterol 7-α-hydroxylase is present, but with a relatively higher load of ribosome. In rat brain, HMG-CoA reductase is not exclusively microsomal (30). In yeast system, most of the activity of HMG-CoA reductase exists in the mitochondria (33, 34). Suzuki (35) presented evidence that HMG-CoA reductase was located in mitochondria and not in the microsomal or supernatant fraction in both fresh and cut sweet potato root tissues, but in diseased tissue the enzyme was locallized in microsomes. Arebalo (36) reported that there is an HMG-CoA reductase activity in cell-free extract of both leaf and callus tissue, and in chloroplasts of Nepeta cataria.

Purification and investigation of the properties of liver HMG-CoA reductase have been very difficult because the enzyme is bound to microsomal membrane. Various procedures have been tried

to solubilize this enzyme from rat liver microsomes. Siperstein used deoxycholate but did not show any purification (37). Linn (38) solubilized HMG-CoA reductase by extracting acetone powders from rat liver. According to Rudney and Kawachi (37), crude microsomal preparations treated with 0.25% sodium deoxycholate was the only procedure which provided soluble enzyme preparation. After solubilization of the enzyme followed with DEAE-cellulose, hydroxyapatite and Sephadex G-200 chromatography, HMG-CoA reductase could be purified up to 320 fold, and it showed the same properties as the enzyme in the yeast system.

Brown in 1973 (39) solubilized the enzyme from rat livers by 3 methods: 1) by using snake venom treatment, 90% of HMG-CoA reductase was released, 2) prolonged incubation with 4 M KCl, the enzyme was released during a 24 hour-period, and 3) freezing and glycerol extraction, 69% of HMG-CoA reductase was released when 50% glycerol was used in a medium. The soluble enzyme was purified up to 130 folds. Heller and Gould (40) described the solubilization technique by slow freezing and thawing. About 80% was solubilized. Higgins et al. (41) observed that the specific activity of the most pure Kawachi-Rudney preps were relatively low when compared to the preps according to Brown et al. (39) and Heller and Gould (40). Ackerman and Redd (42) modified the technique of Linn (38) by extraction with buffer and lyophilizing.

HMG-CoA reductase activity is increased by preincubation with NADPH, and this effect has been utilized for purifying HMG-CoA reductase because this form does not bind to hydroxyapatite (43). Tormanen et al. (44) developed a simple rapid purification method

by using affinity chromatography, and obtained a 975-fold purification. Schrewberry (45) reported a purification of HMG-CoA reductase using slow freeze-thawed for solubilization, a 17-fold increase in activity by using $(NH_4)_2SO_4$, a 139-fold from agarose, and a 350-fold increase from Sephadex. This enzyme was reversibly inactivated at temperatures below 19°. The behavior of this partially purified enzyme on Bio-Gel A appears to be unusual. Multiple forms of the enzyme differing in M.W. were observed. A report (46) which compared the enzyme activity from normal rats and rat treated with cholestyramine showed that cholestyramine treatment produced an increase in concentration of this enzyme. Blue Dextran/Sepharose-4B affinity chromatography showed that HMG-CoA reductase from rat livers can be purified up to 2,747-fold, and it was estimated to be 93% pure based upon spectrophotometric scans of polyacrylamide gels.

A comparison of the enzyme using liver microsomes of rat and chicken from the four methods was performed as follows: 1) freeze-thaw technique, 2) slow freeze-thaw technique, 3) detergent extraction and 4) sonification technique. The slow freeze-thaw procedure produced the higher specific activity (47).

HMG-CoA reductase is the first membrane bound enzyme reported to show reversible cold lability. Heller and Gould (48) showed that soluble HMG-CoA reductase was inactivated reversibly by cold but the microsomal enzyme did not show any cold sensitivity. From this observation he proposed that the enzyme can exist in two forms, a cold inactivated and a heat activated form. HMG-CoA reductase that was solubilized by Brown (39) was irreversibly inactivated at

4°C. In contrast, HMG-CoA reductase that was solubilized by the slow freeze-thaw methods was reversibly inactivated at 4°C. Tormanen et al. (49) concluded that rapid irreversible inactivation is caused by a factor or factors present in crude soluble extract, which is non-dialyzable and is destroyed by heat. This result suggested that the inactivation factor is a protein. Beg et al. (50) found that liver HMG-CoA reductase is diminished when cAMP is added to a microsome, and the preincubation of unwashed rat liver microsomes with MgATP reduced HMG-CoA reductase activity. The time course of inactivation of HMG-CoA reductase by MgATP was shown by Shapiro et al. (51). Inactivation of liver HMG-CoA reductase by Mg^{+2} and ATP *in vitro* is completely reversed by an activator. The activator and inactivator both occur in the liver cytosol. The inactivator is also present in the liver microsomes. Modulation of reductase activity by interconversion of active and inactive forms appear to be a feasible mechanism for the regulation of reductase *in vivo* (52).

Bell et al. (53) reported on the inhibition of HMG-CoA reductase by pure cholesterol and oxygenated cholesterol in hepatoma tissue culture cells. ML-236B which is a fungal metabolite which has been isolated from cultures of *Penicillium citrinum* (52) is a competitive inhibitor of HMG-CoA reductase (53). Brown et al. (54) reported that compactin (ML-236B) increased the cellular content of HMG-CoA reductase in extracts of human fibroblasts. From these observations, it was suggested that when the activity of HMG-CoA reductase is profoundly inhibited in intact cells, the cells responded by increasing the synthesis of the HMG-CoA reductase enzyme. HMG-CoA reductase has also been isolated and purified from *Fusarium oxysporum*

(55).

HMG-CoA reductase and HMG-CoA synthase were shown to be regulated in the adrenal gland of the rat (27); whereas, plasma cholesterol level was lowered by administration of 4-aminopyrolopyrimiaine causing the synthase and reductase to increase. The subsequent intravenous infusion of low density lipoprotein restored the plasma cholesterol level and suppressed synthase and reductase activity in parallel (56). Lin (57) reported the conditions for achieving the steady state of 3-hydroxy-3-methylglutaryl-CoA reductase in culture rat liver cells, and they also found that dexamethasone and hydrocortisome increased HMG-CoA reductase activity of a glucacon decreased the activity.

CHAPTER III

MATERIALS AND METHODS

A. Materials

1. Plants

Fresh spinach (Spinacia oleracea) was purchased from a local grocery store.

2. Animals

Turkey hens (4-16 weeks of age) were fed on tallow diet or corn oil diets (15). The turkeys were kept in a room with lights on from 6:00 a.m. to 6:00 p.m. and lights off from 6:00 p.m. to 6:00 a.m. The turkeys were sacrificed between 8:00 a.m. - 12:00 noon. The livers were removed and used immediately.

3. Radioactive Compounds

DL-3[Glutaryl-3-^{14}C]-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) and DL-5-^{3}H(U) mevalonic acid were purchased from New England Nuclear, Boston, Massachusetts. The HMG-CoA was obtained as an aqueous solution, pH 5.0 with a specific activity of 49.5 millicuries per millimole. The 5-^{3}H mevalonate was obtained as the dibenzoyl-ethylenediamine salt with a specific activity of 5.00 curies per millimole.

4. Enzymes, Substrates and Cofactors

Glucose-6-phosphate dehydrogenase was purchased from Sigma Chemical Company, St. Louis, Missouri. Glucose-6-phosphate as the disodium salt, $NADP^+$ as the sodium salt and NADPH as tetrasodium salt were purchased from Sigma Chemical Company, St. Louis, Missouri.

5. Chemicals and Reagents

Solvents and reagents were of analytical grade or of the highest quality available unless otherwise specified. Insta-gel was purchased from Packard Instrument Company Inc., Downers Grove, Illinois. Whatman number one phase separation paper from Curtin Matheson Scientific Inc. Affi-Gel Blue (100-200 mesh) was purchased from Bio-Rad Laboratories, Richmond, California.

B. Methods

1. Preparation of Microsomes

The turkeys were sacrificed and the livers were removed, placed on ice, and used immediately. The whole turkey liver was weighed, minced with scissors, and then homogenized in a loose fitting Potter-Elvehjem glass tissue grinder with a motor driven, groved teflon pestle in buffer A (2 ml/gram of tissue) containing 0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4, 0.03 M EDTA and 1 mM dithiothreitol at pH 7.2, according to procedure described by Heller and Gould (40). The homogenate was centrifuged twice at 10,000 x g for 15 min, and the pellet was discarded. The supernatant was centrifuged for one hour at 100,000 x g and the pellet was resuspended in one-third of the original amount of

buffer that was used. This suspension was centrifuged again at 100,000 x g for one hour. The pellet was removed and assayed for enzyme activity (Figure 2). The resulting microsomal pellet was transferred to a pyrex tube and frozen in a dry ice-ethanol bath and stored at -20°C.

2. Chloroplast Isolation

Chloroplasts were isolated by the method of R. G. Jensen and J. A. Bassham (58).

3. Assays

HMG-CoA reductase activity was measured by two radioactive assays. The first was the adaption of the method by Huber et al. (59). The reaction contained 10 mM glucose-6-phosphate, 1 mM $NADP^+$, glucose-6-phosphate dehydrogenase (2 units), 1 mM EDTA, 1 mM dithiothreitol, 0.1 M potassium phosphate buffer, pH 7.4, 100 microgram of protein and 3-^{14}C HMG-CoA ($\cong$ 8.0 x 10^4 dpm). The reaction was performed in a total volume of 1.0 ml. The reaction mixture was incubated at 37°C for one hour. 5-^{3}H mevalonic acid ($\cong$ 23-25 x 10^5 dpm/100 µl) was added as an internal standard. The reaction was stopped by quick freezing in a dry ice-ethanol bath and 200 µl of 2N H_2SO_4 was added. The reaction mixture was allowed to stand at room temperature for 10 minutes in order for lactonization of mevalonate to occur. Anhydrous sodium sulfate (6 gm) was mixed with the reaction mixture. After standing for one hour at room temperature, the samples were extracted four times with diethyl ether (1 x 9 ml, 3 x 5 ml).

The combined extracts were evaporated to dryness in a hot water-

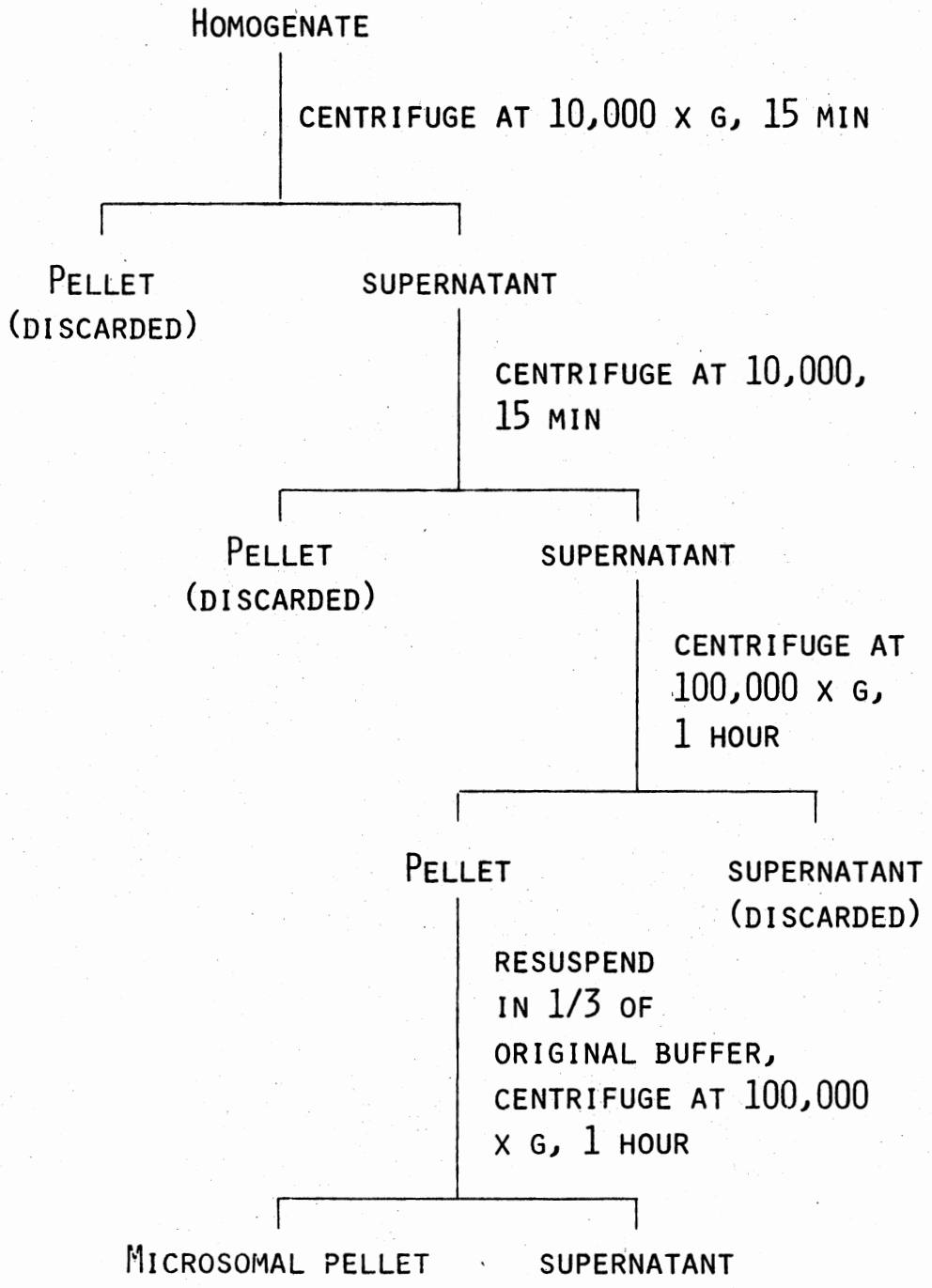


Figure 2. Microsomal Preparation from Turkey Liver

bath. One residue was dissolved in 1.0 ml of water, placed on a Dowex-1 formate column (0.5 x 5 cm) and eluted with 5.0 ml of water. The eluate, which contained the mevalonolactone was collected in a scintillation vial. Only 2.0 ml of the eluate was added to 5.0 ml of Insta-gel. The sample was counted in a Packard PL scintillation spectrometer.

A second assay was based on a method by Ackerman et al. (60) with some modifications. The reaction mixture contained 2 mM NADPH, 20-30 µg of Protein, and buffer A which contained 0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4, 0.03 M EDTA and 1 mM dithiothreitol. The samples were preincubated at 37°C for 30 min. 30 µl of DL-[3-^{14}C]HMG-CoA (≅ 7.9-8.9 x 10^4 dpm/10 µl) was added to each sample, and incubated for 20 min at 37°C. The total incubation volume was 200 µl.

The enzyme reaction was stopped by the addition of 1 ml of 2 N HCl, followed by the addition of 100 µl of 5-^{3}H-MVA (2.3-2.5 x 10^5 dpm). The ^{3}H-MVA served as an internal reference. The samples were allowed to stand overnight at 4°C for lactonization. Each sample was then transferred to a glass scintillation vial, and the small incubation test tube was rinsed with 2 ml of buffer a followed by the addition of 2.3 gm of an equal molar mixture of solid K_2HPO_4-KH_2PO_4. The samples were incubated at 37°C for 30 min.

Chloroform (6 ml) was added, followed by vigorous shaking of the mixture by hand 2-3 times for 10-15 sec. The pressure was then released and the mixture was poured onto a fluted Whatman 1 PS phase separation filter. The chloroform extract was filtered through the phase separating filter and was collected in a scintillation counting vial. The aqueous material that was retained on the filter, was then

transferred with a pasteur pipette back into the original extraction vial and a 6 ml quantity of chloroform was added, followed by shaking and filtering as described above. The second chloroform filtrate was combined with the first chloroform filtrate. The aqueous phase was discarded.

A saturated solution (1 ml) of equal molar KH_2PO_4-K_2HPO_4 was added to the vial and was shaken vigorously, then the contents were poured onto a fresh 1 PS phase separating fluted filter. The chloroform filtrate was collected in a scintillation counting vial.

The chloroform filtrate was evaporated to dryness under a stream of nitrogen. Water (3 ml) was added, and 2 ml of filtrate was added to 4 ml of Insta-gel. The scintillation vial was counted in a Packard PL scintillation spectrometer. The blank need to be subtracted from the sample.

4. Protein

Protein concentration was determined by a modification of the Lowry procedure as described by Hartree (61) using bovine serum albumin as a standard.

5. Solubilization Techniques

Slow freezing and thawing. The freshly prepared microsomal pellet was frozen in a dry ice-ethanol bath by reducing temperature at the rate of 5-10°C per min down 20-30°C, and then was frozen and left overnight at -20°C. Soluble enzyme was prepared the following day.

One pellet was then thawed, suspended in buffer, and centrifuged at 100,000 x g for one hour and the supernatant was removed for assay.

This supernatant is a crude soluble extract (41).

Solubilization by Salt. The freshly prepared microsomal pellet was incubated at room temperature in buffer A containing 4 M KCl for 24 hours, and then centrifuged at 100,000 x g for 60 min. The supernatant was removed for assay (40).

6. Partial Purification of HMG-CoA Reductase

Preparation I: The crude soluble extract by the solubilization technique of Heller and Gould (40) was brought to 30% saturation (170 g/l) with ammonium sulfate and centrifuged at 20,000 x g for 10 minutes. The 30% ammonium sulfate supernatant was brought to 75% saturation by addition of (310 g/l) of ammonium sulfate to the 30% saturated solution and centrifuged at 20,000 x g for 10 minutes.

The 75% pellet was resuspended in buffer, incubated at 37°C with 2 mM NADPH for 30 minutes, followed by heating at 60°C for 10 minutes, and then immediately cooled with ice. The heat-treated solution was centrifuged at 27,000 x g for 15 minutes at room temperature. The supernatant was removed and assayed for HMG-CoA reductase activity (Figure 3).

The 100,000 x g pellet (Figure 4) was solubilized by slow freezing method as described by Heller and Gould (40). NADPH was added to this crude soluble extract to give a concentration of 2 mM. This soluble extract with NADPH was incubated at 37°C for 30 min, followed by heating at 65°C for 8 min, and then cooled immediately in an ice-bath. The heat extract was centrifuged at 27,000 x g for 15 min at room temperature.

The heat-treated supernatant (≅ 3 mg) was applied to an Affi-Gel

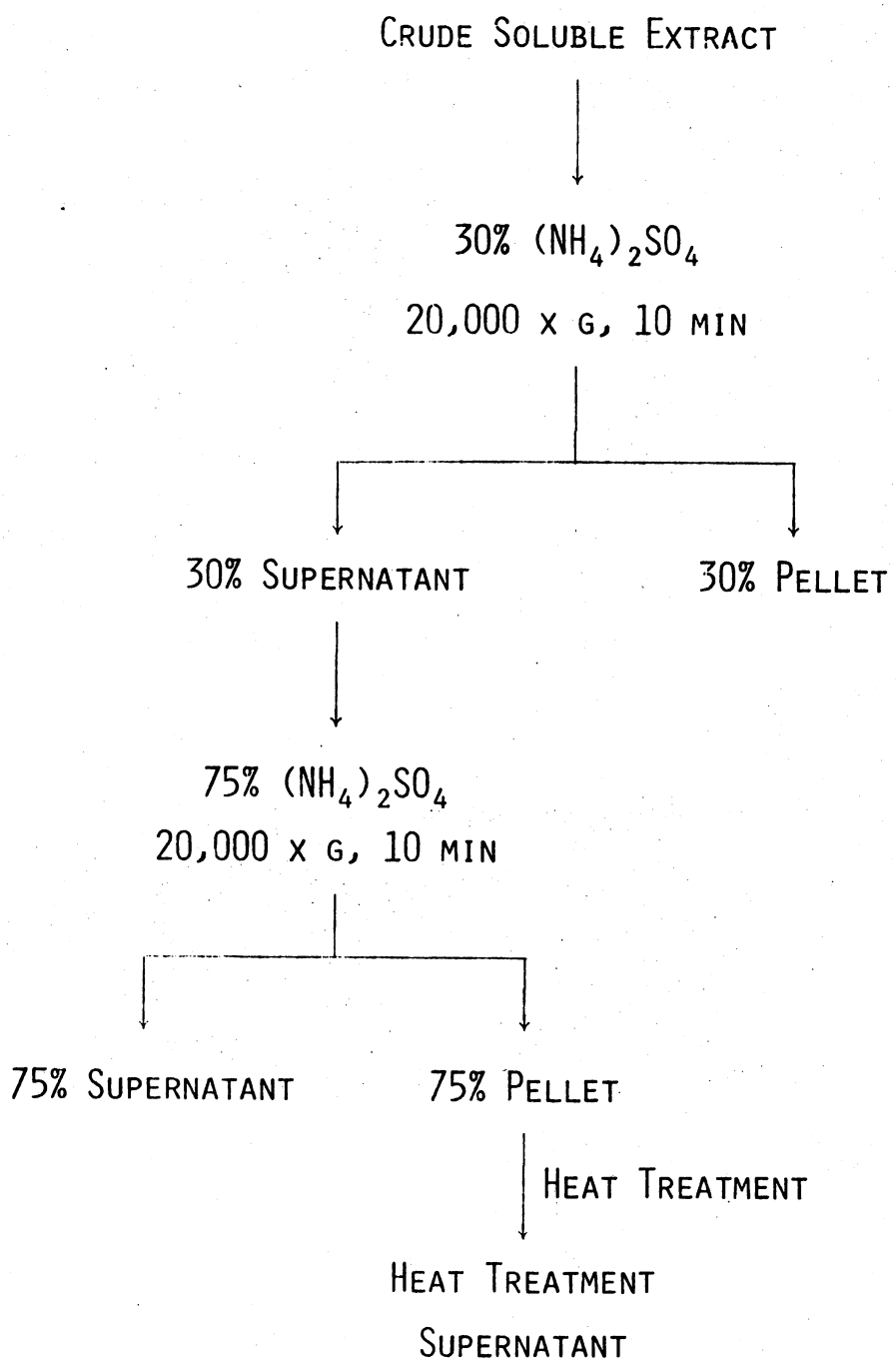


Figure 3. Partial Purification by Ammonium Sulfate Fractionation

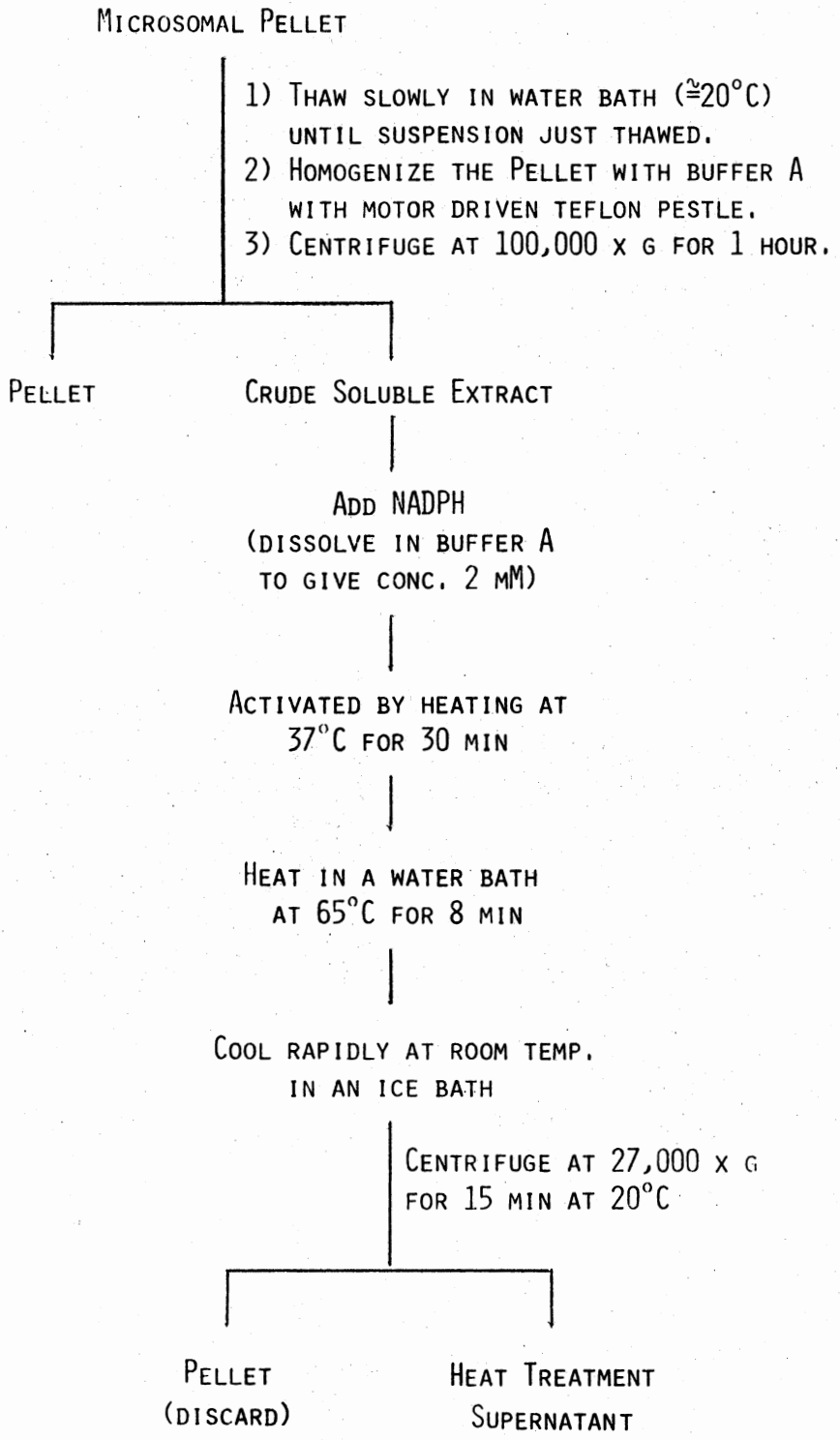


Figure 4. Heat Treatment

Blue affinity column (0.5 x 5 cm) which has been equilibrated and washed with buffer A overnight. The Affi-Gel Blue column was eluted with 4.5 ml of buffer A, 3 ml of buffer A + 0.2 M KCl and finally with 40 ml of buffer A + 0.5 M KCl. A total of ten fractions of 4 ml were collected. Fraction 3 to 8 were pooled and concentrated using a pressure dialysis Amicon Diaflo membrane which had been washed with buffer A containing 2 mM NADPH. HMG-CoA reductase activity was determined (Figure 5).

Preparation II. The same as Figure 4 and 5 but the crude soluble extract was solubilized by incubation with 4 M KCl over 24 hours and centrifuged for 100,000 x g for one hour. This is classified as the crude soluble extract.

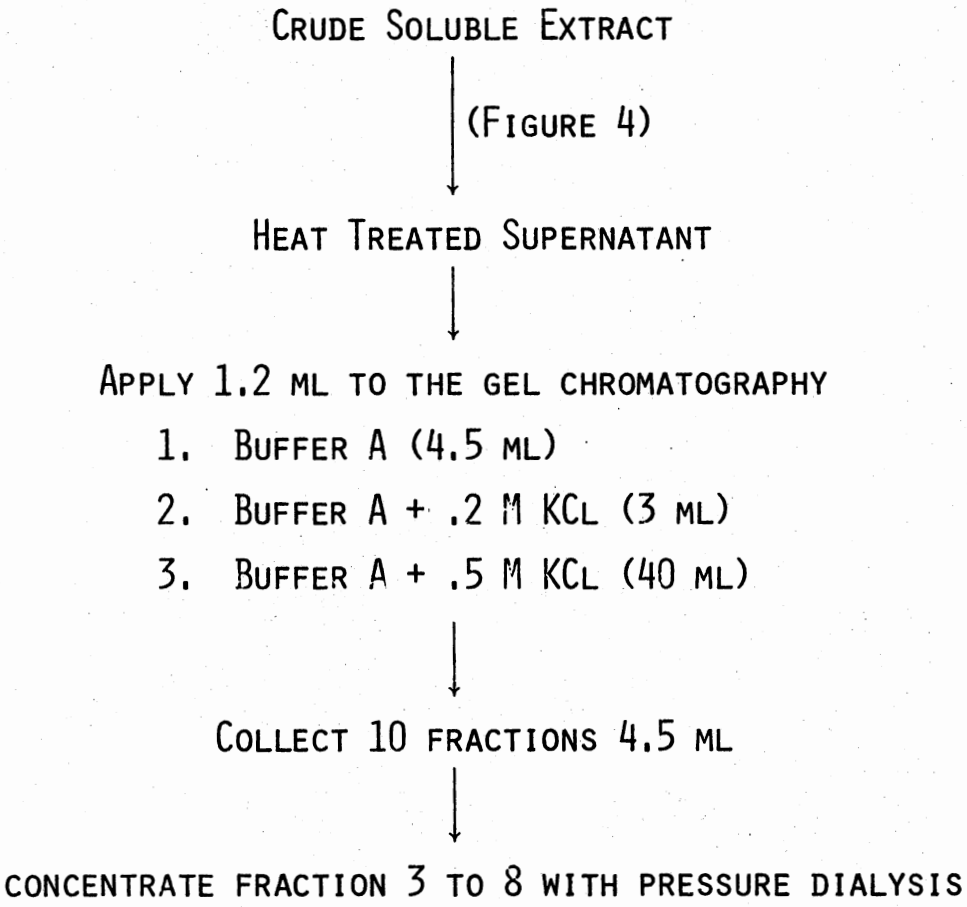


Figure 5. Partial Purification of HMG-CoA Reductase from Turkey Liver

CHAPTER IV

RESULTS AND DISCUSSION

A. Animals

When the procedure as described in Figure 2 was used to obtain a microsomal preparation, HMG-CoA reductase appeared to be loosely bound to the microsomal membrane (Table I). Washed microsomes have less activity than the supernatant. However, HMG-CoA reductase activity is never completely removed from the microsomes.

TABLE I

MICROSOMAL PREPARATION

	protein mg/ml	specific activity nmoles MVA/mg/hr
Washed Microsomal Pellet	8.20	3.55
2nd 100,000 x g supernatant (based upon the first assay)	4.20	5.57

1. Preparation of Soluble HMG-CoA Reductase

All the published techniques that have been used to solubilize HMG-CoA reductase were performed on the microsomal pellet. One of these techniques, which is very simple for effective solubilization of membrane-bound HMG-CoA reductase, is the slow freeze-thaw method as described by Heller and Gould (40). This technique avoids using detergents which inhibit enzyme activity and have to be removed before activity can be measured. The enzyme HMG-CoA reductase from liver microsomes showed a decrease in activity after storing it more than one week at 4°C. Even though the soluble form as prepared by the freeze-thaw method showed an increase in activity when compared to the microsomal suspension, it is not very stable on storage. Whereas the soluble enzyme was completely destroyed when stored overnight at 4°C, but microsomal pellets can be stored overnight without losing activity. Over 80% of HMG-CoA reductase activity was solubilized from liver microsomes by this method.

HMG-CoA reductase can be solubilized more than 90% by incubation with buffer A + 4 M KCl for 24 hours at room temperature as described by Brown et al. (39). 4 M KCl stabilized the enzyme over 24 hours (Table II).

2. Partial Purification of HMG-CoA Reductase

Preparation I: Purification based on solubilization by the slow freeze-thaw method was the first step. The crude soluble extract was fractionated by ammonium sulfate (Figure 3). Ammonium sulfate seemed to do nothing for the purification of HMG-CoA when compared to the

TABLE II

SOLUBILIZATION OF TURKEY LIVER HMG-CoA REDUCTASE

	slow freeze-thaw method		Incubation with buffer A + 4 M KCl	
	specific activity[a] by first assay	specific activity[a] by second assay	specific activity[a] by first assay	specific activity[a] by second assay
Microsomal Pellet	0.30	-	3.22	6.24
Microsomal Suspension	0.34	11.19	4.20	6.69
Crude Soluble Extract	0.38	35.76	13.77	24.30
Pellet	0.06	5.37	1.08	.51
Degree of Solubilization (%)	86.76	86.94	92.73	97.94

[a]Specific Activity Units are given in nanomoles of mevalonate formed per mg of protein per hour at 37°C.

rat liver enzyme (40) which could be purified up to 9 fold. Thus efforts to purify HMG-CoA reductase from turkey liver with ammonium sulfate were unsuccessful (Table III).

As seen in Figure 4, NADPH was added at 37°C to the crude soluble extract in order to activate turkey liver HMG-CoA reductase and stabilize it during a heat step, which did increase the activity (Table IV). Figure 5 shows the elution profile from an affinity column of Affigel-Blue. The ionic strength was increased by using buffer A, followed by buffer A + .2 M KCl and finally with buffer A + .5 M KCl. HMG-CoA reductase activity was in fraction 3 to 10. Partially purified HMG-CoA reductase as reported in Table IV, had a specific activity of 186 nmoles MVA forned mg^{-1} hr^{-1}, an increase in specific activity of only 17 fold.

Preparation II: Purification was based on solubilization by incubation with buffer A + 4 M KCl. NADPH was added at 37°C to the crude soluble extract in order to activate and stabilize HMG-CoA reductase. HMG-CoA reductase retains 90% of its activity when heated in the presence of NADPH for 8 min at temperature up to 65°C and more than 50% of the protein contaminants are denatured and precipitated. HMG-CoA reductase had a specific activity of 2.9 nmoles MVA formed/mg of protein/hr after the heat-treated sample was applied to the affinity column and concentrated in fractions 3 to 8. It is likely that the high salt concentration tended to alter the enzyme by changing the structural conformation so that the enzyme was no longer able to bind to the column. Therefore, the results from Table V showed no purification after applying to the Affi-Gel Blue column.

TABLE III

PARTIAL PURIFICATION OF HMG-CoA REDUCTASE FROM TURKEY LIVER BY AMMONIUM SULFATE

	Volume (ml)	Enzyme Activity (units/ml)	Total Activity (units[a])	Protein mg/ml	Specific Activity[b]	Yield (%)
Freeze-thaw supernatant	25	7.02	176	2.70	2.60	100
Soluble extract	22	5.54	122	1.73	3.20	69
0-30% Pellet	20	4.82	96	1.65	2.92	55
0-30% Suspension	3	4.74	14	1.60	2.96	8
30-75% Pellet	5	4.62	23	1.60	2.89	13
30-75% Suspension	18	.03	<1	.28	.11	<1
Heat treated supernatant	4	3.09	12	1.36	2.27	7

This data was based upon the first assay.

[a]One unit of enzyme activity is defined as the formation of one nanomole of mevalonic acid per hour at 37°C

[b]Specific activity units are given in nanomoles of mevalonate formed per mg of protein per hour at 37°C

TABLE IV

PARTIAL PURIFICATION OF HMG-CoA REDUCTASE FROM TURKEY LIVER USING FREEZE-THAW FOR SOLUBILIZATION

	Volume (ml)	Enzyme Activity (units/ml)	Total Activity (units[a])	Protein (mg/ml)	Specific Activity[b]	Yield (%)	Purification (fold)
Freeze-thaw suspension	25	251	6266	22.40	11	100.00	1
Crude soluble extract	18	89	1609	2.50	36	25.68	3
Pellet	3	112	335	20.80	5	5.30	<1
Heat-treated supernatant	18	114	2048	2.40	47	32.68	4
Affi-Gel Blue concentration	3	24	73	.13	186	1.16	17

This data was based upon the second assay.

[a]One unit of enzyme activity is defined as the formation of one nanomole of mevalonic acid per hour at 37°C

[b]Specific Activity Units are given in nanomoles of mevalonate formed per mg of protein per hour at 37°C

TABLE V

PARTIAL PURIFICATION OF HMG-CoA REDUCTASE FROM TURKEY LIVER USING 4 M KCl FOR SOLUBILIZATION

	Volume (ml)	Enzyme Activity (units/ml)	Total Activity (units[a])	Protein (mg/ml)	Specific Activity[b]	Purification (fold)	Yield (%)
4 M KCl Suspension	10	16.48	164	8.00	2.06	1.00	100.00
Crude soluble extract	8	19.00	152	1.90	10.00	4.85	92.23
Heat treated supernatant	3.30	7.86	25.94	.85	0.25	4.49	15.74
Affi-Gel Blue concentrate fraction 3 to 8	2	.29	.58	.10	2.90	1.40	<1

This data was based upon the second assay.

[a]One unit of enzyme activity is defined as the formation of one nanomole of mevalonic acid per hour at 39°C

[b]Specific Activity Units are given in nanomoles of mevalonate formed per mg of protein per hour at 37°C

B. Plants

1. Chloroplast Isolation

Whole chloroplasts were isolated by a method of Bassham and Jensen (58). Spinach leaves were washed, chilled and the midribs were removed. Leaf tissue was blended with a Waring blender (3 times for 5 seconds each) in solution A, which contained basic stock solution (.33 M Sorbitol, 2 mM $NaNO_3$, 2 mM EDTA, 2 mM sodium ascorbate, 1 mM $MnCl_2$, 1 mM $MgCl_2$, .6 H_2O and .5 mM K_2HPO_4), .50 mM MES (pH 6.1) and 0.2 M NaCl, and then passed through six layers of cheesecloth. The leaf homogenates were centrifuged at 2000 x g for 50 seconds. Whole chloroplasts were identified by light microscopy. The 2000 x g pellet was resuspended in 1 ml of solution B which contained the basic stock solution, .05 M HEPES (pH 6.7) and 0.2 M NaCl, and was homogenized with a pyrex glass tissue homogenizer, then assayed for HMG-CoA reductase activity. The specific activity of the homogenized chloroplasts was 0.16 ± 0.06 nmoles MVA formed/mg of protein/hr. The activity of the enzyme depended upon the freshness of leaves, which varied because they were purchased from the grocery store.

C. Comparison of the Two Assay Systems

β-Hydroxy-β-methyl glutaryl-CoA reductase (HMG-CoA reductase) catalyzed the reduction of NADPH of HMG-CoA to mevalonate. The first assay (60) involved a coupled assay which used glucose-6-phosphate dehydrogenase to convert glucose-6-phosphate and $NADP^+$ to gluconolactone-6-phosphate and NADPH, respectively. The second assay used NADPH directly. A high concentration of protein (up to 100 μg) was used,

but only ≅ 80,000 dpm of 3-^{14}C-HMG-CoA was used in the first assay. However, the second assay used a high concentration of substrate and also a smaller amount of enzyme protein (20-30 µg/assay).

The first assay was incubated for 15 minutes, initiated by the addition of substrate and finally terminated after one hour. This first assay used anhydrous Na_2SO_4 solidified when added to the reaction mixture. Dowex-1 formate was used for the separation of mevalonolactone from the substrate.

The second assay was preincubated for 30 minutes and assayed for 20 minutes. This second assay used an equal molar mixture of K_2HPO_4-KH_2PO_4 and chloroform for extraction. The mixture was poured onto a fluted Whatman 1 PS phase separatory filter. This paper separated HMG which was dissolved in aqueous buffer and mevalonolactone which dissolved in chloroform. The specific activity determined by the second assay was higher than the first method when the same tissue protein was used (Table VI). The difference between the two assays was the extraction and separation procedure. The second assay extracted more mevelonolactone than the first assay. Perhaps the solidified sodium sulfate prevented a good extraction of mevalonolactone. The first assay which used Dowex-1-formate to separate the substrate, HMG-CoA, from mevalonolactone was more effective than the second assay which used a Whatman 1 PS phase separator. The blank of the second assay was found to have high activity whereas the blank of the first assay showed insignificant activity. This suggests that the phase separation technique which is faster is not more efficient and it should only be used as a method for quick assays of many samples.

TABLE VI

COMPARISON OF THE TWO ASSAY SYSTEMS

	Specific Activity[a] by the First Assay	Specific Activity[a] by the Second Assay
Freeze-thaw Supernatant	2.55	28.80
Soluble extract	9.05	75.00
Heat treated supernatant	10.59	78.30

[a]Specific Activity Units are given in nanomoles of mevalonate formed per mg of protein per hour at 37°C

CHAPTER V

SUMMARY

3-Hydroxy-3-methylglutaryl coenzyme A reductase, the major regulatory enzyme in hepatic cholesterol biosynthesis, has been solubilized and partially purified from turkey liver microsome. The HMG-CoA reductase can be removed from turkey liver microsomes by exposing them to high salt concentrations, and a freeze-thaw treatment. No detergents are required. Exposing the microsomes to a freeze-thaw treatment solubilized more than 80% of the enzyme and the high salt concentration solubilized more than 90% of the microsomal HMG-CoA reductase activity. Subsequently, a 17 fold purification has led to an enzyme preparation with a specific activity of 186 nmoles MVA formed per mg of protein per hour by using a freeze-thaw technique, heat treatment and affinity gel chromatography.

The enzyme is also present in isolated spinach chloroplasts demonstrated by these experiments with spinach chloroplasts. Since there was no fractionation of spinach chloroplasts protein, it is difficult to conclude where HMG-CoA reductase is located. At best it could be suggested that HMG-CoA reductase might be somewhat bound to chloroplast thylakoid membranes.

A SELECTED BIBLIOGRAPHY

(1) Schoenheimer, R., and Breusch, F. (1933) J. Biol. Chem., 103, 439-448.

(2) Rittenberg, D., and Schoenheimer, R. (1937) J. Biol. Chem., 121, 235-253.

(3) Siperstein, M. D. (1970) Curr. Top. Cell Regul., 2, 65-100.

(4) Bucher, N. L. R., and McGarrahan, K. (1956) J. Biol. Chem., 222, 1-15.

(5) Knauss, H. J., Porter, J. W., and Wasson, G. (1959) J. Biol. Chem., 234, 2835-2840.

(6) Dietschy, J. M., and Wilson, J. D. (1970) N. Eng. J. Med., 282, 1128-1138.

(7) Bucher, N. L. R., Overath, P., and Lynen, F. (1960) Biochim. Biophys. Acta, 40, 491-501.

(8) Dun, I. F., and Rudney, H. (1960) J. Biol. Chem., 235, 2572-2578.

(9) Steward, R., and Rudney, H. (1966) J. Biol. Chem., 241, 1222-1225.

(10) Rodwell, V. W., McNamara, D. J., and Shapiro, D. J. (1973) Adv. Enzymol., 38, 373-412.

(11) Siperstein, M. D., and Fagen, V. M. (1966) J. Biol. Chem., 241, 602-609.

(12) Linn, T. C. (1967) J. Biol. Chem., 242, 990-993.

(13) Shapiro, D. S., and Rodwell, V. W. (1971) J. Biol. Chem., 246, 3210-3216.

(14) Dietschy, J. M., and Siperstein, M. D. (1967) J. Lipid Res., 8, 97-114.

(15) Hunter, C. F., and Mitchell, E. D. (1978) Fed. Proc., 37, 994.

(16) Ide, T., Akamatsu, H., and Sugano, M. (1978) J. Nutr., 108, 601-612.

(17) Back, P., Hamprecht, B., and Lynen, F. (1969) Arch. Biochem. Biophys., 133, 11-19.

(18) Weis, H. J., and Dietschy, J. M. (1969) J. Clin. Invest., 48, 2398-2408.

(19) Dugan, R. E., Ness, G. C., Lakshmanan, M. R., Nepokroeff, C. M., and Porter, J. W. (1973) Abstr. Am. Chem. Soc., 46.

(20) Oswald, S., and Wiss, V. (1977) Helvetica Chimica Acta, 60, 1967-1968.

(21) Guder, W., Nolte, I., and Wieland, O. (1968) Eur. J. Biochem., 4, 273-278.

(22) Edwards, P. A. (1973) J. Biol. Chem., 248, 2912-2922.

(23) Brown, M. S., and Goldstein, J. L. (1974) J. Biol. Chem., 249, 7306-7316.

(24) Kandutsch, A. A., and Saucier, S. E. (1969) J. Biol. Chem., 244, 2299-2306.

(25) Shapiro, D. S., and Rodwell, V. W. (1969) Biochem. Biophys. Res. Commun., 37, 867-877.

(26) Shefer, S., Hauser, S., Laper, V., and Mosbach, E. H. (1972) J. Lipid Res., 13, 401-412.

(27) Goldfarb, S., and Pitot, H. C. (1971) Cancer Res., 31, 1874-1884.

(28) Higgins, M., Kawachi, T., and Rudney, H. (1971) Biochem. Biophys. Res. Commun., 45, 138-144.

(29) Slakey, L. L., Craig, M. C., Beytia, E., Reiedis, A., Feldbruegge, D. H., Dugan, R. E., Qurishi, A. A., Subbarayan, C., and Porter, J. W. (1972) J. Biol. Chem., 247, 3014-3022.

(30) Aragon, M. C., and Gimenez, C., Valdiveiso, F., and Mayor, F. (1978) J. Neurochem., 30, 649-650.

(31) Goldfarb, S. (1972) FEBS Lett., 24, 153-155.

(32) Metropoulos, K. A., Venkatesan, S., Balasubramanram, S., and Peters, T. J. (1978) Eur. J. Biochem., 82, 419-429.

(33) Shirninzn, I., Nagai, J., Hatanaka, N., and Katsaki, H. (1973) Biochim. Biophys. Acta, 296, 310-320.

(34) Qureshi, N., Dugan, R. E., Nimmannik, S., Wu, W., and Porter, J. W. (1976) Biochem., 15, 4185-4190.

(35) Suzuki, H., and Urikani, I. (1976) Plant & Cell Physiol., 17, 691-700.

(36) Arebalo, R. E. "The Occurrence and Some Properties of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase and Mevalonate Kinase in Nepeta cataria Leaf Tissue." (Unpub. Ph.D. Thesis, Oklahoma State University, 1977.)

(37) Kawachi, T., and Rudney, H. (1970) Biochem., 9, 1700-1705.

(38) Linn, T. C. (1967) J. Biol. Chem., 242, 984-989.

(39) Brown, M. S., Dana, S. E., Dietschy, J. M., and Siperstein, M. D. (1973) J. Biol. Chem., 248, 4731-4738.

(40) Heller, R. A., and Gould, R. G. (1973) Biochem. Biophys. Res. Commun., 50, 859-863.

(41) Higgin, M., Brady, D., and Rudney, H. (1974) Arch. Biochem. Biophys., 163, 271-282.

(42) Ackerman, M. E., Redd, W. L., and Sallen, T. J. (1974) Biochem. Biophys. Res. Commun., 56, 29-35.

(43) Tormanen, C. D., Ackerman, M. E., Redd, W. L., and Scallen, T. J. (1975) Fed. Proc., 34, 560.

(44) Tormanen, C. D., Redd, W. L., Srikantaiah, M. V., and Scallen, T. J. (1976) Biochem. Biophys. Res. Commun., 68, 754-762.

(45) Heller, R. A., and Shrewbury, M. A. (1976) J. Biol. Chem., 251, 3815-3822.

(46) Srikantaiah, V. M., Tormanen, B. C., Redd, L. W., Hargrave, E. J., and Scallen, J. T. (1977) J. Biol. Chem. 252, 6145-6150.

(47) Beg, Z. H., Stanik, J. A., and Brewer, H. B. (1978) Anal. Biochemistry, 86, 531-535.

(48) Heller, R. A., and Gould, R. G. (1974) J. Biol. Chem., 249, 5254-5259.

(49) Tormanen, C. D., Srikantaiah, M. V., and Hardgrave, J. E. (1977) J. Biol. Chem., 252, 1561-1565.

(50) Beg, Z. H., Allmann, D. W., and Gibson, D. M. (1973) Biochem. Biophys. Res. Commun., 54, 1362-1369.

(51) Shapiro, D. J., Nordstrom, J. L., Mitschelen, J. J., Rodwell, V. W., and Schrimke, R. J. (1974) Biochim. Biophys. Acta, 370, 369-377.

(52) Spence, J. T., and Gaylor, J. L. (1977) J. Biol. Chem., 252, 5852-5858.

(53) Bell, J. J., Sargeant, T. E., and Watson, J. A. (1976) J. Biol. Chem., 251, 1745-1758.

(54) Brown, M. S., Faust, J. R., and Goldstein, J. L. (1978) J. Biol. Chem., 253, 1121-1128.

(55) Madhosingh, C., and Orr, W. (1977) Agric. Biol. Chem., 41(7), 1519-1521.

(56) Balasubramarriam, S., Goldstein, J. L., and Brown, M. S. :1977; Proc. Natl. Sci., 74, 1421-1425.

(57) Lin, R. C., and Snodgrass, P. J. (1977) FEBS Lett., 83, No. 1, 89-92.

(58) Jensen, R. G., and Bassham, J. A. (1960) Proc. Natl. Acad. Sci., 56, 1095-1101.

(59) Huber, J., Latzin, S., and Hamprecht, B. (1973) Hoppe-Seyler's Physiol. Chem., 354, 1645-1647.

(60) Ackerman, M. E., Redd, W. L., Tormanen, C. D., Hardgrave, J. E., and Scallen, T. J. (1977) J. Lipid Res., 18, 408-413.

(61) Hartree, E. F. (1972) Anal. Biochem., 48, 422-427.

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