PURIFICATION AND CHARACTERIZATION OF FATTY ACID SYNTHASES FROM THE PEA APHID

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CHAPTER I

INTRODUCTION

Aphids are a major agricultural pest, causing great economic loss and requiring large annual expenditures for pesticides in attempts to control them. Aphids are found on essentially every plant species occurring in the temperate zones. More than one aphid species attacks most crop plants, and some aphid species attack an incredibly broad range of plants. *Aphis gossypii* Glover has been reported to have 220 host species in 46 families (Roy and Behura 1983).

The level of economic impact due to aphids ranges widely from crop to crop, and from year to year. The damage can range from none to total loss of the crop. The level of impact a single aphid species can have is shown by the Russian wheat aphid (RWA). Untreated fields can have yield losses from 35% to 60%, and in some cases the wheat is of too poor a quality to repay harvesting costs. The RWA was first reported from the United States near Muleshoe, Bailey County, Texas in March 1986 (Stoetzel, 1987). By the end of 1986, RWA had been collected in Colorado, Kansas, Nebraska, New Mexico, Oklahoma, Texas, and Wyoming on wheat, barley, oats, rye, triticale, and several grasses (Stoetzel, 1987). For the western United States, the annual economic impact in control costs and yield loss averaged $92 million for the years 1986 to 1989 (Anon., 1990). In the United States, continuing costs and losses total 5% of the gross value of the annual crop (Hughes and Maywald 1990). In a comprehensive review of insect damage to crops in Georgia from 1971 to 1979, the average economic impact due to all aphid species from crop damage and costs of control was 1.27% of the value of the crop, with a range of 0 to 23% (Suber and Todd, 1980; Todd and Suber 1980; Suber et al. 1981a; Suber et al. 1981b).

Understanding the basic metabolic processes of aphids may lead to biorational methods of control, reducing their economic impact and the ecological impact of control measures. Aphids are typically quite small insects, usually under 2 mm long, although some species may reach 8 mm. Most are parthenogenetic with telescoped generations. This means that the developing nymphs already have nymphs developing inside them before their birth, resulting in a very rapid potential rate of increase. This also means that a corresponding high nutrient flux must be taken in, destined for biomass increase of the nymphs with little utilized by the adults. Walters (1992) reported that 35% of radiolabeled acetate injected into Potato aphids was found in the nymphs born in the next 24 hours and, he deduced, the majority of the rest of the label was actually in the unborn embryos remaining in the parent. The aphid's habit of feeding from phloem provides a diet with essentially no fatty acids, and a diet very poor in amino acids relative to the sugar content. Should migration become necessary due to seasonal or host plant changes, the aphid may be required to reduce the rate of nymph production to free up nutrients so that lipids can be synthesized and stored for flight fuel.

The fatty acid composition of insects varies significantly among orders (Thompson 1973). Thompson's review of the reported fatty acids from insects of seven orders found that the orders could be grouped by their percentages of the fatty acids between 14 and 18 carbons long, as well as the ratios of saturated to unsaturated fatty acids. Several factors can change the fatty acid profile of an insect, such as rearing temperature (House et al. 1958), developmental stage (Barlow 1965; Strong 1963), diapause or reproductive activity (Lambremont et al. 1964), flight (Thompson and Bennett 1971), and diet (Barlow 1966; Bergman et al. 1991; Thompson and Barlow 1972). Although the lipid profiles do change, Thompson (1973) concluded that the individual changes were smaller than the differences between orders.

Some aspects of aphid lipid metabolism are unique among the insects, and a few are unique among the animals. Many Aphididae are characterized biochemically by a large proportion of myristic acid (14:0) in their lipids, primarily in the neutral lipids (Bergman et al. 1991; Bowie and Cameron, 1965; Callow et al. 1972; de Renobales, 1984; Fast, 1970; Greenway et al. 1974; Ryan, 1982; Strong, 1963b; Strong, 1967; Thompson, 1973), along with smaller amounts of hexanoic acid (6:0) (Callow et al. 1972; Greenway et al. 1974), and sorbic acid (6:2) (Addae-Mensah and Cameron 1978; Bowie and Cameron 1965; Callow et al. 1972; Fallon and Shimizu 1977; Greenway et al. 1974; Shimizu 1971). The fatty acid aphids synthesize for storage is 14:0, which is not a major membrane lipid (Ryan et al. 1982). This fatty acid is usually a trace constituent of animal tissue. Additionally the aphids have an extremely unusual triglyceride class, found nowhere else, containing sorbic acid (6:2), or hexanoic acid (6:0) in the 2 position. At present the routes of synthesis of 6:2 and 6:0 are uncertain beyond their origin as acetate (Walters, 1992).

Fatty acid synthase [FAS; acyl-CoA:malonyl-CoA C-acyltransferase (decarboxylating, oxoacyl- and enoyl-reducing and thioester hydrolysing), EC 2.3.1.85] is the enzyme responsible for generating fatty acyl groups in the cytoplasm. FAS is very abundant, constituting approximately 0.1% of the wet weight of tissues actively engaged in fatty acid synthesis (Ahmad et al. 1982; Linn 1981). Mattick et al. (1983) characterized it as the most complex of the multifunctional enzymes, possessing 7 enzymatic activities and an acyl carrier prosthetic group.

About 70% of pea aphid (*Acyrthosiphon pisum* (Harris)) fatty acid is myristic acid. The phospholipids are over 80% 18-carbon saturated and unsaturated fatty acids, while the triglycerides are almost 90% myristate (Ryan et al. 1982; Dillwith et al. 1993). Purified FAS produces palmitate and stearate exclusively (Ryan et al. 1982; Aprahamian

et al. 1982). The production of 14:0 is due to the action of thioesterase II (TE II) on FAS (Ryan et al. 1982).

Diet alters the percent composition of 14:0 in aphids (Bergman et al. 1991; Dillwith et al. 1993). The increased production of 14:0 requires either an enzyme activation, the enhanced transcription of its gene, or a decreased use of 14:0 for elongation, metabolism, or export in nymphs. With the large amount of myristic acid present in the pea aphid and its variation with diet, TE II plays a large role in the lipid metabolism of the insect. Given a fixed rate of nutrient intake, routing lipid production by FAS to 14:0 may entail a concomitant reduction in the rate of production of membrane lipids and therefore a reduction in the rate of reproductive biomass increase (Sutherland, 1968). The internal biochemical signal that induces these changes, if it could be artificially manipulated, could provide almost complete control of aphids without adversely affecting non-target organisms the way current pesticides do.

The microsomes of some organisms contain a fatty acid synthase separate from the cytosolic FAS capable of de novo synthesis using malonyl–CoA or methylmalonyl–CoA as the elongation source (Bourre et al. 1977; Khan and Kolattukudy, 1975; de Renobles et al. 1989; Juarez et al. 1992; Peide et al. 1993; Blomquist et al. 1994). Khan and Kolattukudy (1975) isolated a FAS from the microsomes of *Euglena gracilis* by high salt or detergent that synthesized mainly palmitate, but also small amounts of stearate and myristate. In insects this FAS appears to occur primarily in the epidermal tissues (Juarez et al. 1992; Peide et al. 1993), and is able to use methylmalonyl–CoA to generate methyl–branched fatty acids which cytosolic FAS is generally unable to do. Although Blomquist et al. (1994) reported that house fly cytosolic FAS was able to generate small amounts of methyl–branched fatty acids, Peide (personal communication) and Peide et al. (1993) found that purified cytosolic FAS from house flies and cockroaches were both unable to

use methylmalonyl–CoA. Peide (personal communication) also found that substate K_ms, partial tryptic digests, and total amino acid analysis indicated slight differences between the cytosolic and microsomal enzymes.

Ryan (1982) has shown the presence of a cytosolic FAS in pea aphids, and partially purified and characterized it. He also demonstrated the presence of a TE II that alters the product specificity of the FAS. Given the identification of microsomal FAS in a variety of organisms, including other insects, it was likely that it was present in pea aphids as well. This was confirmed by preliminary investigations in our laboratory.

In most organisms fatty acids longer than eighteen carbons are produced by an elongase complex located on the microsomes that uses NADPH and malonyl–CoA to elongate fatty acyl CoAs (Bolton and Harwood 1977; Cook 1991; Seubert and Podack 1973; Vaz et al. 1988a; Vaz et al. 1988b). These enzymes are responsible for the production of fatty acids up to 28 carbons, as well as for the elongation of myristate to palmitate or stearate. In aphids these enzymes generate fatty acids up to 20 carbons, as well as carry out the elongation of 14:0 to palmitate or stearate. It is important to verify that any activity detected on microsomes or fractions isolated from microsomes is in fact a FAS and not an elongase.

Understanding their kinetic parameters could give insight into the partitioning of activity between the two FASes. If one FAS has a much lower or higher K_m for one or more substrates, shifting the intracellular concentration of those substrates could shift the balance of activity. As observed in other organisms, the microsomal FAS may have much higher activity with methylmalonyl–CoA and be responsible for the generation of methyl–branched cuticular hydrocarbons.

Once dietary sugars have been reduced to acetate groups in the glycolytic pathway their enzymatic conversion to fatty acids in the aphid involves the cytosolic fatty acid

synthase, TE II, the elongase complex, and a microsomal fatty acid synthase. The adaptation to different hosts or altered host condition involves changing the metabolic flux through the many enzymes participating in fatty acid synthesis. With the large amounts of lipids synthesized for membrane phospholipids and released in the form of nymphs, for accumulation of 14:0 triglycerides as energy stores, for cuticular lipids and siphuncular lipid stores, and with the variation of fatty acid composition in response to environmental factors, understanding the enzymes involved in fatty acid synthesis in the aphid is crucial to understanding aphid/plant interactions.

OBJECTIVES

The pea aphid is an organism that receives no lipid in its diet, uses lipids as a flight fuel, and has a high reproductive output. Because of the major importance of fatty acid synthesis under these conditions, and the unclear significance of the high 14:0 content which varies with host status in pea aphids I investigated enzymes responsible for the synthesis of fatty acyl chains in the aphid.

HYPOTHESES

There are two fatty acid synthases in pea aphids. These two enzymes have distinct physical and biochemical properties. They produce different products and interact differently with TE II.

To attempt to test these hypotheses the following specific objectives were achieved.

I. The purification and characterization of the cytosolic fatty acid synthase.

A. Purification was by centrifugal separation, $(NH_4)_2SO_4$ precipitation, gel filtration, DEAE Sephacel chromatography, and Reactive Red Sepharose affinity chromatography.

B. Characterization included:

i. Molecular weight by gel filtration, and Laemmli and Weber–Osborn SDS–PAGE.

ii. Kinetic parameters: K_m and V_{max} of malonyl–CoA, acetyl–CoA, NADPH, and methylmalonyl–CoA.

iii. Isoelectric point and pH optimum.

II. The purification and characterization of the microsomal fatty acid synthase performing the same procedures and characterizations.

III. Determination of the products produced by the purified FASes, both alone and in combination with TE II. This involved:

A. Partial purification of TE II, by centrifugal separation and ammonium sulfate precipitation.

B. Incubating the FASes with and without TE II in the presence of radio-labeled malonyl-CoA and determining by radio-HPLC what products were produced.

IV. Preliminary peptide mapping and sequencing of the two fatty acid synthases to determine similarities and differences. This involved cleavage of the FASes with trypsin to generate peptide maps and preliminary comparison by SDS–PAGE, with automated Edman degradation of selected fragments.

CHAPTER II

LITERATURE REVIEW

APHID FATTY ACID VARIATIONS

Bergman et al. (1991) found that the condition of the host plant has a profound effect on the fatty acid composition of spotted alfalfa aphids, *Therioaphis maculata* (Buckton). Aphids from host plants that had not been conditioned by prior aphid feeding had 14:0 contents four fold higher than aphids from chlorotic plants that had been fed upon for ten days. The level of senesence, and perhaps therefore the nutritional quality of the host, influenced the quantity and chain length of fatty acid production.

Dillwith et al. (1993) reported that the levels of 14:0 in pea aphids vary by life stage, morph, and host. The range of 14:0 content can vary five fold when pea aphids are reared on different hosts, and by 20% between alate and apterous aphids. Total lipids also differed nearly four fold between host plants. Febvay et al. (1992) found a 3.5 fold increase of neutral lipids (essentially all 2–hexano–1,3 dimyristin) when pea aphids were reared on an artificial diet.

The over–wintering form of *Thecabius affinis* Koch, a pemphigine aphid, shows a marked difference from the summer form (Sutherland, 1968). Sutherland found that the lipid as a percentage of body weight of these aphids doubles when they shifted to the over–wintering state, and that the amount of nymphal biomass was greatly reduced. Their lipid classes became primarily triglyceride, and their reproductive output dropped by 80%. Greenway et al. (1974) found that over–wintering aphids increased the proportion of hexanoic, sorbic, and myristoleic acids over the summer form.

These changes with season, host, and morph suggest a hormonal control over the length of fatty acids synthesized, as an increase in neutral triglycerides in aphids implies an increase in myristate. The decrease in nymphal biomass could be regulated by the same hormonal signals, or could be due to a decrease of fatty acids suitable for forming membrane lipids for growing embryos. The changes in lipid content and fatty acid chain length with different hosts, seasons, and morphs, also demonstrate the key role FAS and its regulation play in the biology of aphids and their adaptation to their environment.

CYTOSOLIC FATTY ACID SYNTHASE

In animals FAS has a molecular weight of about 500,000 daltons, and is composed of two identical subunits (Wakil et al. 1983). Witkowski et al. (1991) suggested that the malonyl/acetyl transferase and β–ketoacyl synthase of one subunit cooperate with the reductases, acyl carrier protein and TE of the companion subunit in the formation of a center for fatty acid synthesis. This is supported by the monomeric form of FAS being inactive, while the dimer contains two active sites for synthesis (Ahmad et al. 1982; Singh et al. 1984).

The prosthetic group of the fatty acyl carrier protein is a phosphopantetheine group attached to a serine residue in chicken FAS and goose uropygial FAS (Huang et al. 1989; Poulose et al. 1984). Chicken and rat FAS contains two 4'-phosphopantetheine molecules per dimer (Arslanian et al. 1976) which is concordant with the holoenzyme being a homodimer. Polouse et al. (1981) found that the binding of NADPH, which triggers dimerization of FAS monomer, occurred at the enoyl reductase domain, and that FAS from the uropygial gland of the goose is inhibited by a high [NADP]:[NADPH] ratio. The enoyl reductase, ketoreductase, and condensing activity were also affected by the changing ratio.

The native FAS in *Ceratitis capitata* (Wiedmann) (Diptera) is a lipoprotein, with no activity in the delipidated state: this is apparently due to the stabilizing effect of lipids on α-helix segments of the protein (Gavilanes et al. 1979; Gavilanes et al. 1981). However de Renobales et al. (1986) found no lipid associated with the FAS of another dipteran, *Drosophila melanogaster* Meigen.

FAS lengthens acetyl-CoA by a condensation with malonyl-CoA; each condensation increases the acyl chain by two methylene groups, consumes two NADPH, and releases one CO_2 (Wakil et al. 1983). *In vivo*, FAS synthesizes a range of fatty acids, from capric acid (10:0) to stearic acid (18:0), with palmitic acid (16:0) being the most abundant (Municio et al. 1977). Purified FAS produces palmitate and stearate exclusively (Ryan, 1982; Aprahamian, 1982). Palmitate is the primary product of rat FAS, while yeast and chicken FAS produce palmitate and stearate (Aprahamian, 1982). The primer chain length did not affect the product specificity of the animal FAS, while the yeast FAS increased the proportion of stearate produced with longer primers. The V_{max} for animal FAS decreases with increasing primer chain length, and the K_m's for the primers increase (Aprahamian et al. 1982).

In the pea aphid, fatty acids shorter than 16:0 are produced by the action of a separate enzyme, TE II, which cleaves the growing acyl chain at myristate (14:0) (Ryan et al. 1982). However, in *Drosophila melanogaster*, FAS produces 12-18 carbon fatty acids without the interaction of a separate TE: changes in ionic strength vary the chain length specificity (De Renobales and Blomquist 1984).

Branched fatty acids are produced by FAS when methylmalonyl–CoA is incorporated instead of malonyl–CoA. The regulation of branched fatty acid production in goose uropygial gland is by expression of malonyl–CoA decarboxylase, which results in an

increased proportion of methyl malonyl-CoA available for incorporation (Buckner et al. 1978).

Gavilanes, et al. (1983) found that FAS is active in the larval and adult stages of *Ceratitis capitata*, with minimal activity in the pupal stage. They also reported a soluble fraction in supernatant from adults which inhibited FAS activity and was not immuno-precipitated by anti-(fatty acid synthase) antibodies. Lizarbe et al. (1977) reported that the level of FAS as well as its activity varied with instar and adult age in *Ceratitis capitata*. They also found that starvation reduced FAS levels, and refeeding starved insects increased the levels of FAS at a rate dependent on the fat content of the diet.

Control of FAS activity in vertebrates is by transcriptional regulation, with triiodothyronine, insulin, and carnitine acting as transcriptional promoters, and glucagon, hexanoate, and octanoate as inhibitors (Roncero and Goodridge 1992). As would be expected for this cytoplasmic enzyme, Antoniou et al. (1981) found that free and not membrane bound polyribosomes are responsible for FAS synthesis in lactating rabbit mammary gland.

Witkowski et al. (1992) reported that the nutritional state of chicks, ducklings, or goslings influenced transcription of FAS mRNA. High-carbohydrate mash diets caused a steady state of transcription 10 times the initial level after 9 hours. The half life of mRNA was about 4 hours, with most of the increase in transcription occurring within 45 minutes after feeding was initiated. When fed ducklings were starved, the decrease in FAS mRNA occurred more rapidly than the decrease in transcription of the FAS gene, indicating some degree of post-transcriptional regulation.

Hepatic acetyl–CoA carboxylase and FAS have two distinct types of development in the perinatal chick (Fischer and Goodridge 1978). The first increase begins prior to hatching, continues after hatching in the starved chick, and is independent of feeding. The

second increase is caused by feeding and is reversed by starvation. They determined that activity changes in the enzymes were caused by changes in the concentrations of enzyme proteins. Liver tissue culture showed that in the absence of hormones, the cultured cells had an increase of enzyme activity over fresh preparations The addition of insulin or triiodothyronine caused small additional activity increases. Insulin with triiodothyronine caused 8 and 15-fold increases in acetyl-CoA carboxylase and FAS respectively, relative to cells incubated without hormones. Glucagon showed activity only in the presence of insulin plus triiodothyronine, when it inhibited the increase of enzyme activity by about 75% (Fischer and Goodridge 1978).

The rat FAS cDNA sequence has been determined and the order of domains is condensing enzyme—transferase—dehydrase—enoyl reductase—ketoreductase—acyl carrier protein—TE (Amy et al. 1989). The peptide active sites of all except the dehydrase were also determined. Witkowski et al. (1991b) and Amy et al. (1989) assigned amino acids 1-406 to the ß-ketoacyl synthase, 430-802 to the malonyl/acetyl transferase, 1630-1850 to the enoyl reductase, 1870-2100 to the ketoreductase, 2114-2190 to the acyl-carrier protein and 2200-2505 to the TE. Witkowski et al. (1991b) also suggested that the malonyl/acetyl transferase and ß-ketoacyl synthase of one subunit cooperate with the reductases, acyl carrier protein and TE of the companion subunit in the formation of a center for fatty-acid synthesis.

Holzer et al. (1989) reported the complete amino acid sequence of chicken FAS as deduced from the cDNA. They identified sites known to be associated with the enoylreductase NADPH binding site, the acetyl/malonyl transacylase site, the "waiting" site containing cysteine, and a pyridoxal 5'-phosphate binding site, based on sequence homologies to other enzymes. Subrahmanyam et al. (1989) and Chirala et al. (1989) also reported the chicken FAS cDNA sequence, and showed there was a high degree of

identity with the rat FAS cDNA. Yang et al. (1988) published the complete chicken FAS sequence for the TE domain as derived from peptic digestion, while Huang et al. (1989) determined the acyl carrier protein sequence by the same method.

When Pazirandeh et al. (1989) expressed portions of the chicken FAS in *Escherichia coli* using a phage lambda PL promoter expression vector, the recombinant protein was enzymatically active and had the same substrate specificity and kinetic properties as the native FAS. In addition the acyl carrier protein portion of the recombinant molecule was partially pantothenated, indicating that the expressed protein has a similar conformation to the native *E. coli* enzyme and that the folding of the functional domains is independent of the remaining domains of the complete FAS.

MICROSOMAL FAS

In several organisms a separate, microsomally bound FAS has been found, capable of de novo synthesis using malonyl-CoA or methylmalonyl-CoA as the elongation source (Bourre et al. 1977; Juarez et al. 1992; Khan and Kolattakudy, 1975; Peide, 1993). Mouse and rat brain microsomes are capable of de novo fatty acid synthesis using malonyl–CoA and NADPH (Bourre et al. 1977; Aeberhard and Menkes, 1968; Pollet et al. 1973). The activity of the soluble and microsomal FAS can be independent. In the pupae of cabbage loopers, *Trichoplusia ni*, cytoplasmic FAS activity is extremely low, while large amounts of long chain fatty acids and alcohols are being synthesized (de Renobales et al. 1989). The FAS solubilized from *Euglena gracilis* microsomes with high salt or detergent showed two to three times the activity when incubated with 0.4 mg/ml of phosphotidylcholine liposomes (Khan and Kolattakudy, 1975). In both *Trichoplusia ni* and *Euglena gracilis* the microsomal FAS activity seemed to be tightly linked to enzymes that produced fatty alcohols and wax esters.

The integument of the German cockroach, *Blatella germanica*, has a microsomal FAS that uses methylmalonyl-CoA to make methyl branched fatty acids (Juarez et al. 1992; Peide, 1993). Juarez et al. (1992) found no microsomal FAS in the fat body, and the cytosolic FAS was unable to use methylmalonyl–CoA. The lack of detectable methyl–branched fatty acids in the cockroach has led Juarez et al. (1992) to propose a tight linkage to the reductive enzymes that generate the cuticular hydrocarbons. Piede et al. (1993) determined the kinetic constants for soluble and microsomal FAS in cockroach integument and found the microsomal enzyme was able to use methylmalonyl–CoA with a similar K_m to malonyl–CoA but at half the V_{max}. The cytosolic enzyme was unable to use methylmalonyl–CoA.

Musca domestica also has a microsomal FAS that synthesizes methyl-branched fatty acids. In this case the crude cytosolic FAS was able to make limited use of methylmalonyl-CoA while the purified cytosolic FAS was not (Blomquist et al. 1994; Peide personal communication). The microsomal FAS activity was higher in male than female *Musca domestica* when using methylmalonyl-CoA. Partial tryptic digests, total amino acid analysis, and Michaelis–Menten parameters indicated slight differences between the enzymes.

THIOESTERASE II

TE II is a soluble protein that interacts with FAS to release medium length fatty acids instead of the 16 to 18 carbon fatty acids that FAS normally produces (Libertini and Smith 1978; Ryan et al. 1982). Naggert et al. (1988) determined from DNA sequence that the TE I region of FAS had "low, albeit discernable" identity with the TE II's from rat mammary and duck uropygial glands. Rat mammary gland TE II is a monomer of

molecular weight 33,000 and contains a single active site (Libertini and Smith 1978). No evidence was found of significant levels of TE II in lactating rat liver.

Mallard duck (*Anas platyrhyncos*) uropygial TE II is a monomer with Mr=29,500, with an amino acid composition significantly different from those of other TEs (Rogers et al. 1982; de Renobales et al. 1980). Peking duck (*Anas domesticus*) uropygial TE II was found to have a molecular weight of 56,200, whereas SDS-gel electrophoresis of components separated by chromatofocusing showed that the purified enzyme contained enyzmatically active proteins of molecular weights 59,400, 58,300, 56,000, and 55,800: All four had the same N-terminal sequence (Cheesbrough and Kolattukudy 1985).

Wax esters of short chain acids (monomethyl-C6), produced by the uropygial gland of mallard ducks, are replaced by longer chain acyl acids while the bird is molting and reappear when the molt is finished (Kolattukudy et al. 1985). These changes in composition were matched by TE II activity and amounts, and the levels of TE II mRNA. de Renobales et al. (1980) compared the FAS from the uropygial glands of five genera of waterfowl, which generate 2,4,6,8-tetramethyldecanoic acid, 2,4,6-trimethyloctanoic acid, or 2- and 4-monomethylhexanoic acid as major products. In all cases the purified enzyme generated free 16:0 and 2,4,6,8-tetramethyldecanoic acid from malonyl-CoA and methylmalonyl-CoA, respectively, using acetyl-CoA as the primer. The enzymes were immunologically similar, and the molecular and catalytic properties of the enzymes *in vitro* were similar, regardless of the normal *in vivo* products. Gel filtration of mallard uropygial gland 105,000g supernatant showed a protein fraction that interacted with FAS to produce short-chain straight and branched acids. This fraction was fully resolved by DEAE-Sephacel chromatography into a smaller and a larger enzyme. The smaller protein was responsible for the short chain production: the goose gland, which does not produce short-chain acids, contains only the larger enzyme. Phenylmethanesulfonyl fluoride

inhibited FAS produced acyl chains of 16 to 22 carbons. The inhibition was reversed by the smaller, but not the larger TE, and the fatty acids produced were free 10:0-14:0. They suggested that the smaller TE can replace the TE I segment of FAS and cause the release of short-chain acids (de Renobales et al. 1980).

TE II is inhibited by reagents directed against active serine and histidine, indicating it is a serine esterase (Rogers et al. 1982). Rogers et al. also found that TE II is highly sensitive to thiol-directed reagents and high ionic strength. Involvement of an active serine in catalysis was suggested by inhibition of the enzyme by diethylpyrocarbonate, diisopropylfluorophosphate and phenylmethylsulfonyl fluoride (Cheesbrough and Kolattukudy 1985). Witkowski et al. (1992) mutated the active serine to cystine and found that a substitution of cysteine for Ser^{101} retains 90% of its catalytic activity. In the mutant TE dibromopropanone cross-linked the active site Cys^{101} with His^{237}, demonstrating that they are within 0.5 nanometers of each other. They conclude that in normal TE II His^{237} accepts a proton from Ser^{101}, which increases its nucleophilic character and improves the catalytic efficiency of the enzyme. The involvement of a histidyl residue in the catalytic mechanism of TE II has been inferred from studies with the inhibitor diethyl pyrocarbonate (Witkowski et al. 1991a). Prior ethoxycarbonylation of the histidyl residue blocked the ability of the active-site serine to react with phenylmethanesulfonyl fluoride. Witkowski et al. (1992) also speculate on the possibility that the exchange of cysteine and serine active site residues has occurred in the evolution of TEs (Witkowski et al. 1992). The active histidyl residue is the only histidine conserved among similar proteins (Witkowski et al. 1991a). For example, a seven amino acid sequence containing the active serine of duck TE II is identical to that in rat TE II (Safford et al. 1987).

Tai et al. (1993) expressed rat mammary gland TE II in *Escherichia coli* and created several site-directed mutants. Replacing both Ser^{101} and His^{237} with Ala yielded inactive enzymes, suggesting that these amino acids are part of a catalytic triad as in the native FAS TE (TE I). Mutating the conserved Asp^{236} or modifying it with Woodward's reagent K caused a partial (40%) loss of activity, and reduced the response of Ser^{101} and His^{237} toward the specific inhibitors, phenylmethylsulfonyl fluoride and diethyl pyrocarbonate, respectively. Thus Asp^{236} apparently enhances, but is not essential for, reactivity of Ser^{101} and His^{237}. Mutating Leu^{262} at the carboxyl end indicated that it is critical for interaction with FAS. Hydrophobic interactions were involved, as polyethylene glycol increased, but salt reduced, interaction. Inactive mutants and a synthetic C-terminal decapeptide did not compete with normal TE II. They suggested that a TE II-acyl FAS complex forms first, which is then stabilized by the interaction of the hydrophobic C-terminus of TE II with FAS, followed by cleavage and release of the fatty acid (Tai et al. 1993).

These studies provide strong evidence that His^{-237} is involved directly in catalysis and suggest that its role is to increase the nucleophilic character of the active-site Ser^{-101} by acting as a proton acceptor thus facilitating acylation of the seryl residue. The mechanism appears to share certain common features with the charge-relay system characteristic of other esterases (Witkowski et al. 1991). TE II restores activity to FAS which has had the native thioesterase (TE I) inactivated with phenylmethanesulfonyl fluoride or removed by trypsinization (Libertini and Smith 1978). Modifying one cysteine thiol near the carboxyl end of TE II with thionitrobenzoate inhibited interaction with FAS but not with acyl-CoA model substrates (Witkowska et al. 1990). Proteolysis showed the amino acid involved was Cys^{-256}. Their purified TE II showed both full-length and a truncated form of the enzyme missing the carboxyl-terminal Leu-Thr

peptide. The interaction of TE II with FAS was lost in the truncated form (Witkowska et al. 1990). Rat mammary gland TE II is blocked at the N-terminal amino acid with an N-acetyl group (Slabas et al. 1989).

Mikkelsen et al. (1987) studied the interaction between rat mammary gland TE II and fatty acid synthase. Experiments showed that the enzymes do not readily form a complex. TE II hydrolysis of acyl chains from FAS is not inhibited by a large excess of fatty acid synthetase with vacant 4'-phosphopantatheine thiols. This indicates TE II interacts only with FAS that carries acyl chains on the 4'-phosphopantatheine thiols. Column chromatography experiments showed that TE II association with FAS occurs when an acyl chain is present, but dissociates quickly when the acyl chain is cleaved. This differs from avian uropygial gland TE II where the two enzymes form a stable association even when there are no acyl chain substrates (Mikkelsen et al. 1987).

The recombinant proteins of TE I and TE II have been expressed in *Eschericha coli*, and are full-length, catalytically competent TEs with specificities indistinguishable from those of the natural enzymes (Naggert et al. 1991).

Libertini et al. (1979) modified FAS from lactating rat mammary gland by removal of the TE I domains with trypsin or by inhibiting the TE with phenylmethanesulfonyl fluoride. The modified enzymes are able to synthesize long chain acyl moieties (C16-22), but are unable to release the acyl groups as free fatty acids. Their kinetic studies found the modified enzymes rapidly elongated the growing fatty acid to 16:0 and that further elongation to 18:0, 20:0, and 22:0 is progressively slower. TE II was found to form an enzyme-substrate complex with the modified FAS, and release the bound acyl chain (Libertini and Smith 1979).

The site of action of TE II was elucidated by Smith et al. (1979). Their study used two experimental approaches. First, TE I domains were removed from the fatty acid

synthase. Incubation of the FAS with [2-^{14}C] malonyl-CoA, acetyl-CoA, and NADPH, followed by peptic digestion and [^{14}C]acyl-peptide purification, determined the attachment site as a pantetheine thioester. Second, the ability of TE II to hydrolyze cysteine- and cysteamine-containing thioesters was compared. The following preference was found: S-decanoyl-panthatheine>S-decanoyl-CoA>S-decanoyl N-acetylcysteamine. Cysteine thioesters were not effective substrates for TE II. They concluded that the site of action of TE II is the thioester bond linking the fatty acid chain to the 4'-phosphopantatheine prosthetic group of FAS (Smith and Libertini 1979).

The total sequence for mallard duck uropygial TE II was reported by Poulose et al. (1985). Northern blot analysis showed that mature TE II mRNA contained 1350 nucleotides. The nucleotide sequence has an open reading frame coding for a 28.8 kDa peptide. cDNA clones of TE II from rat mammary gland have also been identified (Naggert et al. 1987; Randhawa et al. 1987). They found that it coded for 263 amino acid residues and exhibits sequence homology to mallard uropygial TE II. The 57 amino acid region around the active serine site has a 47% identity between the avian and mammalian enzymes (Randhawa et al. 1987).

MICROSOMAL FATTY ACID ELONGATION COMPLEX

Cytoplasmic fatty acid synthase produces primarily palmitate or stearate. Longer fatty acids are produced by separate elongation systems. There are two fatty acyl chain elongation systems; one is associated with the endoplasmic reticulum and the other in the mitochondria. The elongation can be minor, converting palmitate to stearate (Bolton and Harwood 1977), or extensive, generating up to 28 carbon fatty acids (Vaz et al. 1988b). The endoplasmic reticular system is the more active (Cook 1991). The condensing unit for the microsomal system is malonyl-CoA, and acetyl-CoA for the mitochondria (Bolton

and Harwood 1977; Seubert and Podack 1973). Microsomal elongation is very limited when using acetyl-CoA only, and biotin has no effect on elongation. These facts indicate that acetyl-CoA carboxylase is not involved (Cook 1991). The microsomal system uses either NADH or NADPH, with NADPH being the more active: the mitochondrial system generally shows a need for both NADH and NADPH, with the exceptions needing only NADH (Cook 1991).

The mechanisms for chain elongation in the microsomal system is essentially the same as for fatty acid synthase, but there is evidence that there may be discrete enzymes rather than a multifunctional complex (Cook 1991; Vaz et al. 1988b). Mitochondrial elongation is essentially a reversal of β-oxidation with the substitution of enoyl-CoA reductase for FAD dependent acyl-CoA dehydrogenase for thermodynamic reasons (Seubert and Podack 1973). Mitochondrial elongation is unlikely to have a major role in fatty acid synthesis as it requires anaerobic or high phosphate potential conditions (Seubert and Podack 1973). They considered it to have a role in energy metabolism, a position viewed with suspicion by Cook (1991).

Tissue content of elongating enzymes varies: Vaz et al. found that cockroach epidermal tissue contained 3 times the elongating activity of other body tissues, and that non-epidermal tissues stopped at 22 carbons as opposed to 28 (Vaz et al. 1988b). Housefly heads and thoraxes can only elongate to 20 carbons while the abdomens can elongate to 28 carbons (Vaz et al. 1988a). They found that in *Periplaneta americana* there are very specific elongation systems. 18:0 and 18:2 were elongated to 28 carbons, 18:3 to only 22 carbons, and 18:1 not elongated at all. They suggest the presence of 2 separate elongation systems, one for saturated and one for di-unsaturated substrates (Vaz et al. 1988b). In rat liver only fatty acids 16 or more carbons long are elongated, and fatty acids with 0,1, or >3 double bonds being preferentially elongated (Seubert and Podack 1973).

CHAPTER III

MATERIALS AND METHODS

The materials used in this work were purchased from the following sources. All chemicals were of reagent grade or better.

SIGMA CHEMICAL COMPANY: The following lithium salts of Coenzyme A (CoA) esters: acetyl, propionyl, butyryl-, isobutyryl, pentanyl, isopentanyl, hexanyl, tetradecanyl,and methylmalonyl-CoA, reduced nicotinamide adenine dinucleotide (NADPH), ethylene glycol tetraacetate (EGTA), leupeptin, Tosyl–lysine–chloromethylketone (TLCK), Tosyl-phenylalanine–chloromethylketone (TPCK), benzamidine, phenylmethanesulfonyl fluoride (PMSF), 14% BF_3 in methanol, endoproteinase Glu-C, trypsin, Percoll, ammonium persulfate, trichloroacetic acid, vanillin, trifluoroacetic acid (TFA), tungstosilicic acid, Trizma base (TRIS), ammonium bicarbonate, 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), glycine, Coenzyme A, adenosine triphosphate (ATP), cerulenin, bovine serum albumin (BSA), cross-linked BSA, Sepharose 6B–CL, DEAE Sephacel (diethylaminoethyl cellulose), Reactive Red Sepharose 4B–CL, (N,N,N',N') tetramethylethylenediamine (TEMED), ammonium molybdate, silica gel, gel filtration molecular weight markers kit MW-GF-1000

FISHER SCIENTIFIC: methanol, hexane, and chloroform (all redistilled in glass), sodium chloride, mono- and di-basic potassium phosphate, potassium chloride, glycerin, sulfuric acid, sodium acetate, ferrous sulfate, formic acid

BIORAD: 30% acrylamide/Bis solution 37.5:1, protein assay reagent, sodium dodecyl sulfate (SDS), SDS-PAGE molecular weight markers 161-0317, gel filtration molecular weight markers 151-1901

RESEARCH ORGANICS: dithiothreitol (DTT), ethylene diamine tetraacetate (EDTA), ammonium sulfate, glucose-6-phosphate

PHARMACIA BIOTECH: isoelectric focusing standards and gels, Superose-6, gel filtration columns

ALDRICH: cyanogen bromide in acetonitrile, silver nitrate, *trans*-1-decalone

ALLTECH: Econosil C18 5μM 250 mm x 4.6 mm HPLC column

DIVERSIFIED BIOTECH: Rapid Coomassie stain

PACKARD INSTRUMENTS: Ultima-Flo™ M

NEW ENGLAND NUCLEAR: Sodium [1-^{14}C] acetate, 47 mCi/mmole

BURPEE SEEDS: 'Little Marvel' pea seeds

MALLINCKRODT: sodium carbonate

EM INDUSTRIES: glacial acetic acid

Sodium pyruvate and α-ketoglutarate were a gift from Dr. Franklin Leach.

BUFFER

The buffer used for all procedures was 50 mM K_2HPO_4, pH 7.4, 1 mM ethylene diamine tetra-acetate (EDTA), 1 mM ethylene glycol tetra-acetate (EGTA), 1 mM dithiothreitol (DTT), 10% glycerol, 50 μg/l leupeptin, 0.1 mM TLCK, 0.1 mM TPCK, and 50 mg/l benzamidine (Ryan, 1982; de Renobales et al. 1986; Piede, personal communication). The only change made was the addition of potassium chloride for elution buffers in chromatography.

INSECTS

Pea aphids *Acyrthosiphon pisum* (Harris) were reared on faba beans (*Vicia faba* L.) cv.'Windsor'. These were grown under lights on a 14:10 photoperiod at 25° C between 20 and 50% relative humidity in a commercial potting soil. The aphids were collected by tapping them from the plants onto a sheet of plastic bench cover and kept in petri dishes at 4° C until used (usually within 2 hours). For comparative studies some aphids were reared on alfalfa (*Medicago sativa* L.) cv. 'OK08' under the same conditions.

ASSAY PROCEDURES

FAS ASSAY PROCEDURES

NADPH ABSORPTION ASSAY

FAS activity was assayed at 25°C by measuring the decrease in absorbance at 340 nm with a Beckman DU-65 spectrophotometer in 1 ml cuvettes. The assay used a solution of 60 µM acetyl–CoA, 60 µM malonyl–CoA, and 100 µM NADPH in standard buffer, and enzyme. In order to have repeatable results, room temperature buffer was used in the assays, and the FAS was allowed to sit at room temperature for 1/2 hour or more before assays began. The enzyme was added to buffer along with 10 µl of 2.4 mM acetyl–CoA and 10 µl of 4 mM NADPH for a total volume of 390 µl, vortexed briefly and incubated for 8 minutes. This incubation was also necessary for good activity. The absorbance decrease was measured for 2 minutes, 10 µl 2.4 mM malonyl–CoA added, vortexed, and the rate measured for a further two minutes. The activity due to FAS was the difference between the two rates. One unit of activity equals the consumption of 1 nmole of NADPH/minute/mg protein (Ryan 1982).

DECALONE REDUCTION ASSAY

The assay was derived from Ullman et al. (1978). It measures the NADPH–dependent reduction of cis–decalin–1,4–dione (decalone) by the β–ketoacyl–ACP reductase activity of FAS. This assay detects activity from FAS in the dimer and monomer form, as well as with the TE I function inactivated or cleaved off. This assay was used to detect the active fractions in column chromatographic purifications, as well as to verify that partial activities remained in PMSF treated FAS. It is more sensitive, quicker, and does not use expensive CoA substrates. The assay was conducted in standard buffer that was 1 mM in decalone. The solution must be shaken for several hours for the decalone to fully dissolve in the buffer. Four minutes after FAS was added to the buffer for a total volume of 385 µl, the decrease in absorbance at 340 nm was measured for 2 minutes to determine a background rate. Fifteen µl of 4 mM NADPH were added, and the absorption measured for a further 2 minutes. The difference between the two rates was the activity due to FAS.

THIOESTERASE ASSAYS

TE activity was measured spectrophotometrically by the method of Ellman (1959) using acetyl-CoA or myristyl-CoA as substrates. The assay was conducted in 0.1 M pH 8.0 phosphate buffer. Enzyme, and 10 µl of 2.5 mM DTNB in 0.1 M pH 7.0 phosphate buffer were added to the pH 8 buffer for a total volume of 390 µl, which was briefly vortexed. The rate of absorbance increase was allowed to stabilize for 4 minutes, read for 2 minutes at 412 nm, and then 10 µl of 2.4 mM Coenzyme-A ester added. The

absorbance was read for another 2 minutes, and the difference in the rates of absorbance increase was the activity due to the enzyme.

PROTEIN ASSAYS

Protein content of fractions was determined by the method of Bradford (1976), modified for use in a plate reader. BioRad protein reagent was diluted 4:14, and 180 µl added per well. Protein standards and samples were added, the plate shaken for 10 minutes, and the absorbance measured at 595 and 410 nm. The plate reader was programed to subtract the 410 nm reading from the 595 nm reading to reduce well to well variation. Determinations were made by comparison to the regression calculated from triplicate samples of 0, 1, 3, and 5 µg of BSA.

MARKER ENZYME ASSAYS

LACTATE DEHYDROGENASE ASSAY

Lactate dehydrogenase activity was assayed at 25°C by measuring the decrease in absorbance at 340 nm with a Beckman DU-65 spectrophotometer in 1 ml cuvettes. Sufficient mitochondrial protein was added to give a change in absorbance of between 0.1 and 0.2 AU/min., and an equivalent amount of cytosolic and microsomal protein used (Long 1975). The assay used a solution of 14 mM NADH, 100 mM sodium pyruvate, and 100 mM potassium phosphate, pH 7.5. The protein sample was added for a total volume of 400 µl, and the absorbance decrease was measured for 2 minutes.

GLUTAMATE DEHYDROGENASE ASSAY

The assay was conducted spectrophotometrically at 340 nm in 400µl total volume. The change in absorbance needed to be less than 0.04 AU/min. and measured for less than 1.5 min. to insure linearity. The sample protein was added to 100 mM phosphate pH 7.6 buffer, 5 mM α-ketoglutarate, 50 mM NH_4Cl, 100 µM EDTA, and 1 mg/ml BSA (Fisher 1985) for a total volume of 390 µl. Ten µl of 4 mM NADPH was added and the absorbance read for 1 minute.

GLUCOSE-6-PHOSPHATASE ASSAY

The assay is that of Zakim and Vessey (1973). 50 µl 1 M sodium acetate pH 5.75, 100 µl 0.4 mM glucose-6-phosphate, and 50 µl water were placed in a microfuge tube. The solution was equilibrated at 37° C and the assay started by adding 200 µg protein. After a 5 min. incubation the reaction was stopped by adding 100 µl 10% trichloroacetic acid and the precipitate removed by centrifugation. 100 µl of the supernatant was added to 1 ml 1.6% (w/v) ammonium molybdate in 1 N sulfuric acid followed by 100 µl freshly prepared ferrous sulfate solution, 2.5 g/25 ml 0.15 N sulfuric acid. After shaking, the optical density was read at 660 nm. Zakim and Vessey (1973) stated the color intensity is linear up to an optical density of 1, and a blank for non-specific phosphatase is β-glycerophosphate, but said a blank is usually not necessary and it was not performed.

FAS Purification

Cytosolic FAS

All operations were performed on ice or at 4°C The preparation was assayed for FAS activity at each purification step. Chilled aphids were homogenized (~10 ml buffer per gram aphids) using a Tekmar Tissumizer (Tekmar Co., P.O. Box 37202, Cincinnati, OH) in 4° C protease inhibitor buffer. After centrifugation at 500 g for 5 minutes, 1,200 g for 10 minutes, and 10,000 g for 20 minutes, the supernatant was filtered through a thin layer of glass wool to remove caked lipid. The filtrate was centrifuged at 105,000 g for 1 hour in a Beckman L8-70 or TL-100 ultracentrifuge using a Type 30, an NVT-65, or a TL100.3 rotor, depending on the volume of homogenate (de Renobales et al. 1986; Municio et al. 1977; Ryan 1982; Thompson and Barlow 1976).

The supernatant was again filtered through glass wool to removed caked lipid, and brought to 30% saturation with ammonium sulfate and stirred for 30 minutes on ice (Thompson and Barlow 1976). After pelleting the precipitate by centrifugation at 15,000 g for 10 minutes at 4 ° C, the supernatant was brought to 40% saturation with ammonium sulfate, the stirring and pelleting repeated, and the pellet washed and resuspended in inhibitor buffer (de Renobales et al. 1986; Ryan 1982).

At this point two separate purification protocols were followed. The first method is called "A". Under this scheme the sample was then loaded on a 2.5 x 90 cm Sepharose 6-B column and eluted at .5 ml/min. (Municio et al. 1977). The fractions having FAS activity were combined, loaded on a 1 x 8 cm Reactive Red-Sepharose 4B-CL column at 0.25 ml/min. (Nwokoro and Schachter 1975) to allow equilibration between NADP bound to the enzyme and the dye ligand on the column (Ogunyemi et al. 1978), and the effluent monitored for FAS activity (Miake et al. 1976). The column was washed until absorbance at 280 nm returned to baseline, and the FAS eluted with a gradient of 0 to 1 M KCl in

buffer (Alhama et al. 1991) with a quick ramp to 3 M KCl to clean the column of residual proteins. The active fractions were pooled, desalted or diluted to a KCl concentration of 0.05 M and loaded on a 2 x 15 cm diethylaminoethylcellulose (DEAE Sephacel) column, washed with buffer until the absorbance at 280 nm returned to baseline, and the FAS activity eluted with a gradient of 0 to 1 M potassium chloride in buffer (de Renobales et al. 1986; Municio et al. 1977) and again a ramp to 3 M KCl.

The second protocol, "B", loaded the solubilized 30–40% ammonium sulfate cut onto a 2.5 x 20 cm Reactive Red Sepharose column, the active fractions precipitated with 50% ammonium sulfate, loaded on to a 2 x 20 cm Pharmacia Superose–6 FPLC column, and eluted at 0.1 ml/min.

MICROSOMAL FATTY ACID SYNTHASE

The microsomal FAS assay was adapted from Peide et al. 1993. Aphids chilled to 4° C were homogenized in inhibitor buffer. Cell debris, organelles, and mitochondria were pelleted by a single centrifugation at 4° C in which the speed was increased as follows: 500 g for 5 min., 1200 g for 10 min., and 10,000 g for 20 min. The supernatant was then centrifuged at 100,000 g for 60 min. at 4° C The washed pellet was resuspended gently by re-homogenization in buffer. The purity of the fractions was assayed by the use of lactate dehydrogenase (Long 1975) for the cytosolic proteins, glutamate dehydrogenase for mitochondria (Fisher 1985), and glucose-6-phosphatase (Zakim and Vessey 1973) for the microsomes. The microsomes were then stirred in 1 M KCl on ice for 30 min., and re–centrifuged for 1 hour at 100,000 g. The supernatant then followed the steps in purification scheme A with the exception that the DEAE and Reactive Red steps were reversed, or scheme B.

FAS Molecular Weight Determinations

Gel filtration molecular weights were determined by running carbonic anhydrase (29 KDa), BSA (66 KDa), alcohol dehydrogenase (150 KDa), β-amylase (200 KDa), apoferritin (443 KDa), and thyroglobin (669 KDa), through the Sepharose 6B column and determining a regression for the log of the molecular weight versus the elution volume minus the void volume (log MW vs Ve-V0). For the Superose 6 column thyroglobulin, γ-globulin (158 KDa), ovalbumin (44 KDa), myoglobin (17 KDa), and vitamin B-12 (1.3 KDa) were used as the standards. PAGE molecular weight determinations were conducted in 5% acrylamide gels using the Biorad Minigel system according to the methods of Laemmli (1970) and Weber and Osborn (1969) using Pharmacia broad range standards for the Laemmli system and cross-linked multimers of BSA (Sigma) for Weber-Osborn.

FAS pH optimum Determination

A broad range determination of the cytosolic FAS pH optimum was conducted from pH 4 to 12 using 200 mM buffers. Acetate was used from pH 4.0 to 6, Tris/maleate from 5.5 to 7.5, phosphate from 5 to 9, Tris from 7 to 9.5, and glycine from 9 to 12. A narrow range determination was conducted using purified cytosolic FAS over the pH range 7 to 8 in 0.1 pH unit increments with phosphate buffer. Purified microsomal FAS was tested over the range of 6.5 to 8.2 in 0.1 pH unit increments with phosphate buffer as well. The assays were conducted in 400 µl total volume, 1 mM DTT, 100 µM NADPH, and 60 µM malonyl-CoA and acetyl-CoA.

FAS Isoelectric Point Determination

Isoelectric point (pI) determinations were conducted on a Pharmacia Phast system, using 3 to 9 pH gels, Sigma 3.5-9.3 pI standards, and Pharmacia's published procedures for Pi determinations (Pharmacia bulletin 80-1311-90). The published protocol was modified by reducing the loading voltage to 100 V and increasing the running time to 850 Vh. These changes were needed to prevent streaking and allow for equilibration of the large FAS molecule.

Kinetic characteristics of crude and purified FAS

The kinetic parameters were determined by spectrophotometric assay as detailed in Materials and Methods. Maximum velocities (V_{max}) and Michaelis constants (K_m) of the substrates were calculated for both the crude and partially purified enzymes. The crude cytosolic fraction was the 15,000g supernatant, and the crude microsomal fraction was the 100,000 g supernatant of the KCl extraction. The partially purified enzyme for both microsomal and cytosolic FAS was the Reactive Red fraction from scheme B. The data were plotted as Hanes Woolf plots, substrate in µmoles over the apparent velocity versus substrate concentration (S/v vs S) (Cornish-Bowden, 1979). When a linear regression of the data is determined, the x intercept is $-K_m^{app}$, and the y intercept is K_m^{app}/V_{max}^{app}. Methylmalonyl-CoA inhibition of FAS was also determined, and plotted as apparent velocity over substrate. When plotted as a Hanes Woolf plot, the y intercepts and slopes of the regression lines were replotted vs the concentration of methylmanonly-CoA. (Cornish-Bowden, 1979). The y intercept of the replot regression over the slope of the regression gives K_i for the intercept replot and K'_i for the slope replot.

FAS PRODUCT DETERMINATION

The products of the fatty acid synthases were determined by incorporation of [1-^{14}C]malonyl-CoA prepared according to Roughan (1994) by the use of pea chloroplasts and [1-^{14}C]acetate (47 mCi/mmole, New England Nuclear). The only alteration to the published protocol was to use ammonium acetate buffer in the HPLC purification of the [1-^{14}C]malonyl-CoA and [1-^{14}C]acetyl-CoA. This allowed the last solid phase extraction step to be deleted (Roughan, personal communication). The resulting [1-^{14}C]malonyl-CoA had a specific activity of 20.375 mCi/mMole.

Reactions were performed in 400μL of pH 7.4 phosphate buffer, 100 μM NADPH, 60 μM acetyl-CoA, and 1.88 to 60 μM [1-^{14}C]malonyl-CoA. The lower concentrations of malonyl-CoA were also performed in 3.2 or 6.4 ml total volume with identical concentrations of substrates and enzyme to allow adequate numbers of counts for clear detection to be incorporated.

The incubations were conducted using the Reactive Red fraction of scheme B for the partially purified microsomal and cytosolic FAS, and the 15,000 g supernatant for the crude cytosolic FAS. The crude microsomal FAS was the 100,000 g microsomal pellet, or the 100,000 g supernatant of the KCl extraction for the PMSF inactivation.

The radiolabeled fatty acids were extracted by the method of Bligh and Dyer (1959) with chloroform:methanol:water. They were hydrolyzed with 5% KOH in methanol for 90 min. at 60° C, methylated with 14% BF_3 for 30 minutes, water was added, and the methyl esters extracted into chloroform. The methyl esters were run through $MgSO_4$, dried under nitrogen at 60° C, and then loaded on silicic acid packed Pasteur pipette columns by loading in 200 μl hexane. The samples were cleaned by rinsing the columns with 3 mls of hexane, and eluting the purified methyl esters with 6 ml 5% diethyl ether in hexane. The samples were dried under nitrogen at 60° C and resuspended in

chloroform/methanol. The products were quantitated by radio-HPLC using a Econosil C18 5μM 250 mm x 4.6 mm HPLC column and a 1 ml/min. flow of 80:20 acetonitrile:water for 25 minutes, followed by 23 min. of 85:15 acetonitrile:water. Detection was by a Packard Radiomatic Flow One beta detector fitted with a 1 ml flow cell and using Packard's Ultima Flo M scintillation fluid at a 2:1 fluor/sample ratio.

The interaction of the FASes with TE II was tested by including aliquots of the 40 to 70% ammonium sulfate pellet of pea aphid cytosol in identically conducted assays. The TE fraction was tested for both acetyl and myristoyl TE activity, and verified to have no FAS activity. The TE preparation had an activity of 7.35 nmoles myristyl-CoA/min/μl, and 1.8 nmole acetyl-CoA/min/μl. Fifty μl were used per 400 μl assay mixture. FAS was also poisoned with PMSF to provide a FAS that could only produce a product by interaction with TE II. Following the protocol of Ryan (1986), partially purified FAS was treated with 52 mM PMSF in isopropanol at a ratio of 3 μl /mg protein. The preparation was allowed to sit at room temperature for 45 minutes, and the activity measured. Less than 5% of the original activity remained, and the FAS was separated from the PMSF (which would inactivate TE II if allowed to remain) by precipitation with 50% ammonium sulfate, and centrifugation at 10,000 g for 10 minutes. The pellet was redissolved and used in the same assay as above.

PEPTIDE MAPPING

Cytosolic (Scheme A) and microsomal (scheme B) FAS were subjected to partial digestion with 1:20 trypsin for four hours in 50 mM phosphate buffer, pH 7.4. The digests were the subjected to SDS-PAGE electrophoresis using a 5% gel. The gel was soaked for 10 minutes in a Tris/glycine transfer buffer with 10% methanol and no SDS. The protein fragments were electroblotted onto a PVDF membrane using a BioRad

Transblot system at 100 volts for 2 hours in the same buffer. The blot was stained using coomassie blue and the background destained using methanol. Sequencing of selected bands was conducted on an ABI 470A sequencer following standard protocols.

CHAPTER IV
RESULTS

FAS ASSAYS

The assay used to measure fatty acid synthase activity was validated by measuring the linearity of the change in absorbance over time (Figure 1) and the linearity with protein concentration (Figure 2). The linearity over time was acceptable for greater than 3.5 minutes, which exceeds the 2 minutes integration time used to measure FAS activity. The activity per μg protein was also suitable for up to 70 μg protein, or 7 mg protein/ml in the sample, given the usual addition of 10 μL sample per assay.

FAS ACTIVITY IN APHID MORPHS

Aphids were reared on faba bean and alfalfa, and sorted into alates and apterae. These were individually homogenized in 120 μl of buffer, centrifuged at 10,000 g for 5 minutes, and 100 μl of the supernatant used in FAS assays. The differences between aphids reared on different hosts independent of morph, or between morphs independent of host, were not statistically significant (Figure 3), though alfalfa alates were different from alfalfa apterae. Accordingly, unsorted aphids reared on faba bean were used for subsequent work, as these were the easiest to raise in large quantities.

CYTOSOLIC FAS PURIFICATION

Purification of cytosolic FAS began with homogenization of chilled aphids in 4° C buffer. Care was taken to not allow the homogenizer to churn air into the sample. After the low speed centrifugation the pellet could be seen to be layered, with the mitochondria

laying above cuticular fragments and under a thin loose layer of microsomes and lysosomes. A large amount of yellow lipid was caked at the top of the sample. The homogenate and all subsequent fractions had a yellow color. It was still quite strong in the 30-40% pellet. The yellow color was mostly removed by gel filtration, where it moved somewhat behind the column volume, indicating interaction with the Sepharose. It is apparently a charged compound, as it bound almost irreversibly to DEAE Sephacel, and migrated at the dye front in SDS-PAGE electrophoresis. Until the final purification step, a faint yellow color remained with the protein. This yellow coloration was also reported by Municio et al. (1977) for preparations of Mediterranean Fruitfly FAS.

The 100,000 g centrifugation generated a tightly packed microsomal pellet with a trace of mitochondria at the bottom of the pellet, and an additional small amount of lipid cake. The ammonium sulfate precipitation of 100,000 g supernatant precipitated very little activity until 30% ammonium sulfate was reached, and none remained in solution at 40% ammonium sulfate (Figure 4a). The specific activity was highest for the 30% to 35% pellet (Figure 4b). The 30% to 40% cut was selected for further purification, which gave a 9.4 fold increase in specific activity from this step. At this point the purification procedure diverged between scheme A and scheme B.

SCHEME A

Scheme A proceeded with Sepharose 6B gel filtration which showed the bulk of the activity eluting between fractions 48 and 60, with the peak of activity in fraction 52 (Figure 5). This gave a 1.3 fold increase in specific activity. The highest activity fractions were loaded on a Reactive Red Sepharose column and eluted with a KCl gradient (Figure 6). This gave another 1.3 fold increase in specific activity. The highest activity

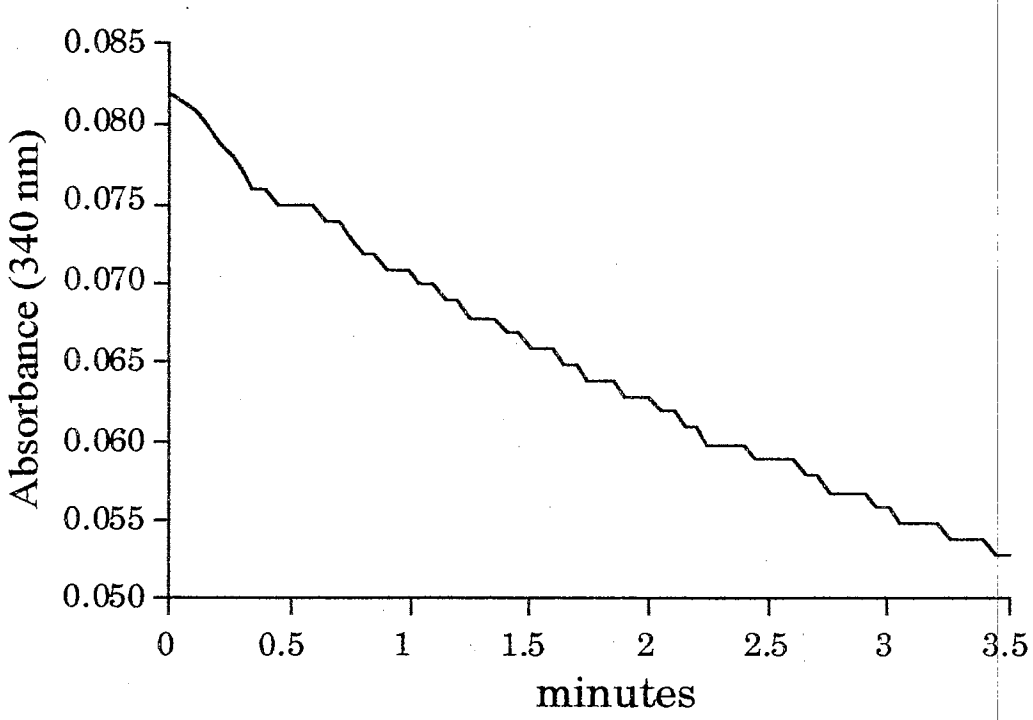


Figure 1. FAS Assay Linearity over Time. The linearity of decrease in absorbance at 340 nm. The reaction was in pH 7.4 buffer, 100 mM NADPH, 60 mM acetyl-CoA, 400 µl final volume. The reaction was started by adding 10 µl of 2.4 mM malonyl-CoA (60 µM final concentration) and the absorbance was measured at 3 second intervals.

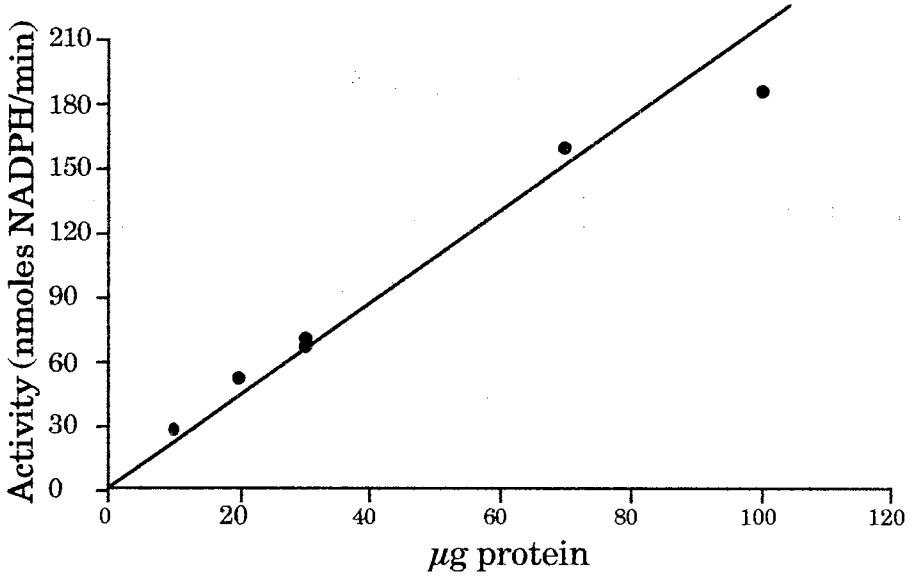


Figure 2. Linearity of Activity with Protein Concentration.. The linearity of response was monitored by adding various amounts of partially purified cytosolic FAS. The reaction was in pH 7.4 buffer, 100 mM NADPH, 60 mM acetyl-CoA, 400 µl final volume. The reaction was started by adding 10 µl of 2.4 mM malonyl-CoA (60 µM final concentration).

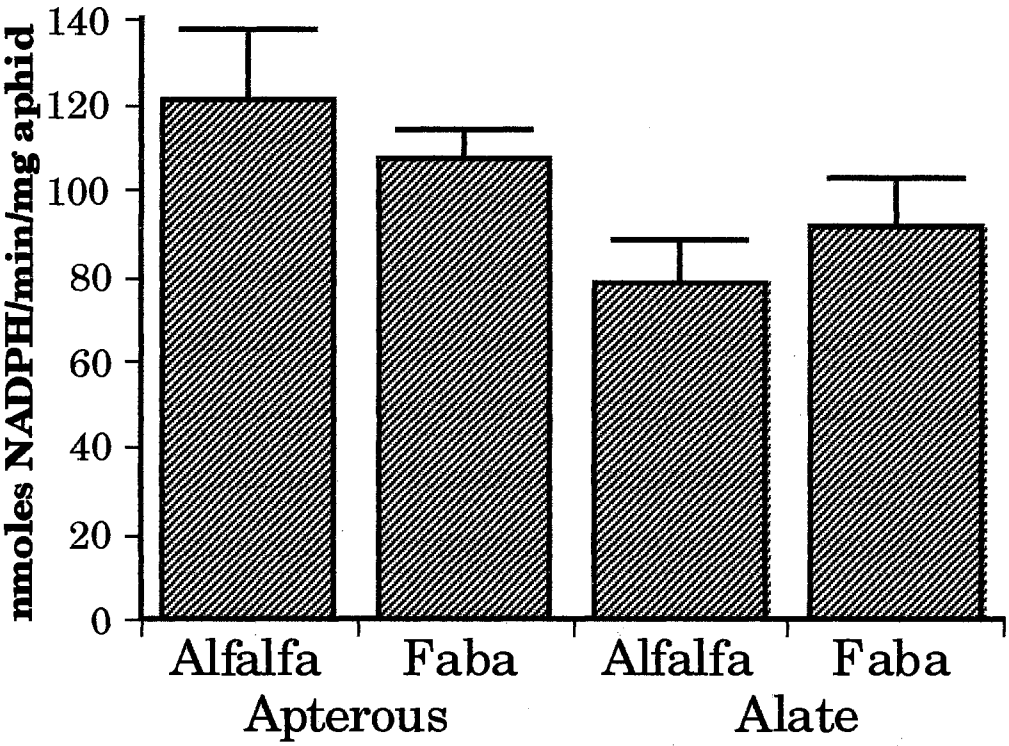


Figure 3. Morph and Host Influence on FAS Activity. Aphids raised on faba and alfalfa were separated into morphs, weighed, and individually homogenized in 120µl buffer. 100 µl of the 10,000g supernatant was used in a standard FAS assay.

fractions were desalted by dialysis against 2 liters of cold buffer overnight. There was some loss of both protein and specific activity in this step. The desalted fractions were loaded onto a DEAE Sephacel column and eluted with KCl (Figure 7) with the FAS actaivity again corresponding to the absorbance peak at 280 nm. The most activefractions were pooled, resulting in a 1.2 fold increase of specific activity. The overall purification gave a final specific activity of 3474 nmoles NADPH/minute/mg protein, an 87 fold purification, and a recovery of 9.5 percent. A typical run is detailed in Table I, and a gel is pictured in Figure 8. Storage of this fraction at -20° C for two days in 10% glycerol resulted in significant breakdown as shown in Figure 9

SCHEME B

Scheme B took the 30-40% ammonium sulfate cut and redissolved it in 40 ml pH 7.4 buffer. This was loaded on a 2.5 x 20 cm Reactive Red Sepharose column at 1 ml/min.

The column was washed with 60 ml buffer, and the activity eluted with a KCl gradient (Figure 10). The active fractions were reprecipitated with 50% ammonium sulfate, and loaded in 200 µl aliquots on a 1 x 20 cm Superose 6 column. The FAS was eluted at 0.1 ml / min with pH 7.4 buffer and 300 µl fractions were collected (Figure 11). Gels of the collected fractions are shown in Figure 12. Multiple runs were made and the fractions pooled. This procedure resulted in a specific activity of 3349 units/mg, a 16 fold purification, and a 9.4% recovery. A typical run is detailed in Table II, and a gel pictured in Figure 13.

DETERMINATION OF MOLECULAR WEIGHT

The M_r of Scheme A purified cytosolic FAS was determined by Sepharose 6B gel filtration, and found to be 231 KDa for the monomer (Figure 14). Under the salt and temperature conditions used, cytosolic FAS was dissociated. Laemmli 5% SDS-PAGE gave a subunit M_r of 228 KDa (Figure 15). This exceeded the largest standard available, so Weber-Osborn PAGE with a 5% gel was run, giving 234 KDa (Figure 16). Scheme B purification gave a result of 256 KDa for the monomer with Superose 6 gel filtration (Figure 17). Under the higher salt content of the Superose buffer, as recommended by Pharmacia, FAS was recovered both as the dimer and monomer, with the dimer being 495 KDa.

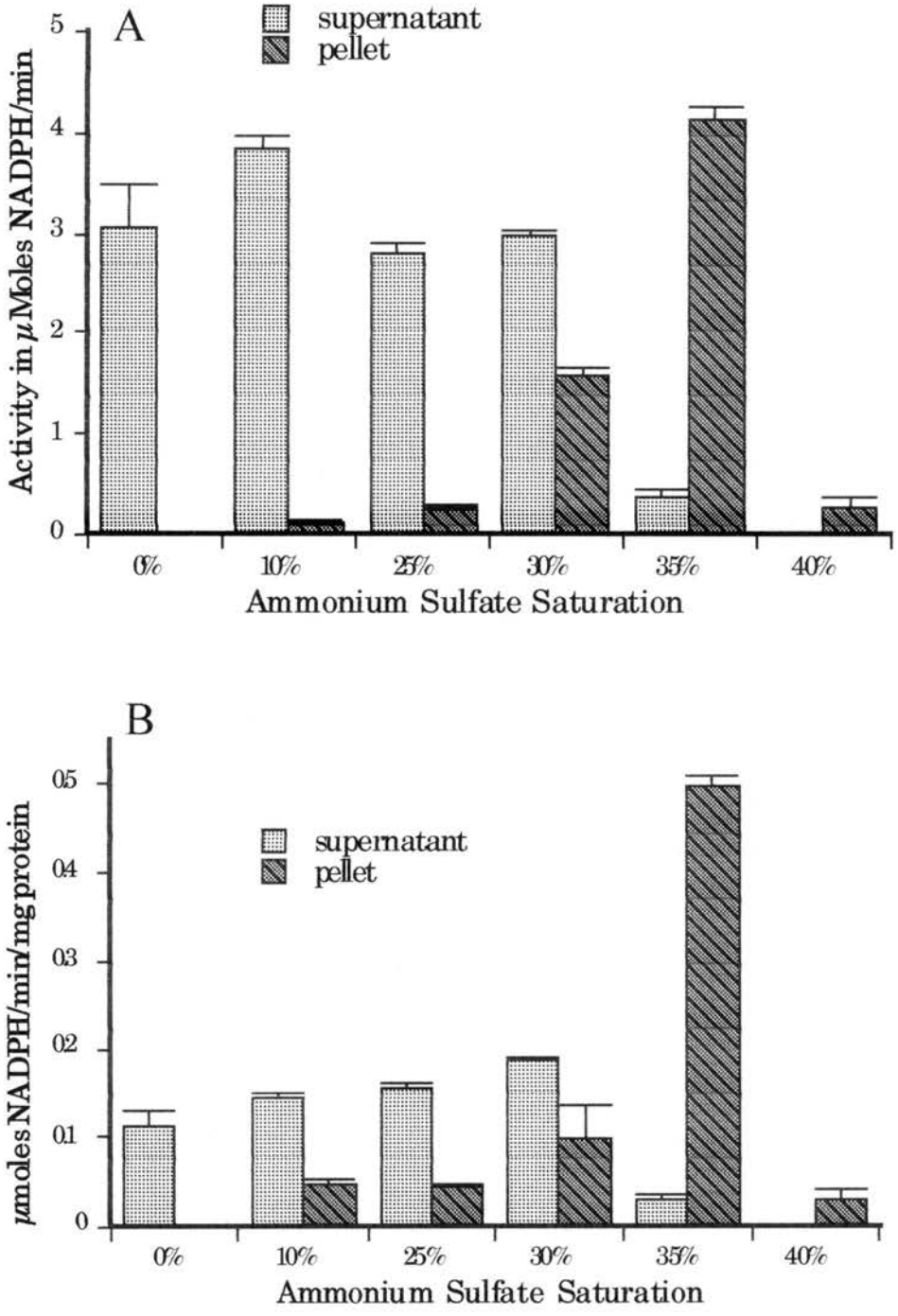


Figure 4. Cytosolic FAS Activity of Ammonium Sulfate Cuts (A) Total activity. (B) Specific activity. 100,000 g supernatant of pea aphid homogenate was subjected to increasing concentrations of ammonium sulfate. Each addition was followed by stirring on ice for 30 min, and 10,000 g centrifugation to separate out the precipitate. The precipitate was redissolved in 1 ml of buffer and the standard assay used.

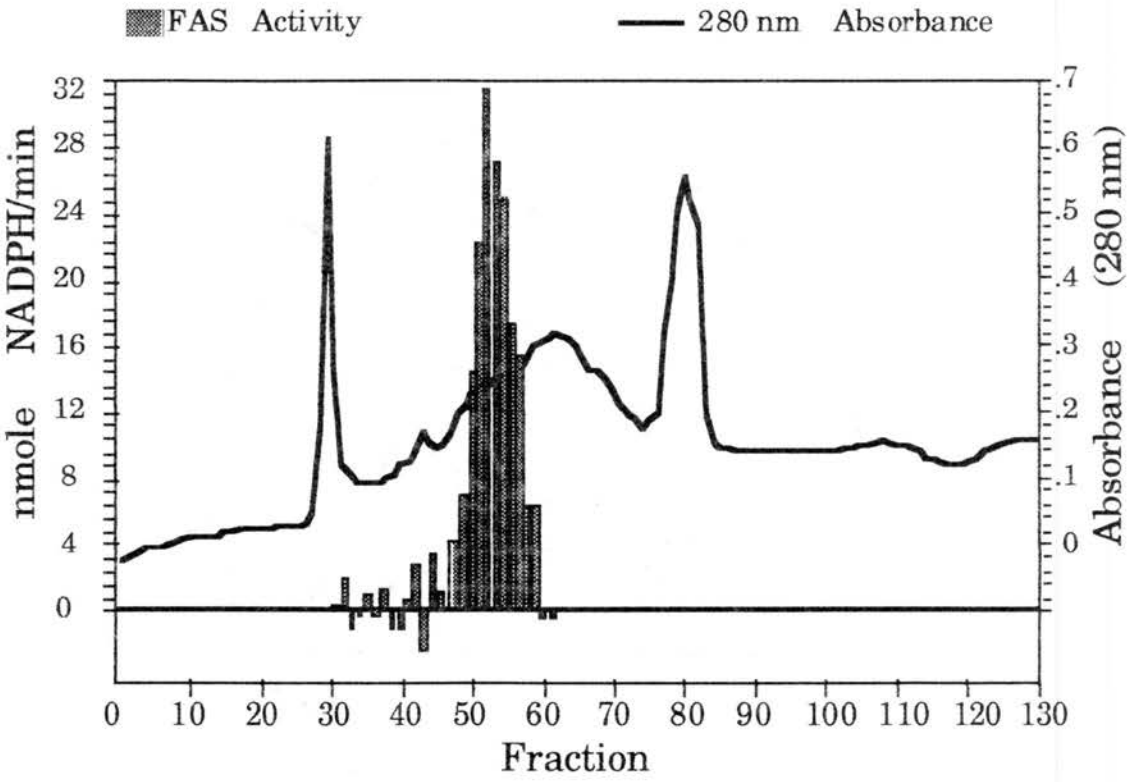


Figure 5. Sepharose 6B Gel Filtration of Cytosolic FAS, Scheme A. The 30%-40% ammonium sulfate precipitate was redissolved in 2 ml buffer. It was then loaded and run at 1 ml/min on a 2.5 x 90cm Sepharose 6B column. 2 ml fractions were collected. 100 µl aliquots of fractions were tested using the standard assay.

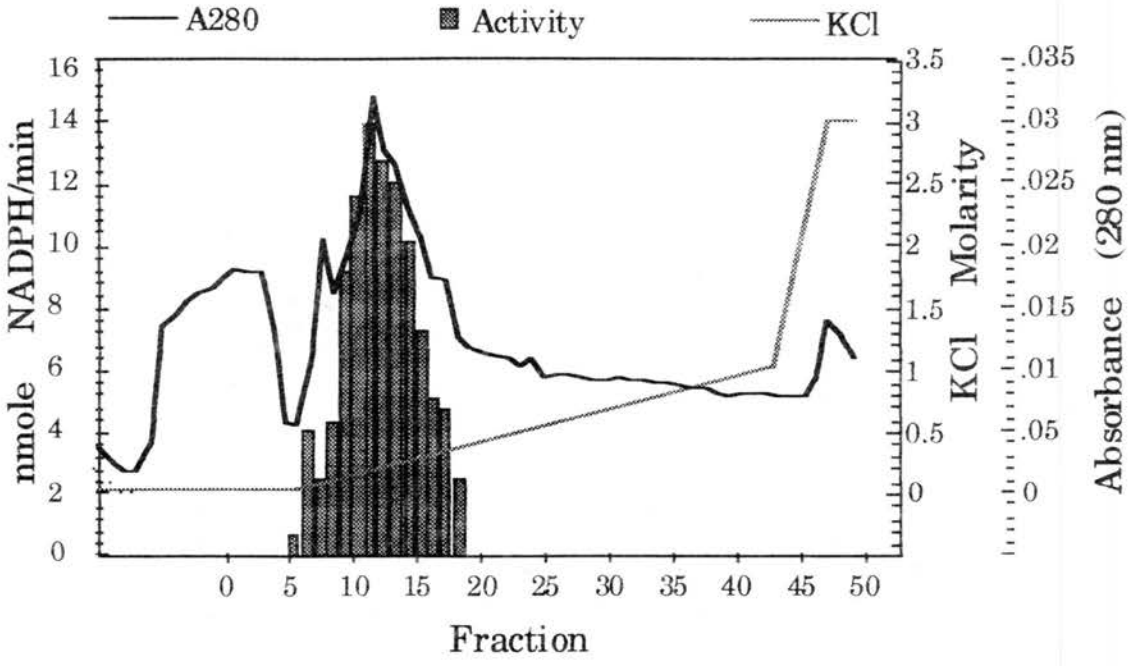


Figure 6. Reactive Red Sepharose Gel Filtration of Cytosolic FAS, Scheme A. Active fractions from Sepharose 6B were loaded at 0.5 ml/min onto a 1 x 15 cm Reactive Red column. The column was washed with 15 ml pH 7.4 50 mM phosphate buffer and the activity eluted with a KCl gradient. 2 ml fractions were collected. Activity was monitored using 50 µl of each fraction in the standard assay.

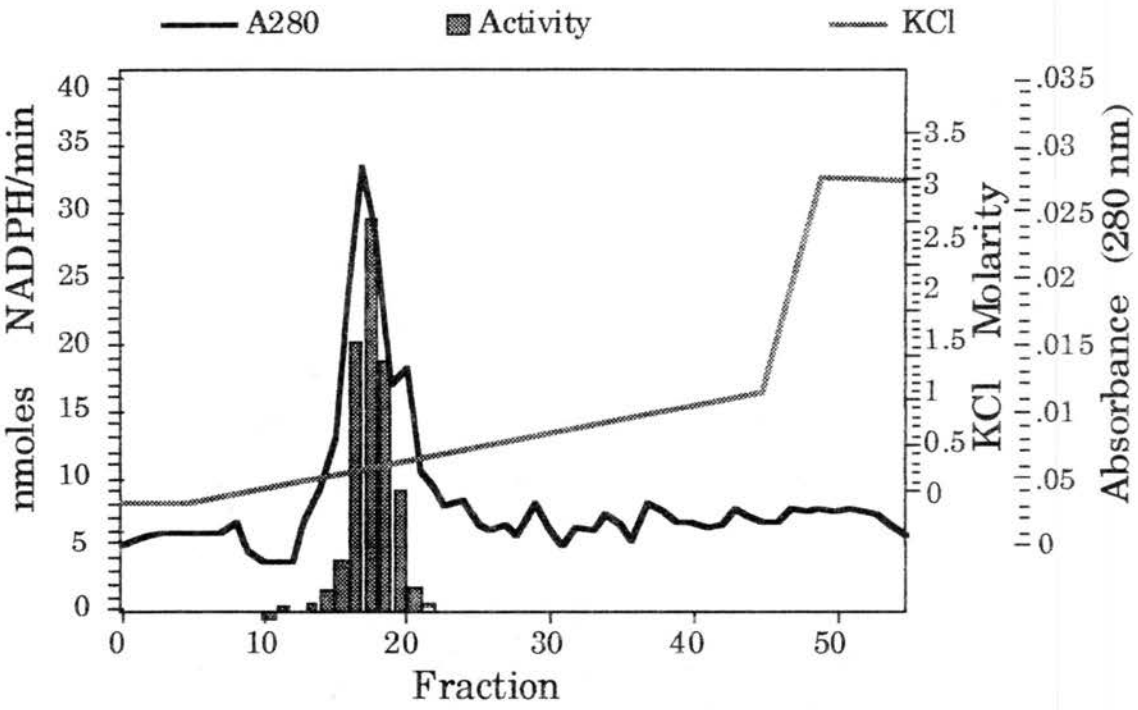


Figure 7. DEAE Sephacel Gel Filtration of Cytosolic FAS, Scheme A. Active fractions from Reactive Red Sepharose were desalted and loaded at 0.5 ml/min onto a 2 x 15 cm column. The column was washed with 15 ml pH 7.4 50 mM phosphate buffer, and the activity eluted with a KCl gradient. 2 ml fractions were collected. Activity was monitored using 50 µl of each fraction in the standard assay.

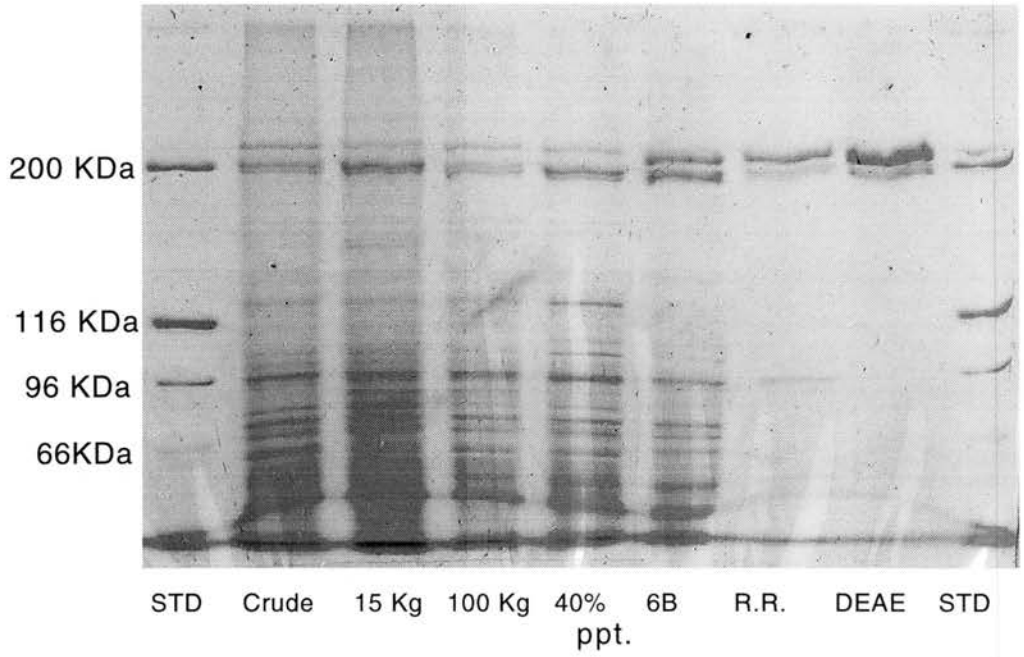


Figure 8. 5% SDS-PAGE Gel of Cytosolic FAS Scheme A Purification. The fractions are the crude homogenate (crude), the 15,000 g supernatant (15 Kg), the 100,000 Kg supernatant (100 Kg), the 30-40% ammonium sulfate pellet (40% ppt.), the combined Sepharose 6B fractions (6B), the combined Reactive Red Sepharose fractions (R.R.), and the combined DEAE Sephacel fractions (DEAE). The standards (STD) are myosin (200 KDa), beta-galactosidase (116 KDa), phosphorylase b (96 KDa), and bovine serum albumin (66 KDa).

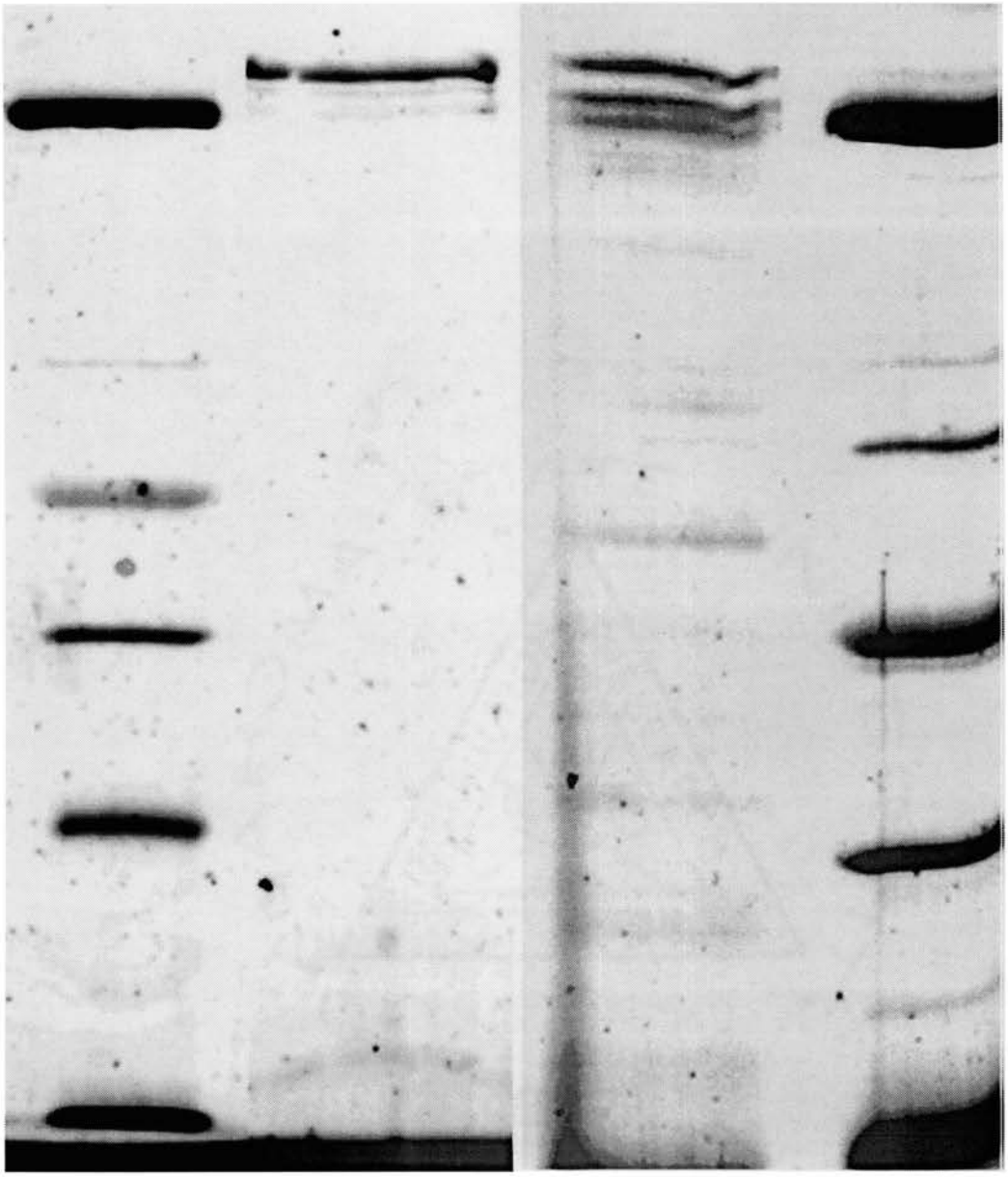


Figure 9. 5% SDS-PAGE Gels Showing the Effect of Freezing on the Integrity of Purified Cytosolic FAS. An aliquot of scheme A purified cytosolic FAS was run on a 5% SDS-PAGE gel, and the balance of the sample was stored at -20° C for 48 hours in pH 7.4 buffer containing 10% glycerol. The sample was then run again under the same conditions. (A) unfrozen (B) post freezing. The standards (Std) are myosin (200 KDa), beta-galactosidase (116 KDa), phosphorylase b (96 KDa), and bovine serum albumin (66 KDa).

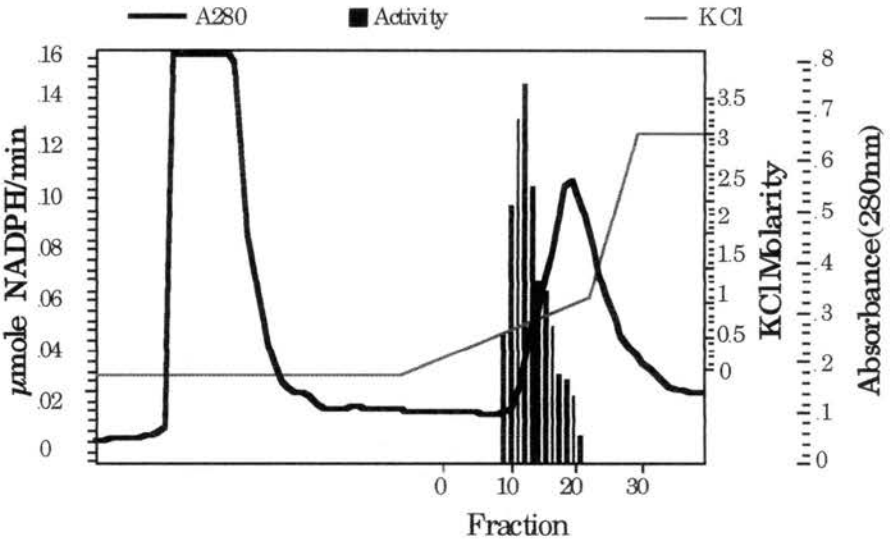


Figure 10. Reactive Red Sepharose Purification of Cytosolic FAS, Scheme B. Redissolved 30% to 40% ammonium sulfate pellet was loaded on a 2.5 x 20 cm column at 1 ml/min, washed with 60 ml pH 7.4 buffer, and eluted with a KCl gradient. 5 ml fractions were collected and 50 µl of each fraction was measured for activity by the standard assay.

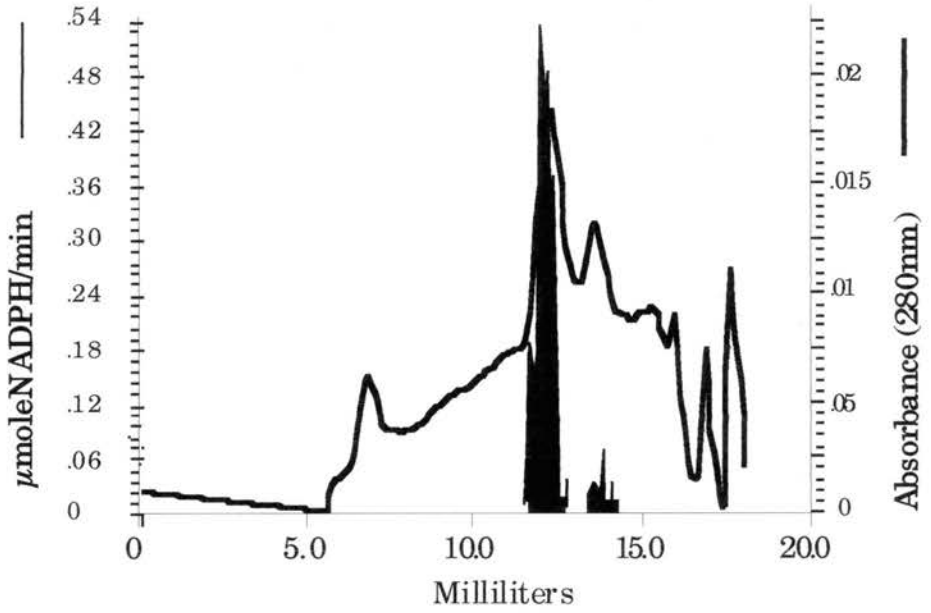


Figure 11. Superose 6 Gel Filtration of Cytosolic FAS, Scheme B. Ammonium sulfate precipitated FAS from the Reactive Red step was loaded on a 1 x 20 cm column and eluted with pH 7.4 buffer at 0.25 ml/min. 0.3 ml fractions were collected, multiple runs pooled, and 50 µl of pooled fractions measured for FAS activity using the standard assay.

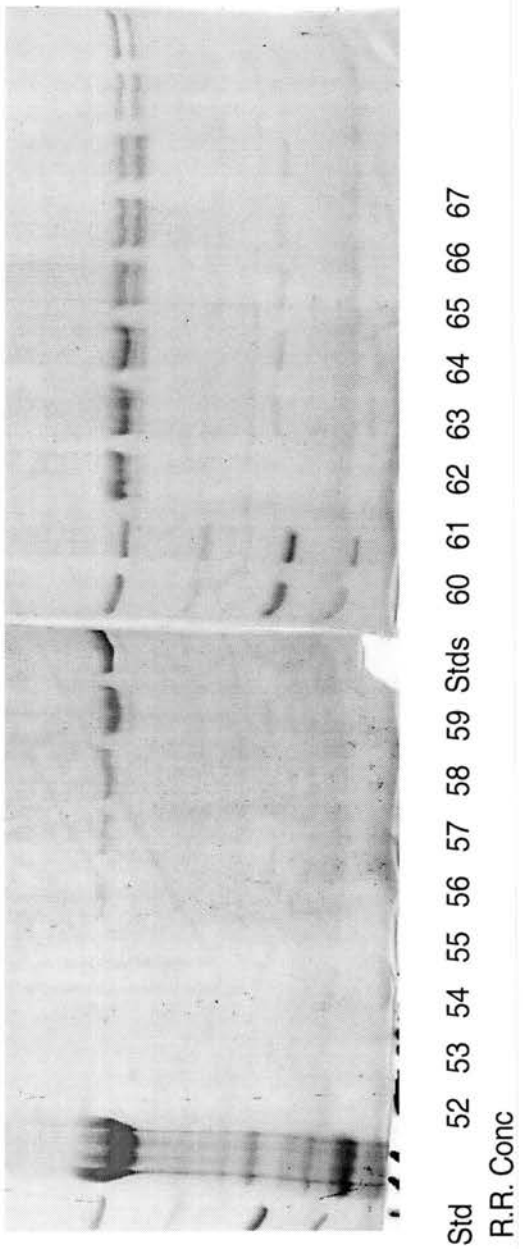


Figure 12. The Scheme B Superose 6 Purification of Cytosolic FAS: A 5 % SDS-PAGE Gel of the Collected Fractions. 20 µl of the fractions from a Superose 6 purification of cytosolic FAS were loaded on a 5% SDS-PAGE gel. The standards (Std) are myosin (200 KDa), beta-galactosidase (116 KDa), phosphorylase b (96 KDa), and bovine serum albumin (66) KDa

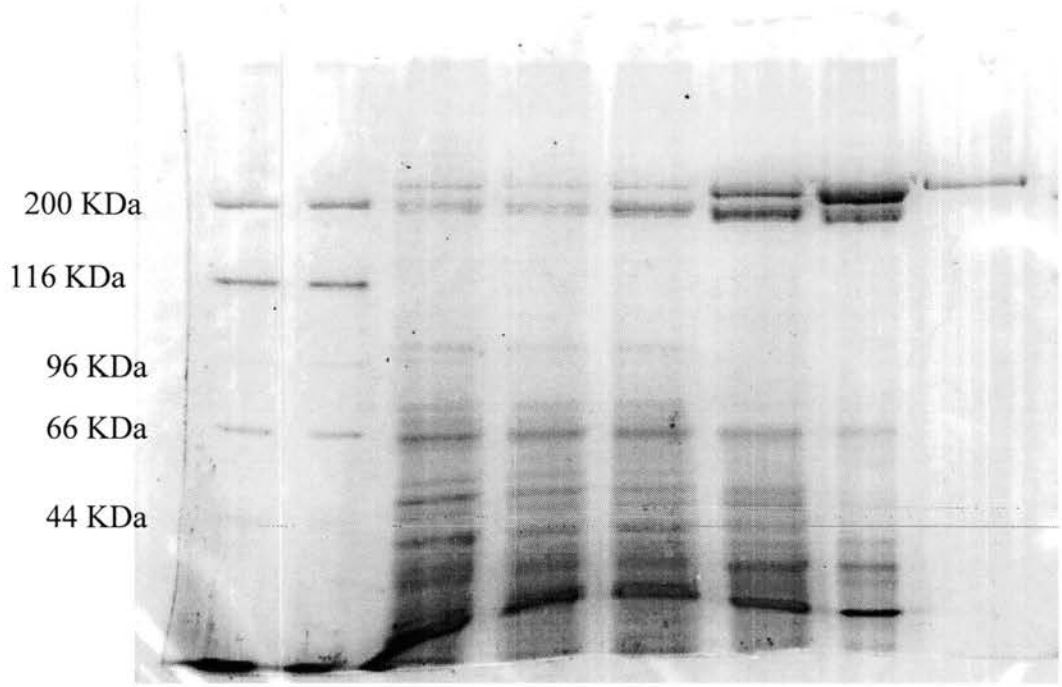


Figure 13. 5% SDS-PAGE Gel of Cytosolic FAS Purification, Scheme B. The fractions are the crude homogenate (Crude), the 10,000 g supernatant (10 Kg), the 100,000 g supernatant (100 Kg), the 30-40% ammonium sulfate pellet (40% ppt.), the combined Reactive Red Sepharose fractions (R.R.), and the combined Superose 6 fractions (Sup. 6). The standards are myosin (200 KDa), beta-galactosidase (116 KDa), phosphorylase b (96 KDa), bovine serum albumin (66 KDa) and ovalbumin (44 KDa).

Table I. Scheme A Cytosolic FAS Purification Table

Fraction	Volume (ml)	Protein mg/ml	Protein mg	Specific Activity units/mg	Total Units	% Recov.	Purif. factor
Crude Homog.	37	9.04	334.5	40	13,383	-	1
15,000 g Sup.	32	4.72	151	123	18,578	100	3.1
100,000 g Sup.	35.64	2.55	90.9	164	14,907	80.2	4.1
30% Sup.	35.64	1.84	65.57	249	16,328	87.9	6.2
40% ppt	1.435	3.5	5	1540	7,700	41.4	38.5
6B-CL	59.4	.0457	2.7	2408	6501	35	60.2
Reactive Red	34.77	.0358	1.24	3206	3975	21.4	80.2
R. R. Desalted	20	.04	.8	3016	2413	12.98	75.4
DEAE	7	.0732	.51	3474	1772	9.5	86.9

Table II Scheme B Cytosolic FAS Purification Table

Fraction	Volume (ml)	Protein mg/ml	Protein mg	Specific Activity units/mg	Total Units	% Recovery	Purif. factor
Crude Homog	150	35.8	5370	215	1,154,550	—	1
15,000 g Sup.	128	22.35	2860	340	911,200	—	1.5
100,000 g Sup.	123	14.33	1763	795	1,401,585	100	3.7
30% Sup.	123	9.56	1176	874	1,027,824	73.3	4.1
40% ppt	12.9	12.2	157.4	1367	215,138	15.3	6.4
Reactive Red	62	1.174	72.78	2807	204,293	14.6	13
R. R.40 % ppt	1.7	63.1	63.9	2627	167,865	12	12.2
Superose 6	20.4	1.92	39.23	3349	131,381	9.4	16

DETERMINATION OF pH OPTIMUM AND PI

Determination of pH optimum and isoelectric point: The pH optimum for FAS was determined on both the 100,000 g supernatant and on the Scheme A purified FAS. The optimum for the supernatant was 7.5 (assayed in 0.5 pH unit increments), and 7.4 for the purified material (assayed in 0.1 pH unit increments) (Figure 18). The Pi was determined to be 7.194 using the Pharmacia Phast system with the pH 3 to 9 range gel (Figure 19). Pharmacia's recommended protocol was modified by reducing the loading voltage to 100 V and increasing the running time to 850 Vh. These changes were needed to prevent streaking and allow for equilibration of the large FAS molecule.

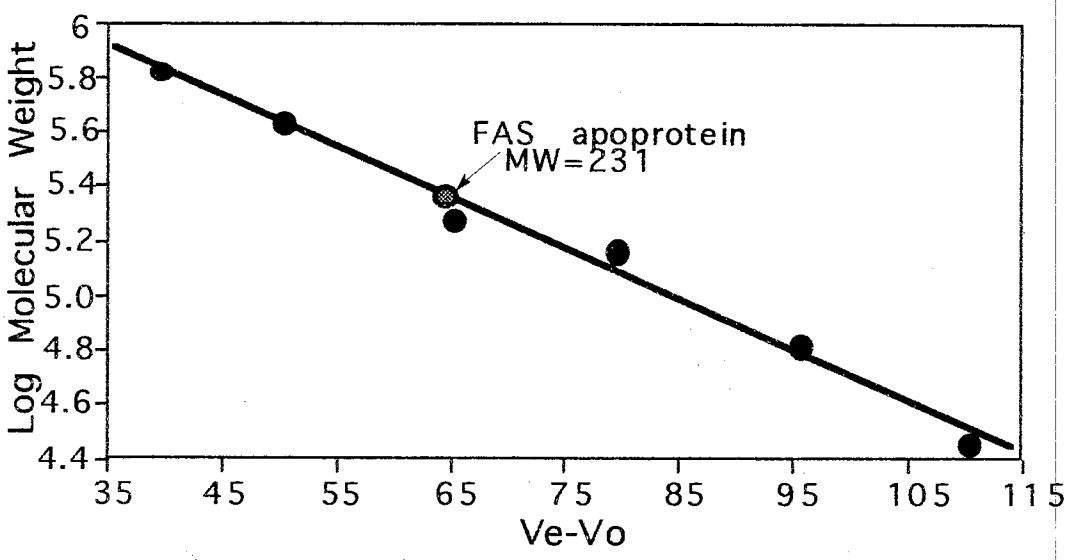


Figure 14. Sepharose 6B Gel Filtration Molecular Weight Determination of Cytosolic FAS. A sample of 40% ammonium sulfate precipitate was run at 0.5 ml/min on a 2.5 x 90 cm Sepharose 6B column in pH 7.4 buffer. Standards are carbonic anhydrase (29 KDa), bovine serum albumin (66 KDa), alcohol dehydrogenase (150 KDa), beta-amylase (200 KDa), apoferritin (443 KDa), and thyroglobulin (669 KDa). Sample was Sepharose 6B purified FAS.

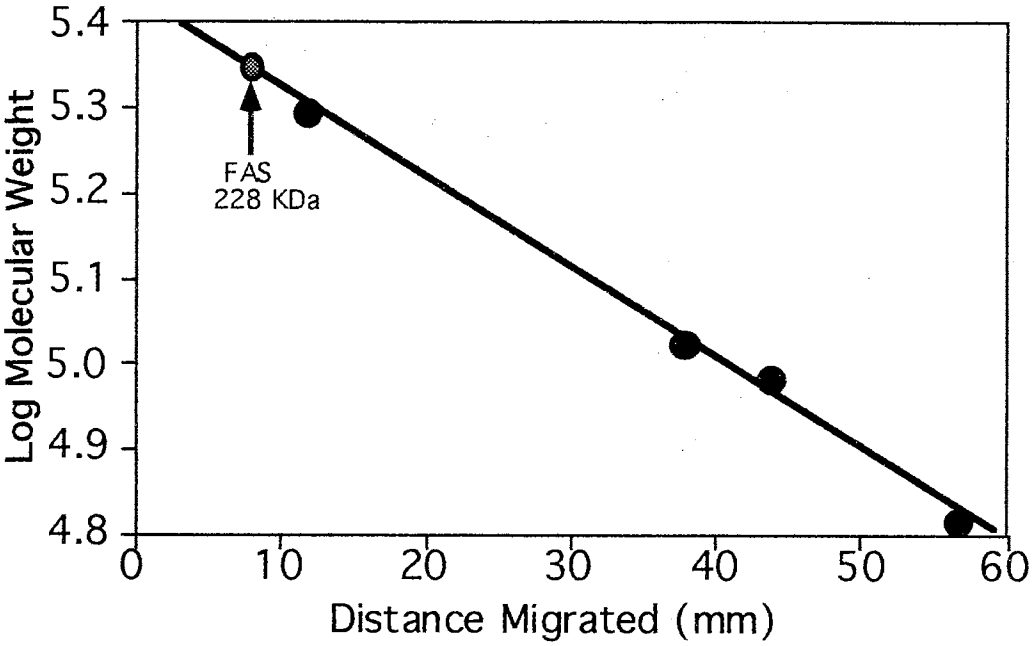


Figure 15. Molecular Weight Determination of Cytosolic FAS by Laemmli SDS-PAGE 5% Gel. The FAS was Sepharose 6B purified. Standards are myosin (200 KDa) beta-galactosidase (116 KDa), bovine serum albumin (97.4 KDa), and ovalbumin (45 KDa). Sample was Sepharose 6B purified FAS.

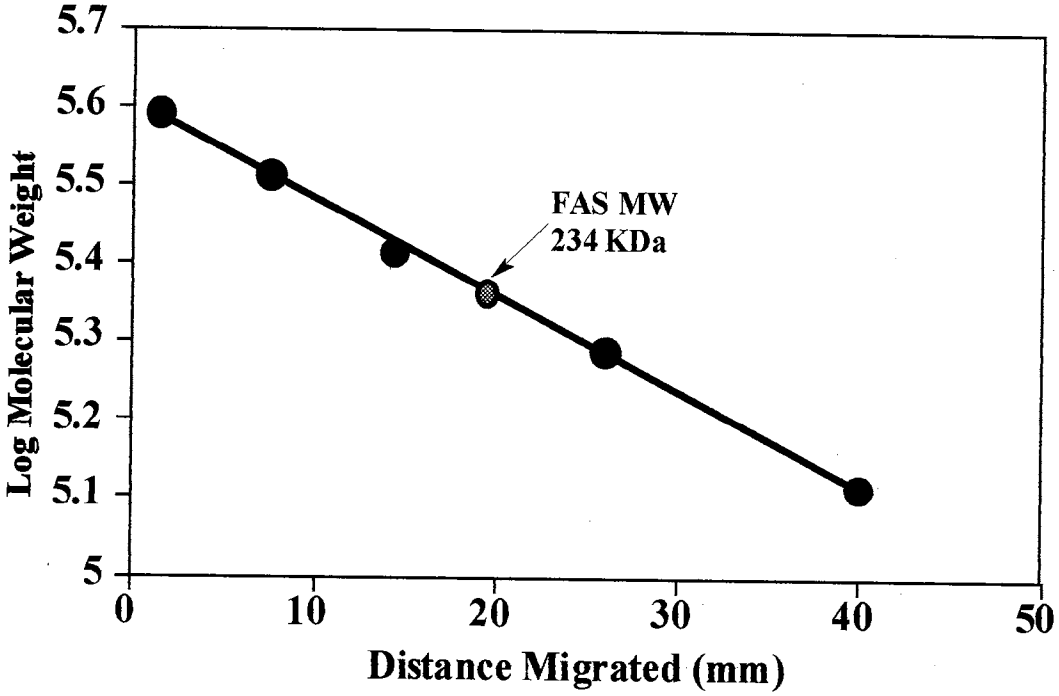


Figure 16. Molecular Weight Determination of Cytosolic FAS by Weber-Osborne PAGE 5% Gel. Standards are cross-linked multimers of bovine serum albumin, 132, 198, 264, 330, and 396 KDa. Sample was Sepharose 6B purified FAS.

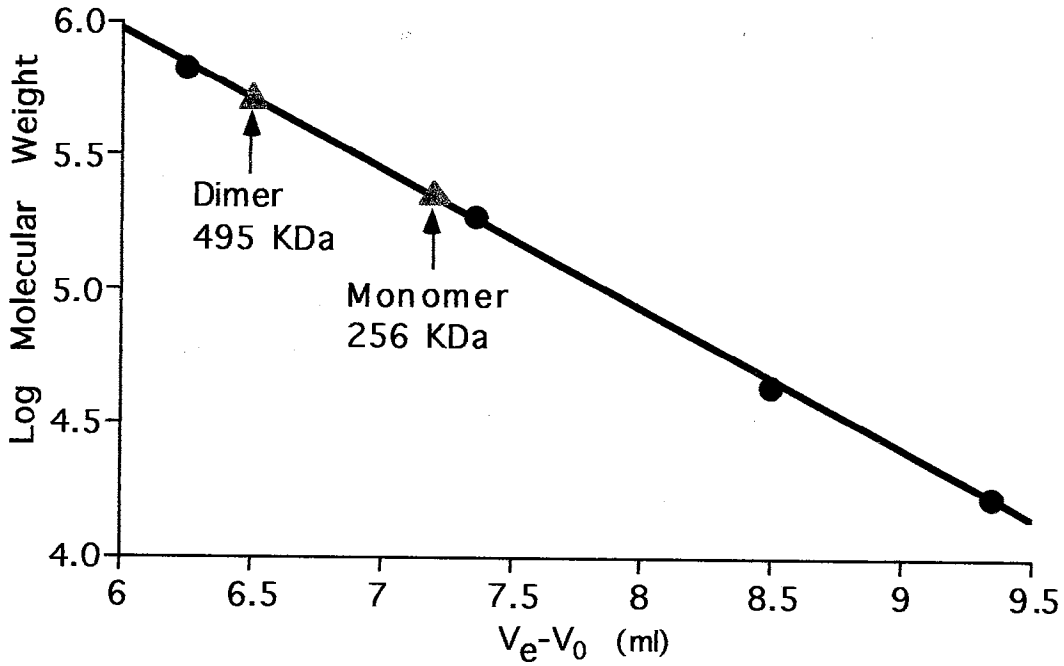


Figure 17. Molecular Weight Determination of Cytosolic FAS by Superose 6 Gel Filtration. Standards are thyroglobulin (670 KDa), gamma globulin (158 KDa), ovalbumin (44 KDa), and myoglobin (17 KDa). Sample was Scheme B Reactive Red purified FAS. Column was run at room temperature.

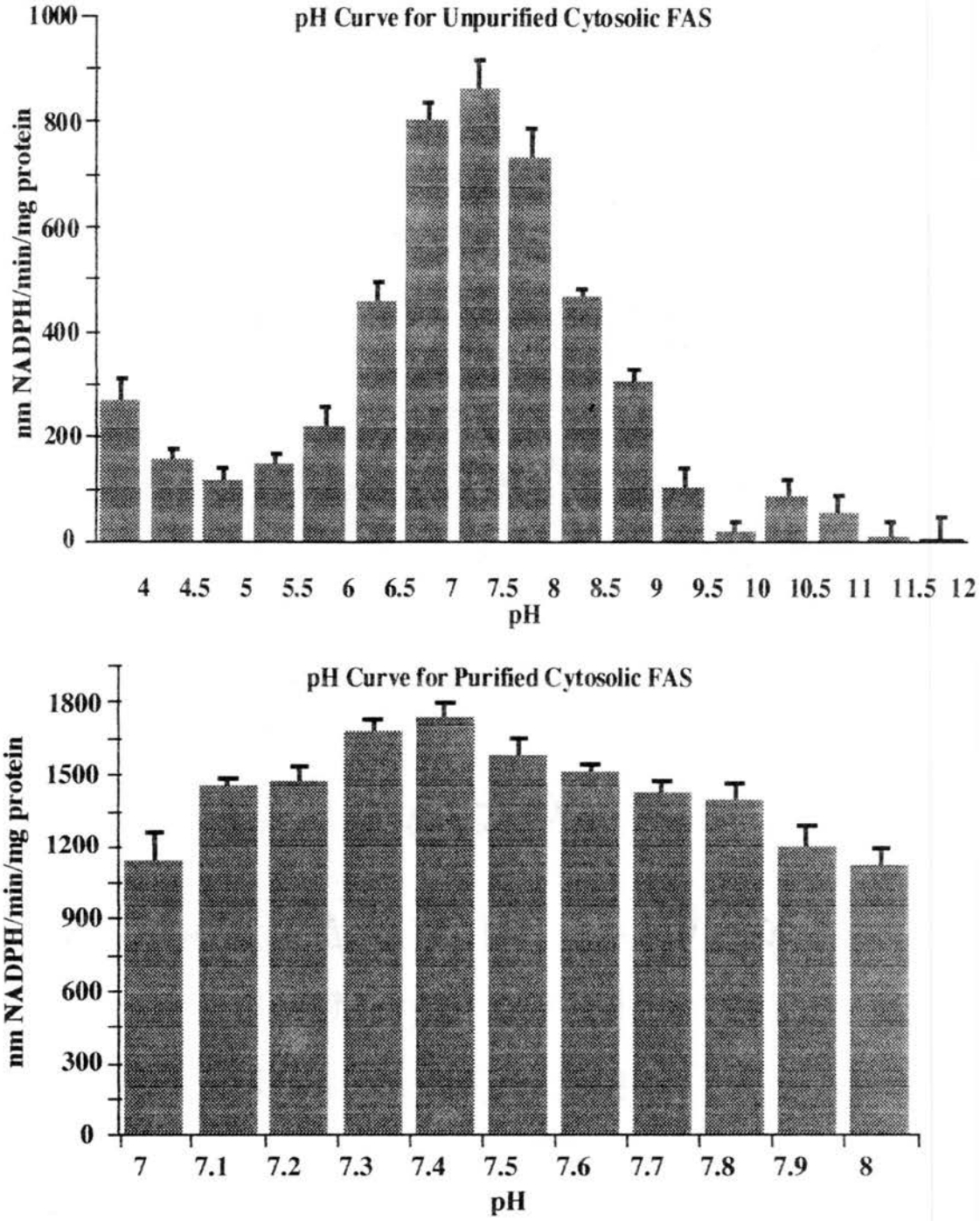


Figure 18. pH Optimum of Cytosolic FAS. Standard Assays were conducted using 0.2 M buffers of the various pH. See Materials and Methods for buffers and substrate concentrations used. The unpurified FAS assay was conducted on 100,000 g supernatant, and the assay of purified FAS on Scheme A purified FAS protein.

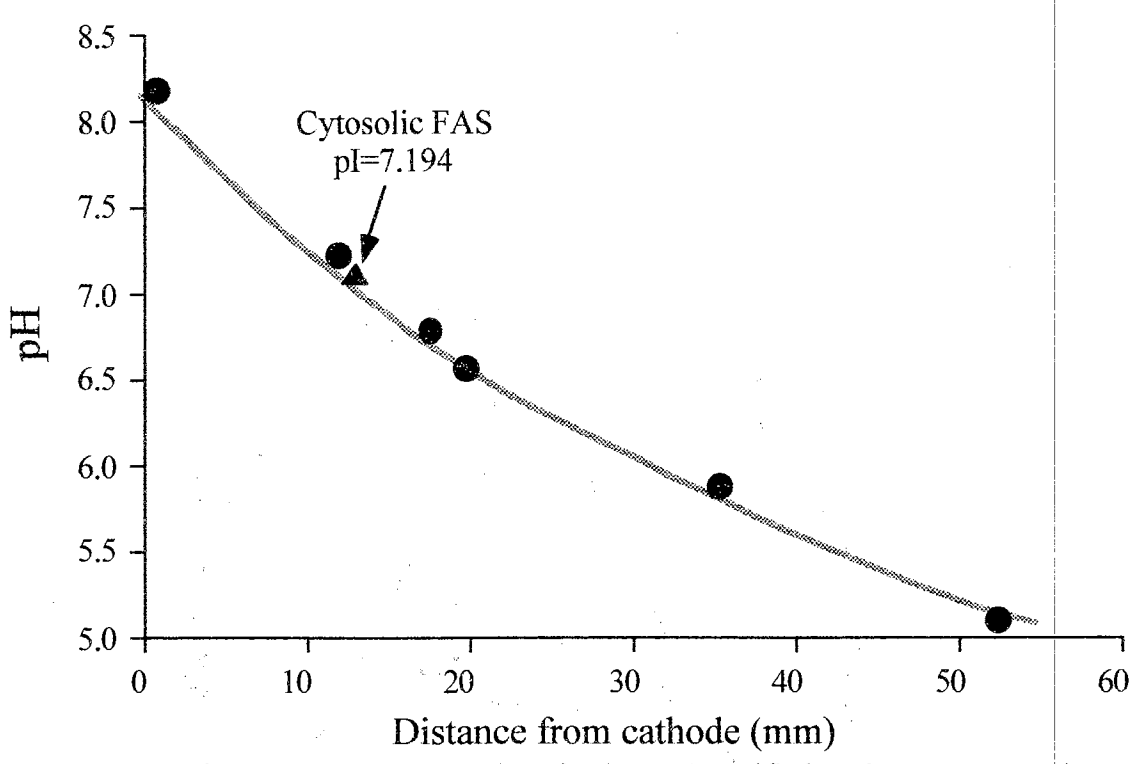


Figure 19. pI of Cytosolic FAS. Samples of Scheme A purified FAS were run on the Pharmacia Phast system on a pH 3-9 gel as per manufacturers protocols with Pharmacia isoelectric focusing standard.

KINETIC CHARACTERISTICS OF CRUDE AND PURIFIED CYTOSOLIC FAS

The kinetic parameters were determined by spectrophotometric assay as detailed in Materials and Methods. Maximum velocities (V_{max}) and Michaelis constants (K_m) were calculated for both the crude and partially purified enzyme. Hanes-Woolf plots for acetyl-CoA, malonyl-CoA, and NADPH showed similar K_m's and V_{max}'s between the crude and purified forms (Figures 20 and 21). Methylmalonyl-CoA could not be used by cytosolic FAS as the sole elongation substrate. Figure 22 compares the saturation curves for malonyl-CoA and methylmalonyl-CoA for both crude and purified enzymes.

Increasing concentrations of acetyl-CoA showed a hyperbolic increase in activity of cytosolic FAS (Figures 23A and 24A). Methylmalonyl-CoA acted as an inhibitor, lowering the reaction rate. It acted as both a competitive and uncompetitive inhibitor, as shown by the effect on both slope and intercept in the Hanes-Woolf plots (Figures 23B and 24B). The K_m for the crude and purified forms was 10 μM and 6.6 μM respectively. The replots (Figures 25 and 26) give the competitive inhibition constant K_i as 11.4 μM for the crude form and 11.6 μM for the purified, and the uncompetitive inhibition constant K'_i as 8.4 μM for the crude and 9.5 μM for the purified.

Malonyl-CoA showed similar effects (Figures 27 and 28). The K_m's were 5.7 μM and 5 μM for the crude and purified enzyme respectively. Methylmalonyl-CoA acted as aa purely competitive inhibitor (Figures 29 and 30). The K_i was 16 μM for the crude and 14 μM for the purified.

The NADPH K_m for the crude enzyme was 20.6 μM and 5.8 μM for the pure enzyme. The replots show there is no competitive inhibition (Figure 33 and 34) in line with the proposed mechanisms for this enzyme. They show the inhibition to be purely uncompetitive with a K'_i of 11 μM for the crude enzyme and 8 μM for the purified. The kinetic results are summarized in Table III.

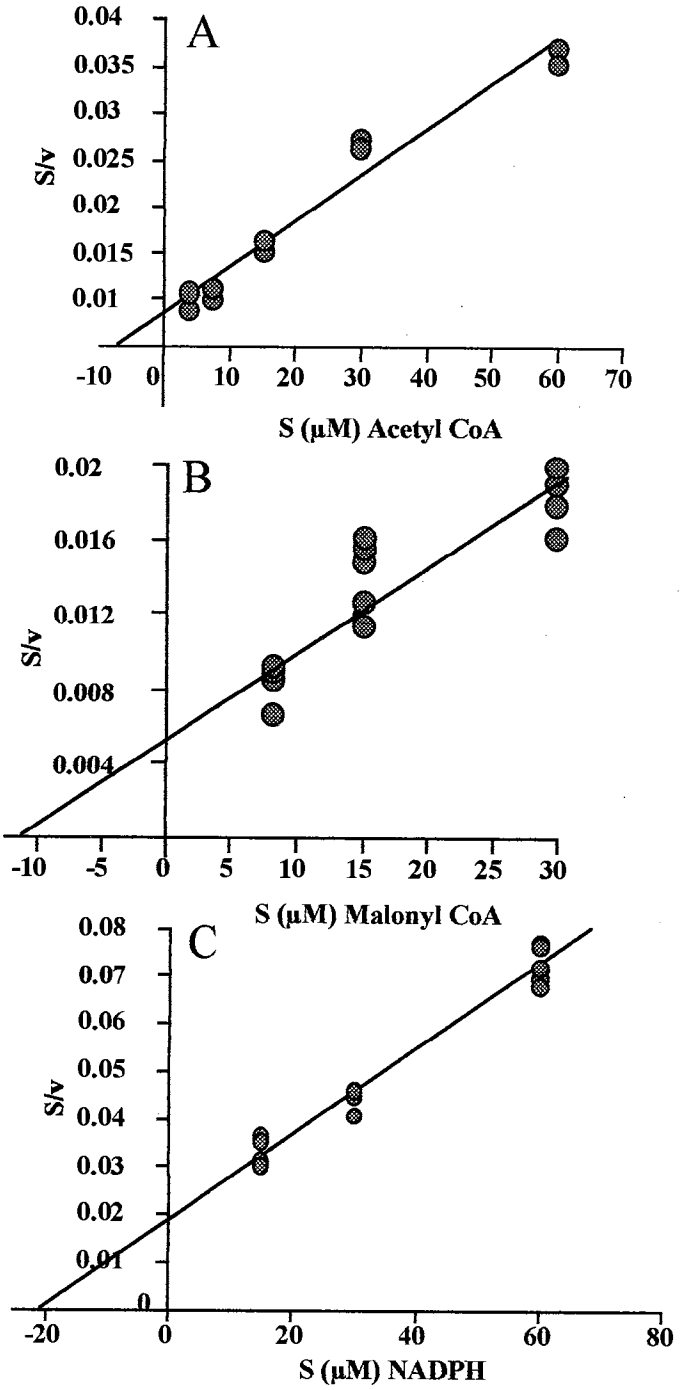


Figure 20. Hanes-Woolf Plots of Crude Cytosolic FAS. Obtained by varying (A) acetyl-CoA, (B) malonyl-CoA, or (C) NADPH between 7.5 and 60 µM. The fixed substrate concentrations were 100 µM NADPH, 60 µM malonyl-CoA, and 60 µM acetyl-CoA in a final volume of 400 µl pH 7.4 buffer. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

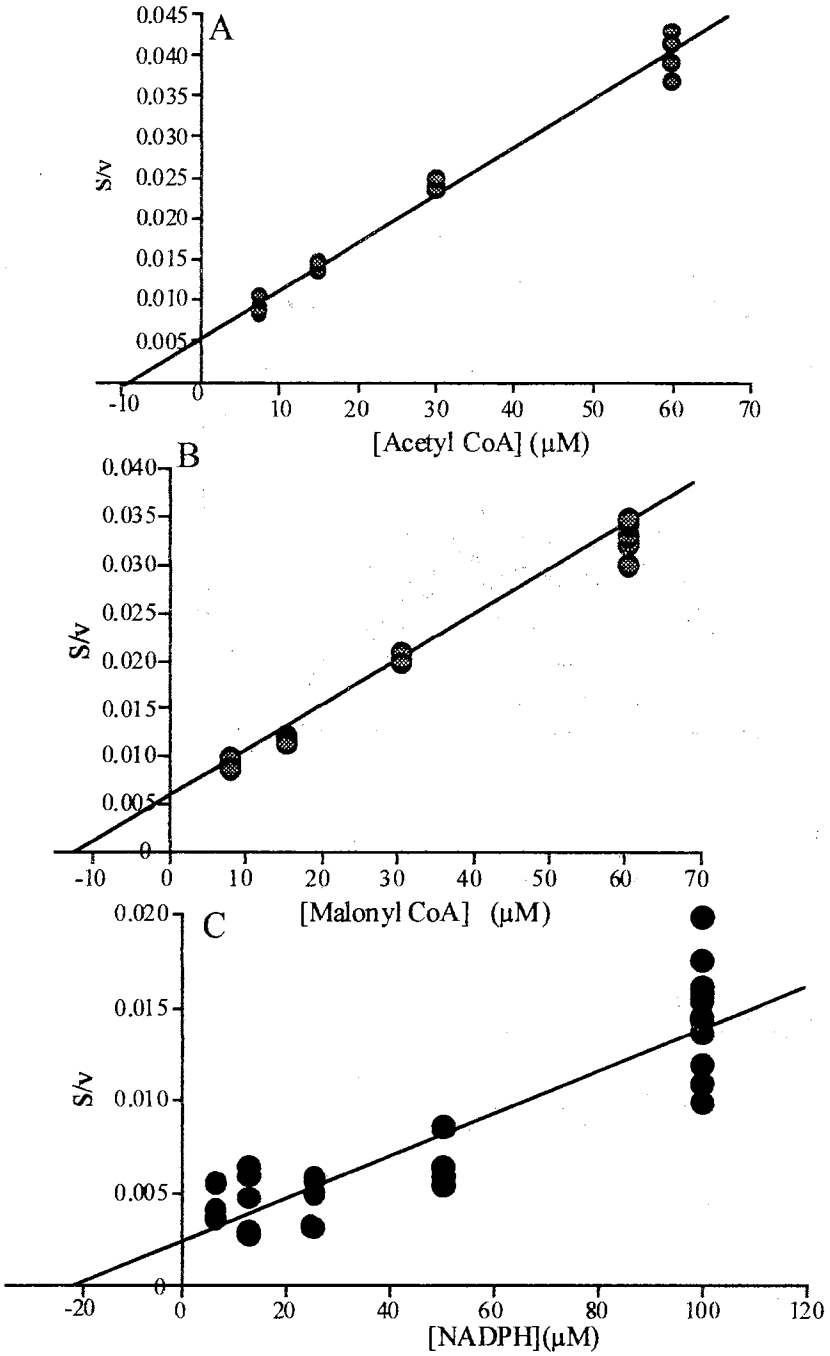


Figure 21. Hanes-Woolf Plots of Purified Cytosolic FAS. Obtained by varying (A) acetyl-CoA, (B) malonyl-CoA, or (C) NADPH between 7.5 and 60 μM. The fixed substrate concentrations were 100 μM NADPH, 60 μM malonyl-CoA, and 60 μM acetyl-CoA in a final volume of 400 μl pH 7.4 buffer. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

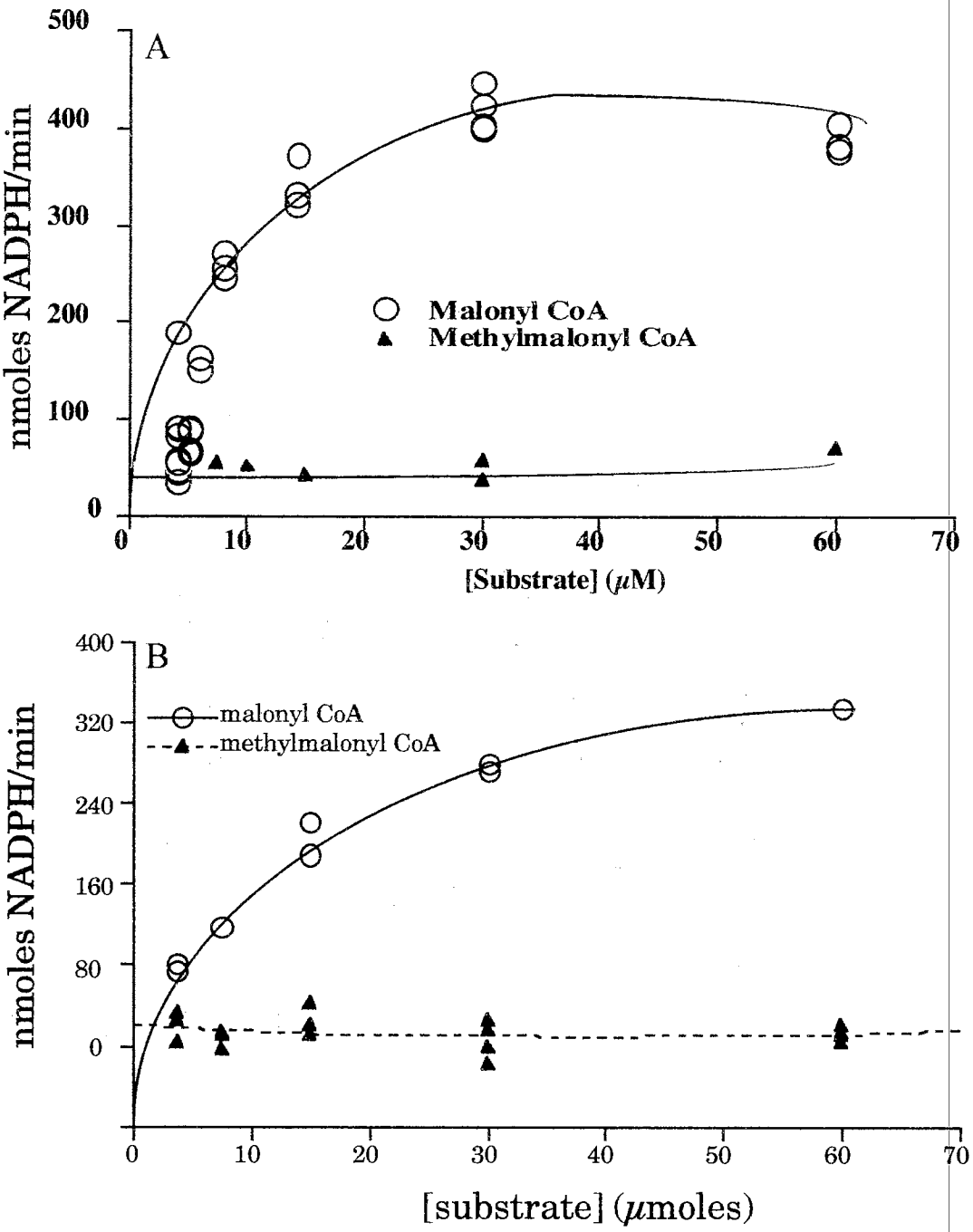


Figure 22. Saturation Curves of Malonyl and Methylmalonyl-CoA Using (A) Crude or (B) Purified Cytosolic FAS. Obtained by varying malonyl-CoA or methylmalonyl-CoA between 3.75 and 60 μM. The fixed substrate concentrations were 100 μM NADPH and 60 μM acetyl-CoA in a final volume of 400 μl pH 7.4 buffer. The reaction was started by adding malonyl-CoA or methylmalonyl-CoA to the reaction mixture and monitored spectrophotometrically.

Table III K_m and V_{max} for Crude and Purified Cytosolic FAS.

	K_m (μM)				V_{max} (μMoles/min/mg protein)			
FAS	NADPH	Acetyl–CoA	Malonyl –CoA	Methyl malonyl-CoA	NADPH	Acetyl–CoA	Malonyl –CoA	Methyl malonyl-CoA
crude	20.6	10	5.7	–*	91	142	27	–
purified	5.8	6.6	5	–	118	166	50	–

*Reaction rates with methylmalonyl-CoA as the sole elongating substrate were too low to be measured.

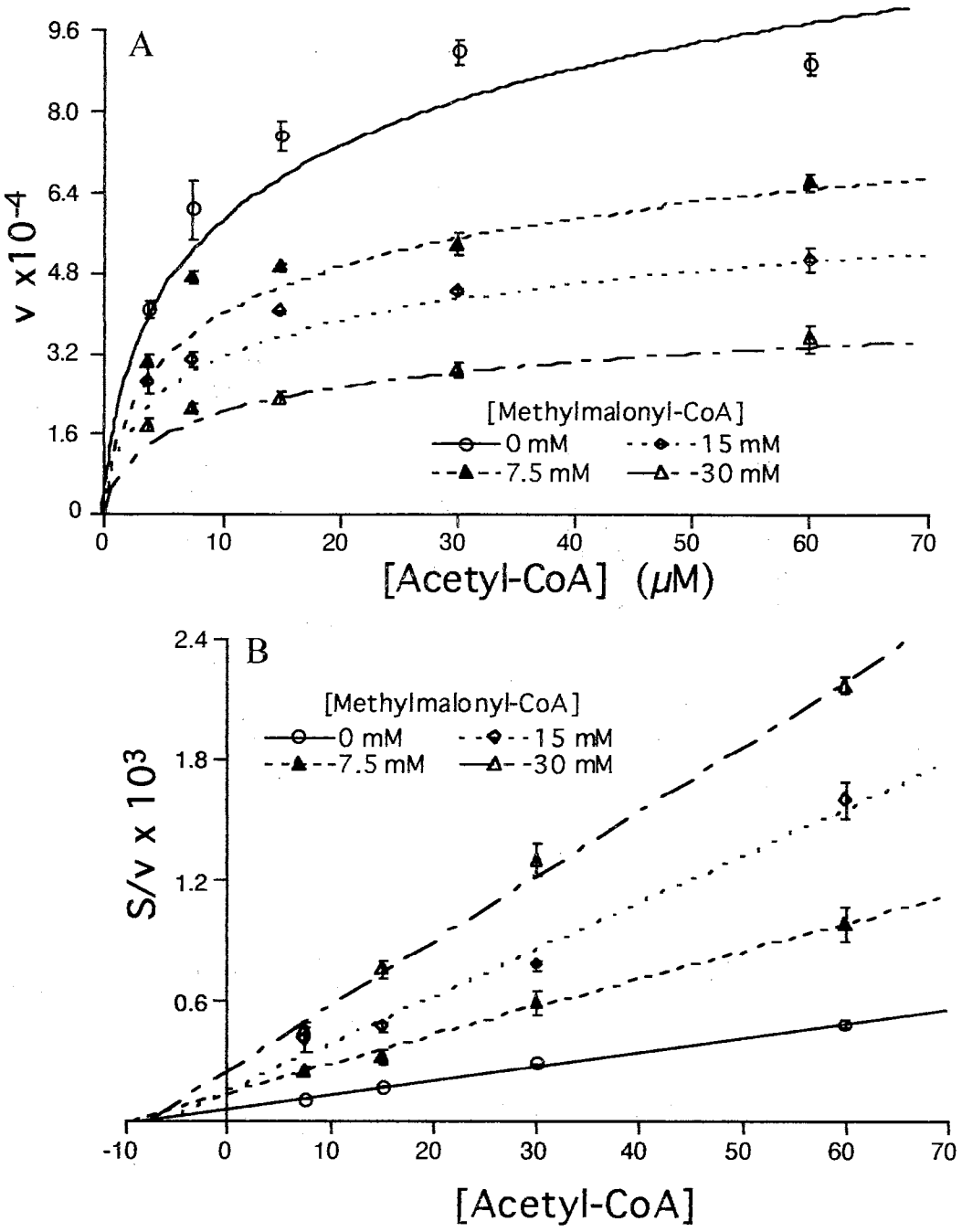


Figure 23. The Effect of Methylmalonyl-CoA with Acetyl CoA on Fatty Acid Synthesis by Crude Cytosolic FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl-CoA, 0, 7.5, 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl-CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

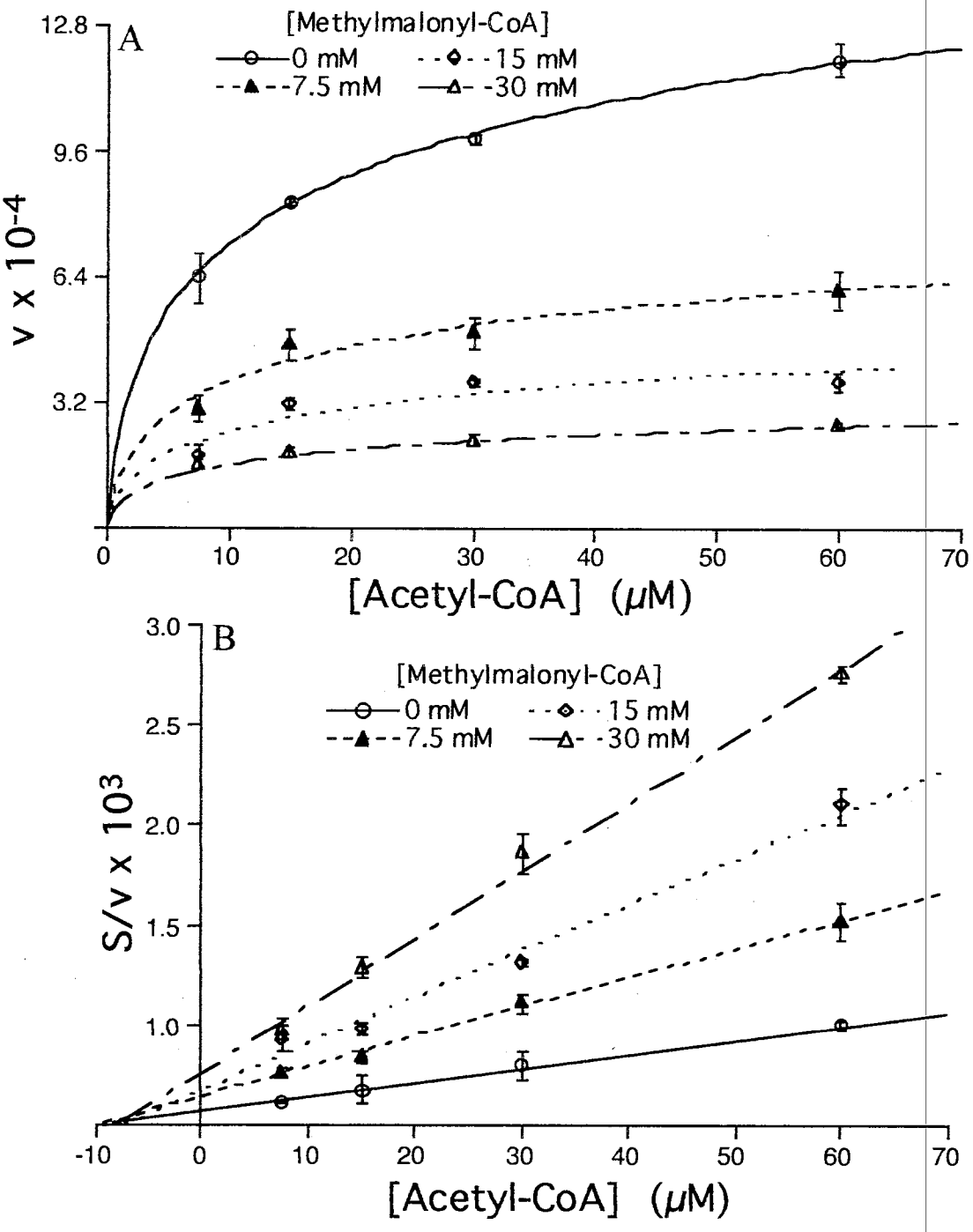


Figure 24. The Effect of Methylmalonyl-CoA with Acetyl-CoA on Fatty Acid Synthesis by Purified Cytosolic FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl-CoA, 0, 7.5 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl - CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

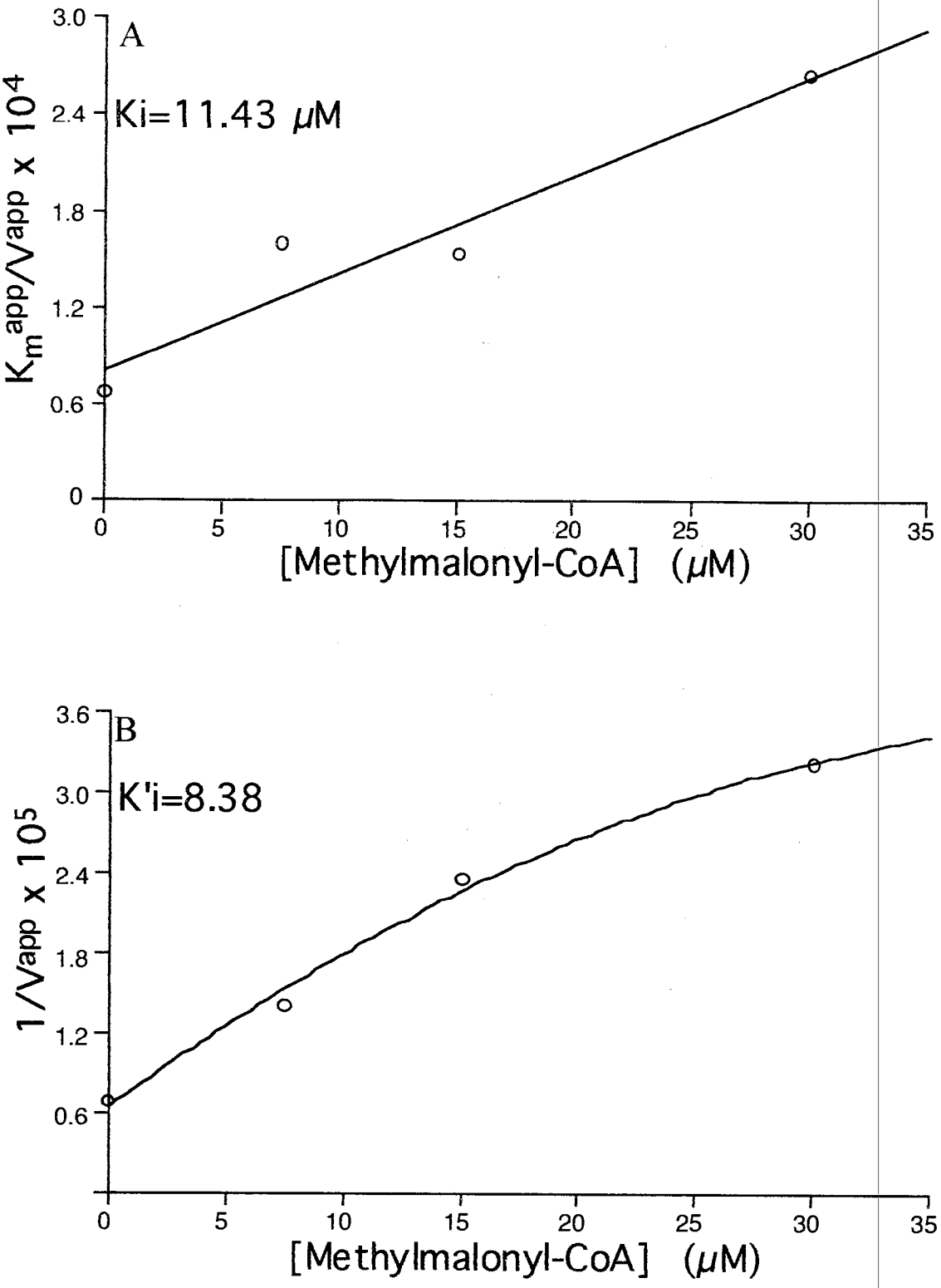


Figure 25. Replots of the Effect of Methylmalonyl-CoA with Acetyl-CoA on Fatty Acid Synthesis by Crude Cytosolic FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I].

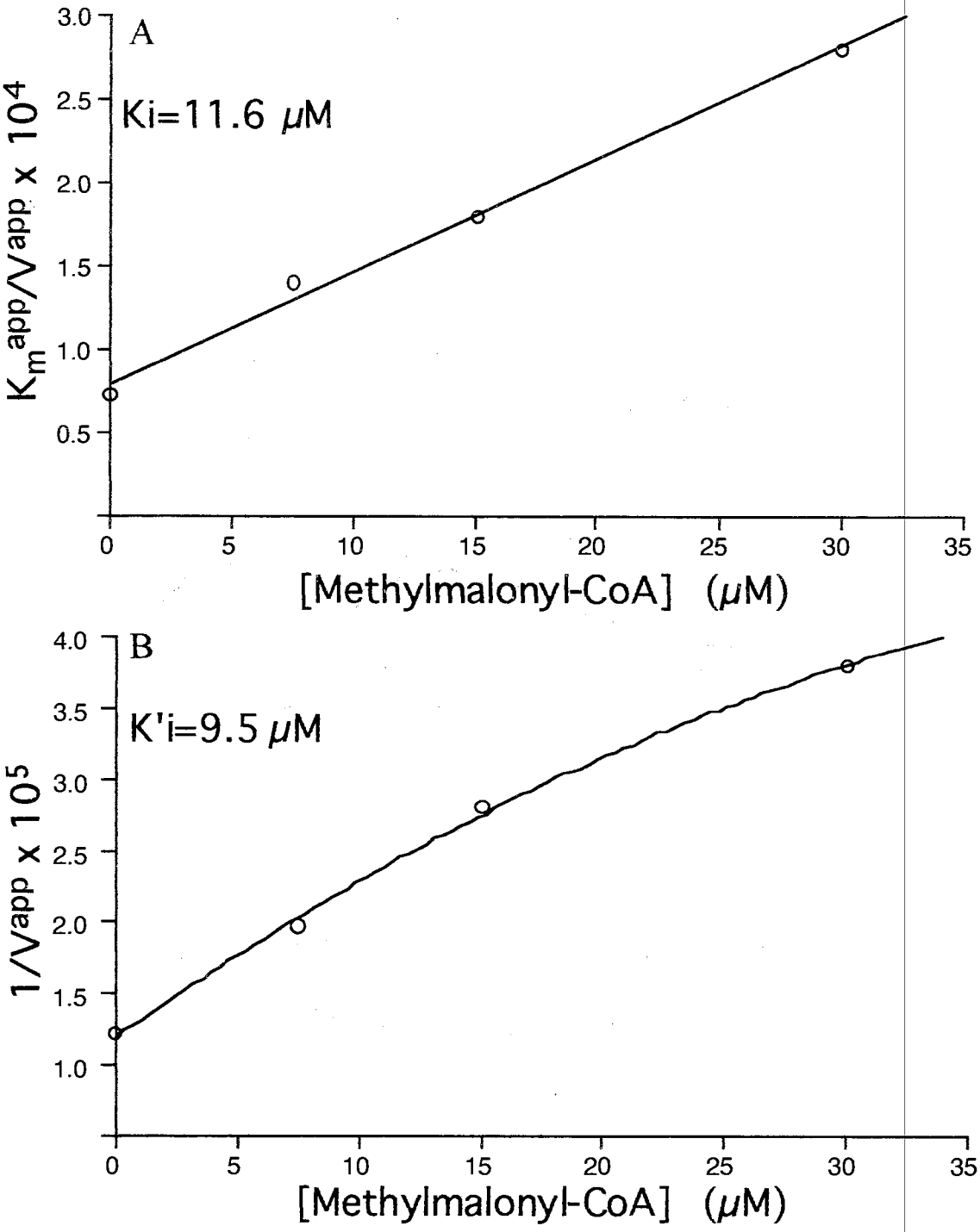


Figure 26. Replots of the Effect of Methylmalonyl-CoA with Acetyl-CoA on Fatty Acid Synthesis by Purified Cytosolic FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I].

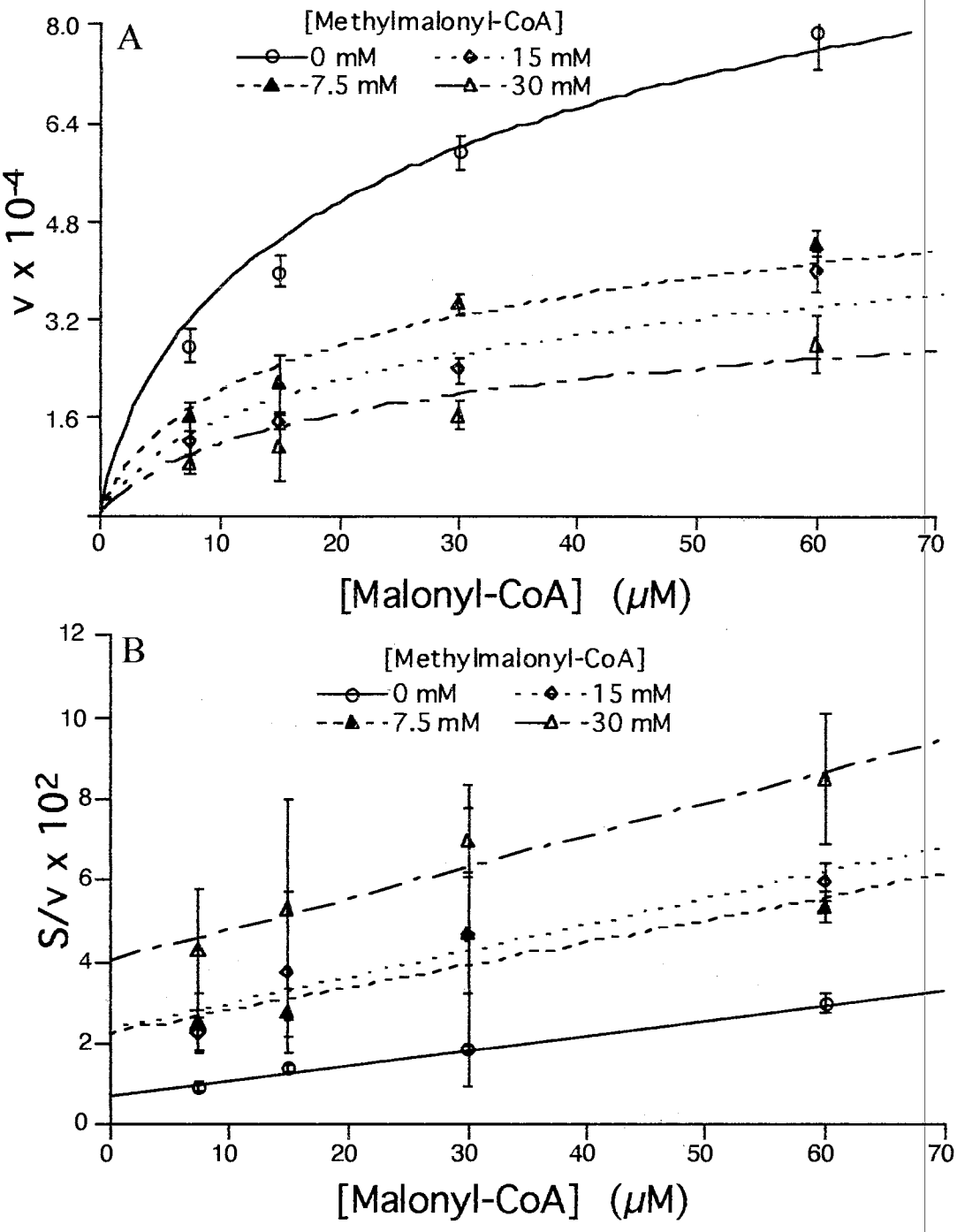


Figure 27. The Effect of Methylmalonyl-CoA with Malonyl-CoA on Fatty Acid Synthesis by Crude Cytosolic FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl-CoA, 0, 7.5, 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl-CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

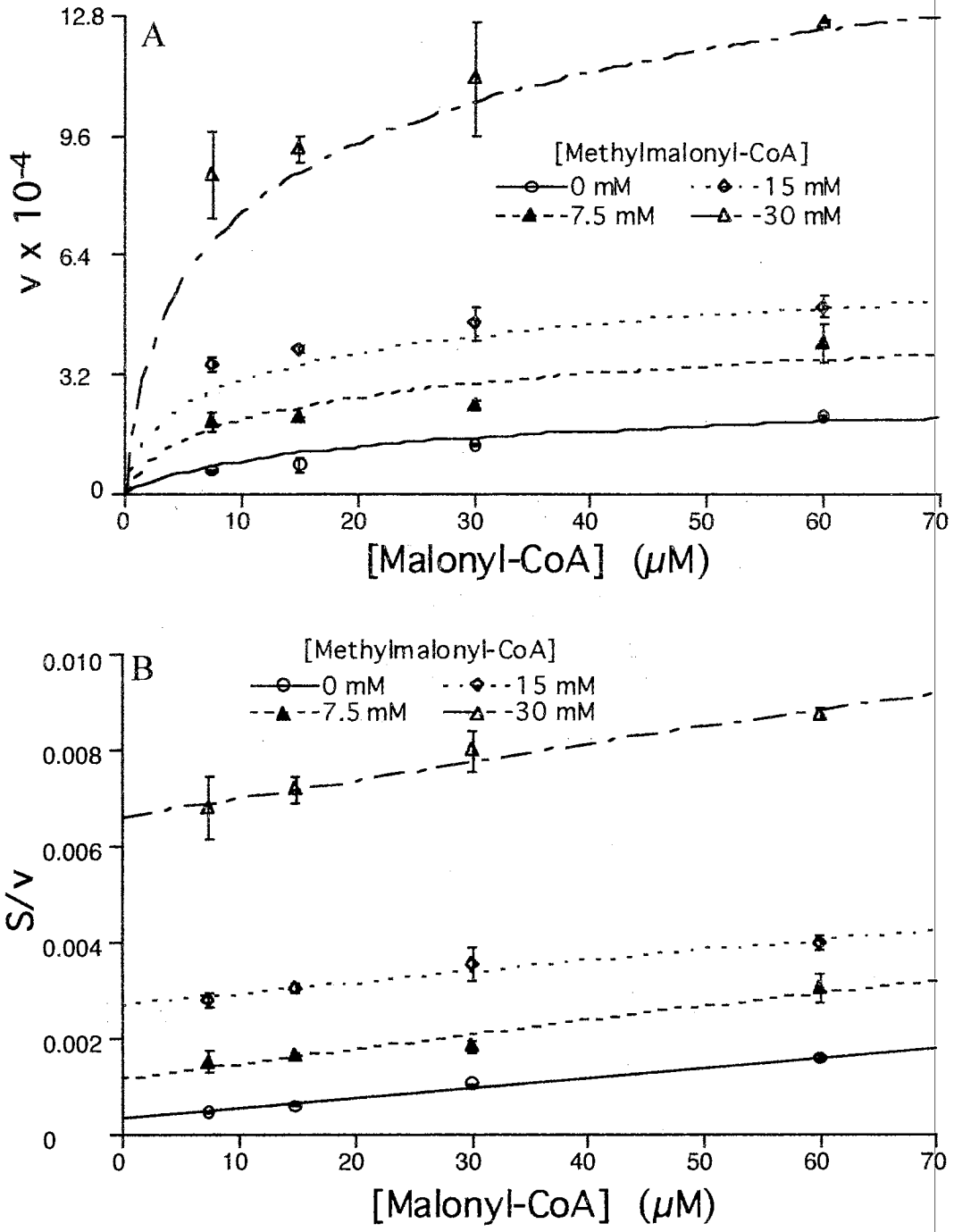


Figure 28. The Effect of Methylmalonyl-CoA with Malonyl-CoA on Fatty Acid Synthesis by Purified Cytosolic FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl-CoA, 0, 7.5, 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl-CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

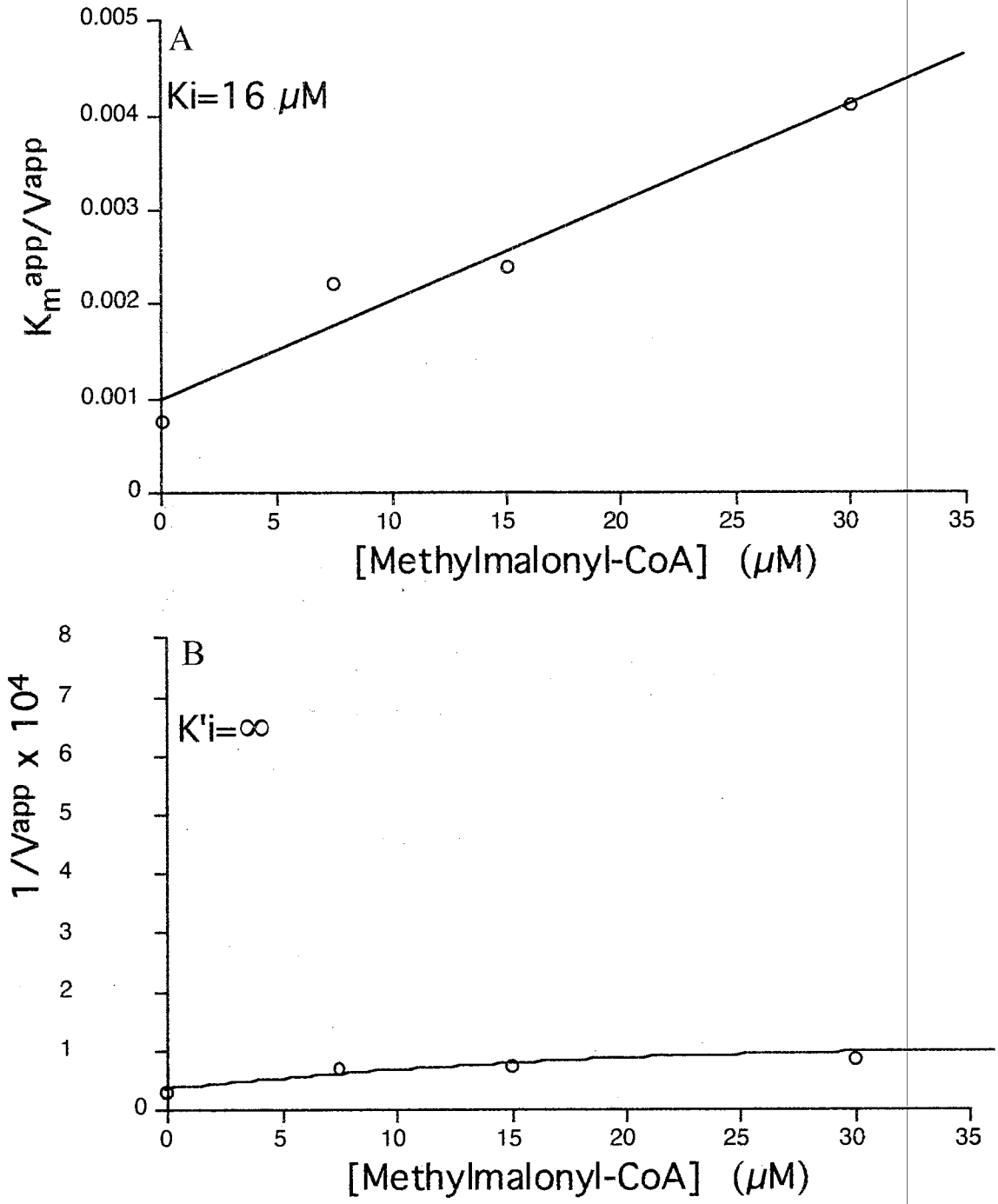


Figure 29. Replots of the Effect of Methylmalonyl-CoA with Malonyl-CoA on Fatty Acid Synthesis by Crude Cytosolic FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I].

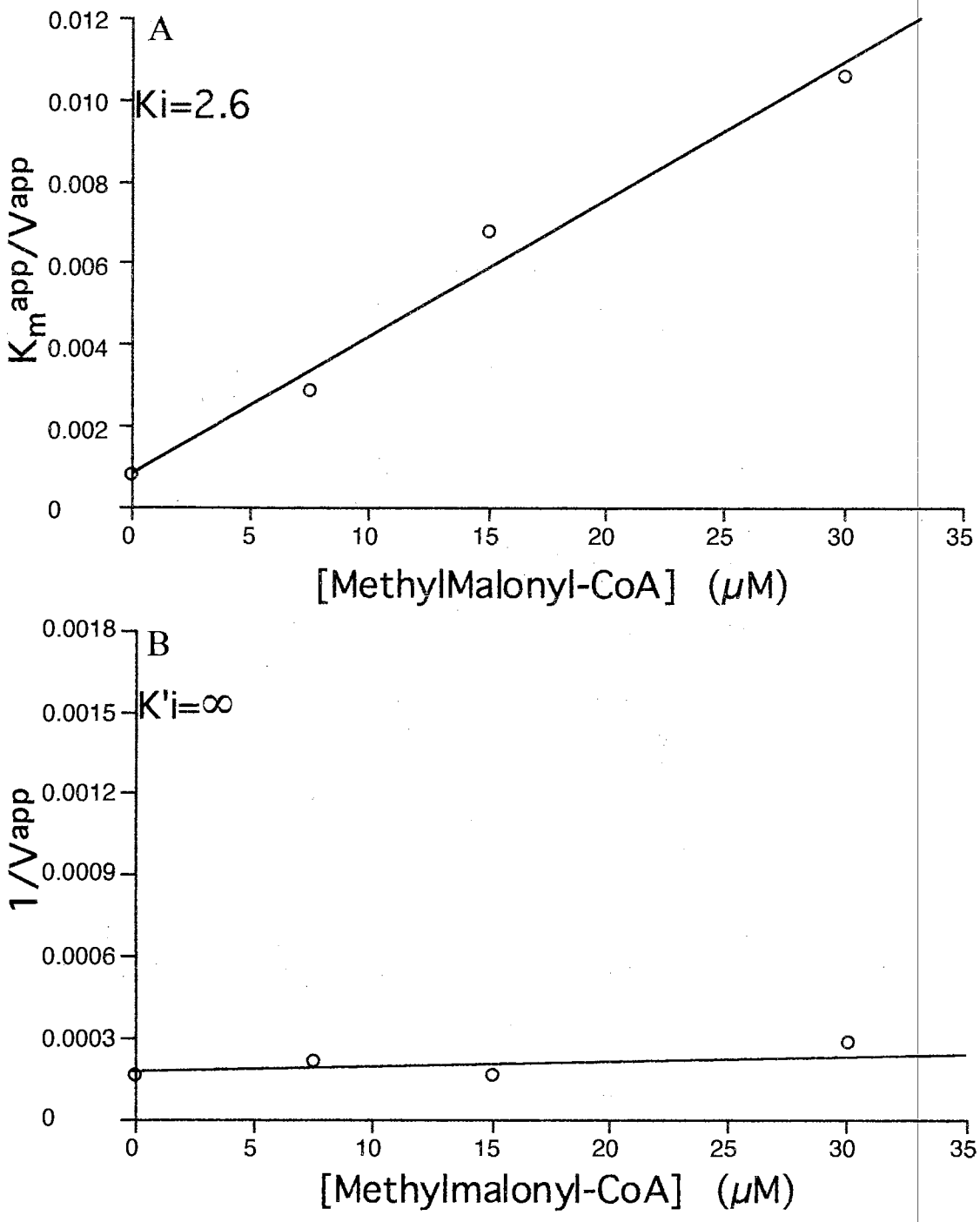


Figure 30. Replots of the Effect of Methylmalonyl-CoA with Malonyl-CoA on Fatty Acid Synthesis by Purified Cytosolic FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I].

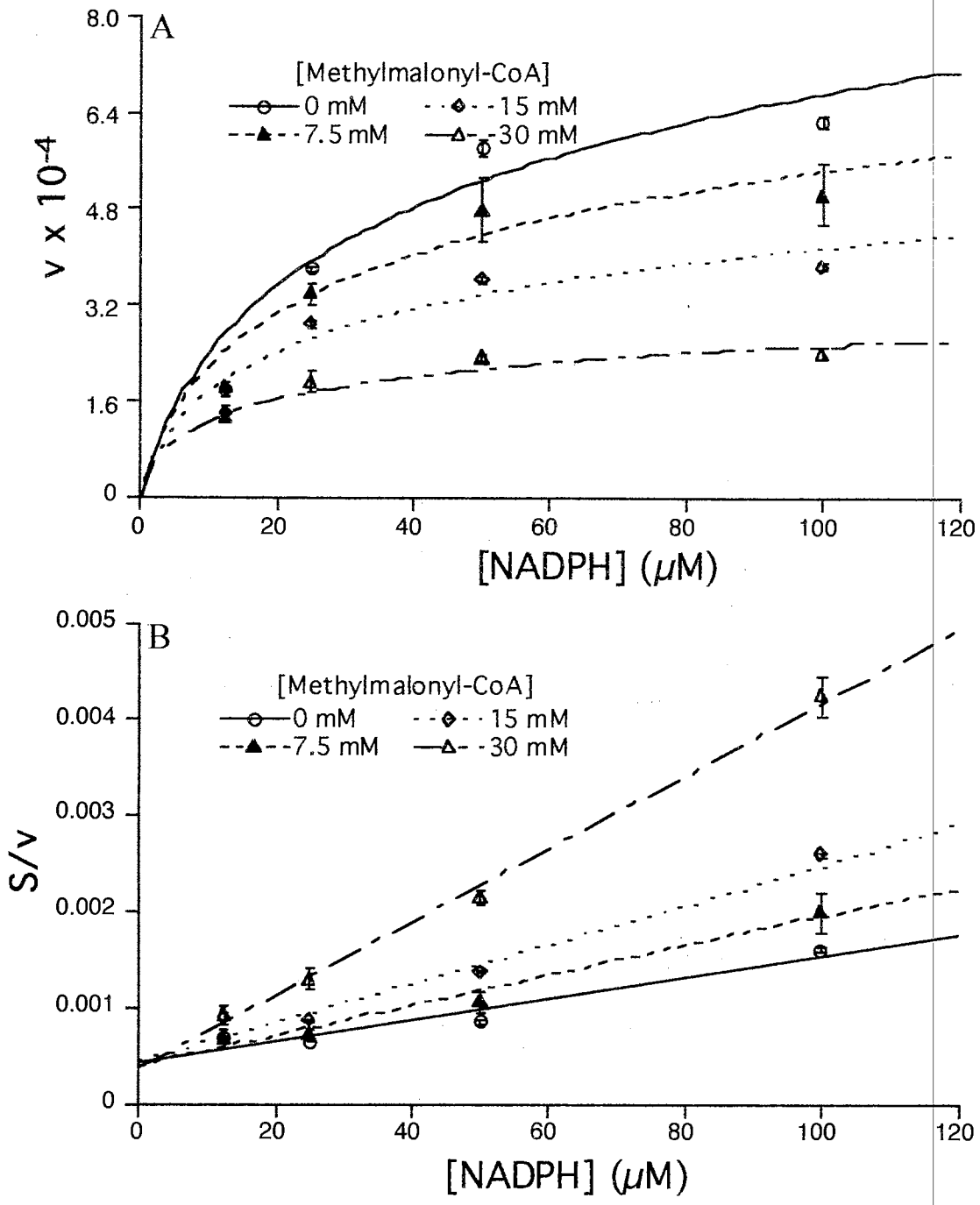


Figure 31. The Effect of Methylmalonyl-CoA with NADPH on Fatty Acid Synthesis by Crude Cytosolic FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl-CoA, 0, 7.5 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl-CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

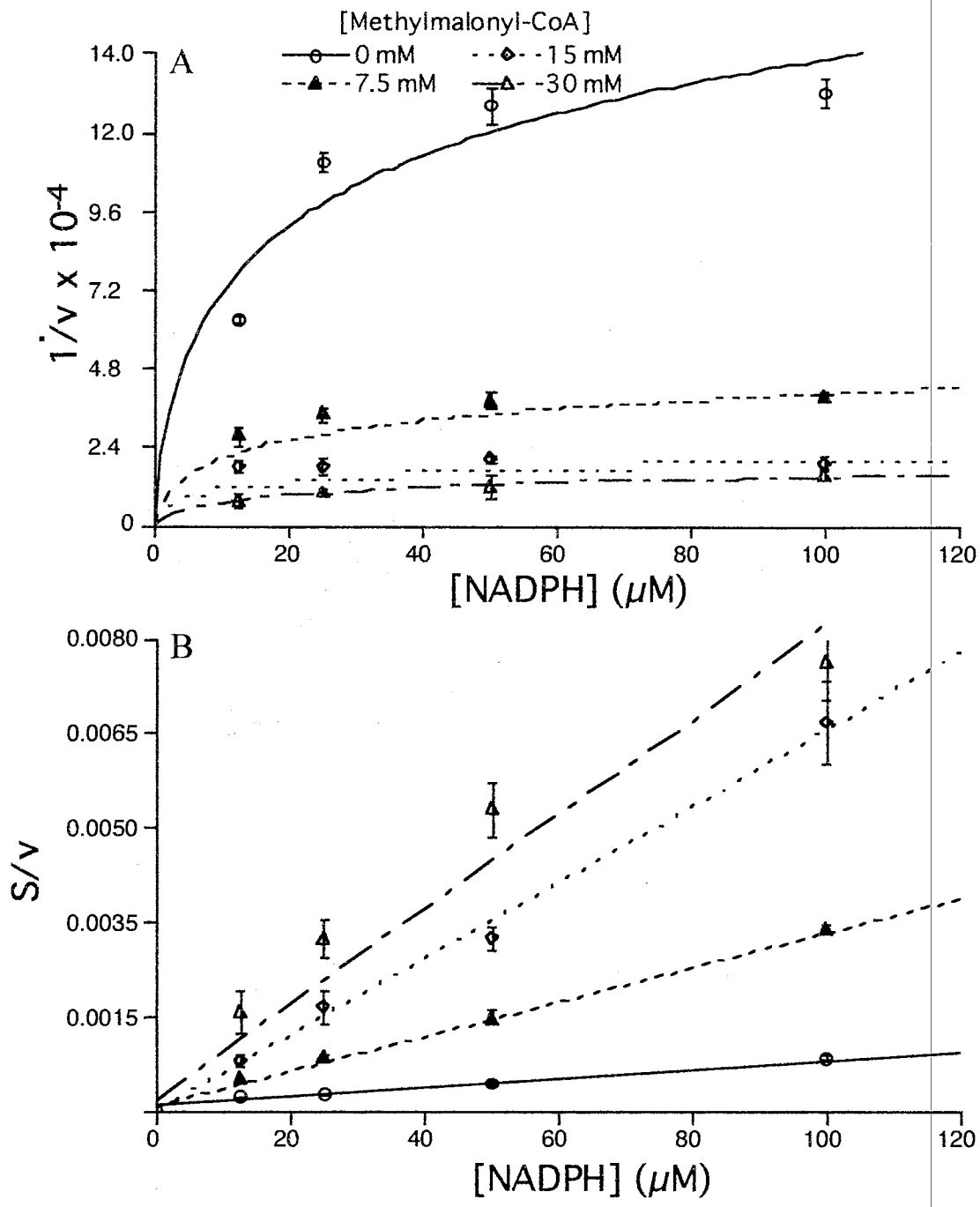
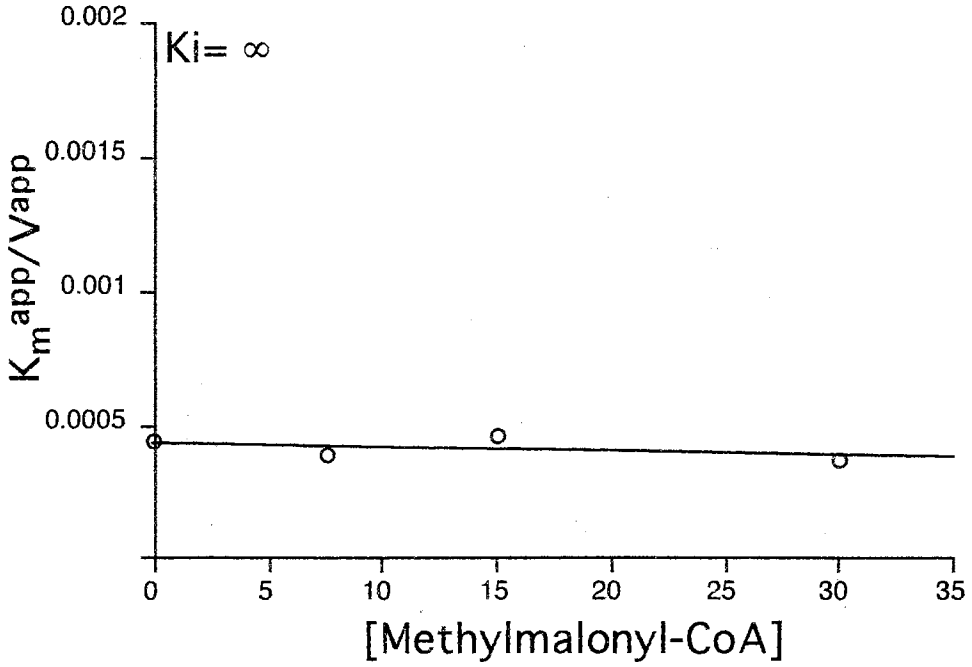


Figure 32. The Effect of Methylmalonyl-CoA with NADPH on Fatty Acid Synthesis by Purified Cytosolic FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl-CoA, 0, 7.5 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl-CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.



A

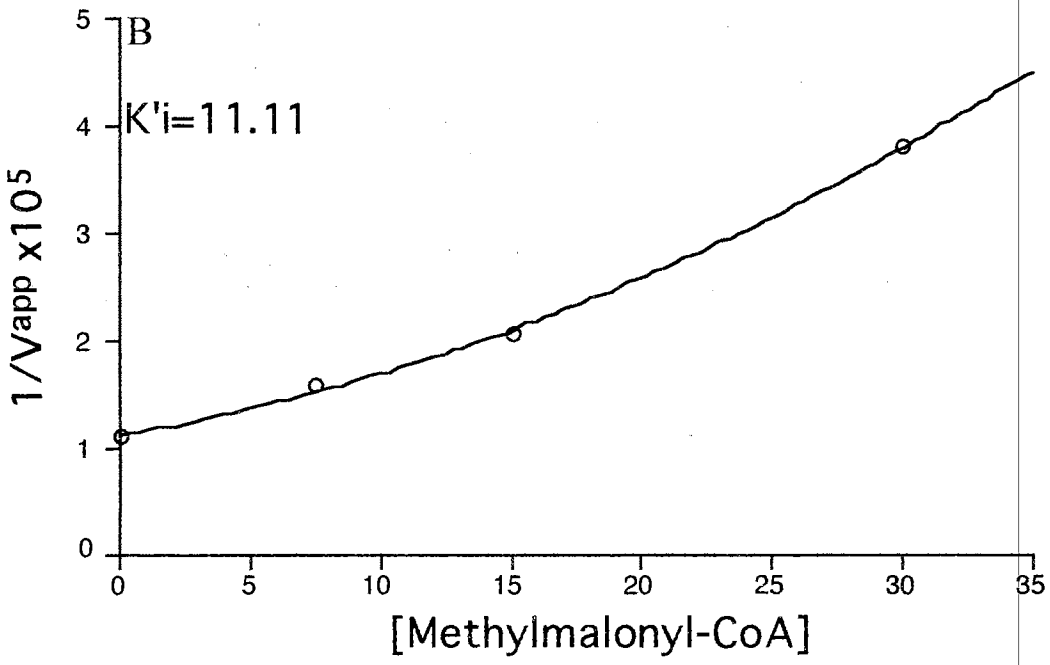


Figure 33. Replots of the Effect of Methylmalonyl-CoA with NADPH on Fatty Acid Synthesis by Crude Cytosolic FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I].

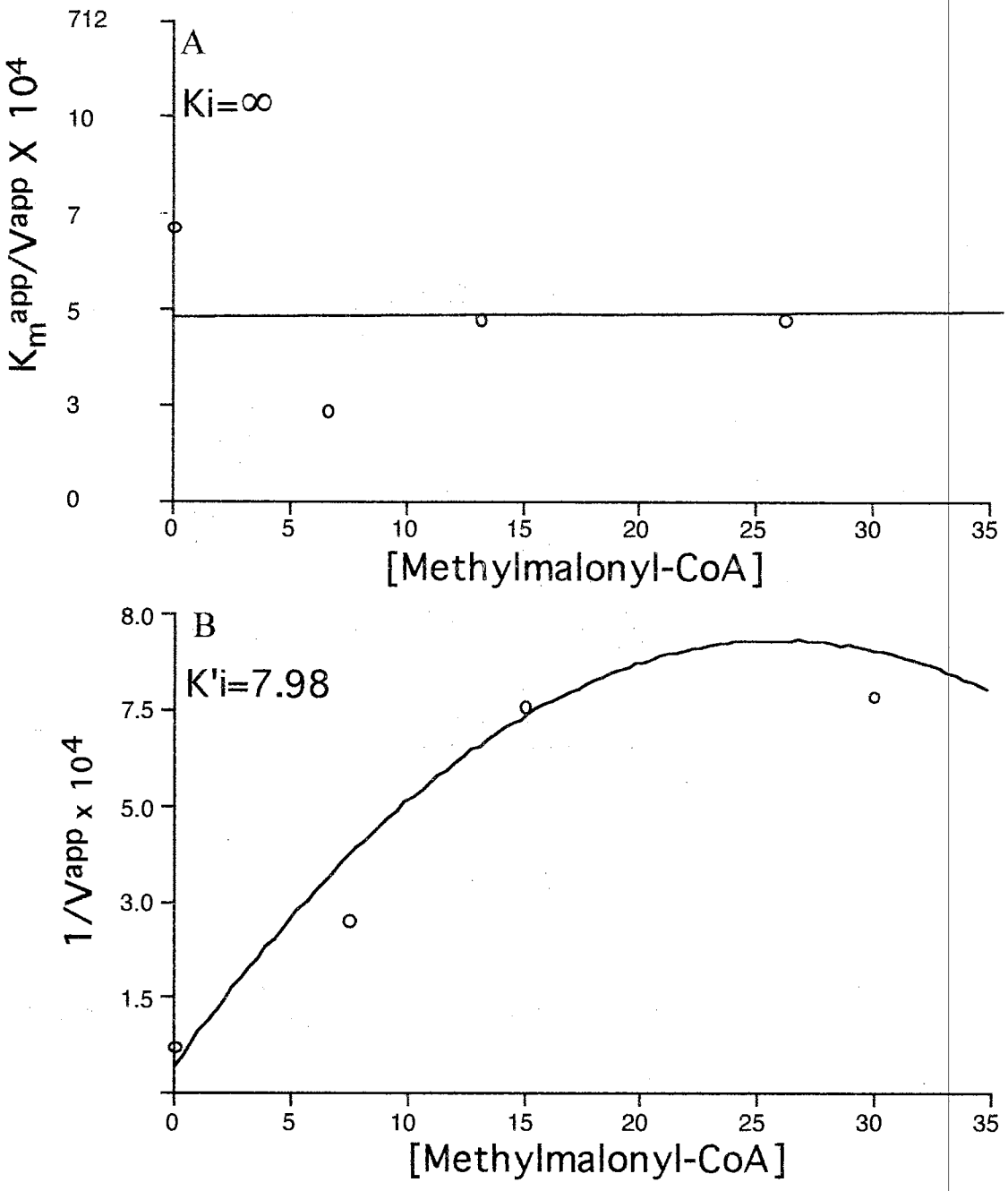


Figure 34. Replots of the Effect of Methylmalonyl-CoA with NADPH on Fatty Acid Synthesis by Purified Cytosolic FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I]].

MICROSOMAL FAS

SUB-CELLULAR FRACTIONATION

The characterization of the sub-cellular fractionation showed lactate dehydrogenase solely in the cytosol. Glutamate dehydrogenase was evenly split between the microsomes and the mitochondria. Glucose-6-phosphatase was 40% in the microsomes, 30 % in the mitochondria, and 20 % in the cytosol. This indicates that the mitochondrial and microsomal fractions were free of cytosolic contaminations, and that the mitochondria were significantly broken in the homogenization process. The spread of glucose-6-phosphatase activity is not suprising given its labile nature. As the purpose of the separation was to resolve microsomal from cytosolic proteins, this arrangement was deemed satisfactory.

When investigating the existence of a microsomal FAS, it is necessary to insure that the assay used is detecting a true fatty acid synthase, and not an elongase. Elongases located on the microsomes use NADPH and malonyl–CoA to elongate fatty acyl CoAs but cannot initiate fatty acyl chains using acetyl-CoA. These elongases could use fatty acyl-CoAs and the added malonyl-CoA in the FAS assay, consuming NADPH. This would give a positive response, making the interpretation of results difficult. If NADPH consumption occurred with the addition of malonyl-CoA first but not acetyl-CoA alone this would be indicative of an elongase activity. The order of addition had no significant effect on the result, indicating that an elongase, which would be active independently of the presence of acetyl-CoA in the assay mixture, was not a significant component and that a true FAS was present on the microsomes (Figure 35).

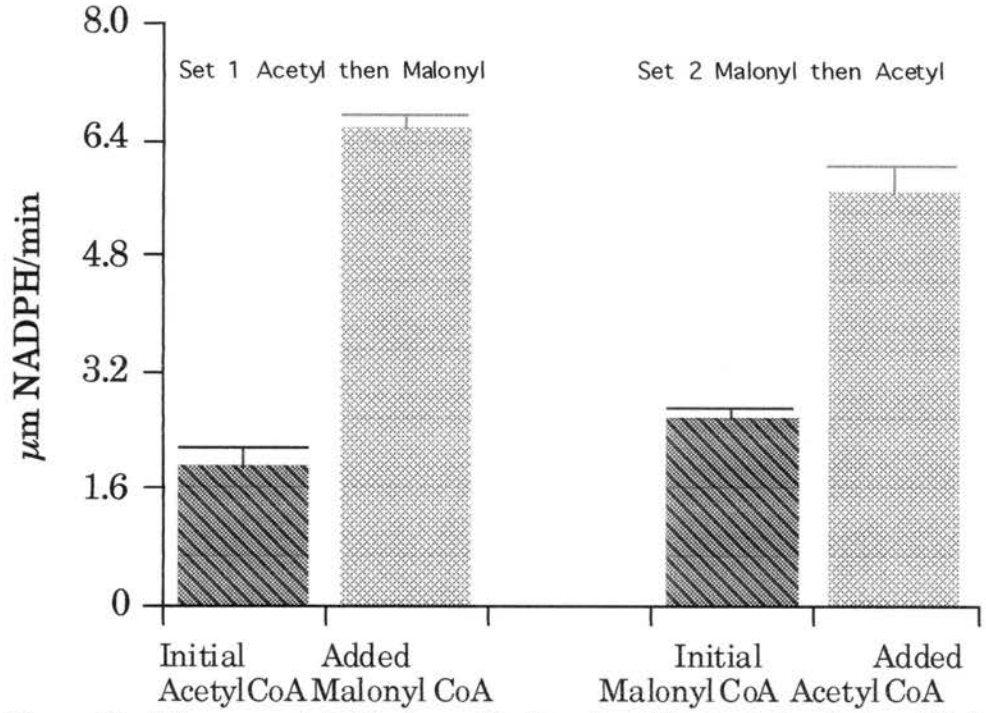


Figure 35. Microsomal FAS Assay Testing the Effect of the Order of Substrate Addition on Activity. Crude microsomes were incubated with 100 µM NADPH and 60 µM of either acetyl CoA or malonyl CoA. Activity was measured spectrophotometrically, 60 µM of the other substrate added, and the activity re-measured.

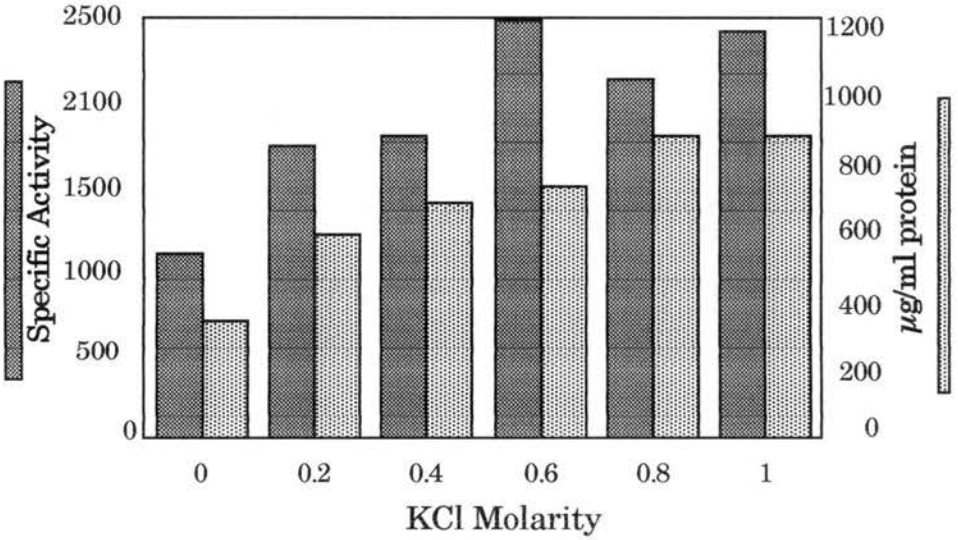


Figure 36. KCl Extraction of Microsomal FAS Activity. Microsomes were shaken at room temperature for 1/2 hour at different concentrations of KCl, centrifuged at 150,000 g for 90 minutes, and the supernatants assayed for FAS activity.

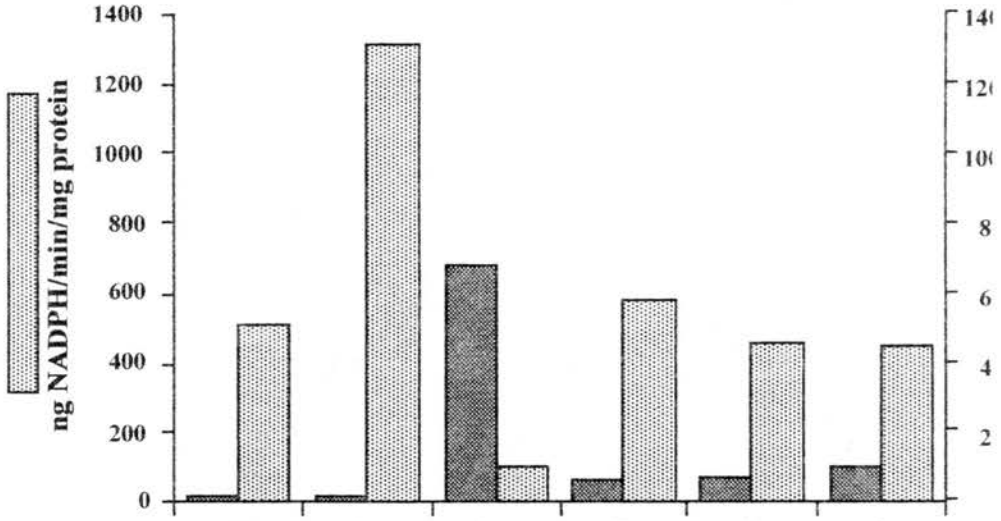


Figure 37. Ammonium Sulfate Precipitation of the KCl Extract of Microsomal FAS. 100,000 g supernatant of KCl extracted microsomes was subjected to increasing concentrations of ammonium sulfate. Each addition was followed by stirring on ice for 30 min, and 10,000 g centrifugation to separate out the precipitate. Standard assay was used on the precipitate.

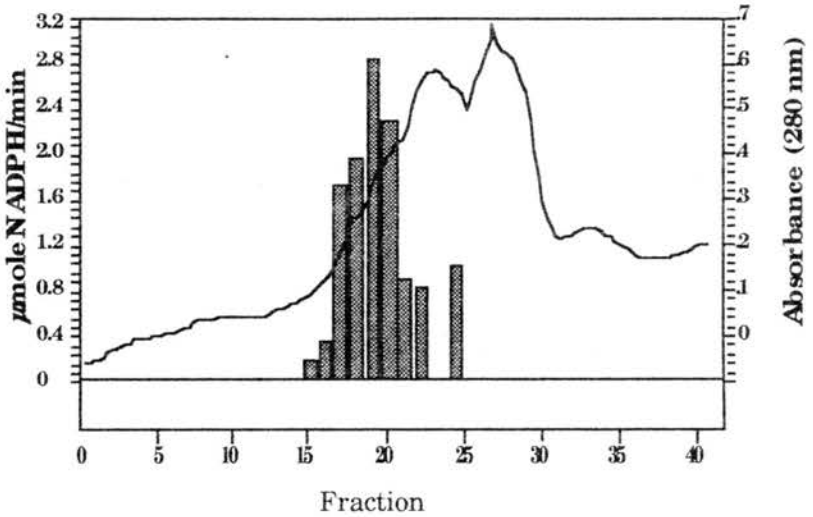


Figure 38. Sepharose 6B-CL Purification of Microsomal FAS, Scheme A. The 30% 45% ammonium sulfate pellet was dissolved in 2 ml phosphate buffer and loaded on a 90 by 1.5 cm column run at 1 ml/min using standard pH 7.4 buffer. Five ml fractions were collected and 50 µl aliquots were assayed for FAS activity.

Following the work of others (Khan and Kolattukudy, 1975; Peide et al. 1993; Blomquist et al. 1994) salt extraction was used to attempt to solubilize the FAS. Microsomes were shaken at differing concentrations of KCl at room temperature for 1/2 hour, centrifuged at 150,000 g for 90 minutes, and the supernatants analyzed for FAS (Figure 36). Based on these results, 1 molar KCl was chosen as the concentration to extract microsomal FAS.

The KCl extract was next subjected to ammonium sulfate fractionation (Figure 37). There was a large amount of potassium sulfate that precipitated out, as this has low solubility in both high concentrations of ammonium sulfate and glycerol. The 30 to 45% ammonium sulfate fractions were selected for further purification. The apparently more attractive 20% fraction was not consistent, and will be discussed further in the next chapter.

SCHEME A

The ammonium sulfate pellet was redissolved in 2 ml of buffer and loaded onto a Sepharose 6B column. The bulk of activity eluted early, preceeding the bulk of the A_{280} absorbance (Figure 38). The active fractions were loaded onto a DEAE Sephacel column and eluted with a KCl gradient, giving one main peak of activity (Figure 39). The active fractions were diluted 5 fold and loaded onto a Reactive Red Sepharose column. The activity eluted in a sharp peak that corresponded with the peak of absorbance (Figure 40). The result of this purification was a 3% recovery of activity, a specific activity of 1013 nmoles NADPH/min/mg protein, and a purification factor of 151 (Table IV) and is pictured in Figure 41.

SCHEME B

In order to use a preparation comparable to the purified cytosolic FAS used for kinetic measurements, Scheme B was used for the microsomal preparation as well. The 30-50% ammonium sulfate cut was loaded onto a Reactive Red Sepharose column and eluted with a KCl gradient. The peak of activity co-eluted with the main absorbance peak (Figure 42). The active fractions were precipitated with 50% ammonium sulfate, redissolved in a minimum of buffer, and loaded in 200 µl aliquots onto a Superose 6 column giving 2 peaks of activity (Figure 43). The minimum between 10 and 11 ml was tested repeatedly and is not an artifact. The final result of this purification ,the gel of which is pictured in Figure 44, was a 7% recovery of activity, a specific activity of 3600 nmoles NADPH/min/mg protein, and a purification factor of 124 (Table V).

pH OPTIMUM and pI

The pH optimum of the purified microsomal FAS was determined by testing over the range of pH 6.5 to 8.2 using 200 mM phosphate buffer, with the maximum activity occurring at pH 7.6 (Figure 45). The pI was determined to be 7.163 using the Pharmacia Phast system with the pH 3 to 9 range gel (Figure 46). The same running conditions were used as for the cytosolic FAS.

MOLECULAR WEIGHT

The molecular weight of microsomal FAS by Sepharose 6B gel filtration was 437 KDa for the homodimer (Figure 47). The monomeric molecular weight was determined to be 240 KDa by Laemmli SDS-PAGE (Figure 48) and 245 KDa by Weber-Osborn SDS-PAGE (Figure 49). Superose 6 gel filtration gave a dimer molecular weight of 485 KDa and a monomer weight of 257 (Figure 50).

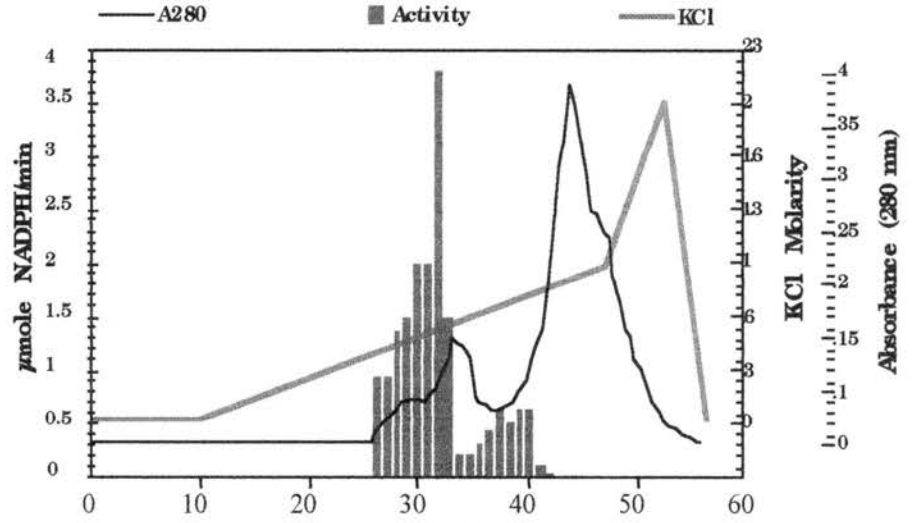


Figure 39. DEAE Purification of Microsomal FAS, Scheme A. Active fractions from Sepharose 6B were loaded at 0.5 ml/min onto a 2 x 15 cm column. The column was washed with 15 ml buffer, and the activity eluted with a KCl gradient. 2 ml fractions were collected. Activity was monitored using 50 µl of each fraction in the standard assay.

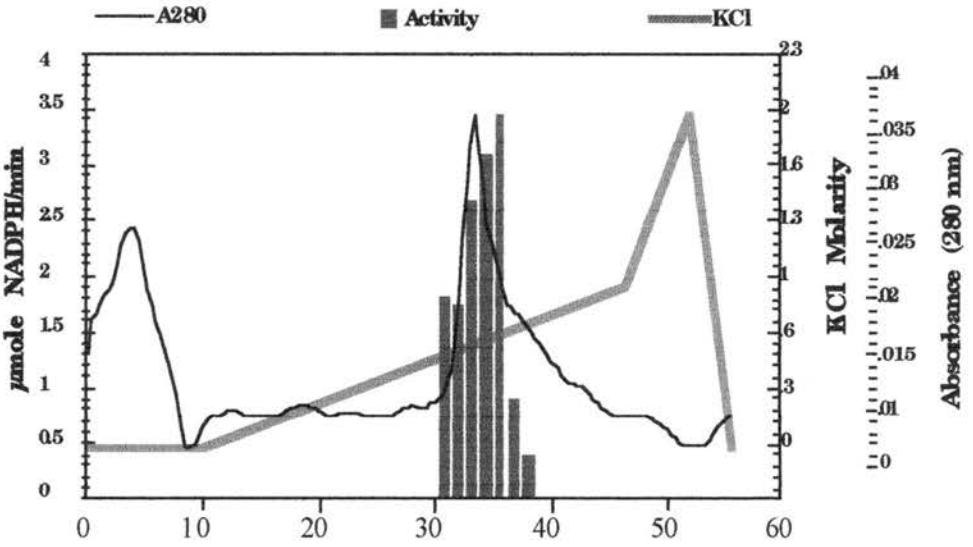


Figure 40. Reactive Red Sepharose Purification of Microsomal FAS, Scheme A. Active fractions from DEAE Sephacel were loaded at 0.5 ml/min onto a 1 x 15 cm Reactive Red column. The column was washed with 15 ml buffer, and the activity eluted with a KCl gradient. 2 ml fractions were collected and activity was monitored using 50 µl of each fraction in the standard assay.

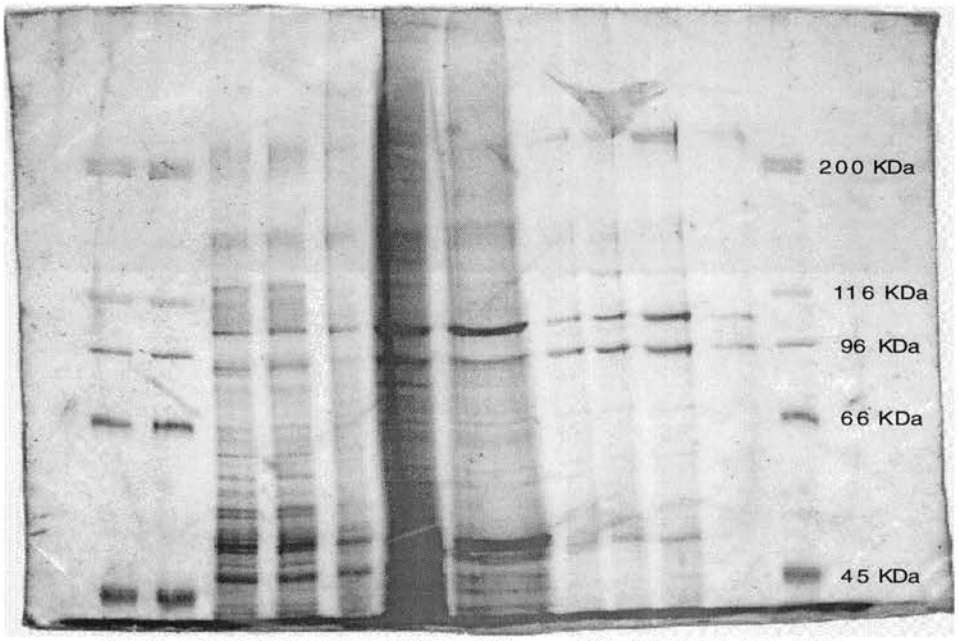


Figure 41. 5% SDS-PAGE Gel of Microsomal FAS, Scheme A Purification. The fractions are crude homogenate(Crude), 10,000 g supernatant (10Kg), microsomes (Micro), KCl extract, 30-50% ammonium sulfate pellet (50% ppt.), combined Sepharose 6B fractions (6B), combined DEAE Sephacel fractions (DEAE), and combined Reactive Red Sepharose fractions (RR). Standards are myosin (200 KDa), beta-galactosidase (116 KDa), phosphorylase b (96 KDa), bovine serum albumin (66 KDa), and ovalbumin (45 KDa).

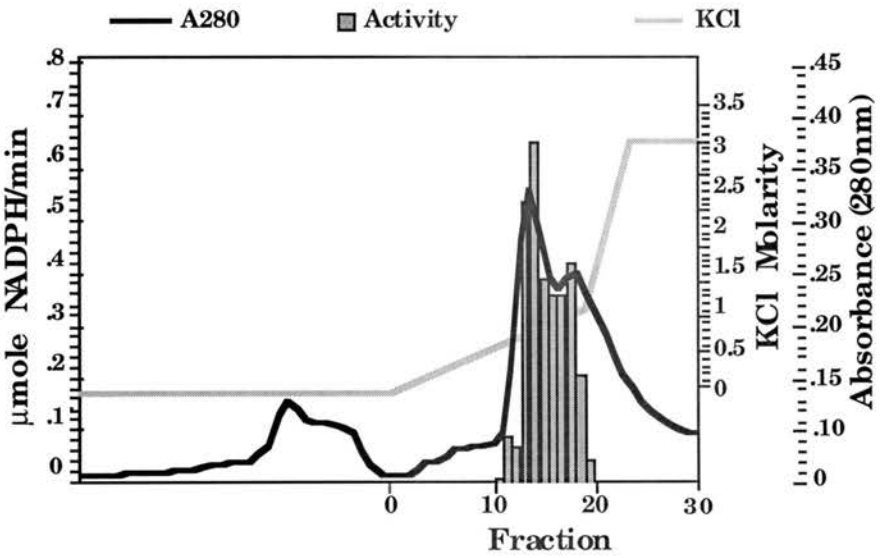


Figure 42. Reactive Red Sepharose Gel Filtration of Microsomal FAS, Scheme B Redissolved 25% to 50% ammonium sulfate pellet was loaded on a 2.5 x 20 cm column at 1 ml/min, washed with 60 ml pH 7.4 buffer, and eluted with a KCl gradient. 50 µl aliquots of 2 ml fractions were measured for activity by the standard assay.

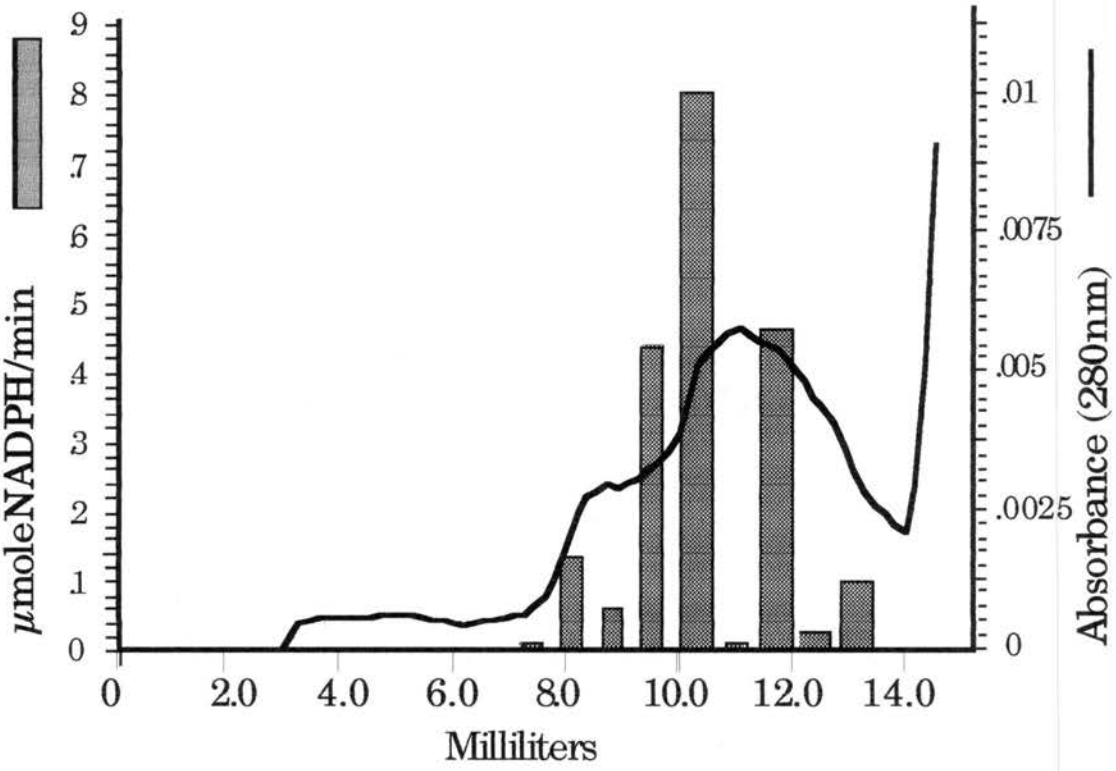


Figure 43. Superose 6 Gel Filtration of Microsomal FAS, Scheme B. Ammonium sulfate precipitated FAS from the Reactive Red step was loaded on a 1 x 20 cm column and eluted with pH 7.4 buffer at 0.1 ml/min. 0.3 ml fractions were collected, multiple runs pooled, and 50 µl aliquots were measured using the standard assay.

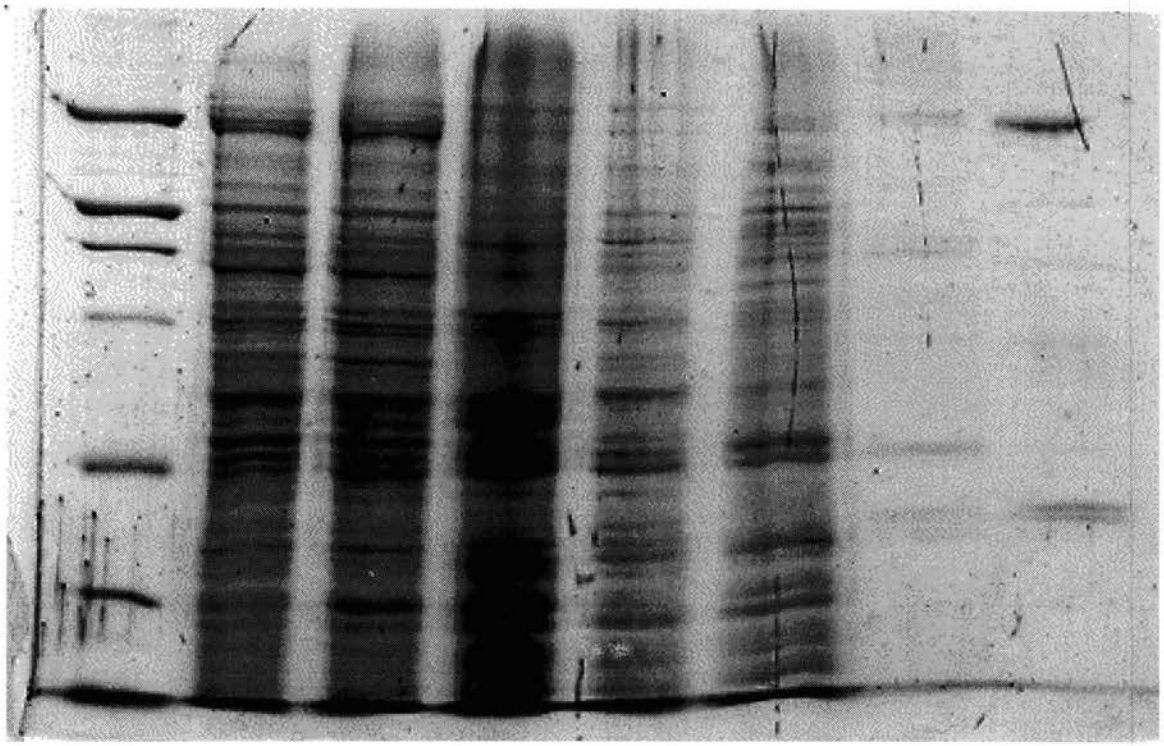


Figure 44. 5% SDS-PAGE Gel of Microsomal FAS Scheme B Purification. The fractions are crude homogenate (Crude), 10,000 g supernatant (10Kg), microsomes (Micro), KCl extract, 30-50% ammonium sulfate pellet (50% ppt.), combined Reactive Red Sepharose fractions (RR), and combined Superose 6 fractions (Sup 6). Standards are myosin (200 Kda), beta-galactosidase (116 KDa), bovine serum albumin (97.4 KDa), and ovalbumin (45 KDa).

Table IV Purification Table for Microsomal FAS, Scheme A

Fraction	Volume ml	Protein mg/ml	Protein mg	Specific Activity units/mg	Total Units	% Recovery	Purif. factor
Crude Homog.	75	37.2	2790	15.1	42,129	-	-
15,000 g Sup.	72.1	29.6	2134	21.6	46,094	-	-
microsomes	21.2	22.47	476.4	6.7	3192	-	1
KCl extract	20.2	11.82	238.8	17.5	4179	100	2.6
30% sup.	20.2	5.00	101	37.5	3787	91	5.6
40% ppt	2	10.9	21.8	96	2093	50	14.3
6B-CL	50.5	.034	1.72	380	654	16	56.7
DEAE	11.2	.046	.52	493	256	6	73.6
Reactive Red	4	.03	.12	1013	122	3	151.2

Table V Purification Table for Microsomal FAS, Scheme B

Fraction	Volume ml	Protein mg/ml	Protein mg	Total Units (1000s)	Specific Activity U/mg	% Recov	Purif. factor
Crude	150	4.45	667.5	52950	79.3	-	
15,000 g Sup.	140	1.53	214.2	53480	249.7	-	
microsomes	10	3.59	35.9	1040	29.0	100	1.0
KCl extract	8.1	2.03	16.4	976	59.5	94	2.0
50% ppt.	1.4	2.2	3.08	484	157.1	47	5.4
Reactive Red	25	.09	.21	270	1285.7	26	44.4
Superose 6	11.2	.02	.11	72	3600	7	124

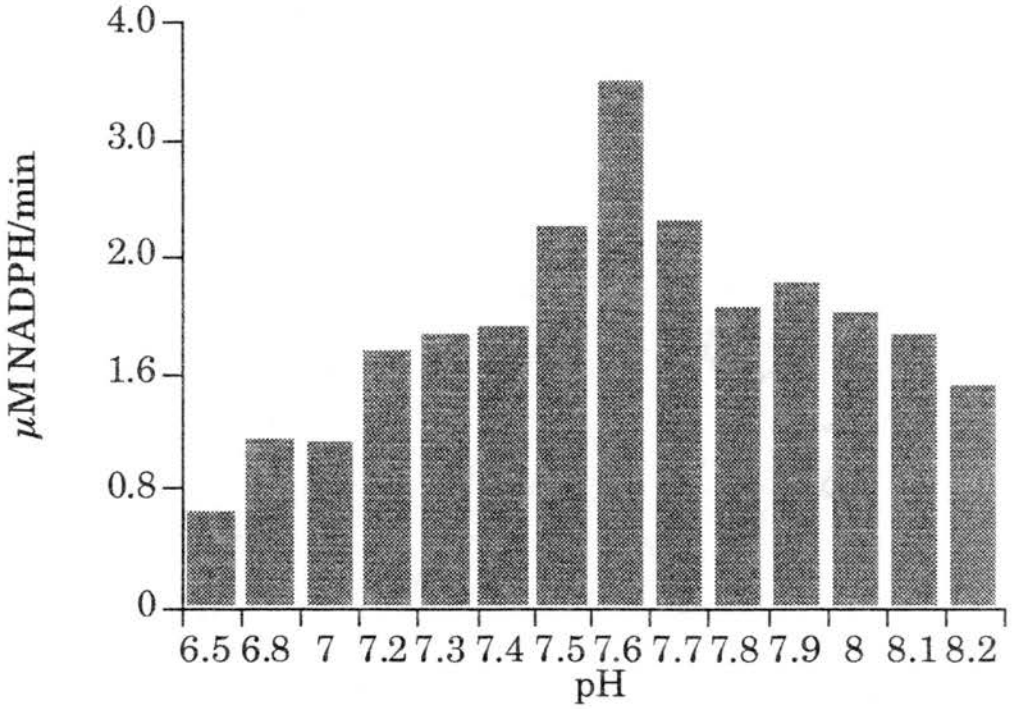


Figure 45. pH Curve of Scheme B Purified Microsomal FAS. Assayed at 25° C, using 0.2 M phosphate buffer at labeled pHs, 100 μM malonyl and acetyl-CoA, and 150μM NADPH, in 300 μl final volume.

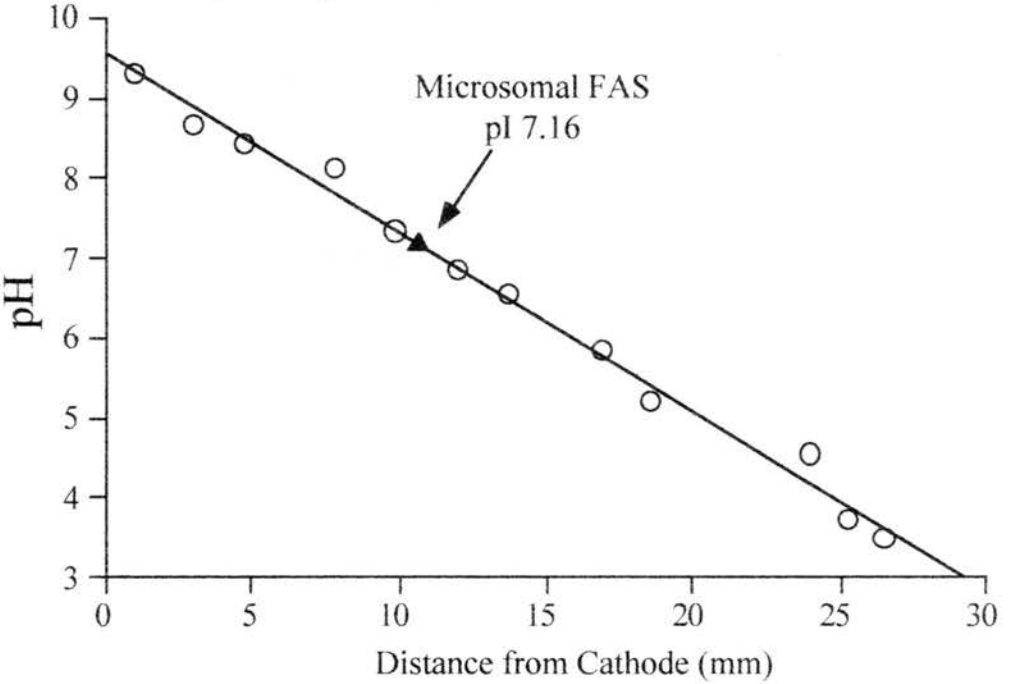


Figure 46. pI of Microsomal FAS. Samples were run on the Pharmacia Phast system on a pH 3-9 gel as per manufacturer's protocols with Pharmacia isoelectric focusing standard.

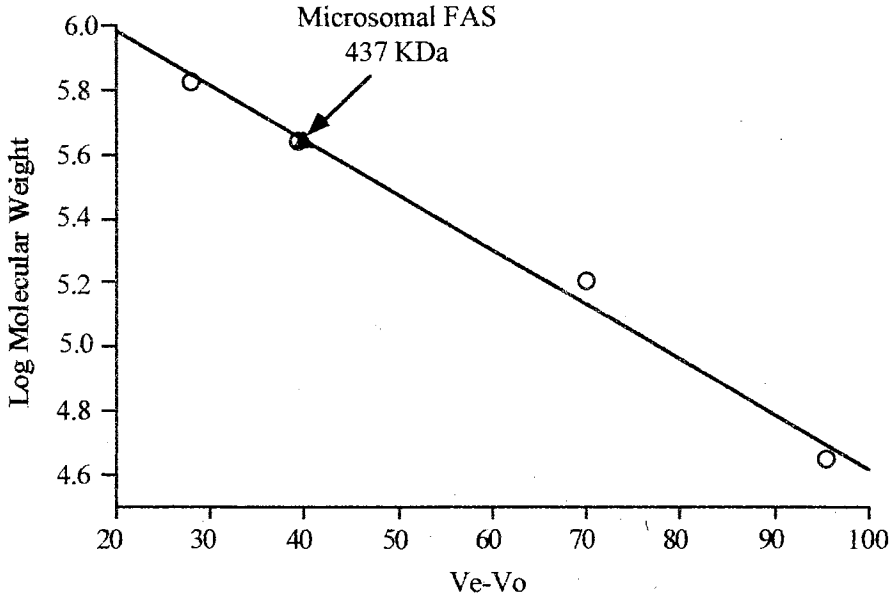


Figure 47. Sepharose 6B Gel Filtration Molecular Weight Determination of Microsomal FAS. The 50% ammonium sulfate precipitate was run at 0.5 ml/min on a 2.5 x 90 cm Sepharose 6B column in pH 7.4 buffer. Standards are ovalbumin (44 KDa), gamma globulin (158 KDa), apoferritin (443 KDa), and thyrogobulin (669 KDa). ▲ microsomal FAS.

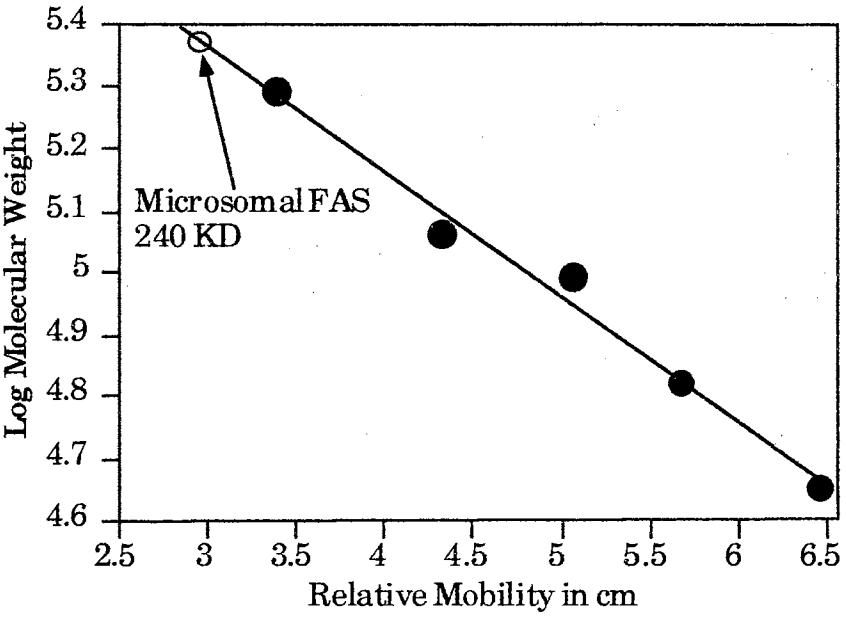


Figure 48. Molecular Weight Determination of Microsomal FAS by Laemmli SDS-PAGE, 5% Gel. Reactive Red purified FAS used. Standards are Standards are myosin (200 KDa) beta-galactosidase (116 KDa), bovine serum albumin (97.4 KDa), and ovalbumin (45 KDa).

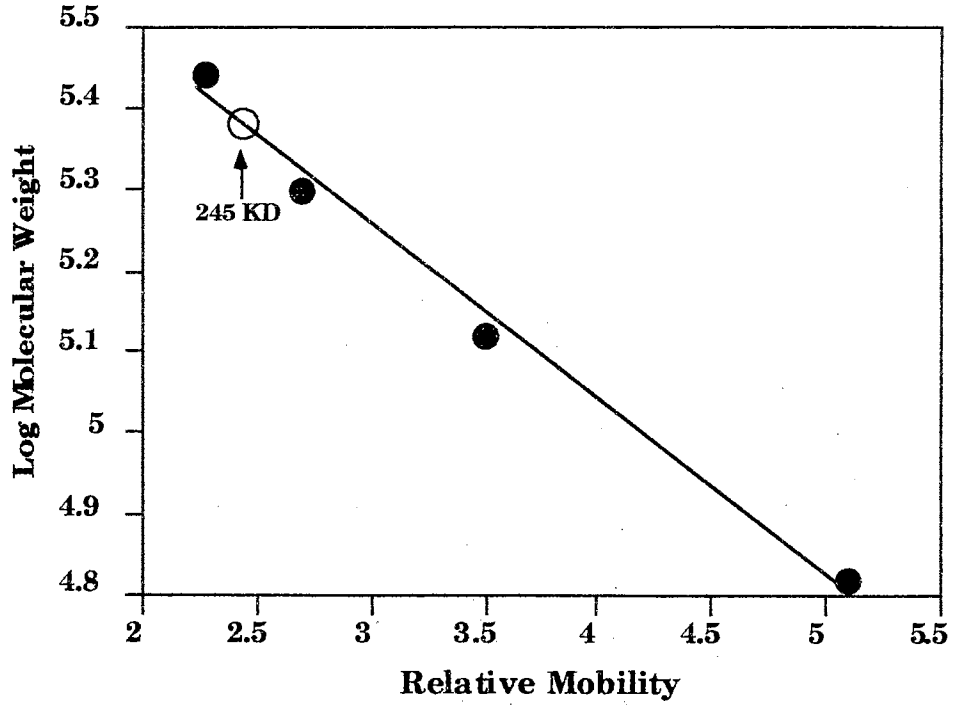


Figure 49. Molecular Weight Determination of Microsomal FAS by Weber-Osborne PAGE 5% Gel. Standards are multimers of cross-linked bovine serum albumin, 66, 132, 198, and 264 KDa.

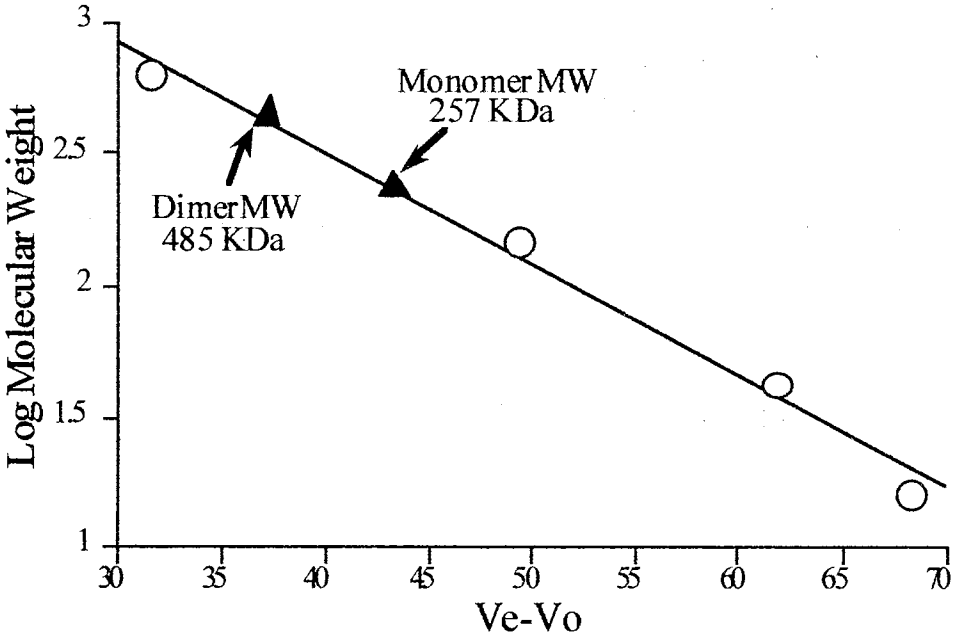


Figure 50. Molecular Weight Determination of Microsomal FAS by Superose 6 Gel Filtration. Standards are thyroglobulin 670 KDa, gamma globulin 158 KDa, ovalbumin 44 KDa, and myoglobin 17 KDa.

KINETIC CHARACTERISTICS OF CRUDE AND PURIFIED MICROSOMAL FAS

The kinetic parameters were determined by spectrophotometric assay as detailed in Materials and Methods. Maximum velocities (V_{max}) and Michaelis constants (K_m) were calculated for both the crude and partially purified enzyme.Hanes-Woolf plots for acetyl-CoA, malonyl-CoA, NADPH, and methylmalonyl-CoA showed similar K_m's and V_{max}'s between the crude (Figures 51 and 52) and purified forms (Figures 53 and 54). The results are summarized in Table VI.

Table VI Km and V_{max} for Crude and Purified Microsomal FAS

	K_m(μM)				V_{max} (μM/min/mg protein)			
FAS	NADPH	Acetyl–CoA	Malonyl –CoA	Methyl malonyl-CoA	NADPH	Acetyl–CoA	Malonyl –CoA	Methyl malonyl-CoA
crude	18	7.1	20	11	100	143	200	5
purified	12.5	6.9	20.8	12	166	250	249	2

Increasing concentrations of acetyl-CoA showed a hyperbolic increase in activity of microsomal FAS (Figures 55 and 56). Methylmalonyl-CoA acted as an inhibitor, lowering the reaction rate. It acted soley as an uncompetitive inhibitor, in contrast to the cytosolic where it acted as a mixed inhibitor. shown by the effect on slope and intercept (Figures 55 and 56). The acetyl-CoA K_m for the crude and purified forms was 7.1 μM and 6.9 μM respectively. The replots (Figures 57 and 58) give the K'_i as 7.9 μM for the crude microsomal FAS and 12.6 μM for the purified enzyme.

Malonyl-CoA showed similar effects (Figures 59 and 60). The K_m's were 20 μM and 20.8 μM for the crude and purified enzyme respectively. Here, methylmalonyl-CoA acted as both a competitive and uncompetitive inhibitor (Figures 61 and 62). The K_i was 3.2 μM for the crude enzyme and 11 μM for the purified.. The K'_i was 5.3 μM for the crude and 5.4 μM for the purified FAS.

The NADPH K_m for the crude enzyme was 18 μM and 12.5 μM for the pure enzyme. The replots show there is no competitive inhibition (Figure 65 and 66) in line with the proposed mechanisms for this enzyme.. They show the inhibition to be purely uncompetitive with a K'_i of 20.8 μM for the crude enzyme and 7.8 μM for the purified. A summary of the kinetic parameters for the crude and purified cytosolic and microsomal FAS are in Table VII.

THE PRIMER SPECIFICITY OF CYTOSOLIC AND MICROSOMAL FAS

We also investigated the primer specificity of cytosolic and microsomal FAS. The usual primer for FAS is assumed to be acetyl-CoA, and both FASes showed high activity with acetyl-CoA (Figure 67). However, cytosolic FAS showed slightly less activity with propionyl-CoA, while microsomal FAS showed significantly higher activity with propionyl-CoA than with acetyl-CoA. Cytosolic FAS showed much less activity with longer primers, while microsomal FAS showed significant activity with longer primers. In particular isobutyryl and pentanyl-CoA were not significantly worse primers than acetyl-CoA for microsomal FAS, while they were quite a bit lower for cytosolic FAS. The primers tested, with the exception of acetyl and isopentanyl-CoA showed higher activity with microsomal FAS than with cytosolic.

THE PRODUCTS OF CYTOSOLIC AND MICROSOMAL FAS

The presence of two similar enzymes in distinctly different cellular locations led me to ask if part of the reason for having two enzymes is that they produce different products. An additional question is the interaction of TE II with FAS. While it is known the TE II interacts with the cytosolic enzyme, microsomal FAS may be unable to interact

with it, either inherently or by virtue of its association with the microsomes. I decided to test the products produced by microsomal FAS both on and off the microsomes, with and without added TE II.

Purified cytosolic and microsomal FAS were incubated with 1.875 μM radiolabeled malonyl-CoA along with cold malonyl-CoA in total concentrations from 60 μM to 1.875 μM. NADPH and acetyl-CoA were at 100 μM and 60 μM respectively. When the products were analyzed, both microsomal and cytosolic FAS produced primarily stearate at high concentrations of malonyl-CoA, while palmitate predominated at low concentrations (Figures 68 and 69). At the lowest concentration of malonyl-CoA cytosolic FAS had myristate as its second most abundant product, while microsomal FAS produced more stearate. When crude cytosolic FAS, which should contain naturally occurring TE II, was incubated in the same concentrations of malonyl-CoA, it also showed a similar progression, however at 60 μM malonyl-CoA its primary product was palmitate instead of stearate, and at 1.875 μM it produced primarily myristate (Figure 70).

To investigate if the microsomal FAS was also able to interact with TE II, it was incubated with and without added TE II both on the microsomes and in the soluble state. Purified cytosolic FAS was also tested at the same time (Figure 71). All preparations produced primarily palmitate without TE II and primarily myristate with it. This shows that both the microsomal and cytosolic FAS are able to interact with TE II to produce shortened products, and the microsomal FAS interacts with TE II independent of its assocation with the microsomes. An alternative method of testing the interaction of TE II with the two FASes was to inactivate the integral TE I of crude microsomal and cytosolic FAS with PMSF. The two FASes were then partially purified by

ammonium sulfate precipitation to remove any remaining PMSF, and then incubated with TE II. Both FASes were reactivated and produced mostly myristate and palmitate (Figure 72).

FAS Peptide Mapping

The cytosolic and microsomal FAS may be the same protein fixed in different subcellular locations by post-translational modification, or two different gene products. This can be investigated by protease digestion and separation of the products. Another purpose of peptide mapping is to provide peptides that can be sequenced, giving further information on the structure of the proteins and allowing oligonucleotide probes to be designed for DNA sequencing.

Limited trypsinization was conducted on microsomal and cytosolic FAS. The products were run out on 5% SDS-PAGE gels, blotted on to PVDF, and stained with Coommassie blue (Figure 73). The molecular weights of the bands were determined by reference to the standards and the results were co-plotted for direct comparison (Figure 74). The two most intense bands from the cytosolic blot were selected for sequencing. The 194 and 107 KDa bands of cytosolic FAS (see Figure 74) had the same N-terminal sequence, HARFPQDNQQ–TSNGAVNGN.... The 103 KDa and 90 KDa bands of microsomal FAS were selected because they had the possibility of being related to the 107 KDa band from cytosolic FAS and having some sequence identity, allowing me to make definitive statements about whether or not microsomal and cytosolic FAS are different gene products. Unfortunately this turned out not to be the case. The 103 KDa band had an N terminal sequence of KNG–NEGV.... The 90 KDa microsomal band had the sequence PTVQQISKWDWEKQRDII.... The blank cycles could be cysteine, due to the samples not having been alkylated before sequencing. This

information, although not allowing me to make a statement about the degree of sequence similarity between cytosolic and microsomal FAS, will allow the construction of primers to probe a cDNA library.

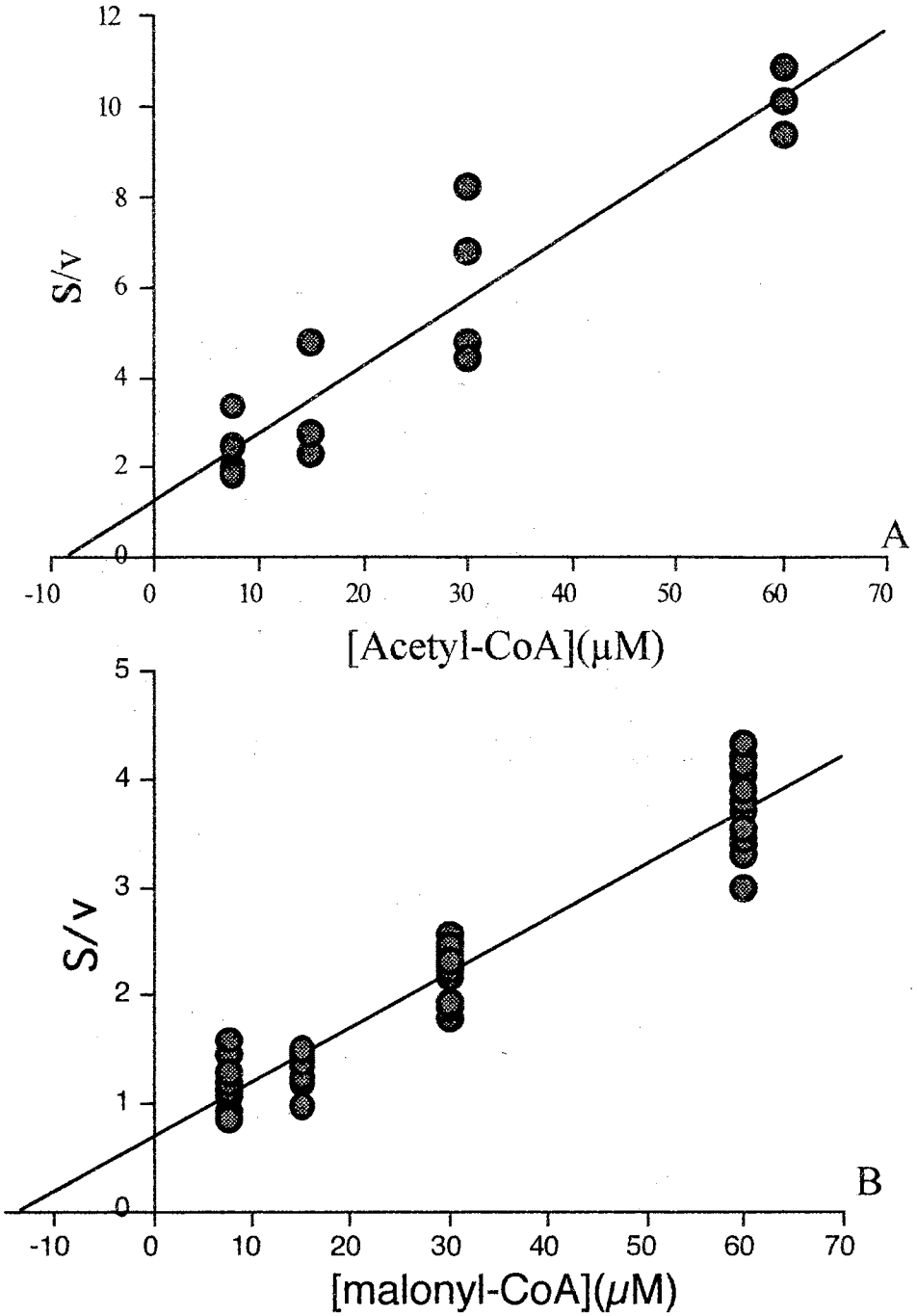


Figure 51. Hanes-Woolf Plots of Crude Microsomal FAS, Acetyl-CoA and Malonyl-CoA. Obtained by varying (A) acetyl-CoA, (B) malonyl-CoA, between 7.5 and 60 μM. The fixed substrate concentrations were 100 μM NADPH, 60 μM malonyl-CoA, and 60 μM acetyl-CoA in a final volume of 400 μl pH 7.4 buffer. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

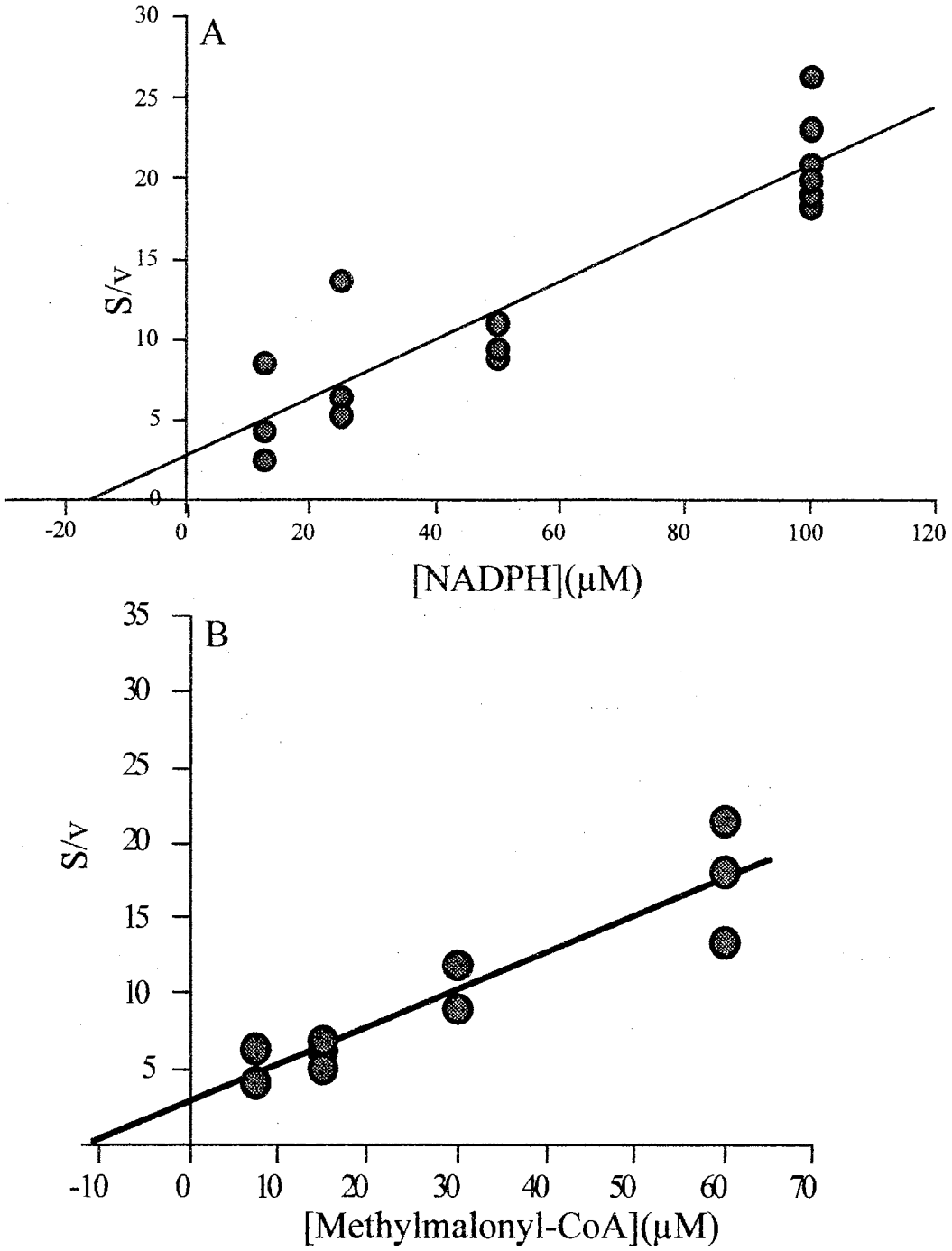


Figure 52. Hanes-Woolf Plots of Crude Microsomal FAS: NADPH and Methylmalonyl-CoA. (A) NADPH was varied between 12.5 and 100 μM, with 60 μM malonyl-CoA, and 60 μM acetyl-CoA in a final volume of 400 μl pH 7.4 buffer. (B) Methylmalonyl-CoA was varied between 7.5 and 60 μM. The fixed substrate concentrations were 100 μM NADPH and 60 μM acetyl-CoA in a final volume of 400 μl pH 7.4 buffer. The reaction was started by adding malonyl-CoA or methylmalonyl-CoA.

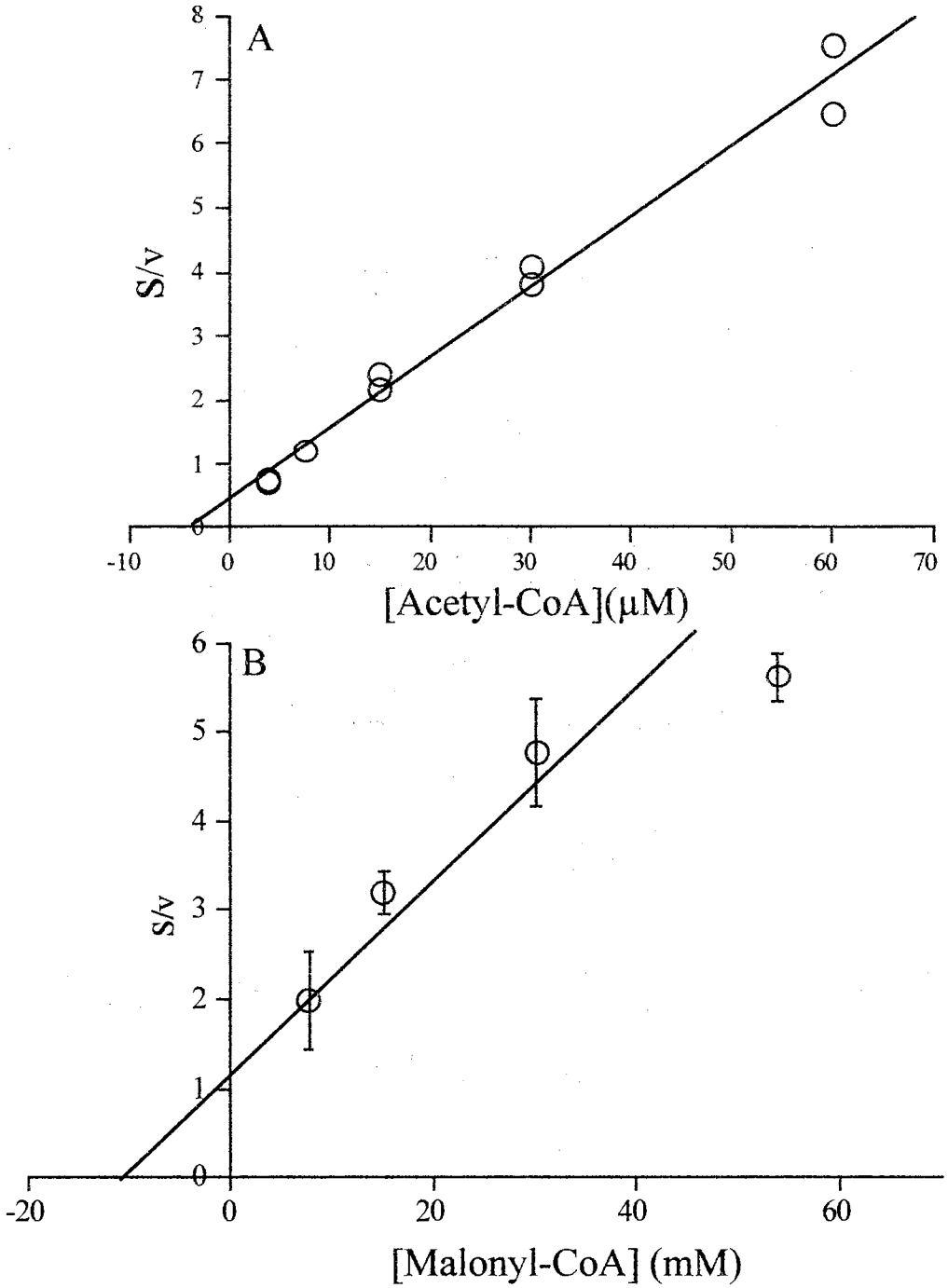


Figure 53. Hanes-Woolf Plots of Purified Microsomal FAS: Acetyl-CoA and Malonyl-CoA. (A) Acetyl-CoA was varied between 7.5 and 60 μM, with 60 μM malonyl-CoA, and 100 μM NADPH in a final volume of 400 μl pH 7.4 buffer. (B) Malonyl-CoA was varied between 7.5 and 60 μM. The fixed substrate concentrations were 100 μM NADPH and 60 μM acetyl-CoA in a final volume of 400 μl pH 7.4 buffer. The reaction was started by adding malonyl-CoA.

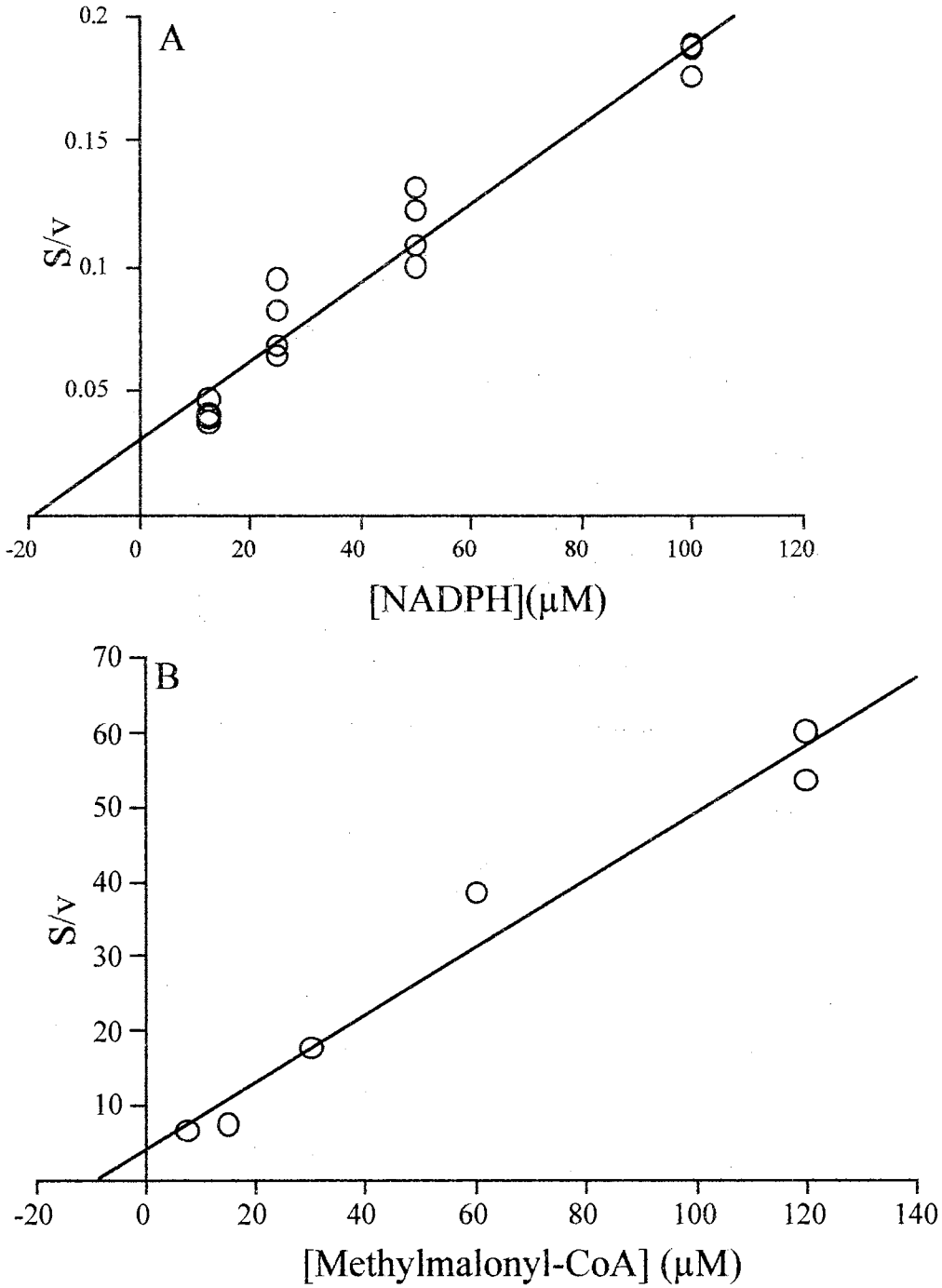


Figure 54. Hanes-Woolf Plots of Purified Microsomal FAS: NADPH and Methylmalonyl-CoA. (A) NADPH was varied between 12.5 and 100 μM, with 60 μM malonyl-CoA, and 60 μM acetyl-CoA in a final volume of 400 μl pH 7.4 buffer. (B) Methylmalonyl-CoA was varied between 7.5 and 60 μM. The fixed substrate concentrations were 100 μM NADPH and 60 μM acetyl-CoA in a final volume of 400 μl pH 7.4 buffer. The reaction was started by adding malonyl-CoA or methylmalonyl-CoA.

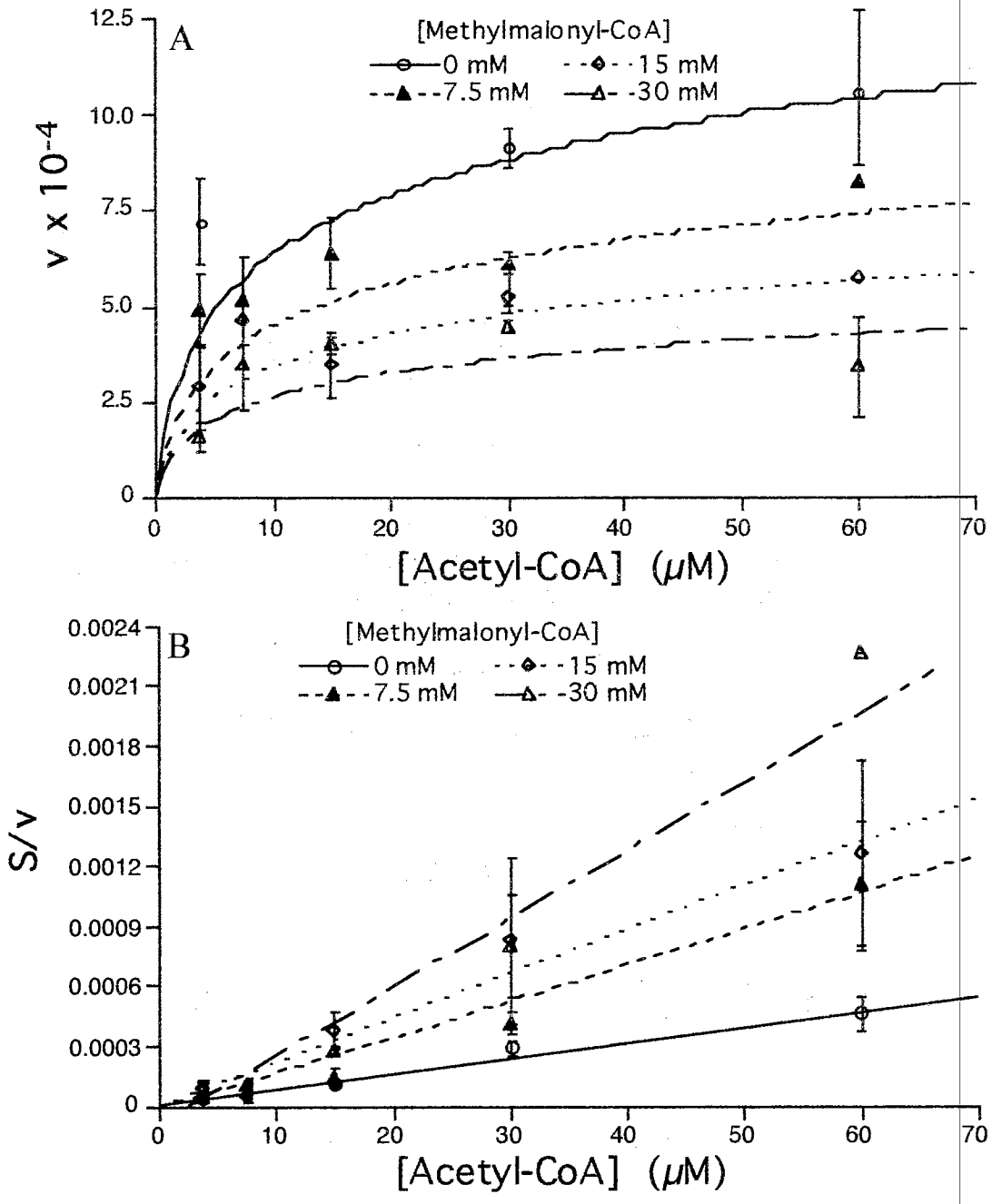


Figure 55. The Effect of Methylmalonyl-CoA with Acetyl-CoA on Fatty Acid Synthesis by Crude Microsomal FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl-CoA, 0, 7.5 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl - CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

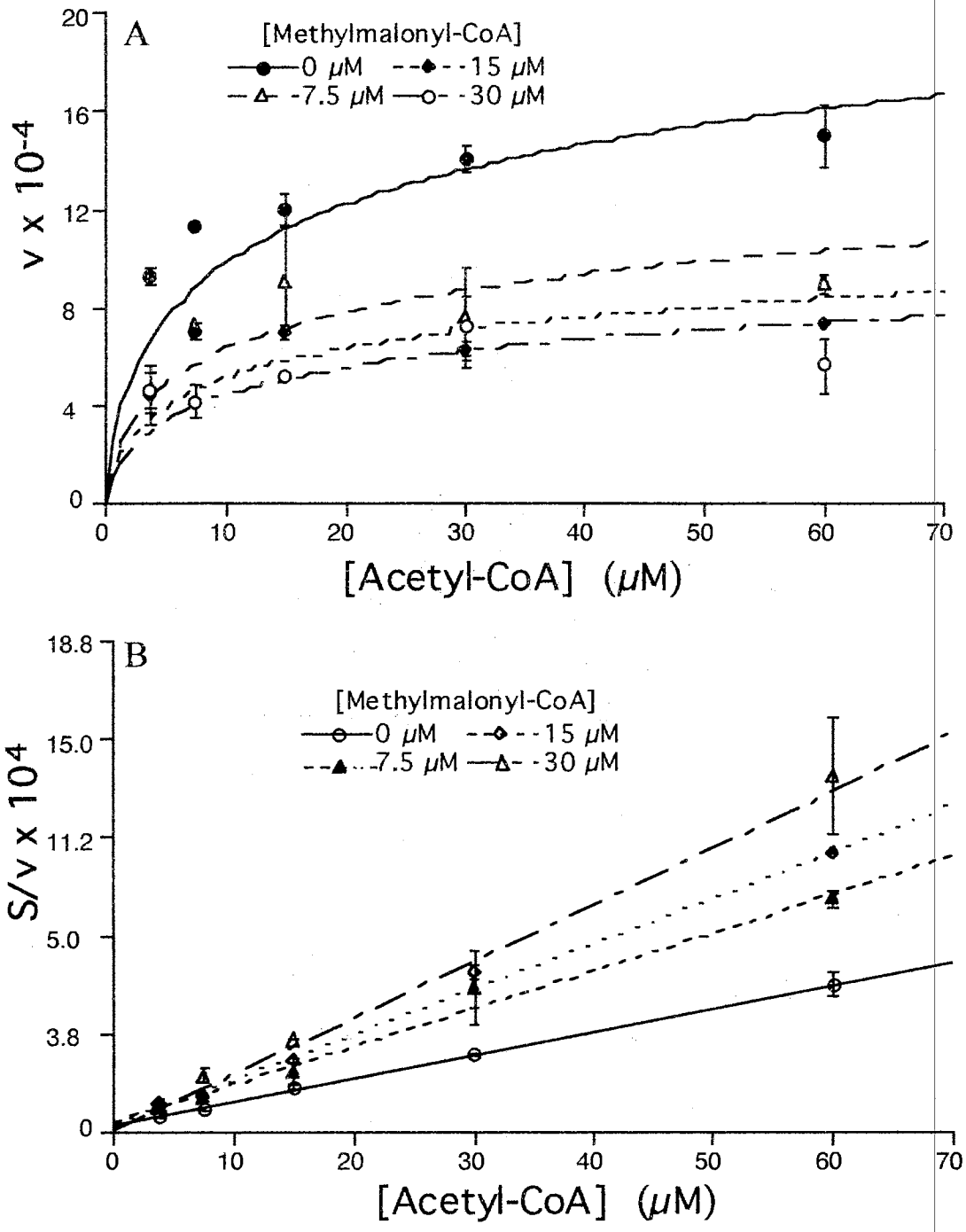


Figure 56. The Effect of Methylmalonyl-CoA with Acetyl-CoA on Fatty Acid Synthesis by Purified Microsomal FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl-CoA, 0, 7.5 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl - CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

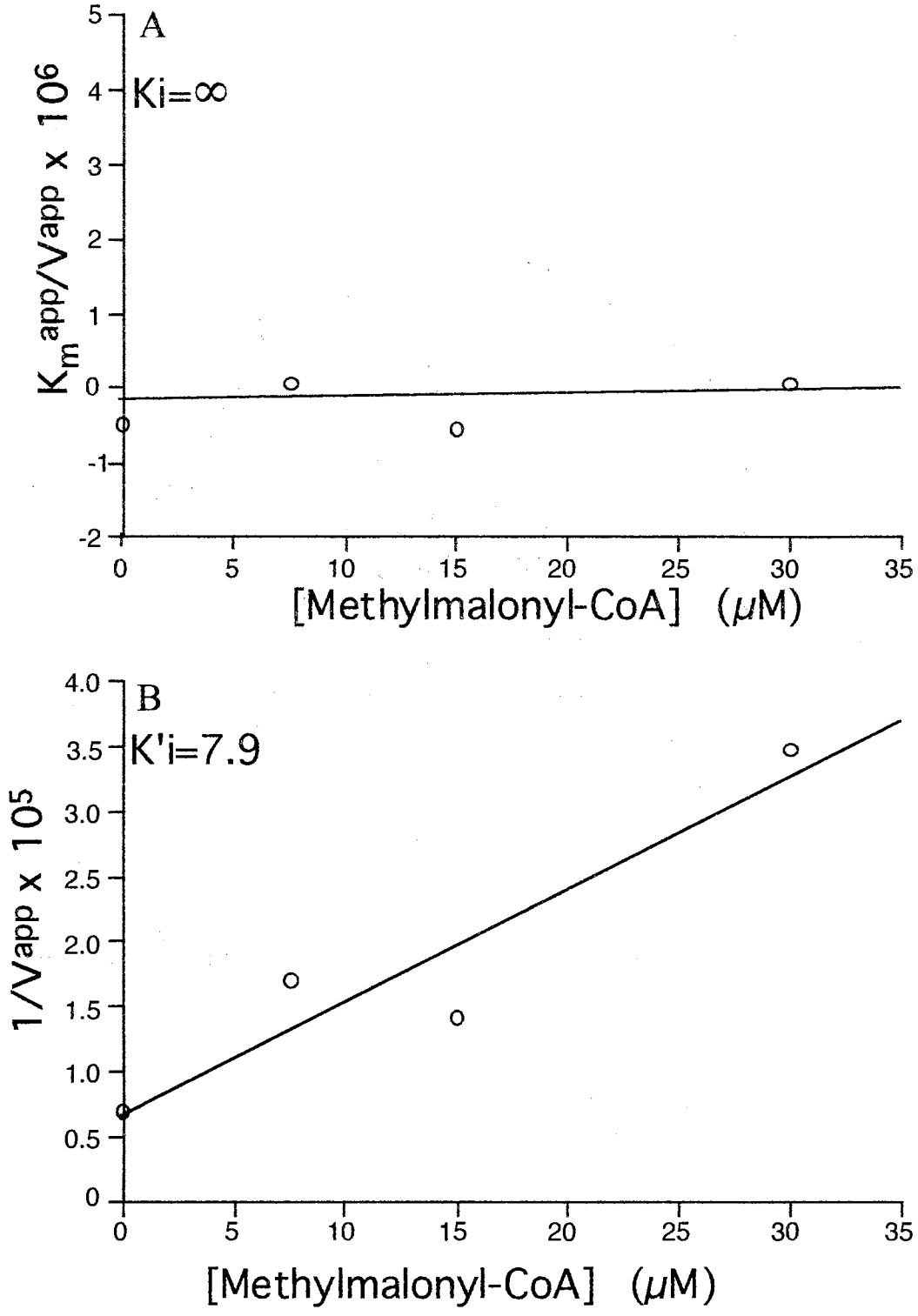


Figure 57. Replots of the Effect of Methylmalonyl-CoA with Acetyl-CoA on Fatty Acid Synthesis by Crude Microsomal FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I].

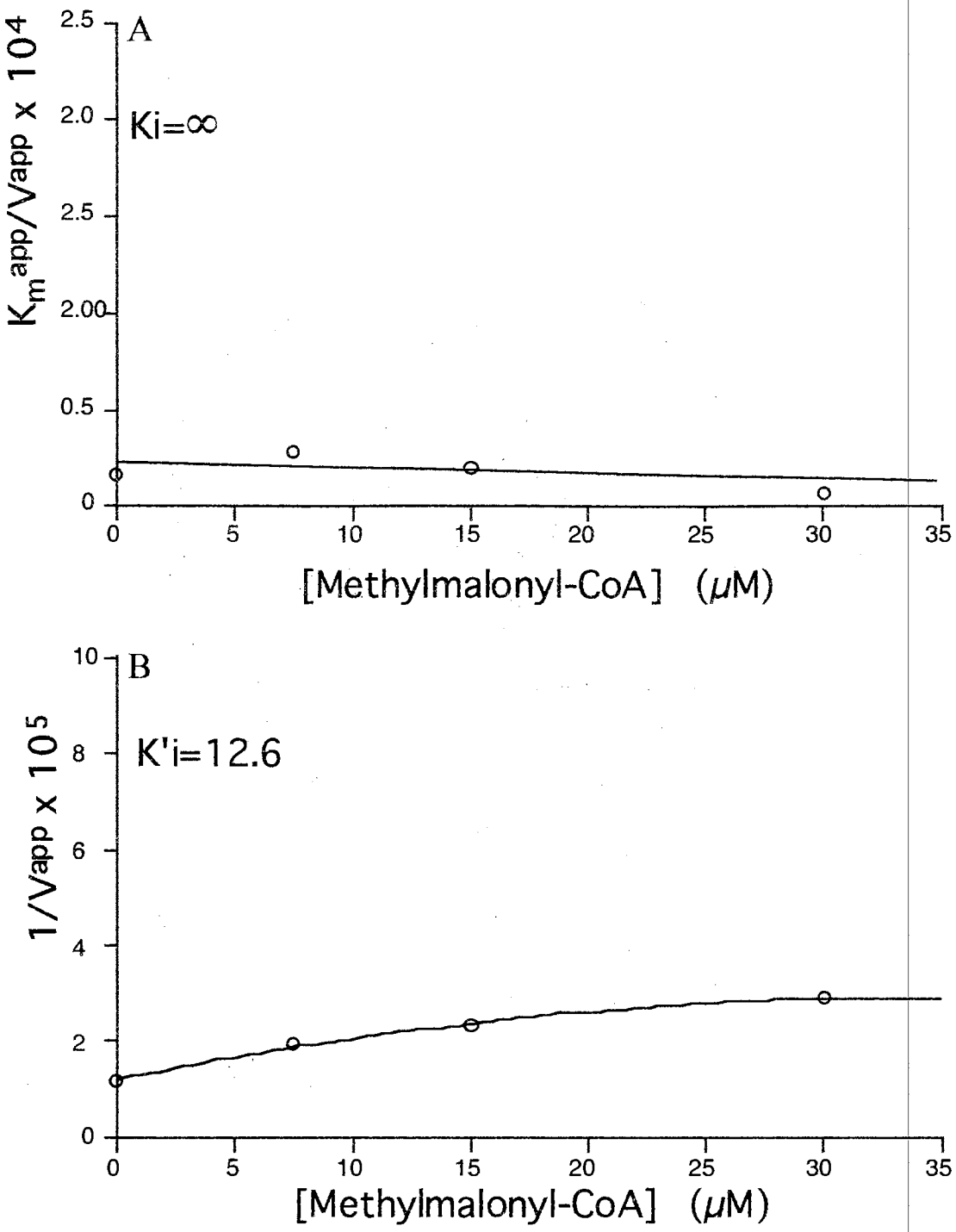


Figure 58. Replots of the Effect of Methylmalonyl-CoA with Acetyl-CoA on Fatty Acid Synthesis by Purified Microsomal FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I].

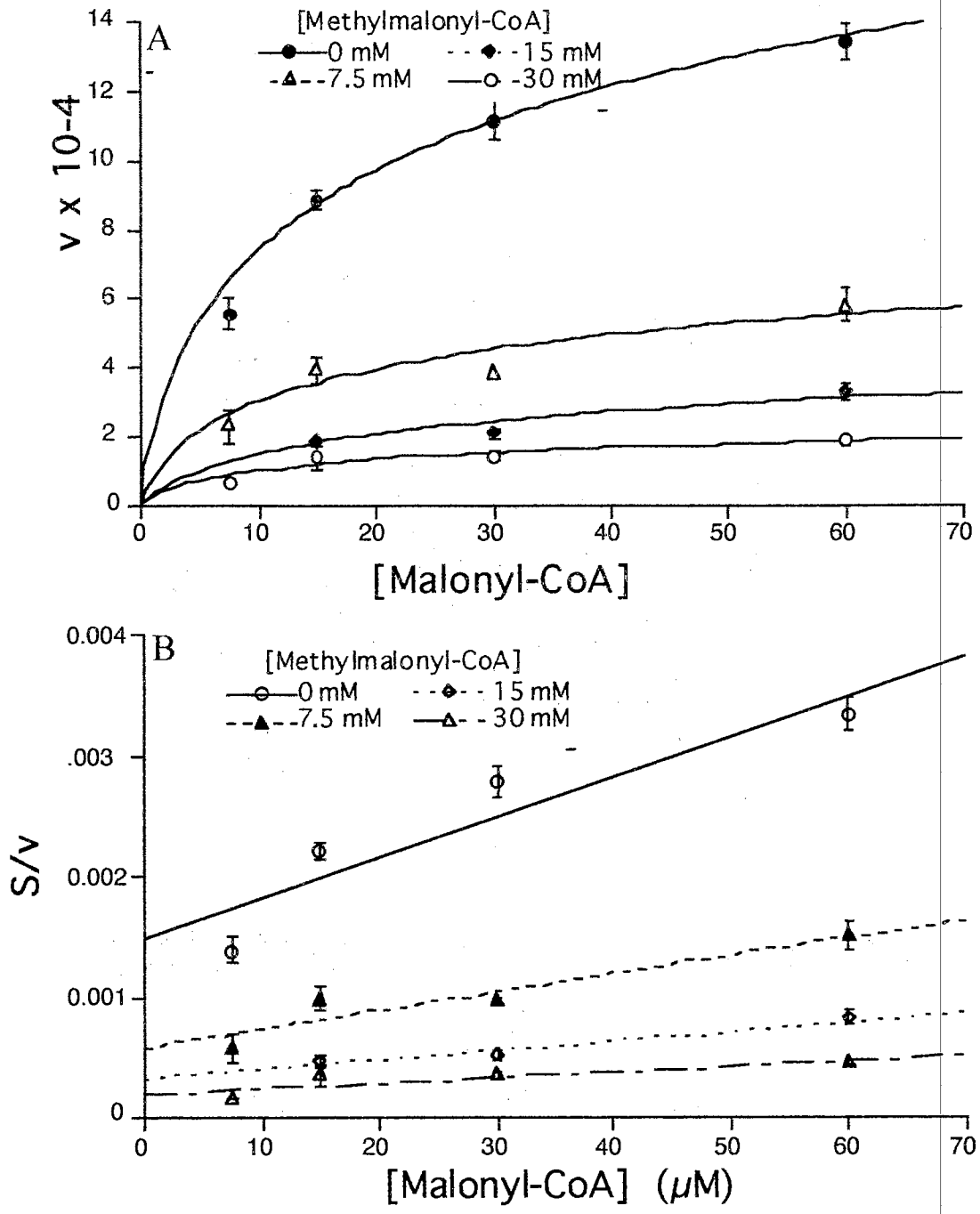


Figure 59. The Effect of Methylmalonyl-CoA with Malonyl-CoA on Fatty Acid Synthesis by Crude Microsomal FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl-CoA, 0, 7.5, 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl - CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

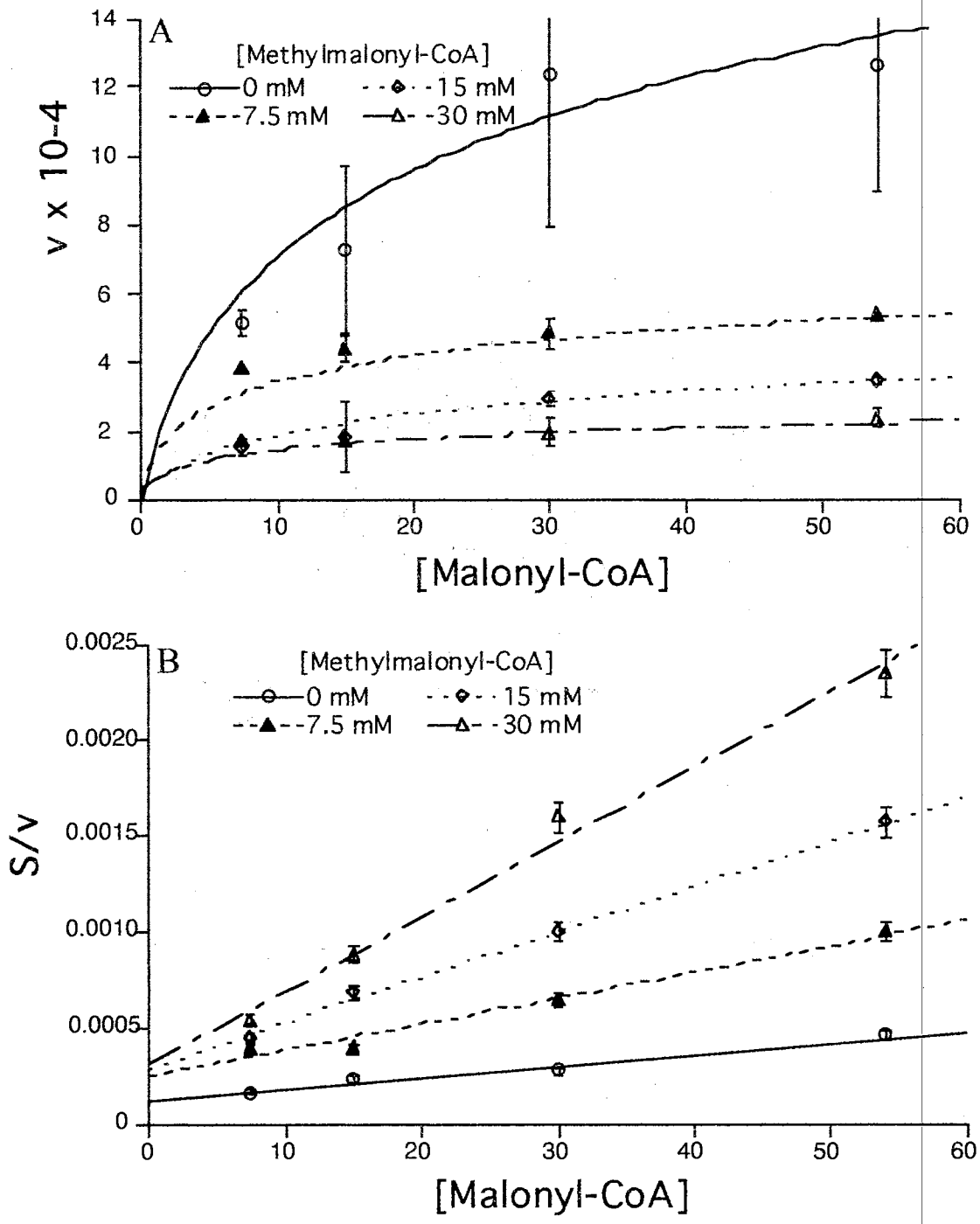


Figure 60. The Effect of Methylmalonyl-CoA with Malonyl-CoA on Fatty Acid Synthesis by Purified Microsomal FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl-CoA, 0, 7.5 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl - CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

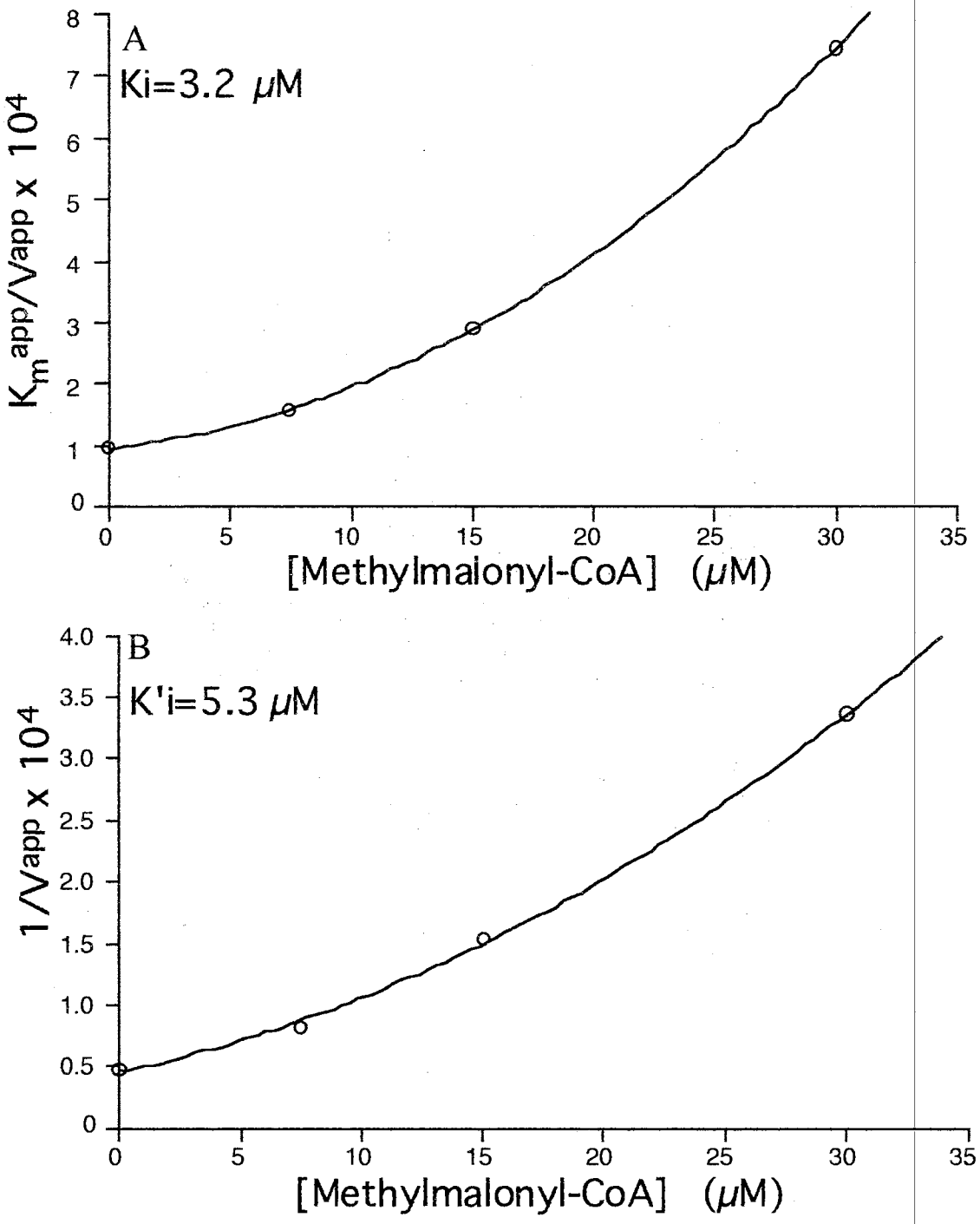


Figure 61. Replots of the Effect of Methylmalonyl-CoA with Malonyl-CoA on Fatty Acid Synthesis by Crude Microsomal FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I].

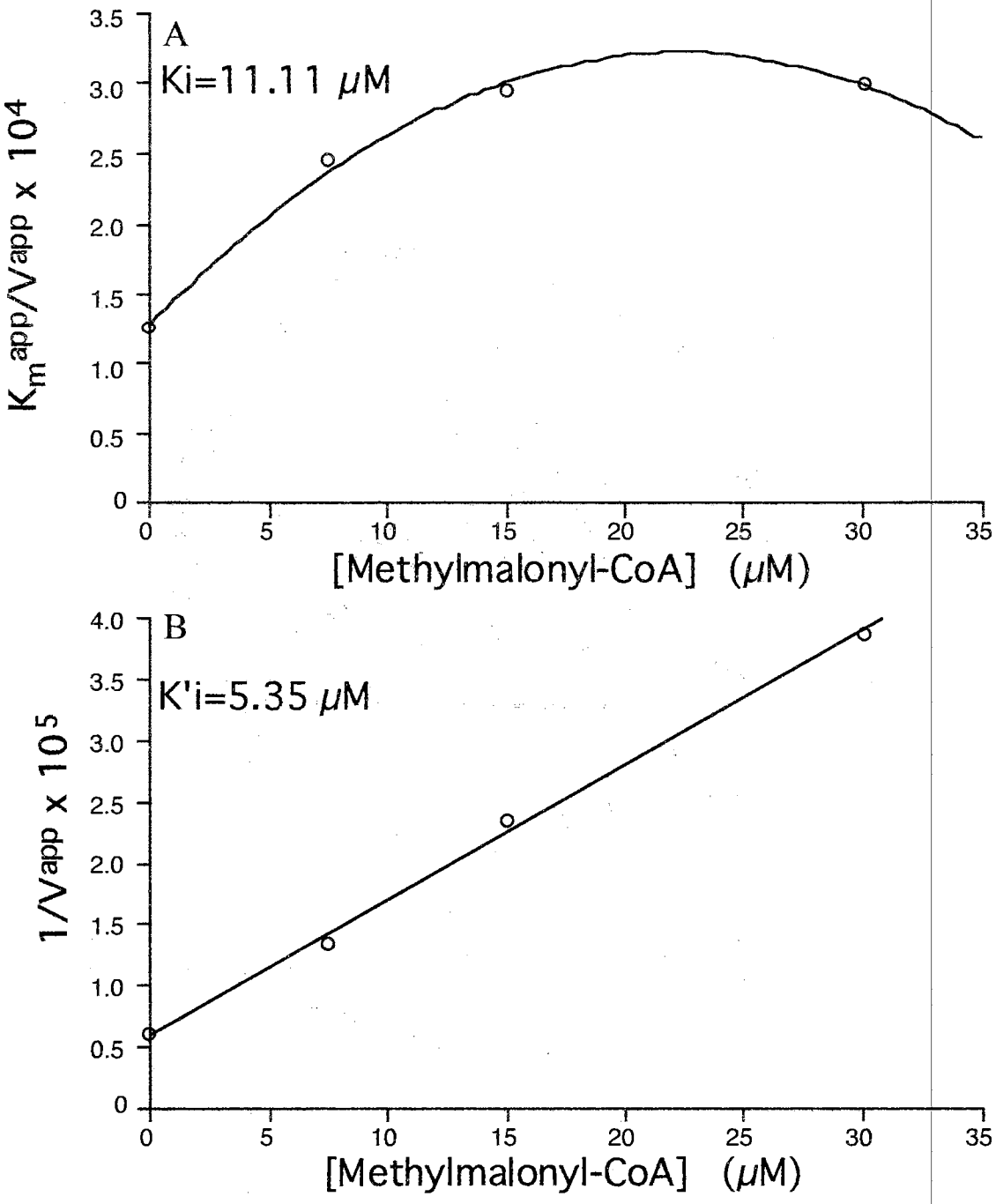


Figure 62. Replots of the Effect of Methylmalonyl-CoA with Malonyl-CoA on Fatty Acid Synthesis by Purified Microsomal FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I].

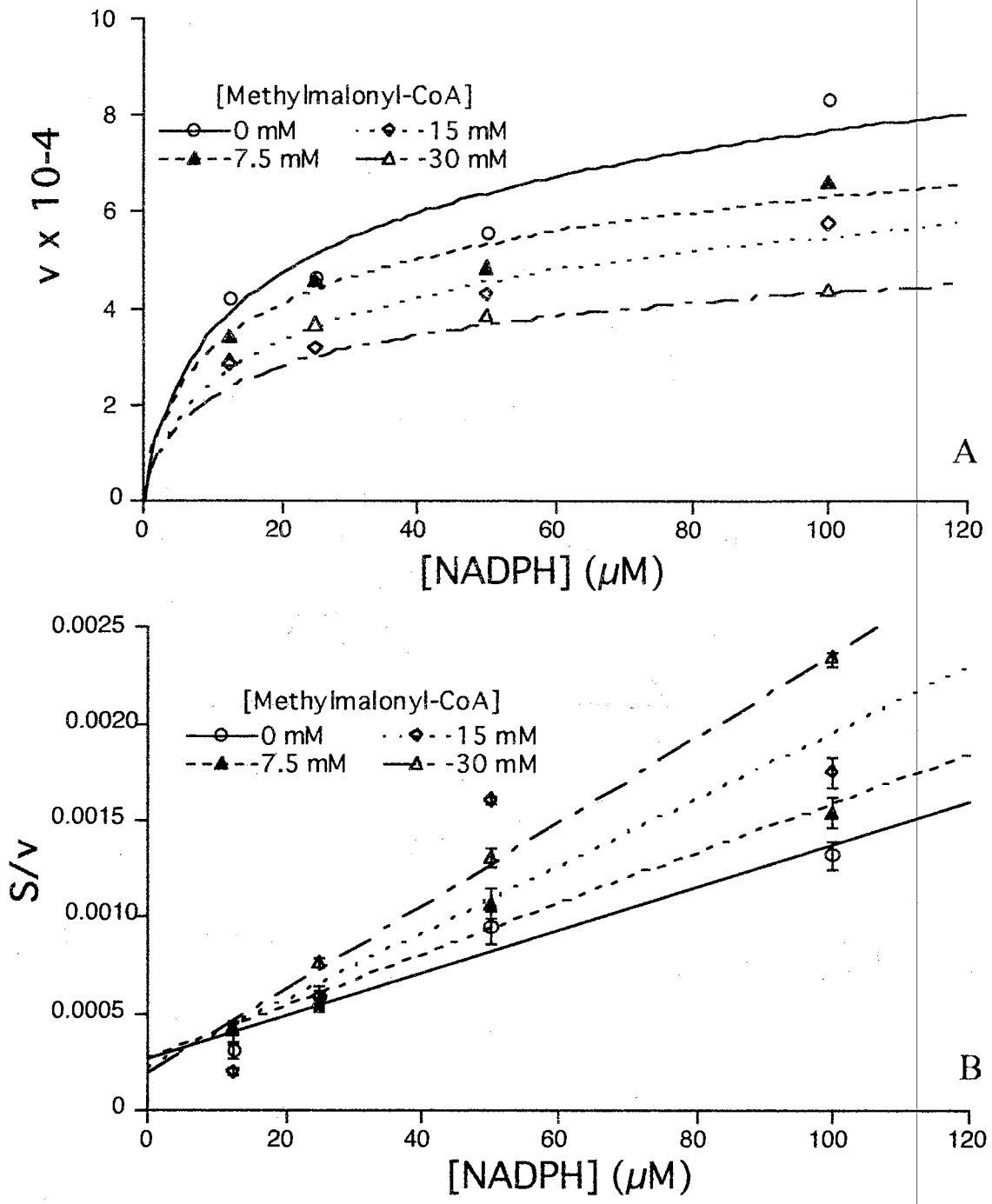


Figure 63. The Effect of Methylmalonyl-CoA with NADPH on Fatty Acid Synthesis by Crude Microsomal FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl - CoA, 0, 7.5, 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl-CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

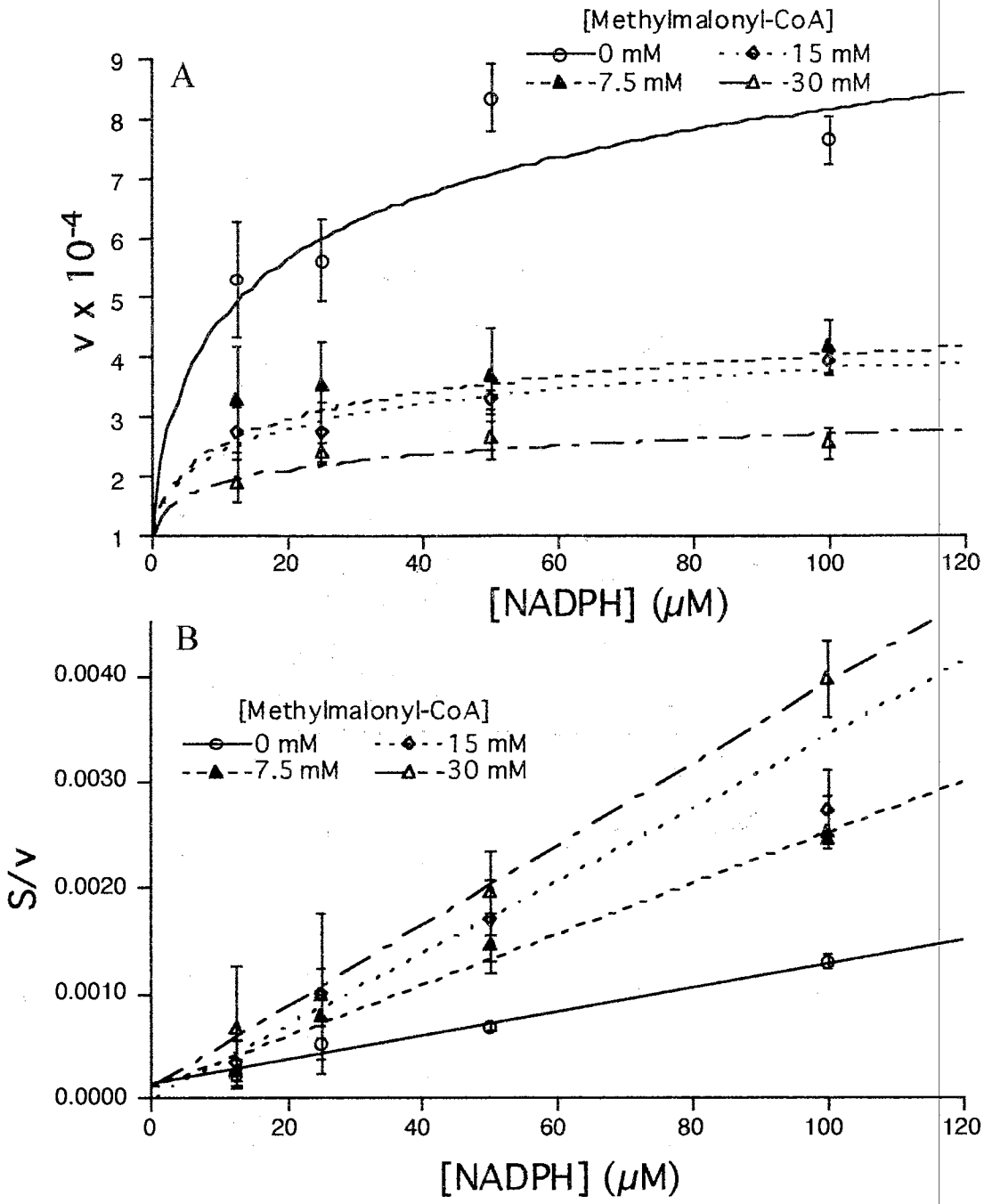


Figure 64. The Effect of Methylmalonyl-CoA with NADPH on Fatty Acid Synthesis by Purified Microsomal FAS. A) V/[S] plot (V=nMoles/min/mg protein) B) Hanes-Woolf plot. The reaction mixture contained pH 7.4 buffer, 100 μM NADPH, 60 μM malonyl-CoA, 0, 7.5, 15, or 30 μM methylmalonyl-CoA, and from 7.5 to 60 acetyl-CoA in a total volume of 400μl. The reaction was started by adding malonyl-CoA to the reaction mixture and monitored spectrophotometrically.

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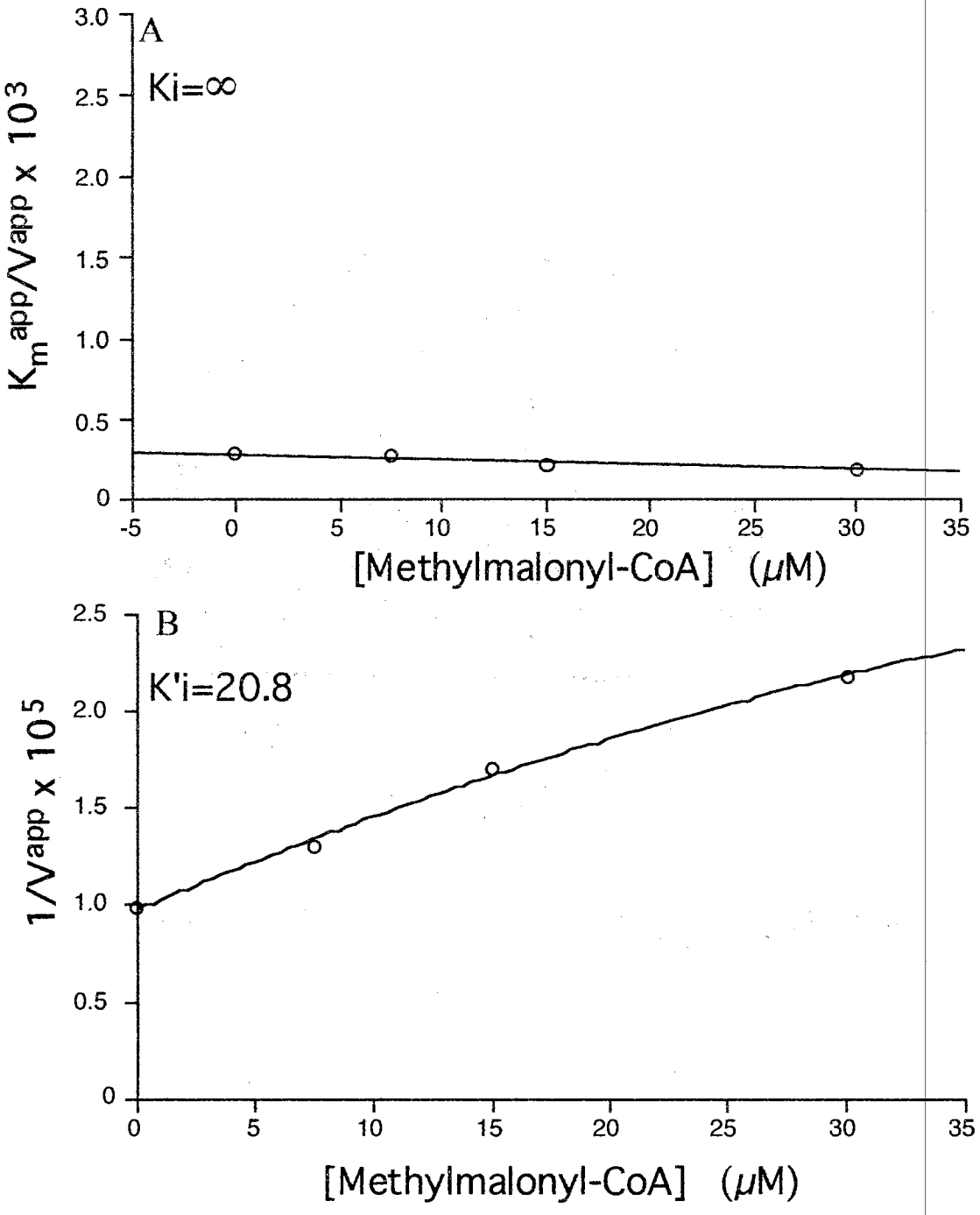

Figure 65. Replots of the Effect of Methylmalonyl-CoA with NADPH on Fatty Acid Synthesis by Crude Microsomal FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I].

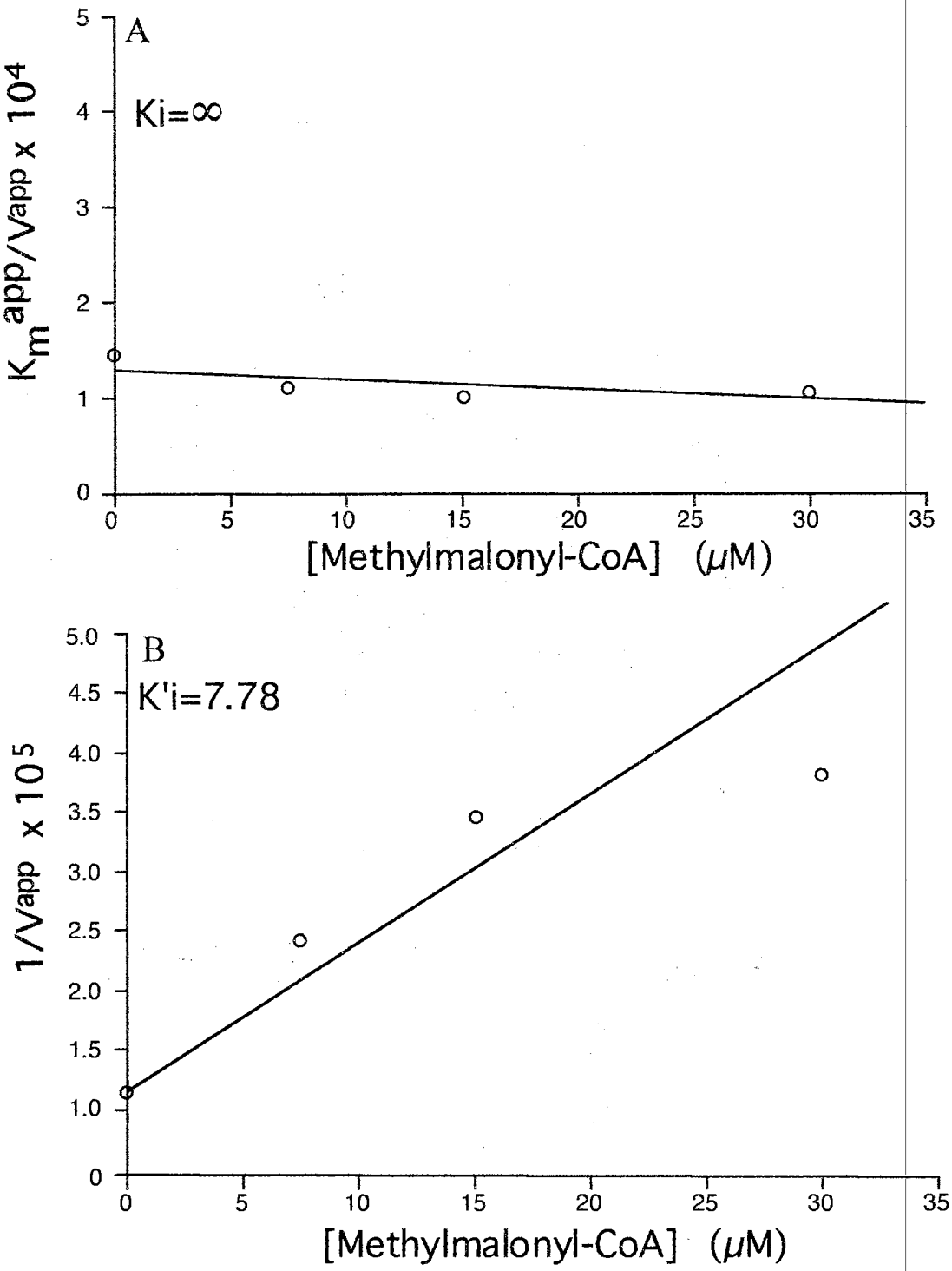

Figure 66. Replots of the Effect of Methylmalonyl-CoA with NADPH on Fatty Acid Synthesis by Purified Microsomal FAS. A) K_m^{app}/V^{app} vs [I] B) $1/V^{app}$ vs [I]].

Table VII Kinetic Parameters for Crude and Purified Cytosolic and Microsomal FAS

Cytosolic

	crude				pure			
Substrate	K_m	V_{max}	K_i	K'_i	K_m	V_{max}	K_i	K'_i
acetyl	10	142	11.4	8.4	6.6	166	11.6	9.5
malonyl	5.7	27	16	∞	5	50	12	∞
NADPH	20.6	91	∞	11	5.8	118	∞	8
methylmalonyl	*	-	-	-	*	-	-	-

Microsomal

	crude				pure			
Substrate	K_m	V_{max}	K_i	K'_i	K_m	V_{max}	K_i	K'_i
acetyl	7.1	143	∞	7.9	6.9	250	∞	12.6
malonyl	20	200	8	4	20.8	249	8	6
NADPH	18	100	∞	20	12.5	166	∞	14
methylmalonyl	11	5	-	-	12	2	-	-

* Rate too low to be determined. V_{max} is in μM/min/mg protein. K_m. K_i, and K'_i are in μM.

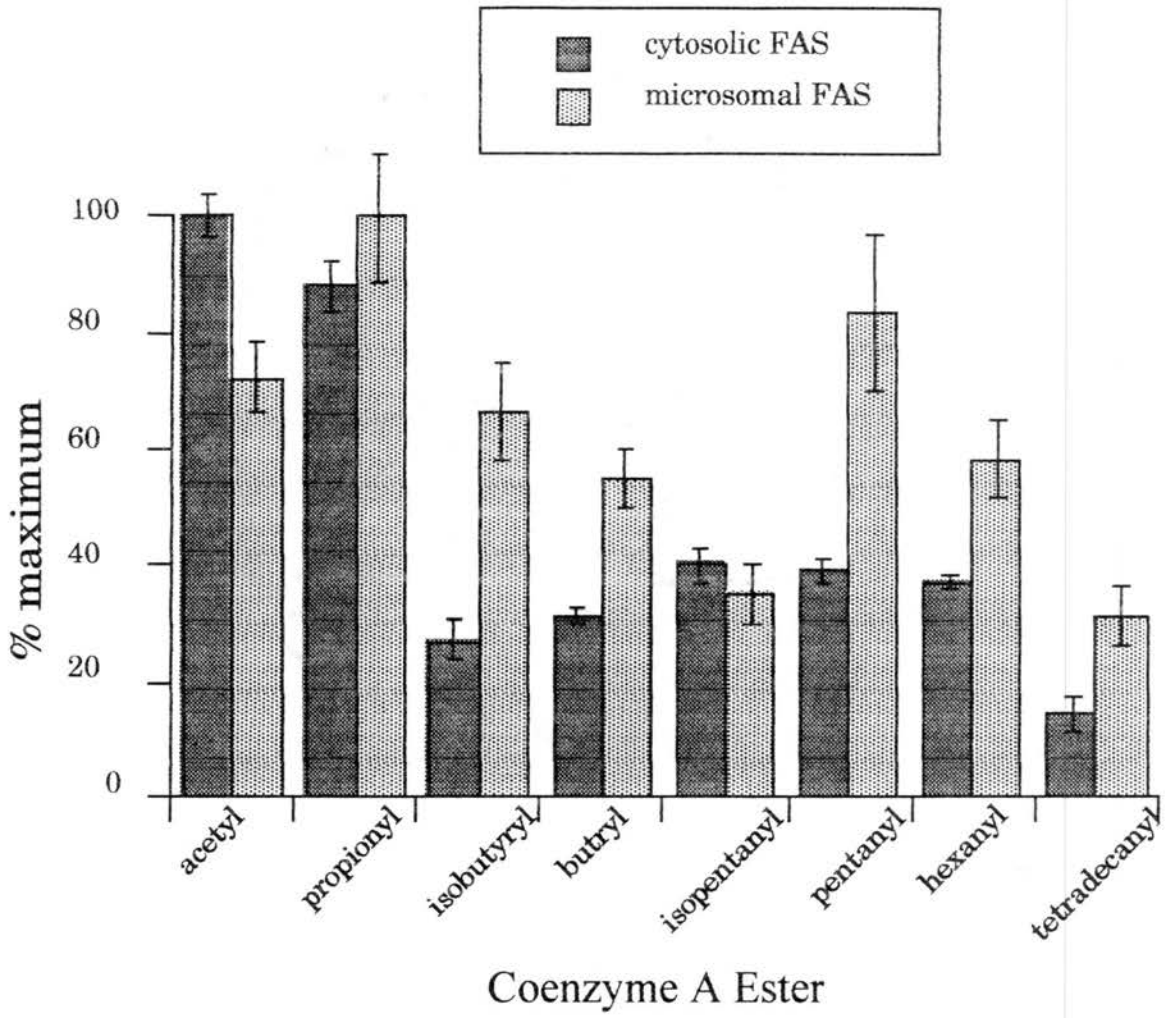

Figure 67. Primer Specificity of Microsomal and Cytosolic FAS. FASes were incubated with 100 μM NADPH, 60 μM malonyl-CoA, and 60 μM of the individual primers. Rates of consumption of NADPH were measured by spectrophotometry, and the results for each enzyme scaled to its most active primer.

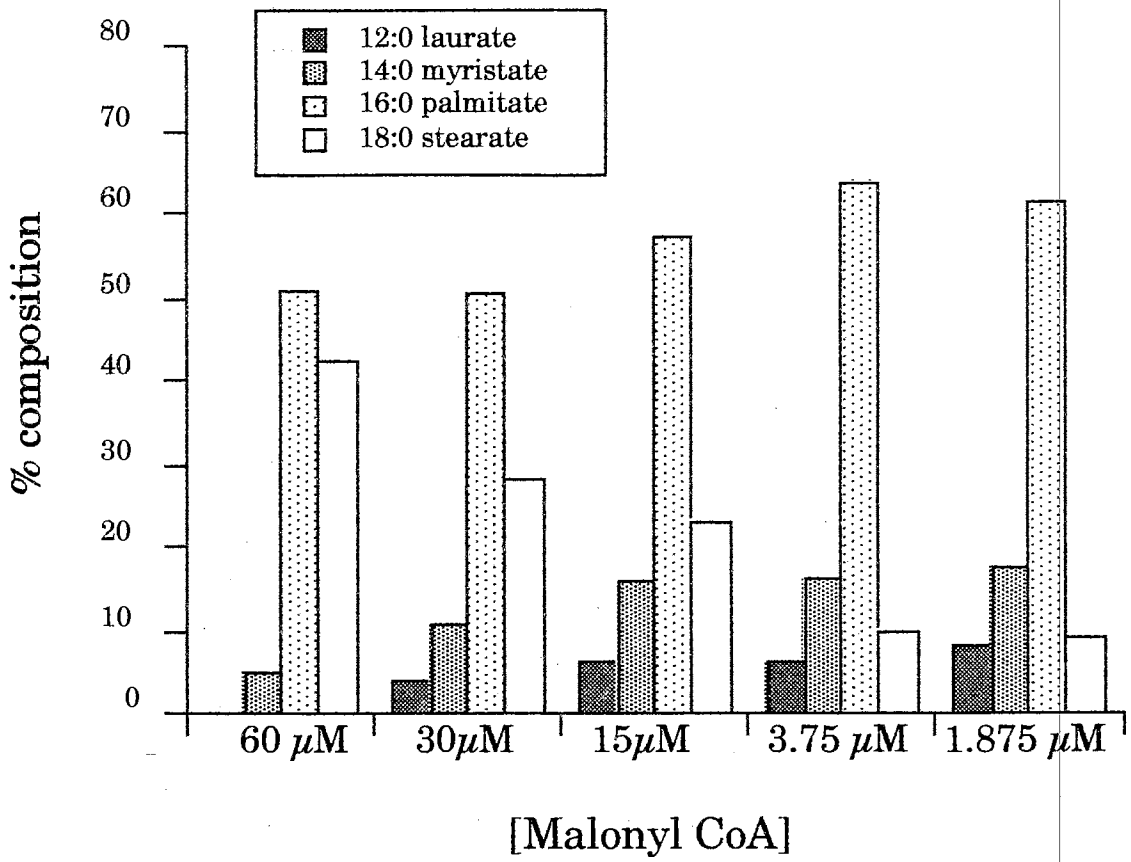

Figure 68. Products of Purified Cytosolic FAS. The FAS was incubated with 100 μM NADPH, 60 μM acetyl-CoA, and 1.875 μM ^{14}C radio-labeled malonyl-CoA with the balance being unlabeled malonyl-CoA. Products were extracted, methylated, and analyzed by radio-HPLC.

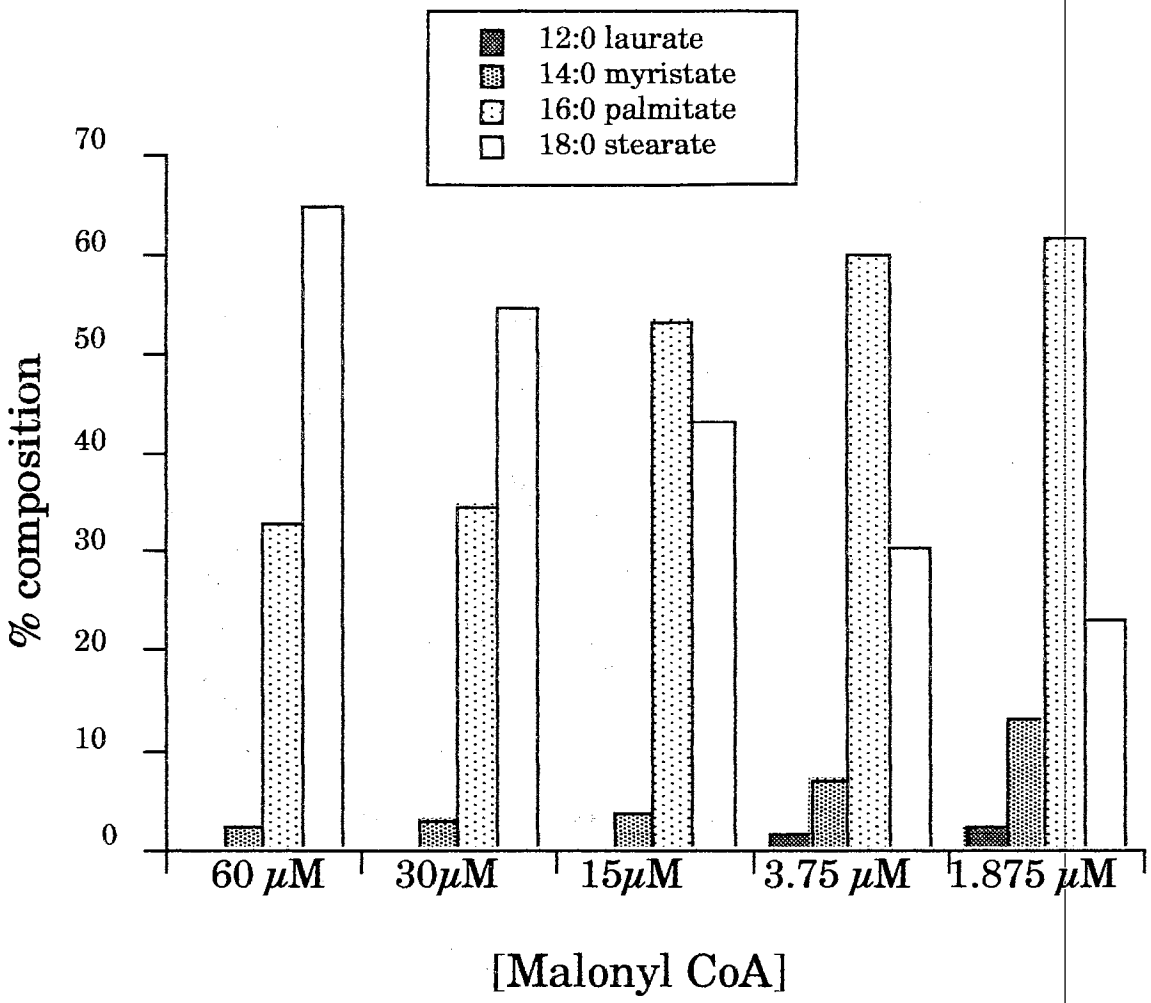

Figure 69. Products of Purified Microsomal FAS. The FAS was incubated with 100 μM NADPH, 60 μM acetyl-CoA, and 1.875 μM 14C radio-labeled malonyl-CoA with the balance being unlabeled malonyl-CoA. Products were extracted, methylated, and analyzed by radio-HPLC.

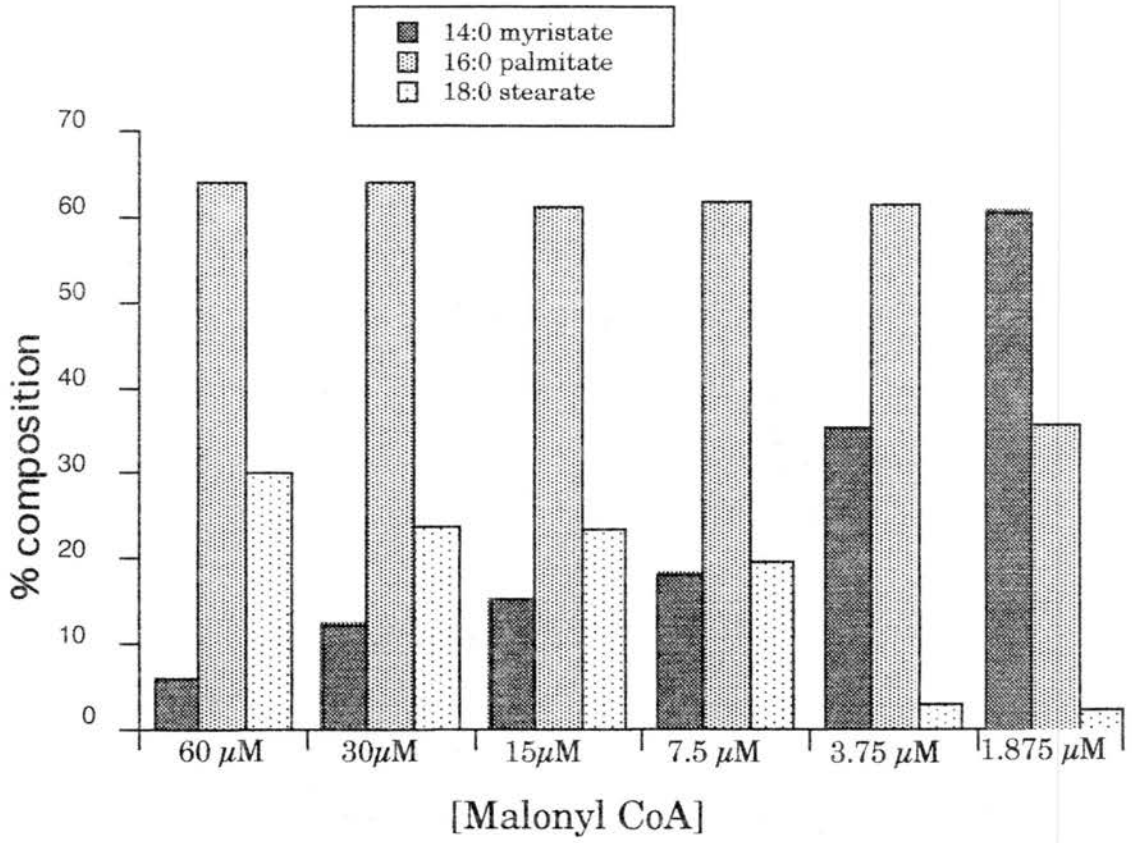

Figure 70. Products of Crude Cytosolic FAS. The FAS was incubated with 100 μM NADPH, 60 μM acetyl-CoA, and 1.875 μM 14C radio-labeled malonyl-CoA with the balance being unlabeled malonyl-CoA. Products were extracted, methylated, and analyzed by radio-HPLC.

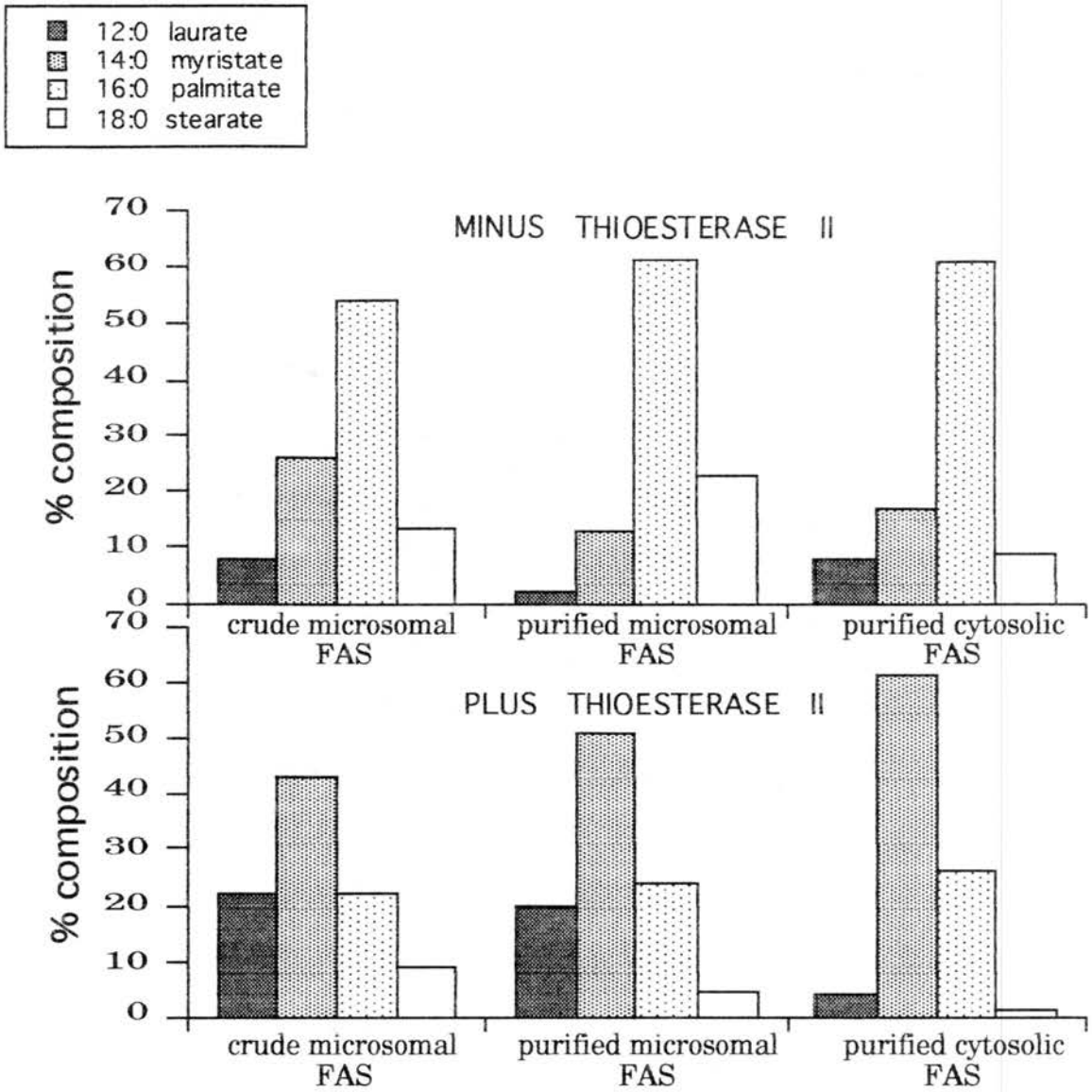

Figure 71. Products of Microsomal and Cytosolic FAS With and Without Added Thioesterase II Containing Cytosol Fraction. The FASes were incubated with 100 μM NADPH, 60 μM acetyl-CoA, and 1.875 μM 14C radio-labeled malonyl CoA. Products were extracted, methylated, and analyzed by radio-HPLC.

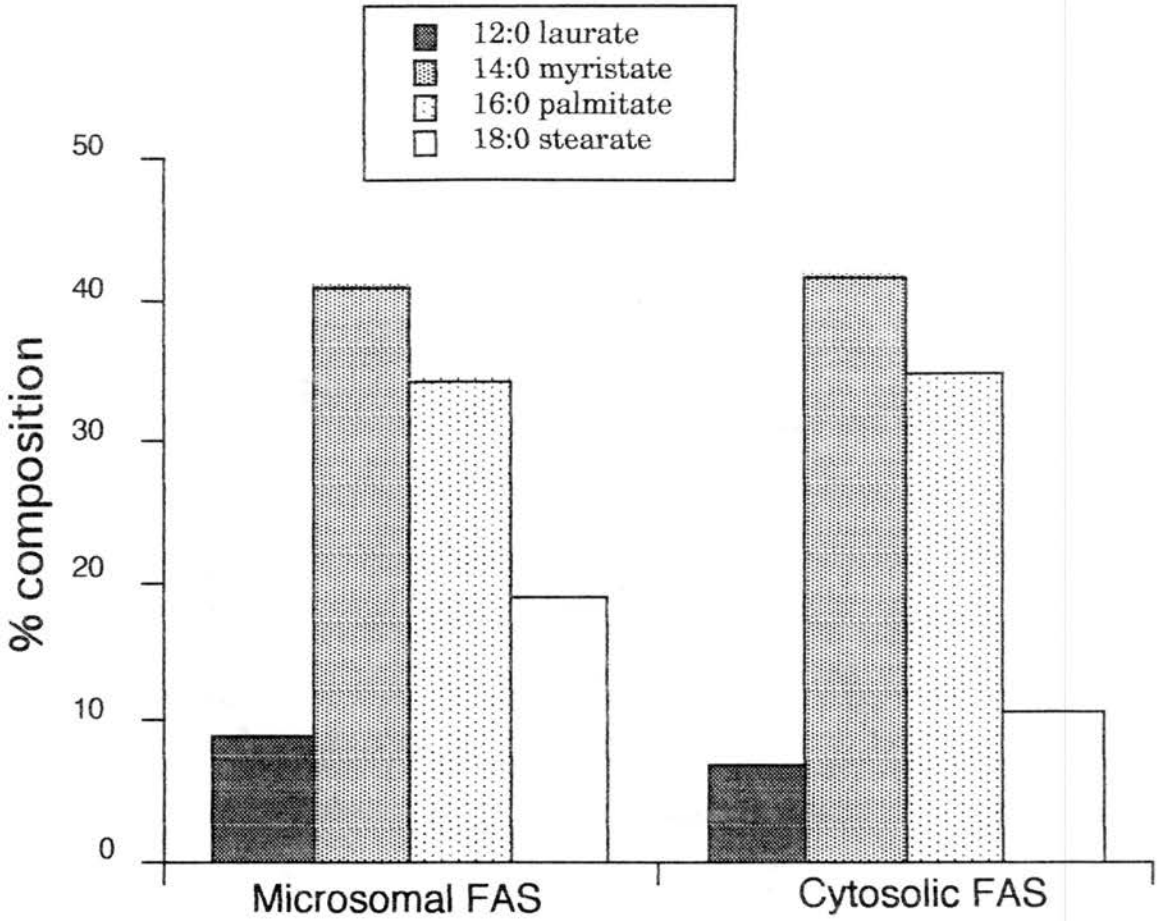

Figure 72. Products of Poisoned Microsomal and Cytosolic FAS. The crude FASes were treated with 200 ng of PMSF until no detectable activity remained. They were then partially purified by precipitation with ammonium sulfate, and incubated with a TE II enriched cytosolic fraction, 100 μM NADPH, 60 μM acetyl-CoA, and 1.875 μM 14C radio-labeled malonyl-CoA. Products were extracted, methylated, and analyzed by radio-HPLC.

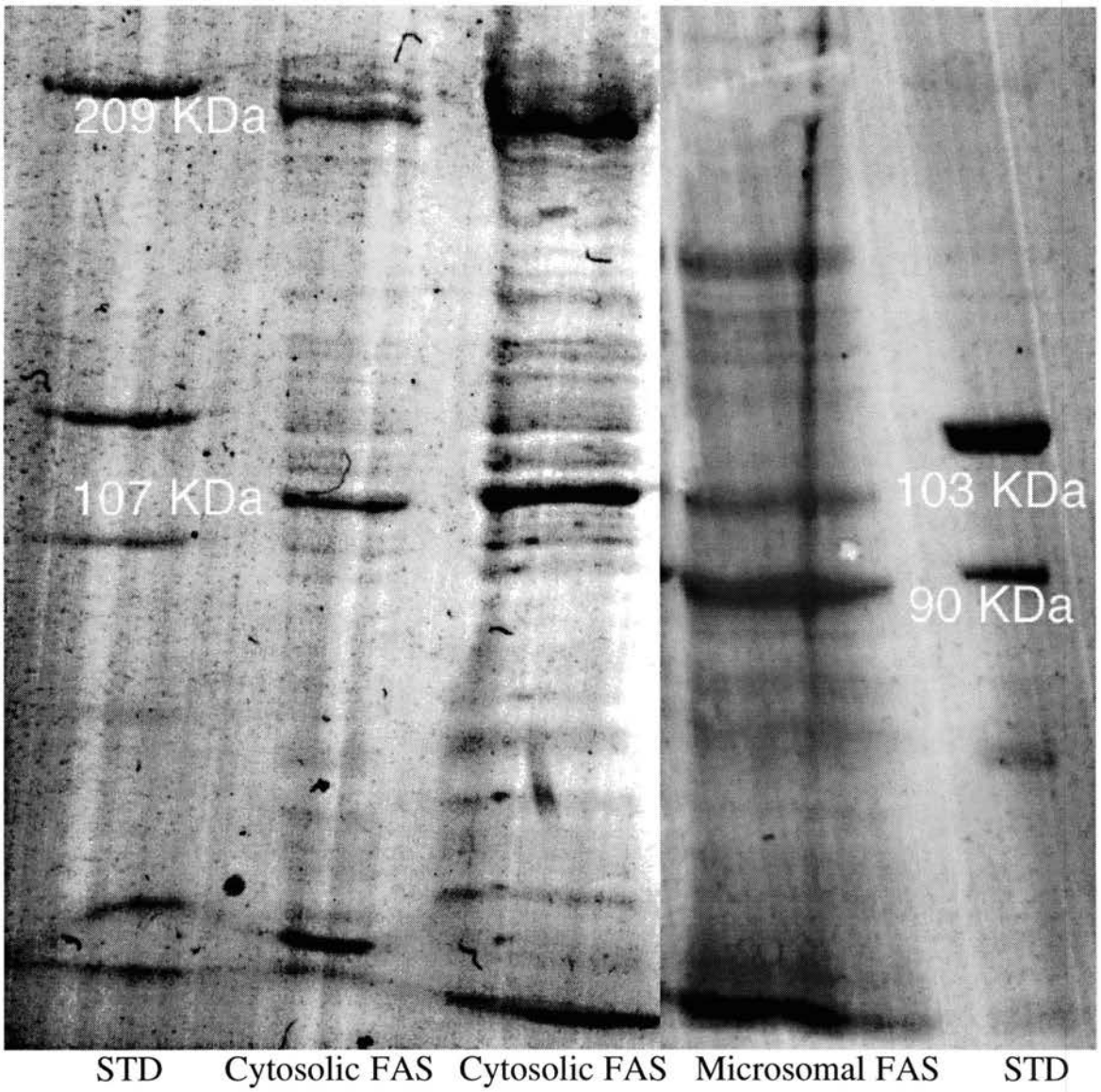

Figure 73. Partial Trypsinization of Cytosolic and Microsomal FAS: 5% SDS-PAGE PVDF Blots. Cytosolic and microsomal FAS were treated with 1:20 trypsin for 4 hours at room temperature, and then run on separate 5% SDS-PAGE gels. The gels were blotted onto PVDF membrane, stained with Coommassie blue, and photographed. The standards are at 200, 116, 97.4, 66, and 45 kilodaltons. Labeled bands were sequenced.

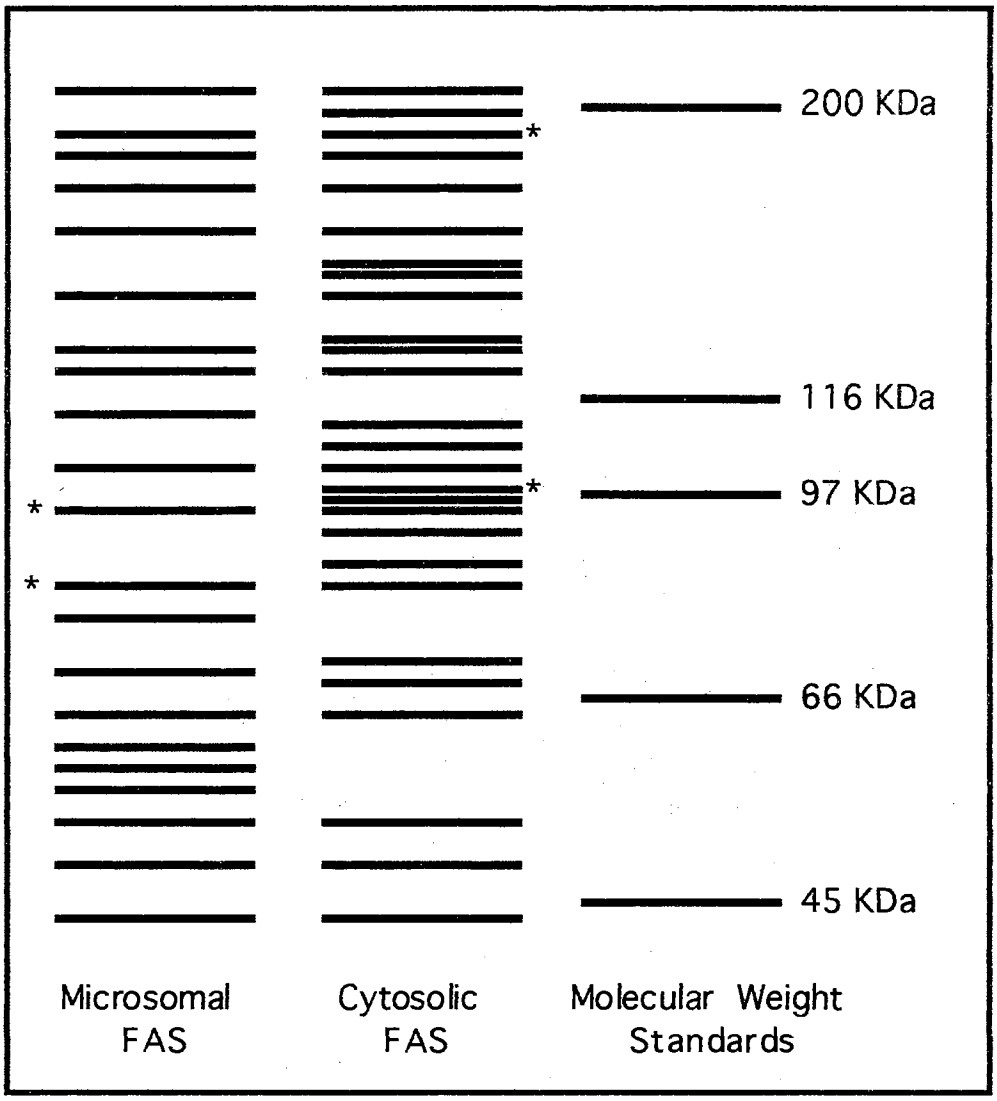

Figure 74. Partial Trypsinization of Microsomal and Cytosolic FAS: Plotting of the Bands in Figure 73. The blots in Figure 73 were analyzed, the Rf's of the bands converted to molecular weight, and the results co-plotted. * Indicates bands that were sequenced.

CHAPTER V

DISCUSSION

A variety of factors appear to have dramatic effects on FAS. A number of papers have discussed the buffer conditions that are optimal for FAS activity. A brief summary of these, and observations on what effects various buffer changes had on pea aphid FAS may guide future exploration.

Kashem and Hammes (1988) determined that FAS dissociated at 0°C with a $t_{1/2}$ of 150 minutes, and reassociated at 25°C in 3 hours, or 5 minutes if 1 mM NADPH was added. As both forms of pea aphid FAS were isolated and stored at 4° C, it was necessary to allow samples to incubate at room temperature to get stable, repeatable assay results.

Smith and Abraham (1971) reported that lack of DTT led to loss of activity, both in the dimer and in the re-associated monomer. They found that addition of 1 to 10 mM DTT restored activity to both forms. Linn (1981) also reported that activity was restored if DTT was added. In the course of this research we found that fresh DTT restored activity to stored preparations of aphid FAS as well.

Kumar et al. (1972) reported that for pigeon liver FAS a pH near neutrality and phosphate buffer above 10 mM gave the best retention of dimer and activity. Kashem and Hammes (1988) reported that phosphate was superior to Tris/glycine in retaining activity. A majority of researchers use the phosphate buffer, either potassium or sodium. However, when potassium salts were used for purification of Pea aphid FAS wee encountered difficulties in subsequent SDS-PAGE analysis and during the ammonium sulfate precipitation of microsomal FAS. Therefore using sodium phosphate and sodium chloride instead may improve FAS purification. The 50 mM potassium phosphate buffer

caused slight problems with precipitation of SDS during sample preparation for electrophoresis. Once potassium chloride was used, either for gradients in column chromatography or microsomal extraction, the problem was severe. The use of potassium chloride for extraction of microsomal FAS led to difficulties during ammonium sulfate precipitation as well. Potassium sulfate has low solubility in cold solutions, and much less in strong ammonium sulfate solutions. The result of this was the precipitation of a large amount of potassium sulfate along with the protein during ammonium sulfate fractionation. The potassium sulfate was, for the most part, resolved into a separate layer during centrifugation, but it was difficult to re-dissolve the protein without including a large amount of the salt as well. This extra salt needed to be removed before affinity or ion exchange chromatography could proceed. Coprecipitation with potassium sulfate may produce an undetermined denaturing effect on the enzyme.

Kashem and Hammes (1988), Linn (1981), Peide (personal communication), de Renobales et al. (1986), and Ramakrishna and Benjamin (1983), all used 10 % glycerol as osmotic additives in their buffers. Hsu and Yun (1970) found 20% glycerol was necessary for complete stability of chicken liver FAS. Peide et al. (1993), Juarez et al. 1992, and Ramakrishna and Benjamin (1983), used glucose instead, while Stoopes et al. (1978), Ahmad et al. (1982), and Kahn and Kolattakudy (1975) used no osmotic additive. During preliminary investigations, when glycerol was left out of the buffer during the purification of cytosolic FAS the enzyme lost activity at a significantly higher rate. All buffers thereafter included 10% glycerol which returned activity decline in cytosolic FAS to its lower initial rate, and whether microsomal FAS is stabilized by this addition was not tested.

Kumar et al. (1972), Stoops and Wakil (1982), and Smith and Abraham (1971) reporting on chicken liver, pigeon liver, and rat mammary FAS respectively, found that

hydrophobic interaction, and reduced electrostatic repulsion due to high ionic strength, are responsible for keeping FAS in the active dimer form, and that low ionic strength and cold cause the gradual dissociation of FAS into inactive subunits. We found assays to be more repeatable when room temperature buffer was used in the assays, the FAS was allowed to sit at room temperature for 1/2 hour or more before assays began, and incubated about 8 minutes in 100 μM NADPH, 50 mM potassium phosphate buffer at pH 7.4 with 10% glycerol and 1 mM DTT immediately before measurements were taken. Varying the parameters may give better results, but these proved workable, and were directly comparable to previously reported studies.

As a further note, when malonyl-CoA is used in enzyme kinetic determinations it is important to check the purity of the malonyl-CoA purchased. Aprahamian et al. (1982) found that commercial malonyl-CoA contained as much as 10% acetyl-CoA. The lot used in this research had 1.5% acetyl-CoA as reported by the manufacturer. Instead of removing acetyl-CoA from the malonyl-CoA (by HPLC), its presence was taken into account when determining substrate concentrations for kinetics.

Differences in triglyceride content of pea aphids different morphs and on different hosts led us to investigate if the differences were due to differences in FAS activity under these conditions. Neese (1995) found that alates had a 16% higher triglyceride content than apterae, and that aphids reared on alfalfa had a 40% higher triglyceride content than those reared on faba bean. This led to the hypothesis that the higher triglyceride content could represent a higher FAS activity. Figure 3 shows that alfalfa reared aphids do not have significantly higher FAS activity than faba bean reared aphids, and that FAS activity in apterae is not significantly different from alates. The finding that there was no significant difference in FAS activity between morphs or hosts allowed us to justify using the more easily available unsorted faba bean reared aphids for all subsequent analyses.

Consistent with the reports of Khan and Kolattakudy, (1975) and Peide et al. (1993, personal communication), microsomal FAS was solubilized by stirring with high salt buffer, indicating as Peide et al. stated, that primarily ionic interactions hold microsomal FAS to the endoplasmic reticulum. Both the cytosolic and microsomal FAS were efficiently precipitated by ammonium sulfate, although the microsomal FAS did not fully precipitate until 50%, as opposed to 40% for the cytosolic.

During ammonium sulfate precipitation of microsomal FAS, the 20% pellet occasionally contained fairly clean, high specific activity FAS. Attempts to repeat this reliably failed, and a working hypothesis is that under just the right concentrations of KCl, and ammonium sulfate addition rates, FAS is selectively adsorbed to the fresh surfaces of the rapidly crystallizing potassium sulfate, perhaps similar to what occurs when calcium phosphate gels are used to purify proteins.

The two purification schemes for cytosolic FAS gave a specific activity of around 3400 nmoles NADPH/min/mg protein, a 16 to 87 fold purification, and a recovery of 9.5%. These figures are comparable to Ryan's (1982) partial purification of pea aphid cytosolic FAS with a specific activity of 1,700 units/mg , a 26 fold purification factor, and a yield of 5.4%. House fly FAS was reported to have a specific activity of 7428 units/mg (Peide, personal communication) , and *Drosophila* 4500 units/mg (de Renobales et al. 1986). Further activities are summarized in table VIII.

Microsomal FAS showed a higher specific activity by scheme B, around 3600 U/mg, as compared to 1000 U/mg for scheme A. This is consistent with the observation that microsomal FAS tended to lose activity during the course of kinetics assays much faster than cytosolic FAS. The quicker purification of scheme B preserved more of the activity than scheme A. The purification was around 140 fold, with a 3-7 % yield.

Peide et al. (personal communication) working with the house fly reported a specific activity of 6477 U/mg, a 1440 fold purification, and a 1.5% recovery of activity in House fly FAS..

We found that isolated FAS rapidly degraded to a significant extent. Because pea aphid FAS (as apparently all FASes are) is extremely susceptible to proteolytic degradation adaptations to the purification protocols needed to be made. First, a protease inhibitor cocktail needed to be used throughout the purification. Additionally, all characterizations needed to be performed on freshly isolated FAS. All FASes are unstable, however, insect FASes may be even more sensitive to breakdown. Several authors (Vernon and Hsu, 1983; Linn, 1981; Buckner and Kolattukudy, 1976: Hardie et al. 1981) report vertebrate FASes to be stable frozen for several months, or even at room temperature for several days. In this study, pea aphid FAS was not stable either at 4° C or frozen in 10% glycerol. An example of the fragile nature of FAS is shown in Figure 9. Purified FAS was run on a 5% SDS-PAGE gel, and the remainder of the sample stored at -70° C in 10% glycerol for 48 hours. Upon thawing, another aliquot was run, and was found to have broken down considerably. The same fragments were produced when the preparations were kept at 4° C (data not shown). This is similar to the findings of de Renobales et al. (1986), who reported Drosophila FAS to lose 25% of its activity overnight (75 % without an inhibitor cocktail), and was so subject to proteolytic degradation, despite the use of an inhibitor cocktail, that complete characterization of the protein was not possible. Photographs of their final product show equal quantities of intact FAS and the 200 KDa fragment resulting from loss of the TE portion, along with small quantities of other bands. de Renobales et al. made the point that the low molecular weight bands present after Sepharose gel filtration indicate that FAS is undergoing continual breakdown during the purification. Thompson et al. (1975) found Blowfly FAS

to lose 50% of its activity in 55 hours at 5° C. Stoops et al. (1978) reported that at least 10% of FAS was proteolytically degraded, or more if DEAE chromatography was delayed. They also found that a PMSF inhibitable protease co-migrated with FAS through gel filtration, lagging slightly behind the leading edge of the FAS peak. Peide et al. (personal communication) showed a second high molecular weight band in their purification of housefly microsomal and cytosolic FAS but were able to eliminate it in their final products.

The Sepharose 6B separations showed large numbers of low molecular weight peptides in the high activity, high molecular weight fractions. Given that the column separation was reasonably efficient, these smaller peptides must have been associated with the FAS. Proteolytic cleavage of the peptide backbone may have little effect on the overall structure of the intact protein, with hydrophobic and ionic forces holding the tertiary structure intact. This collection of associated degradation products would then migrate similarly to the intact FAS and reveal its fractured nature under SDS-PAGE electrophoresis.

Under the scheme A purification protocol the reactive red Sepharose and DEAE Sephacel removed most of the other peptides, but the putative 200 KDa breakdown product remained. These techniques succeeded in removing low molecular weight contaminants. If the hypothesis that attractive forces were holding proteolytically degraded FAS together, why then would affinity and ion exchange chromatography remove these putative associations? The answer could be that the high salt used to elute the FAS, while enhancing hydrophobic interactions, would weaken ionic attractions. If this is the case, then cleaved FAS is primarily held together by ionic attractions.

The primary breakdown of most FASes is to lose the TE portion of the molecule, leaving a ~200 KDa and a ~30 KDa fragment (Mattick et al. 1983). The 30 KDa fragment

was fairly easily removed in the course of the purifications done in this research, but the 200 KDa fragments co-purified with the intact FAS, and in fact were augmented by their continual generation. Figure 8 shows that the final step in purification scheme A still retains the 200 KDa fragment to a significant extent. Switching to purification scheme B, which used Reactive Red Sepharose followed by Superose 6, gave a cleaner separation (Figure 13). However, this required throwing away a significant portion of the FAS activity that was contaminated with the 200 KDa fragment.

It is possible the difficulty of removing this contaminant may stem from the tendency of FAS to exist as a homodimer under conditions of high salt. FAS folds into 3 major domains, the C-terminal domain consisting of the endogenous TE, TE I. This is connected to the rest of the monomer by a section of alpha helix that is the first area cleaved by almost all of the proteases examined (Mattick et al. 1983; Wakil et al. 1983). The areas of FAS that contribute to binding (areas of and around the two reductases (Kashem and Hammes, 1988; Kumar et al. 1972) on domains 1 and 2 are not affected by the cleavage of the C-terminal TE, and thus the two electrostatically and hydrophobically bound molecules elute together, separating when denatured for SDS-PAGE electrophoresis. This is supported by the fractions that elute from the high resolution size exclusion Superose 6 column (Figure 12) The higher molecular weight intact dimer elutes first, followed by the heterodimer, and perhaps by the homodimer of the 200 KDa fragment.

Summarizing, the 30 KDa TE is rapidly cleaved and as rapidly disassociates from the rest of the FAS molecule. The remaining intact monomer and the now 200 KDa monomer are still held together by their normal interactions, and co-purify. The success in using Superose 6 to producing a homogenous protein is due to the ability to resolve an intact 450 KDa protein from a proteolytically degraded 420 KDa protein.

The use of protease inhibitors was essential for good yields in purification, and high activities in kinetic experiments. In the future, as more protease inhibitors come out, they should be tried to see if the protease nicking problem may be reduced to a more manageable level. On this line, Linn (1981) reported that rabbit serum, probably due to the α2-macroglobulins, greatly reduced nicking. Macroglobulins are now available commercially, and may be effective with a purification scheme that removes the complexed proteases before a high salt step that would release them. This might entail switching to polyethylene glycol as the initial precipitant, something both Ryan (1983) and Linn (1981) found gave acceptable preliminary purification. It would be interesting to see if the co-eluted (although un-isolated) protease reported by Stoops et al. (1978) is removed by macroglobulins.

Microsomal FASes have been found in a wide variety of organisms now. They have been reported from *Euglena*, cockroaches, houseflies, moths, aphids, rat and mouse brains. The question arises as to the purpose of having a separate FAS associated with the microsomes, when it produces essentially the same products as the soluble cytosolic FAS. The following comparisons can be made.

1. The pI for the cytosolic and for the microsomal FAS of the pea aphid were not significantly different. The microsomal FAS had a pI of 7.16 and cytosolic was 7.19.
2. The pea aphid pH optimums were different, with cytosolic FAS having a pH optimum of 7.4, and microsomal FAS showing maximum activity at pH 7.6. Insect FASes appear to have a higher pH optimum than vertebrate FASes. Vertebrate FASes seem to cluster around pH 6.8, while insect FASes (some inferred from the buffer pH used) cluster around 7.4.

3. Consonant with the reports for FASes other than waterfowl, cytosolic FAS is unable to use methylmalonyl-CoA as a sole elongation substrate, while microsomal FAS does generate fatty acids using only methylmalonyl CoA.

4. The kinetic parameters for pea aphid FAS are summarized in table VII. While the Km, the competive and noncompetitive inhibition constants, and maximum velocities for the various substrates do differ somewhat, a major difference is that for acetyl-CoA methylmalonyl-CoA is a mixed inhibitor for the cytosolic enzyme and an uncompetitive inhibitor for the microsomal enzyme. With malonyl-CoA, methylmalonyl-CoA is a competitive inhibitor for the cytosolic FAS, and a mixed inhibitor for the microsomal FAS. Another significant difference is the ability of microsomal FAS to use methylmalonyl-CoA as an effective elongation substrate. Additionally, the microsomal FAS has a lower Vmax for the substrates, much lower for NADPH.

5. The molecular weight of microsomal FAS is higher than that of cytosolic FAS, both in the pea aphid and the house fly.

Returning to the reason for having a microsomally bound FAS capable of generating methyl-branched fatty acids of odd and even chain length, insects do use methyl-branched fatty acids in the production of cuticular compounds. However, the degree of methyl-branched fatty acid use in *Euglena*, and mouse and rat brain is likely to be quite low, which indicates there must be another use. The evidence for this microsomal production of methyl-branched products from microsomal FAS in insects is good. *Trichoplusia ni* pupae produce methyl-branched hydrocarbons and alcohols at a time when cytosolic FAS activity is unmeasurably low (de Renobales et al. 1989).Peide et al.

(1993 and personal communication) found that houseflies and cockroaches, both of which use methyl-branched hydrocarbons in their cuticles, produce these compounds by way of the microsomal, not the cytosolic, FAS. Although pea aphids do not have large amounts of methyl-branched compounds in their cuticles, the percentage varies among the aphid species.

Pea aphid microsomal FAS differs from cytosolic in its primer specificity. Cytosolic FAS is most active with acetyl-CoA as a primer, uses propionyl slightly less effectively, and has low efficiency for longer primers. Microsomal FAS in contrast uses propionyl-CoA more effectively than acetyl-CoA, and has significantly more activity with longer primers than cytosolic FAS does. The capability to use acetyl, propionyl, and isobutryl-CoA as chain initiators, coupled with the ability utilize methylmalonyl-CoA as elongating substrate means microsomal FAS products could be converted to almost any combination of odd or even hydrocarbons and alcohols, with methyl-branches from position 2 on up.

Methyl-branched fatty acids are not generally found in insect tissues (Juarez et al. 1992), although the products of these fatty acids are abundant in the cuticle. An explanation for the presence of a separate FAS, capable of utilizing unusual primers and generating methyl-branched fatty acids, restricted to the microsomes, may involve close association with the elongase, desaturase, and reductase complexes on the endoplasmic reticulum. These unusual fatty acids, released in the vicinity of the enzymes that would elongate and form them into their final cuticular lipid state, might then be rapidly taken up and converted. This would prevent them from entering the general lipid pool as free fatty acids and perhaps causing metabolic difficulties. This arrangement would also explain their scarcity in tissue extracts, when their products are so abundant.

Microsomal and cytosolic FAS both have the ability to interact with TE II. The microsomal FAS does tend to produce somewhat higher proportions of longer chain fatty acids than cytosolic FAS under identical conditions, but the difference is small. If microsomal FAS were unable to interact with TE II, this would mean there was a protected source of long chain fatty acids to insure a dependable supply of palmitate and longer fatty acids, and their desaturated analogs, for membrane synthesis and other essential metabolic processes which would not be affected by changing levels of TE II. As this is not the case then four possibilities present themselves. The tissues may express TE II at levels that allow production of sufficient long chain fatty acids from the ocassional molecules that randomly miss interacting with the TE II. The elongases on the microsomes may be immune from TE II activity. TE II levels may cycle, allowing bursts of long chain fatty acids to be made. Finally, TE II expressing tissues may get their long chain fatty acids from non-expressing tissues by way of lipid transport molecules.

In line with the microsomal and cytosolic FASes from cockroaches (Gu et al. 1993), pea aphid cytosolic FAS has a higher Vmax with malonyl-CoA than the microsomal does: 2180 versus 1040 U/mg. They reported that cockroach microsomal FAS had a Vmax for methylmalonyl-CoA one half that for malonyl-CoA with similar Kms. pea aphid microsomal FAS had a Vmax for methylmalonyl-CoA 1/3 that for malonyl-CoA, again with similar Kms. Cytosolic FAS, in both their study and this one, was unable to use methylmalonyl-CoA effectively as the sole elongating substrate.

Gu et al. (1993) found methylmalonyl-CoA to be a competitive inhibitor with respect to malonyl-CoA, as did others (Buckner, and Kolattukudy, 1975; de Renobales et al. 1986) pea aphid FAS has both a competitive and uncompetitive component to its inhibition by methylmalonyl-CoA with respect to malonyl-CoA as well as to acetyl-CoA. Methylmalonyl-CoA showed pure uncompetitive inhibition with respect to

NADPH. This indicates that there are two ways that methylmalonyl-CoA acts to inhibit the enzyme. Direct competition for the two binding sites, the acyl carrier protein and the acetyl/malonyl trans-acylase , make up one. Peide et al. (1993) bring up 3 points: that the Km for malonyl-CoA is nearly the same for both enzymes, that the Kis are approximately the same, and that the values of Ki and Km are close. They draw the conclusion that the difference between the two enzymes is not in the affinity for methylmalonyl-CoA, but in the turnover ratio of methyl-branched intermediates.

If the reductases, the transferase, the hydrolase, or the TE are sensitive to the presence of a methyl branch, this would account for difference between the two enzymes. The transferase function is know to be sensitive to chain length, growing slower with increasing chain length, and stopping at 22 carbons. This is part of the mechanism by which chain length specificity is regulated. As the completed fatty acid is holding longer and longer on the acyl carrier protein the TE has more and more time to effect the cleavage. The very low production of fatty acids shorter than 14 carbons even under very slow synthesis rates indicates that part of the selectivity resides in the TE portion of the enzyme. Investigating the chain length of fatty acids produced by both enzymes in the presence and absence of methylmalonyl-CoA could indicate if the transferase is an area of difference between the cytosolic and microsomal FAS. On the other hand, it is easy to imagine the presence of a methyl branch sterically hindering the active site of the reductases, hydrolase, or TE. To investigate this, large amounts of actively synthesizing enzymes could be stopped quickly by boiling or trichloroacetic acid, and analyzed for accumulation of labeled intermediates when methylmalonyl-CoA is included.

Further work on this enzyme should include more peptide mapping and sequencing to design DNA probes for amplifying and completely sequencing both the microsomal and the cytosolic enzyme. Then the true differences between them could be

examined, looking at active sites and overall structure for the features that influence substrate binding and cellular localization. In addition, probes could be synthesized and antibodies could be produced for *in situ* hybridization to examine tissue specific expression of the two forms of fatty acid synthase.

Having the complete sequence for the microsomal and cytosolic FAS would allow comparison between the active domains of each enzyme. The structure of the genes for vertebrate FASes indicate that the multifunctional enzyme found in animals has been assembled from genes coding for the individual enzymatic activities, the condition in plants and bacteria. Having the ability to examine the separate domains would allow some interesting questions to be asked. Did the two enzymes evolve from a common ancestral product or did they originate from assembling different subunits? Have the different subunits evolved at similar rates, or have certain sections show conservation or hypervariability?

Having the sequences would also allow developing DNA probes that could detect the presence of microsomal FASes in other organisms, leading to more information on the differences and similarities between microsomal and cytosolic FASes. Eventually we may be able to answer a currently unaswerable question: Why do animals have two FASes?

Table VIII Comparison of Reported Characteristics of Animal FASes

Source	Acetyl-CoA Km	Malonyl-CoA Km	NADPH Km	Spec. Act.	Mol. Wt. KDa	‡ pH opt.	Reference
rat liver	18	26	12	2100	480	6.8	Aprahamian 1982
pig liver	50	50	200		478	6.5-6.8	Kim 1981
chicken liver	14	8	6	2750	460/220		Stoops 1978
pigeon liver	39	21	300		450	6.7	Kumar 1972
human liver		2.5			410		Mooney 1982
rat adipose	3	22	31	1800	-/250	*6.6*	Raakrishna 1983
rat mammary	22	13	34	2600	470/220	6.5-6.8	Ahmad 1982
guinea pig mammary	24	27			400	6.6	Strong 1972
rabbit mammary	9	29			512/252	6.6	Carey 1970
bovine mammary	9		19	941	530		Kumar 1981
goat mammary	9.2			3200		*6.8*	Nandedkar 1969
goose uropygial gland		40	20	1200	547/269	6.5-7.5	Kolattukudy 1981
blowfly *Lucilia sericata*	11	4	17	6870	500	*7.1*	Thompson 1975
Ceratitis capitata adult	11	80	30		560/260	7.4	Municio 1977
Ceratitis capitata larva	11	80	15		560/260	7.4	Municio 1977
Drosophila melanogaster	7	24	9	3800	480/226	*7.2*	de Renobles 1986
House fly cytosol	10	39	15	7428	405/201	*7.4*	Peide in prep.
House fly microsomes	7.6	34.8	24.6	6477	419/206	*7.4*	Peide in prep.
Cockroach cytosol	.7	24				*7.4*	Peide 1993
Cockroach microsomes	2.6	30.4				*7.4*	Peide 1993
Pea aphid cytosol (Ryan)	7.7	9	11	1800		*7.4*	Ryan 1982
Pea aphid cytosol	6.87	5.7	20.6	3400	495/231	7.4	
Pea aphid microsomes	9	12	18	3600	485/245	7.6	

‡ Italicized pH optima are inferred from the buffer used.

Table IX Comparison of Reported Animal FAS Methylmalonyl-CoA Kis

Source *	Acetyl-CoA	Malonyl-CoA	NADPH	Reference
human liver		8.4		Mooney 1982
goose uropygial gland		16		Kolattukudy 1981
Blatella germanica cytosol	17.6	21		Peide 1993
Blatella germanica microsomes	26.9	10		Piede 1993
Pea aphid cytosol	8	12	15	
Pea aphid microsomes	18	7	14	

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