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PART I. ISOLATION AND PARTIAL CHARACTERIZATION OF AN INACTIVATING FACTOR FOR FATTY ACID SYNTHETASE

PART II. ISOELECTRIC FOCUSING IN DENSITY GRADIENTS IN THIRTY-FIVE MINUTES

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ABSTRACT

PART I. ISOLATION AND PARTIAL CHARACTERIZATION OF AN INACTIVATING FACTOR FOR FATTY ACID SYNTHETASE

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An inactivating factor for fatty acid synthetase has been partially purified from the microsomal fraction of pig liver. It is of low molecular weight (less than 750 daltons), is heat stable, contains no detectable sulfhydryl groups or disulfide bonds, is of net neutral or slightly negative charge, and apparently consists of several closely related compounds. The inactivation rate is dependent upon both temperature and inactivating factor concentration. The first-order inactivation is prevented by reducing agent and is slowed by enzyme substrates and EDTA. The enzyme aggregates nonspecifically shortly after its inactivation.

Related projects involving improvements in the methodology for isolation, assay, and analysis of fatty acid synthetase are also described. Particularly significant is the definition of a procedure for isoelectric focusing in as little as thirty-five minutes. Also presented are a new purification procedure for ferritin and characteristics of the purified protein.

DEDICATION

To Sandy, and my parents

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The author wishes to express his appreciation to Dr. William C. Deal, Jr., for suggestions, discussions, and guidance throughout this research. The author also thanks Drs. Kindel and Sadoff for their time and efforts as members of his guidance committee.

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LIST OF ABBREVIATIONS

CM-cellulose	carboxymethyl-cellulose
d	diameter
DEAE-cellulose	diethylaminoethyl-cellulose
EDTA	ethylenediaminetetraacetic acid
FAS	fatty acid synthetase, from pig liver unless otherwise indicated.
h	height
min	minute
meq	milliequivalent
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
o. d.	outside diameter
PAGE	native polyacrylamide gel electrophoresis
PED buffer	0.2 M potassium phosphate (pH 7.4) containing 1 mM EDTA and 1 mM dithiothreitol
PMSF	phenylmethanesulfonylfluoride
rpm	revolutions per minute
TE(D) buffer	50 mM tris(hydroxymethyl)aminomethane (pH 7.4) containing 1 mM EDTA (and 1 mM dithiothreitol)
TEMED	N,N,N',N'-tetramethylethylenediamine
v/v	volume per volume
w/v	weight per volume (grams per milliliter)
x g	times gravitational acceleration

INTRODUCTION

The most important accomplishment described in this thesis is the partial purification and characterization of an inactivating factor for fatty acid synthetase. Several related projects were also undertaken; these involved improvements in the methodology for isolation, assay, and analysis of the enzyme. One particularly significant result from these studies was the definition of a procedure for isoelectric focusing which allows focusing in as little as thirty-five minutes. The organization of the results from these various studies is outlined below.

Improved procedures for purification and assay of fatty acid synthetase are presented in Chapter 2. A portion of this material has been published ("Fatty Acid Synthase from Pig Liver," by I. C. Kim, Gary Neudahl, and W. C. Deal, Jr., Meth. Enzymol. 71C, 79 (1981)). The initial work toward purification and characterization of the inactivating factor is presented in Chapter 3. Most of this work was done with pellet fraction suspensions, which are relatively crude preparations of the inactivating factor. Chapter 4 describes the further purification and characterization of the inactivating factor extracted from the microsomal pellet fraction. Chapter 5 presents the procedure for isoelectric focusing; this work is prepared for submission for publication ("Isoelectric Focusing in Thirty-five Minutes," by Gary A. Neudahl and W. C. Deal, Jr., Anal. Biochem.). Chapter 6 presents a new purification procedure for ferritin from pig liver. Ferritin copurifies with fatty acid synthetase, and this work was undertaken to determine whether ferritin had any effect upon, or interaction with, fatty acid synthetase.

These chapters are presented in the general format of independent, complete scientific papers, with the exception of a common list of references, which is at the end of the thesis.

CHAPTER 1

LITERATURE REVIEW

This chapter reviews the literature concerning the purification, assay, and control of fatty acid synthetase and the purification and characterization of ferritin, since the work in this thesis is related to these areas. In particular, the regulation of fatty acid synthetase activity, both *in vivo* and *in vitro*, is covered.

FATTY ACID SYNTHETASE

A major step in the elucidation of fatty acid biochemistry was the demonstration of fatty acid oxidation in the mitochondria of rat liver (Kennedy and Lehninger, 1948). It seemed reasonable at that time that fatty acid biosynthesis might occur as the reverse of the oxidative process, and perhaps in the same organelle. Indeed, the first demonstration of fatty acid biosynthesis used enzymes partially purified from mitochondrial material (Seubert *et al.*, 1957). These enzymes, which included only those of the fatty acid oxidation system and an NADPH-specific enoyl reductase, produced short chain (eight carbons or less) fatty acids.

Meanwhile, Brady and Gurin (1952) had shown that all the enzymes necessary for fatty acid biosynthesis were present in the particle-free supernatant of pigeon liver homogenate. Subsequently, complete systems for the synthesis of fatty acids were found in the particle-free cytoplasm of rat liver (Langdon, 1955, 1957), adipose tissue (Martin *et al.*, 1961), and lactating mammary gland (Popjak and Tietz, 1955). The significance of the bicarbonate requirement in these systems was realized when Wakil (1958) isolated a malonic acid derivative as an intermediate in the reaction. Lynen (1959) and Formica and Brady (1959) confirmed the enzymatic carboxylation of acetyl-CoA as the first step in the synthetic sequence. The enzyme catalyzing this reaction is now known as acetyl-CoA carboxylase. The participation of malonyl-CoA as an intermediate in the cytosolic fatty acid-synthesizing system established that fatty acid synthesis did not occur as the reverse of fatty acid oxidation. The malonyl-CoA-dependent fatty acid-synthesizing system, now

known as fatty acid synthetase (FAS), yielded palmitate, which constitutes 20 to 25% of the total fatty acid in most mammalian tissues.

The fatty acid synthetases isolated to date have been divided into several major groups (Bloch and Vance, 1977): Type I (aggregated FASs), Type II (nonaggregated FASs), and Type III (microsomal and mitochondrial FASs). Type I fatty acid synthetases are of the greatest interest here, so they will be described later in greater detail.

Type II fatty acid synthetases are those which have seven individual, separable, protein moieties. They are found in plants (Rutkoski and Jaworkski, 1978), eukaryotic algae, and most bacteria. The best characterized of the Type II enzymes are those from Escherichia coli (Vagelos et al., 1969). The overall reaction carried out by these enzymes is:

$$\text{ACETYL-CoA} + 7\ \text{MALONYL-CoA} + 14\ \text{NADPH} + 14\ \text{H}^+ \longrightarrow$$

$$\text{PALMITATE} + 14\ \text{NADP}^+ + 8\ \text{CoA} + 7\ \text{CO}_2 + 6\ \text{H}_2\text{O}\ .$$

There is no evidence that the enzymes catalyzing this reaction are associated in vivo, although a specific acyl carrier protein (ACP) has been localized near the surface of the cell membrane (Volpe and Vagelos, 1973). This acyl carrier protein and six individual enzymes (Goldman et al., 1961; Lennarz et al., 1962) are required for the overall reaction. Each of these enzymes, when purified, catalyzes a specific reaction in the biosynthetic sequence. The acyl carrier protein has a central role in the overall reaction in that all intermediates are covalently bound to it through its prosthetic group, 4'-phosphopantetheine. The enzymes involved in the overall fatty acid-synthetic reaction are: (1) acetyl-CoA-ACP transacylase (Alberts et al., 1964), which catalyzes the transfer of the acetyl group from CoA to ACP, (2) malonyl-CoA-ACP transacylase (Alberts et al., 1964), which catalyzes the transfer of the malonyl group from CoA to another molecule of ACP, (3) β-ketoacyl ACP synthetase (Alberts et al., 1965), which catalyzes the synthesis of β-ketoacyl thioesters of ACP from malonyl-ACP and fatty acyl-ACP with concomitant loss of CO_2 and release of free ACP, (4) β-ketoacyl ACP reductase (Majerus et al., 1965), which catalyzes the NADPH-dependent reduction of β-ketoacyl-ACP to β-hydroxyacyl-ACP, (5) β-hydroxybutyryl-ACP dehydratase (Majerus et al., 1965, 1969), which catalyzes the dehydration of short chain β-hydroxyacyl-ACP to trans-α,β unsaturated acyl-ACP, and (6) enoyl-ACP reductase (Weeks and Wakil, 1968), which cata-

lyzes the NAD(P)H-dependent reduction of enoyl-ACP to fatty acyl-ACP.

Many of these reactions have been studied using model substrates (Lynen, 1961; Trams and Brady, 1960) for the physiological ones, including acylthioester derivatives of CoA, pantetheine, and N-acetylcysteamine. Some of the enzymes have very rigid specificities, so the model substrates could not be used (Yang et al., 1967). Since 4'-phosphopantetheine is a component of both ACP and CoA, the importance of the apoprotein structure of ACP in confering increased reactivity to the bound intermediates is clear (Majerus, 1967). Model substrate and other studies on these enzymes have shown that a sulfhydryl residue on ACP, other than that of the 4'-phosphopantetheine group, is involved in both β-ketoacyl-ACP synthetase (Vagelos et al., 1969) and acetyl-CoA-ACP transacylase (Williamson and Wakil, 1966) reactions.

Type III fatty acid synthetases are microsomal or mitochondrial membrane-associated fatty acid elongation systems (Bloch and Vance, 1977). The microsomal enzymes modify fatty acids formed by Type I enzyme (see below) and also modify the essential unsaturated fatty acids (Nugteren, 1965; Seubert and Podack, 1973). The desaturation (Marsh and James, 1962) and elongation (Guchhait et al., 1966) functions performed by these enzymes yield polyunsaturated fatty acids such as arachadonic acid. Brain is a particularly good source of these Type III fatty acid synthetases; it has at least three separate Type III FASs which are apparently involved in myelinogenesis (O'Brien, 1965).

The mitochondrial fatty acid synthetases elongate short chain-length fatty acids by the reverse of β-oxidation, except an enoyl-CoA reductase replaces acyl-CoA dehydrogenase (Seubert and Podack, 1973; Wakil, 1961; Hinsch et al., 1976). These enzymes are most active when the in vivo ATP level is high and the oxygen level is low (Hinsch and Seubert, 1975; Whereat, 1971).

The type I fatty acid synthetases are those which contain multiple catalytic activities on single polypeptide chains (Stoops et al., 1978; Mattick et al., 1981). They are found in mammals, birds, insects (Municio et al., 1977), yeast, and some fungi (Giompres and Packter, 1978) and bacteria (Kawaguchi and Okuda, 1977). The fatty acid synthetase complex from yeast (Lynen, 1961) was the first of the Type I enzymes to be extensively studied. The reactions required for overall fatty acid-

synthetic activity are identical to those catalyzed by the dissociable Type II enzymes, except the acyl intermediates in Type I fatty acid synthetase remain bound to a single 4'-phosphopantetheine group within the complex until synthesis of the fatty acid is completed. The "acyl carrier protein" of Type I fatty acid synthetase is thus an integral part of a multifunctional polypeptide chain in the complex. Also, in yeast, the synthesized fatty acids are released as acyl-CoAs, rather than as free fatty acids.

The yeast fatty acid synthetase complex is a high molecular weight (2.3×10^6) substance of subunit composition $\alpha_6\beta_6$ and overall dimensions 21 x 25 nm (Lynen, 1969). The nonidentical subunits have been shown by genetic mapping to be coded by two unlinked gene loci designated fas1 and fas2 (Schweizer and Bolling, 1970; Schweizer et al., 1973; Kuhn et al., 1972; Knobling et al., 1975). The fas1 locus codes for the β subunits, which has enoyl reductase, dehydratase, acetyl transferase, and malonyl (palmitoyl) transferase activities. The fas2 locus codes for the α subunit, which has condensing enzyme (β-ketoacyl synthetase) and β-ketoacyl reductase activities and the acyl carrier protein (Schweizer et al., 1975). The assignment of activities to one or the other subunit has been verified by protein chemical studies (Wieland et al., 1979; Engeser et al., 1977; Kresze et al., 1976). The molecular weights of the α and β subunits are 213,000 and 203,000, respectively (Stoops and Wakil, 1978). The complex will utilize NADH in the reductive steps, but NADPH is preferred; also, FMN serves as a hydrogen carrier in the reaction catalyzed by the enoyl reductase activity (Lynen, 1969). The complex has optimal activity at pH 6.5 to 7.0. The Km values for acetyl-CoA, malonyl-CoA, and NADPH are 28, 8, and 67 μM, respectively. Many of the partial reactions, which together comprise the overall fatty acid-synthetic activity, have been studied using model substrates (Lynen, 1961, 1962, 1967), and the structural-functional relationships in the complex have been examined in detail (Lynen, 1980; Stoops and Wakil, 1981a).

Type I fatty acid synthetases from mammalian and avian sources differ somewhat from those of yeast and various bacterial sources: (1) the overall fatty acid-synthetic reaction is the same as for the Type II enzyme; that is, palmitate and other free fatty acids are produced,

rather than acyl-CoAs, (2) FMN is not utilized by the complex, (3) NADPH specificity is more stringent, (4) the complex has subunit structure α_2 or $\alpha\beta$, and (5) there is a palmitoyl (long chain acyl) transacylase activity separate from the malonyl transacylase activity.

Genetic studies similar to those performed with yeast have not been possible with mammalian and avian fatty acid synthetases, so identity or nonidentity of the two subunits which comprise the native FAS complex in animal tissues has instead been suggested on the basis of physicochemical and catalytic properties of the subunits. Messenger RNA for fatty acid synthetase is of sufficient size to code for complete subunits (Lau et al., 1979; Nepokroeff and Porter, 1978), and in cell-free translation systems it produces proteins of the appropriate size and immunological characteristics (Flick et al., 1978; Mattick et al., 1981). Two types of subunits have been obtained from pigeon liver and have been separated and characterized (Lornitzo et al., 1974, 1975). Each subunit catalyzed some, but not the same, partial reactions; this suggested that the subunits were different. The evidence for nonidentical subunits in fatty acid synthetase complexes from other tissues and species has been based on finding only one mole of 4'-phosphopantetheine per mole of complex (Smith and Abraham, 1975a; Yun and Hsu, 1972; Nepokroeff et al., 1975). Other studies have given two moles of 4'-phosphopantetheine per mole of complex, however (Paskin and Mayer, 1976; Alberts et al., 1975; Buckner and Kolattukudy, 1976; Hardie and Cohen, 1978), and have suggested that limited proteolysis of identical subunits is responsible for both the low 4'-phosphopantetheine content and differing subunit activities reported by some workers (Stoops et al., 1978). In any case, the subunits, if not identical, are of very similar molecular weight and amino acid content for complex from any given source. Also, the subunits cannot be genetically coded in the same manner as those of yeast, as both subunits lack β-ketoacyl synthetase activity; this activity requires domains of both subunits (Kumar et al., 1970; Stoops and Wakil, 1981b).

Mammalian and avian fatty acid synthetases have been isolated from a large number of species and tissues (Bloch and Vance, 1977), including red blood cells (Jenik and Porter, 1981), pigeon liver (Hsu et al., 1969), chicken liver (Arslanian and Wakil, 1975), rat liver

(Nepokroeff et al., 1975), pig liver (Kim et al., 1977a), lactating rat mammary gland (Smith and Abraham, 1975b), and rabbit mammary gland (Dils and Carey, 1975; Hardie and Cohen, 1978). These complexes all have molecular weights between 488,000 and 555,000 in their native forms. The specific activities of the purified enzymes, although determined under varied conditions, are all between 200 and 1400 nmoles NADPH oxidized per minute per milligram protein, with optimal pH for the overall activity assay between 6.5 and 7.0. Substrate Km values for the complexes are between 2.3 and 25, 10 and 50, and 20 and 70 μM for acetyl-CoA, malonyl-CoA, and NADPH, respectively. The major product of fatty acid synthesis is palmitic acid for all except the mammary gland enzyme, which also produces large amounts of butyryl-CoA (Strom et al., 1979), apparently the result of high catalytic efficiency in the reduction of acetoacetyl-CoA and trans-crotonyl-CoA (Kumar and Dodds, 1981). All the complexes are malonyl-CoA dependent, but some prefer butyryl-CoA over acetyl-CoA as a primer (Maitra and Kumar, 1974; Lin and Kumar, 1972; Smith and Abraham, 1971). Methylmalonyl-CoA was viewed by most workers as a competitive inhibitor of fatty acid synthetase (Frenkel and Kitchens, 1977), but when it replaces malonyl-CoA in an assay, branched chain fatty acids are formed, although at a slow rate (Buckner et al, 1978). Most studies using methylmalonyl-CoA as a substrate were performed with goose uropygial gland fatty acid synthetase (Buckner and Kolattukudy, 1976).

Several mechanisms for control of fatty acid synthetase activity have been proposed. Although acetyl-CoA carboxylase was originally thought to be the critical regulatory enzyme in fatty acid biosynthesis, it now seems likely that under certain dietary conditions (Porter et al., 1971; Guynn et al., 1972; Butterworth et al., 1966; Burton et al., 1969), and especially in developing tissues (Vagelos, 1974), fatty acid synthetase is the rate limiting enzyme (Chang et al., 1967; Smith and Abraham, 1970; Volpe and Kishomoto, 1972). Indeed, the capacity of developing mouse liver to synthesize fatty acids from acetate correlates best with changes in fatty acid synthetase activity (Ahmad et al., 1979).

Allosteric regulators of Type I fatty acid synthetases are unknown at this time. Because these enzyme complexes have bound intermediates, it is unlikely that any one step is rate-limiting (Bloch and Vance, 1977); the overall fatty acid-synthetic rate is not dependent on the Km values of individual intermediates, but rather on the rate of

transfer between sites (Bloch, 1977). Nevertheless, reagents external to the complex can affect product distribution and the overall reaction rate. Lower malonyl-CoA to acetyl-CoA ratios yield shorter chain-length fatty acids (Smith and Abraham, 1975a, 1975b; Dils and Carey, 1975), as do specific chain length modifying proteins from mammary tissue (Smith and Abraham, 1975a; Carey, 1977). Palmitoyl-CoA inhibits the complex (Hsu et al., 1969; Tubbs and Garland, 1963; Lynen et al., 1964; Lust and Lynen, 1968; Robinson et al., 1963), apparently by a nonspecific detergent-like denaturation of the complex (Dorsey and Porter, 1968; Taketa and Pogell, 1966). Recently CoA was shown to activate fatty acid synthetase at very low concentrations, so low that the activation was not believed to be of physiological significance (Linn et al., 1980).

Control of the activity of fatty acid synthetase by covalent modification of several types has been reported. Qureshi et al. (1975,1978) gave evidence for a phosphorylation/dephosphorylation control mechanism, but the presence of a phosphorylated form of the complex has not been verified (Rous, 1974; Hardie and Cohen, 1978). Also, excessive malonylation of the enzyme has been shown to inactivate its overall activity (Kumar and Srinivasan, 1981). The complex has two catalytic sites for the binding of malonyl-CoA. When exposed to high levels of malonyl-CoA (more than 375 moles per mole complex), six moles of malonate are bound to the complex. Binding is apparently near a catalytic site, thereby inactivating the enzyme. Inactivation of fatty acid synthetase by this mechanism is not universal, however (Roncari, 1975). For at least some fatty acid synthetases (e.g., those from rat and pigeon liver), interconversion of the apo- (no 4'-phosphopantetheine group) and holo- (covalently attached 4'-phosphopantetheine) forms of the complex has been put forth as a mechanism for the in vivo control of enzyme activity. Indeed, the turnover rate of the 4'-phosphopantetheine group is more rapid than that of the remainder of the FAS complex (Tweto et al., 1971; Tweto and Larrabee, 1972). Also, the enzymes responsible for the transfer of this group from CoA to the apocomplex (Yu and Burton, 1974a,b) and for the hydrolytic release of this group from the holocomplex (Roncari, 1975; Kim et al., 1977b) have been partially characterized (Sobhy, 1977, 1979).

Another proposed mechanism for control of fatty acid synthetase activity involves dissociation of the complex into subunits. This has been demonstrated *in vitro*, especially at temperatures around 4° (Muesing and Porter, 1975; Kumar *et al.*, 1972; Yun and Hsu, 1972; Smith and Abraham, 1971b). Invariably, the purified enzyme is cold labile in low ionic strength solutions, especially in the absence of reducing agents (Strom and Kumar, 1979; Dils and Carey, 1975). There is no evidence for dissociated subunits *in vivo*, although inactive FAS has been reported (Liou and Donaldson, 1977; Strawser and Larrabee, 1976).

The long term (adaptive) control of mammalian and avian fatty acid synthetases *in vivo* (control by variation in the rates of protein synthesis and degradation) occurs through both dietary and hormonal channels. Dietary effects on FAS levels are greatest in liver, small in lung (Kumar, 1977), and nil in brain (Volpe and Vagelos, 1973). A diet high in saturated fatty acids lowers hepatic FAS levels (Romsos and Leveille, 1974), as does fasting (Liou and Donaldson, 1973; Allmann and Gibson, 1965). The lower levels of FAS are the result of decreased translation rather than an increased degradation rate for the complex (Volpe and Vagelos, 1973; Craig *et al.*, 1972; Fischer and Goodridge, 1978). The only dietary condition thus far shown to significantly affect the degradation rate of FAS is transfer from a fat-free to a linoleate-rich diet; the transfer causes a decrease in the half-life of rat liver FAS from 3.8 to 1.8 days (Flick *et al.*, 1977). Upon refeeding with a fat-free diet following a fast, a sharp rise in the amount of fatty acid synthetase-messenger RNA, and then FAS peptide associated with polysomes, is observed (Nepokroeff *et al.*, 1979; Flick *et al.*, 1978; Taylor and Schimke, 1974). After 48 hours, the maximal supranormal level of fatty acid synthetase activity is reached; the enzyme level then drops over about a week to normal levels (Alberts *et al.*, 1975). Weaning of rat and mouse pups (Volpe and Kishomoto, 1972) and hatching of chicks (Zehner *et al.*, 1977) increase liver FAS levels as the result of the involuntary change from a high fat diet to a high carbohydrate diet.

Hormonal regulation of FAS levels *in vivo* is quite organ specific. Prolactin raises mammary gland FAS levels (Speake *et al.*, 1976), and estrogen raises oviduct FAS levels (Aprahamian *et al.*, 1980) with little effect on liver FAS levels. Prednisolone increases FAS levels in brown

fat, but has no effect on hepatic FAS levels (Hahn et al., 1978). Adrenal steroids in general lower adipose tissue FAS levels, but have no effect on liver FAS levels (Flick et al., 1977). Insulin, the most widely studied hormonal effector of FAS levels, increases FAS levels in liver, especially upon administration to diabetic (naturally or chemically induced) animals or their cultured explants (Joshi and Aranda, 1979; Nepokroeff et al., 1979; Lakshmanan et al., 1972; Dahlen et al., 1968; Gibson and Hubbard, 1960). The absolute requirement for insulin in raising fatty acid synthetase levels in vivo has been questioned (Kukulansky and Yagil, 1979).

FERRITIN

Iron is a required nutrient for cells in almost all phyla (Munro and Linder, 1978). Because ferrous ion in aqueous solution at physiological pH tends to oxidize and form insoluble ferric hydroxide or oxyhydroxide, and then precipitate, a means for intracellular storage and transport of iron is needed. Although low molecular weight polyphosphates have been implicated in the small scale transport and storage of iron (Jacobs, 1977; Konopka and Romslo, 1980; Konopka et al., 1981), the protein ferritin has been found to be the major intracellular iron storage depot. Ferritin maintains the iron for cellular metabolic needs in a soluble and nontoxic form. In addition to its function as an iron storage protein, it participates directly in the transfer of iron from plasma membrane to the mitochondria (Ulvik et al., 1981; Mazur and Carleton, 1963; Nunez et al., 1980; Ulvik and Romslo, 1978, 1979), where the iron is transferred to a ferrochelatase (Ulvik and Romslo, 1981) and then inserted into protoporphyrin IX to complete heme biosynthesis (Ulvik, 1981).

The first preparation of an iron-containing protein was obtained from pig liver. This poorly defined, denatured "ferratin" contained about 5% iron by weight (Schmiedeberg, 1894). It was in deference to this "ferratin" that the first pure iron-containing protein, isolated from horse spleen, was named. Horse spleen ferritin (Laufberger, 1937) is the best characterized of the subsequently isolated ferritins. Among the other tissues and organisms from which ferritin has been isolated are

Escherichia coli (Yariv et al., 1981), fungi (David and Easterbrook, 1971; Bozarth and Goenaga, 1972), legumes (Crichton et al., 1978), plants (Seckbach, 1972), dog, cat, jackal, Guinea pig, rabbit, tuna-fish, dolphin, and human spleen (Kuhn et al., 1940; Granick, 1943; Muraoka et al., 1966; Kato et al., 1968; Kato and Shimada, 1970; Wagstaff et al., 1978), rat and human heart (Valimiri et al., 1977; Wagstaff et al., 1978), and amphibian red blood cells (Thiel, 1973). A summary of the relative occurrences of ferritin in various animal tissues has also been published (Michaelis, 1947).

Ferritin consists of an approximately spherical hollow shell of protein with a core containing up to 4500 iron atoms (Crichton, 1973). The core consists of iron in the same crystalline form as the mineral ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$), with appropriately adsorbed phosphate (Towe, 1981; Chukhrov et al., 1973; Harrison, 1963; Michaelis et al., 1943). The iron is present in a layered structure with flattened octahedral arrangement of oxygen about each iron atom (Heald et al., 1979), with iron in the ferric state (Blaise et al., 1965). The phosphate is primarily on the surface of the core (Granick, 1942; Fishbach et al., 1969) and typically constitutes 1 to 1.5% of the total mass of the core (Granick and Hahn, 1944), although as the core increases in size, the relative amount of phosphate present decreases (Treffry and Harrison, 1978).

The roughly spherical protein shell about the core consists of 24 subunits, arranged such that the shell belongs to the F_{432} space group (1980b). In mammalian ferritin, the shell is about 2.4 nm thick and has an outer diameter of 12-13 nm and inner diameter of 6-7 nm. Six channels, located at the fourfold symmetry axes of the shell, provided for the entry and exit of small molecules about the interior (Banyard et al., 1978). These channels are 0.9-1.2 nm wide at the outer surface of the shell and 1.7-2.0 nm at the inner surface (Jones et al., 1978.

The subunits comprising the shell are of two types (Linder et al., 1974; Urushizaki et al., 1971) designated H and L (Russel and Harrison, 1978; Adelman et al., 1975; Ishitani et al., 1975). The respective molecular weights of these subunits are approximately 21,000 and 19,000 daltons, giving an overall molecular weight of the shell of 440,000 to 480,000 daltons, depending on the proportion of L and H subunits (Hoare

et al., 1975; Banyard et al., 1978; Crichton and Bryce, 1970; Bjork and Fish, 1971; Crichton et al., 1973a; Drysdale et al., 1975). These subunits have distinct immunological properties despite their many common structural features. Each subunit is of cylindrical shape and consists of a bundle of four long helices lying parallel (or antiparallel) to one another, together with two much shorter helices (Clegg et al., 1980a) and a long loop over the outside surface of the subunit which forms a short section of β-pleated sheet with the loop from a neighboring subunit (Clegg et al., 1980b). The L subunits have a more highly ordered secondary structure than the H subunits; the L subunit is about 50% α-helix and the H subunit is about 30% α-helix (Otsuka et al., 1981). In either case, the subunit conformations bear resemblance to those of myohemerythrin, hemerythrin, tobacco mosaic virus, and cytochromes b-562 and c' (Clegg et al., 1980b). The amino acid sequence of a subunit from horse spleen ferritin has been determined (Heusterspreute and Crichton, 1981).

The ratio of L to H subunit in ferritin shells varies with species and tissue (Crichton et al., 1973b; Massover, 1978; Kohgo et al., 1980). Iron poor tissues, such as heart, intestine, and pancreas, have a lower ratio of L subunit to H subunit in their ferritin shells than iron-rich tissues (Bomford et al., 1981; Arosio et al., 1978). Ferritin shells with higher L to H subunit ratios have been shown to have longer turnover times in the cell, and also to have a lesser surface charge (Kohgo, et al., 1980).

Under physiological conditions, iron is transferred to and from ferritin in the ferrous state, although it is stored in the ferric state (Niederer, 1970). Deposition of iron in the shell involves oxidation of ferrous ion to the ferric state followed by hydrolysis (Treffry and Harrison, 1980a; Macara et al., 1972; Crichton et al., 1977). Initiation of deposition apparently occurs as follows: two ferrous ions bind to catalytic sites on adjacent polypeptide chains, followed by bonding of dioxygen by the ferrous ions. The iron is oxidized to the ferric state, reducing the oxygen to a peroxo- complex. Incoming ferrous ion then displaces the peroxo- complex, resulting in the hydrolysis and deposition of the ferric ion (Crichton et al., 1980). After initiation of the core, iron is then deposited directly onto the preexisting core; consequently, the deposition rate is greatest in ferritin shells which

are half-filled, containing about 1400 atoms of iron (Ulvik et al., 1981). Certain carboxyl groups, located in residues 53-59 of the 174 amino acid subunit of horse spleen ferritin (Wetz and Crichton, 1976; Vandamme, 1980) have been implicated in the binding of iron and catalysis of oxidation. The reductive mobilization of iron from ferritin in vivo by oxidoreductases in the cytosol and microsomes (Zaman and Verwilghen, 1977), by an NAD(P)H: flavin oxidoreductase (Sirivech et al., 1977), and by a mitochondrial ubiquinol-FMN oxidoreductase (Ulvik et al., 1981; Ulvik and Romslo, 1981) have been suggested. A variety of biological reducing agents also release iron from ferritin; these include cysteine, ascorbate, glutathione (Dognin and Crichton, 1975) and dihydroflavin (Jones et al., 1978; Sirivech et al., 1974). The apparent mediators of transfer of iron from ferritin in biological systems are flavins, since $FMNH_2$ generated at the mitochondria by a ubiquinol-FMN oxidoreductase seems to reduce iron in ferritin to the ferrous state, and then transport it to a ferrochelatase in the inner mitochondrial membrane (Jones and Jones, 1969; McKay et al., 1969).

An increase in flow of iron into cells stimulates ferritin synthesis in all cells studied to date with the exception of lymphocytes (Lema and Sarcione, 1981). Ferritin levels in the cell can be increased up to ten-fold through the use of iron-rich media or diet (Hoy and Jacobs, 1981). Distinct messenger RNAs for the H and L subunit types (Watanabe and Drysdale, 1981b) are produced in disproportionate amount under iron-rich conditions: the greater the iron concentration, the greater the proportion of L subunit formed (Otsuka et al., 1981). Under normal dietary conditions, ferritin messenger RNA is the most common messenger RNA in polysomes and mRNP particles. It is present in sufficient quantity to be translated immediately upon entry of iron to the cell (Watanabe and Drysdale, 1981a). As iron concentration rises in the cell, the amount of polysome-associated ferritin messenger RNA increases even further (Zahringer et al., 1976) and translation of the ferritin messenger RNA occurs at an increased rate (Schaefer and Thiel, 1981). The ferritin produced, as well as having a higher L to H subunit ratio, also has a lower isoelectric point, apparently due to its greater incorporation of iron (Treffry and Harrison, 1980c; Harrison, 1959; Banyard et al., 1978; Bomford et al., 1981; Kohgo et al., 1980) rather than its higher L to H subunit ratio (Treffry and Harrison, 1980b; Hoy and Jacobs, 1981).

CHAPTER 2

IMPROVED PARTIAL REACTION ASSAY AND PURIFICATION PROCEDURE FOR FATTY ACID SYNTHETASE

ABSTRACT

This chapter presents improved procedures for assay and for purification of fatty acid synthetase from pig liver. The decalone reductase activity assay used in this laboratory at the beginning of this work had limited applicability because of precipitation of the substrate, trans-1- decalone, at high salt and protein concentrations. Increasing the ethanol concentration and decreasing the trans-1-decalone concentration in the assay now allows the assay to be used at any step in the purification of fatty acid synthetase. This assay is about fifteen times more sensitive than the overall activity assay.

The procedure for the purification of fatty acid synthetase used in this laboratory at the beginning of these studies provided enzyme of high purity in good yield, but required five days for completion. Addition of 5% isopropanol to the homogenization buffer, modification of the procedure for DEAE-cellulose chromatography, and elimination of the calcium phosphate gel treatment have significantly reduced the time and materials required for purification of the enzyme. The enzyme is now purified in three days without compromise of purity or yield.

The purified enzyme is highly susceptible to air-oxidation. The oxidation is readily reversed by the addition of reducing agent to the enzyme.

INTRODUCTION

Dutler et al. (1971) described several substrate analog model compounds specific for the 3-oxo-acyl carrier protein-reductase component of fatty acid synthetase. The Km and Vmax values for (9R)-trans-decal-1,4-dione were 1.2 mM and 163 sec^{-1}, respectively. The Km and Vmax values for (9R)-trans-1-decalone were 0.17 mM and 51.5 sec^{-1}, respectively. They used the (±) trans-decal-1,4-dione dissolved in diethyleneglycol dimethyl ether to assay for the β-ketoacyl reductase component of fatty acid synthetase, apparently because of its higher Vmax value. A partial reaction assay for fatty acid synthetase using (±)-trans-1-decalone was reported by this laboratory (Kim, Unkefer, and Deal, 1977). The commercially available trans-1-decalone was dissolved in absolute ethanol because, unlike diethyleneglycol dimethyl ether, ethanol is stable indefinitely. Although the value for Vmax of trans-1-decalone is only a third of that for trans-decal-1,4-dione, the decalone reductase assay for fatty acid synthetase is still about fifteen times more sensitive than the overall assay.

The decalone reductase assay reported earlier by this laboratory worked well for relatively pure preparations of enzyme at low ionic strength. However, samples with high salt or protein content, e. g., crude liver fractions and fractions with added ammonium sulfate, could not be assayed for decalone reductase activity because trans-1-decalone precipitated upon its addition to the incubation mixture. Part of this chapter describes an analysis of the precipitation problem and an improved assaying procedure which eliminates precipitation of the trans-1-decalone.

The purification procedure for fatty acid synthetase described by Kim, Neudahl, and Deal (1981) yielded high purity enzyme in good yield, but required a large investment of time (five long days) and gave enzyme which was not as stable as desired. Therefore, studies aimed both at obtaining enzyme that was more stable and at obtaining high purity enzyme in good yield in a lesser period of time were performed. The result was an improved procedure for purification of the enzyme.

MATERIALS AND METHODS

Reagents. Acetyl-CoA, malonyl-CoA, and NADPH were obtained from P-L Biochemicals. Dithiothreitol was a product of Eastman-Kodak and trans-1-decalone of Aldrich Chemical Co. Absolute ethanol was obtained from Aaper Alcohol and Chemical Co. Bovine serum albumin, gum arabic, bromphenol blue, and Coomassie Brilliant Blue R were obtained from Sigma Chemical Co. Tannic acid was obtained from Baker, and DEAE-cellulose (standard, 0.91 meq/g) was obtained from Schleicher and Schuell. Ammonium persulfate, N,N,N',N'-tetramethylethylenediamine was a product of Canalco. Riboflavin was a product of Calbiochem, and 2-mercaptoethanol was a product of Mallinckrodt. All other reagents were of analytical reagent grade.

Liver. Pig livers were obtained from Peet Packing Company (Chesaning, MI) within an hour of slaughter and were frozen on dry ice. The livers were stored at -30°.

Protein Determination. Protein was determined by the method of Massey and Deal (1973). The stock solutions were as follows (distilled, deionized water was used throughout):

1. Tannin reagent: Liquified phenol (2 ml, 88%) was dissolved in 98 ml of 1 M HCl. Tannic acid (10 g) was then dissolved in the solution. The reagent was stored at 4°. Before use, the reagent was brought to room temperature and was stirred until any gum present had dissolved. After filtering through Whatman No. 1 filter paper, the reagent was ready for use.
2. Arabic reagent: Gum arabic (100 mg) was dissolved in 100 ml water. The reagent was stored at 4° and was brought to room temperature prior to use.
3. Protein standard: Crystalline bovine serum albumin (10.0 mg) and sodium chloride (900 mg) were dissolved in 100 ml water. This solution was stored at 4°.
4. Diluent: Sodium chloride (900 mg) was dissolved in 100 ml water.

The protein determinations were carried out as follows:

1. A set of six 15 x 125 mm test tubes, which contained 0, 10, 20, 40, and 50 μg bovine serum albumin in 0.9% NaCl, were prepared.
2. Several dilutions of the unknowns were prepared, such that at least one dilution for each unknown had between 10 and 100 μg

protein per ml sample. The samples, of 0.50 ml volume, were placed in 15 x 125 mm test tubes. Estimates of protein concentration were made by measuring absorbance at 280 nm; an absorbance of 1 represents a protein concentration of about 1 mg/ml.

3. Tannin reagent (0.50 ml) was added in a defined sequence to each tube at regular intervals of 45 seconds. Immediately after adding the tannin reagent, the solutions were thoroughly mixed.

4. Twenty minutes after adding tannin reagent, arabic reagent (1.00 ml) was added to each tube. The solutions were thoroughly mixed after addition of the arabic reagent.

5. The apparent absorbances of the turbid suspensions were measured at 650 nm. The sample containing only NaCl, tannin reagent, and arabic reagent was the reference solution. A standard curve was constructed using the absorbances of the protein standards. Protein concentrations for the unknowns were obtained using this curve.

Since this protein determination is a turbidimetric one, absorbance measurements should be made as soon as possible in order to minimize errors due to settling of the turbid suspension. The standard curve was linear between 5 and 50 μg protein per assay. Above 50 μg protein per assay, absorbance per μg protein rapidly decreases. For this reason, the testing of several different dilutions of unknown is required.

Conductivity and pH Measurements. Conductivities of solutions were measured using a Radiometer CDM2e conductivity meter with a CDC114 cell . The values of pH were determined at room temperature using a Radiometer PHM4c pH meter equipped with a GK2321C electrode.

Native Polyacrylamide Gel Electrophoresis. Native polyacrylamide gel electrophoresis was performed using the method of Clark and Switzer (1977) with minor modification. The solutions required for PAGE were the following (Staining and destaining solutions were stored at room temperature; ammonium persulfate solution was prepared fresh; all other solutions were stored at 4°; deionized, distilled water was used throughout):

1. Solution A: Tris(hydroxymethyl)aminomethane (36.6g), 48 ml of 1M HCl, and 0.23 ml TEMED were taken to 100 ml with water. The pH of this solution was approximately 8.9.

2. Solution B: Tris(hydroxymethyl)aminomethane (1.5 g), 11.7 ml of 1 M HCl, and 0.13 ml TEMED were taken to 25 ml with water. The pH of this solution was 6.9.

3. Solution C: Acrylamide (56.0 g) and N,N'-methylenebis(acrylamide) (1.47 g) were taken to 200 ml with water. The solution was filtered through Whatman No. 2 filter paper before use.

4. Solution D: Acrylamide (5.0 g) and N,N'-methylenebis(acrylamide) (1.25 g) were taken to 50 ml with water. The solution was filtered

through Whatman No. 2 filter paper prior to use.

5. Solution E: Riboflavin (1.25 mg) was dissolved in 25 ml water.
6. Solution F: Sucrose (40 g) was taken to 100 ml with water.
7. Solution G: Ammonium persulfate (1.12 mg/ml) was prepared and deaerated by aspiration immediately prior to use.
8. Electrophoresis buffer: Tris(hydroxymethyl)aminomethane (6.0g) and glycine (28.8 g) were taken to one liter with water. This buffer is diluted ten-fold with water prior to use.
9. Tracking dye solution: Bromphenol blue (0.5 mg) was dissolved in 5 ml water and 5 ml glycerol was then added.
10. Staining solution: Coomassie Brilliant Blue R (1.3 g) was dissolved in a solution containing 265 ml 95% ethanol, 50 ml glacial acetic acid, and 235 ml water. The staining solution was reused indefinitely.
11. Destaining solution: Glacial acetic acid (280 ml) and 95% ethanol (300 ml) were taken to four liters with water.

Gel tubes were prepared by cutting 7 mm (o. d.) Pyrex tubing into 13 cm lengths and then fire-polishing both ends. Marks were etched 10 cm for one end of the tubes. These markings indicate the filling positions for the resolving gel solution.

Polyacrylamide gels were formed one to seven days prior to electrophoresis. The tubes were sealed at the ends opposite the markings with Parafilm and were mounted vertically. Resolving gel solution (about 1.8 ml per tube) was then prepared on ice by mixing one volume Solution A, two volumes Solution C, and five volumes Solution G. The gel tubes were filled to the etched markings using a Pasteur pipet. The resolving gel solution was overlayered with about 0.1 ml water.

During polymerization of the resolving gel, the stacking gel solution was prepared. One volume Solution B, two volumes Solution D, one volume Solution E, and four volumes Solution F were placed in an aluminum foil-wrapped test tube. A volume of 0.2 ml of the resulting solution is required for each tube.

After polymerization of the resolving gel, the water overlayer was removed using a Pasteur pipet. Stacking gel solution (0.2 ml per tube) was then applied. Again, a water overlayer was applied; this overlayer promotes the formation of a sharp, flat boundary upon polymerization of the gel solutions. The tubes were placed under a fluorescent lamp until the stacking gel polymerized.

Immediately before a PAGE experiment, the water overlayer was removed

from the gels. After moistening the tops of the tubes with glycerol, the tubes were inserted in the electrophoresis apparatus. The two reservoirs and the space in the tubes above the stacking gels were then filled with the ten-fold diluted electrophoresis buffer.

Samples were prepared for electrophoresis by adding at least a tenth-volume of tracking dye solution. Sample size was limited to about 200 µl by the dimensions of the gel tubes. Removal of salt from the samples prior to electrophoresis was not required, but high salt concentrations increased the amount of time required for electrophoresis. The samples (with tracking dye) were applied just above the stacking gels using an Eppendorf pipet.

After all samples were applied, the chambers were attached to a Heath-kit Model IP-32 regulated power supply. The negative lead was attached to the upper reservoir, the positive lead to the lower reservoir, and a 200 volt potential was applied. Electrophoresis was continued until the dye band was about 5 mm from the bottom of the resolving gels. After turning off the power supply, the tubes were removed from the apparatus. Gels were removed from the tubes using a stream of water pointed at the bottom of the tube, between the gel and the wall of the tube. The stream of water was produced by an 18 gauge needle attached to a water faucet by a length of Tygon tubing. The gels were placed in 16 x 150 mm screw-cap culture tubes containing staining solution. Until the staining solution had been used eight times, gels were stained for about two hours; later, overnight staining was used.

After staining, the staining solution was removed and the gels were rinsed with distilled water. Destaining solution was then added to the tubes, and the tubes were left on a mechanical shaker overnight. The destaining solution was then discarded and fresh destaining solution was added to the tubes. The destaining solution changes were continued until stain was no longer released from the gels.

Assays for Fatty Acid Synthetase. The assays described below (Kim, Unkefer, and Deal, 1977) were the standard assays for fatty acid synthetase in use in this laboratory when this work was begun. They therefore were the starting point for the development of improved assays.

Initial Standard Decalone Reductase Assay. This partial reaction assay was routinely used in partially purified preparations of fatty acid

synthetase. The stock reagents for this assay (final concentrations in parentheses) are given below:

potassium phosphate, 0.2 M, pH 6.10 (0.1 M)
trans-1-decalone, 200 mM, in ethanol (10 mM)
NADPH, 2 mM, in 1 mM NaOH (50 μM)
enzyme (about 4 μg/ml)

The reaction was carried out at 25°. It was initiated by the addition of trans-1-decalone. The final assay volume was 0.4 ml. Activity values were measured from the initial slopes of the absorbance curves. One unit of activity was the amount of enzyme that catalyzed the oxidation of one μmol NADPH per minute.

Initial Standard Overall Assay. The overall reaction assay was used primarily to determine the specific activity of purified fatty acid synthetase. The stock reagents for this assay (final assay concentrations in parentheses) are given below:

potassium phosphate, 0.4 M, pH 6.89 (0.1 M)
acetyl-CoA, 2 mM (50 μM)
malonyl-CoA, 2 mM (50 μM)
NADPH, 4 mM, in 1 mM NaOH (200 μM)
bovine serum albumin, 20 mg/ml (1 mg/ml)
enzyme (about 60 μg/ml)

The reaction was carried out at 25°. It was initiated by the addition of malonyl-CoA. The final assay volume was 0.4 ml. Activity values were measured from the initial slopes of the absorbance curves. Activity units are as described for the decalone reductase assay. Specific activity is expressed as units per milligram of protein.

RESULTS

The Decalone Reductase Assay. The initial standard decalone reductase assay was about fifteen times more sensitive for fatty acid synthetase than the overall assay. This sensitivity would make it the preferred assay for crude preparations of the enzyme; smaller sample sizes would be required, and any contaminating enzyme activities would be proportionately reduced. Also, the decalone reductase assay required fewer and less costly reagents than the overall assay. Unfortunately, trans-1-decalone precipitated when samples with high concentrations of protein or of salt were assayed for decalone reductase activity. Studies aimed at extending the usefulness of the decalone reductase assay to samples

of high protein or of high salt concentration were therefore undertaken.

Development of an Improved Assay. Apparently negative activities were obtained when the initial standard decalone reductase assay was used with samples of high salt or protein concentration. The cause was the addition of trans-1-decalone, which produced precipitation (Table 1).

Table 1. Determination of the Optimal Concentrations of trans-1-decalone and Ethanol in the Decalone Reductase Assay for Samples Containing Up To 1.4 M Ammonium Sulfate.

Initial Contents of the Cuvette[a] (310 µl)	Sample (50 µl)	Solution Added (40 µl)	Precipitation?[b]
KPO_4, NADPH, H_2O	H_2O	200 mM TDEC in ethanol	yes
KPO_4, H_2O	H_2O	200 mM TDEC in ethanol	yes
KPO_4, NADPH, H_2O	H_2O	ethanol	no
KPO_4, H_2O	H_2O	ethanol	no
KPO_4, NADPH, H_2O	H_2O	100 mM TDEC in 95% ethanol	yes
KPO_4, NADPH, H_2O	H_2O	100 mM TDEC in ethanol[c]	no
KPO_4, NADPH, H_2O	1.4 M $(NH_4)_2SO_4$	100 mM TDEC in ethanol	yes
KPO_4, NADPH, H_2O	1.4 M $(NH_4)_2SO_4$	50 mM TDEC in ethanol[d]	no

[a]Concentrations of NADPH and potassium phosphate buffer (KPO_4) were as indicated in Materials and Methods for the decalone reductase assay.

[b]Precipitation was measured as an increase in apparent absorbance at 340 nm upon addition of the substrate (ethanol-containing) solutions; absorbance normally decreases as the enzyme oxidizes NADPH.

[c]This is a previous stock solution of trans-1-decalone (TDEC) for the assay.

[d]This is the present stock solution of trans-1-decalone for the assay.

Addition of ethanol alone to the assay mixture did not cause precipitation. The precipitation upon addition of the trans-1-decalone stock solution was prevented by increasing the ethanol concentration in the assay mixture to 10%. This was accomplished by dilution of the trans-1-decalone stock solution to 100 mM trans-1-decalone in ethanol. Precipitation again occurred, however, when samples other than water were used in this modified assay

Two alternatives were considered for the elimination of the precipi-

tation: increasing the ethanol concentration, or decreasing the trans-1-decalone concentration. Since the Km for trans-1-decalone is 0.17 mM (Dutler et al., 1971), the concentration of trans-1-decalone in the assay was reduced. At concentrations of up to 5 mM trans-1-decalone (with 10% ethanol) in the assay, one-eighth volume of 0.33 saturated ammonium sulfate (the highest ionic strength reached in the purification of fatty acid synthetase) did not induce precipitation (Table 1). Consequently, a stock solution of 50 mM trans-1-decalone in ethanol was selected for the assay. This stock solution yields final concentrations of 5 mM trans-1-decalone and 10% ethanol in the assay.

Enzyme Concentration and Activity. Proportionality tests between enzyme concentration and reaction rate in both the decalone reductase and overall assays were performed. The overall activity of the enzyme was linearly proportional to enzyme concentration over at least a ten-fold range of enzyme concentration. This proportionality was retained even when bovine serum albumin was eliminated from the assay and the NADPH concentration in the assay was reduced to 100 mM. For this reason, the present standard overall activity assay has the following final concentrations of reagents in the assay:

potassium phosphate, 0.1 M (pH 6.89)
acetyl-CoA, 50 μM
malonyl-CoA, 50 μM
NADPH, 100 μM
enzyme (about 0.006 units/ml)

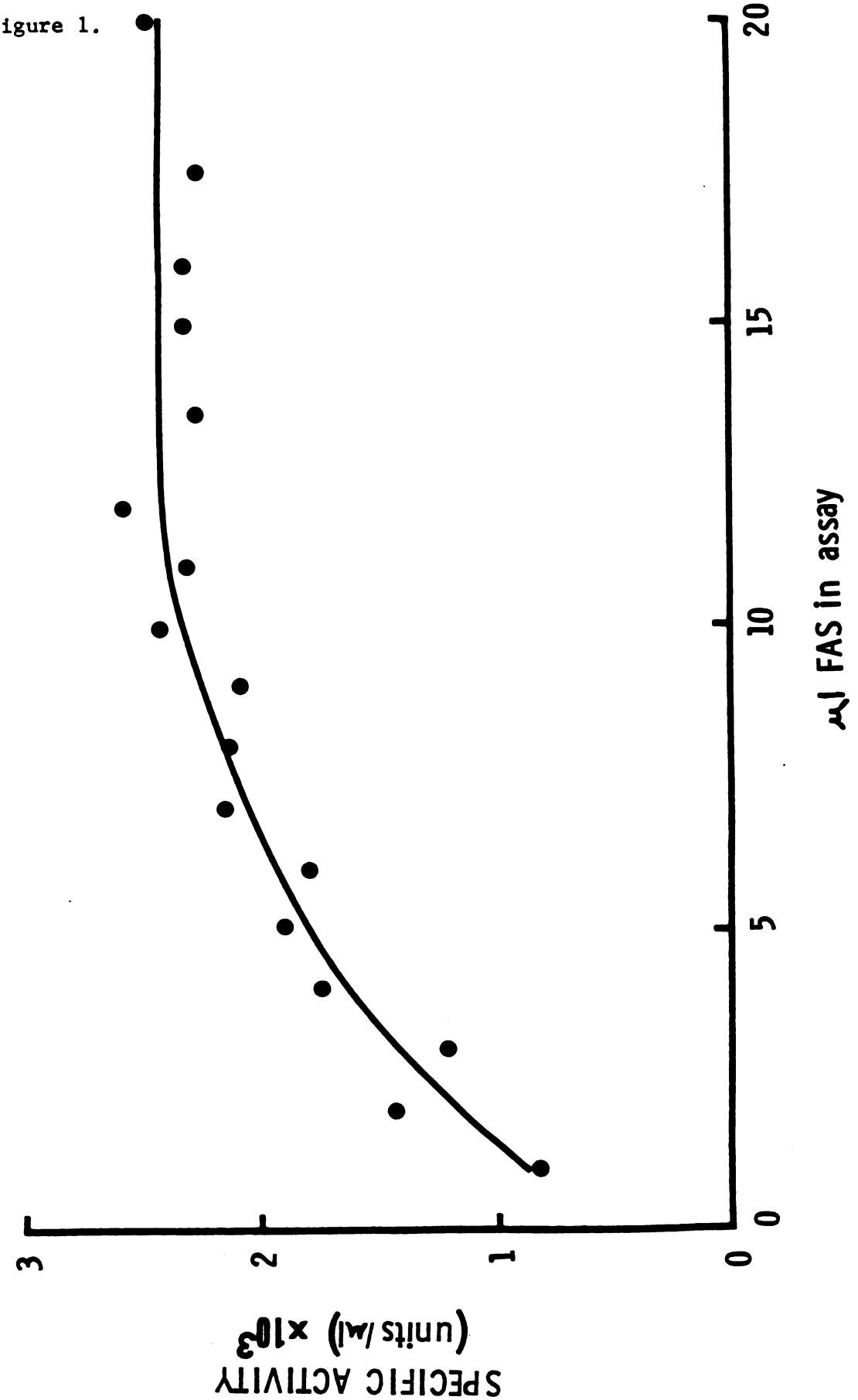
All other details of the assay are as for the initial standard overall assay.

The specific activity of fatty acid synthetase as measured by decalone reductase activity increased significantly with enzyme concentration up to 0.015 units of activity in the assay (Figure 1). Above this level, activity is proportional to the concentration of enzyme over more than a two-fold range of enzyme concentration. The decreasing specific activity at lower enzyme concentrations is apparently due either to dissociation of the enzyme into inactive (or less active) subunits or to inactivation of the enzyme by trans-1-decalone. The improved decalone reductase assay has the following stock reagents (final concentrations in the assay in parentheses):

-potassium phosphate, 0.2 M, pH 6.10 (0.1M)
-trans-1-decalone, 50 mM, in ethanol (5 mM)

Figure 1. Specific Activity of Fatty Acid Synthetase as a Function of Enzyme Concentration Using the Decalone Reductase Activity Assay. The standard decalone reductase assay presented at the beginning of Results was used to assay various amounts of purified fatty acid synthetase. A stock solution of the enzyme was diluted ten-fold with 0.13 M potassium phosphate buffer (pH 6.10); various volumes of this solution were then used in the assays.

Figure 1.


SPECIFIC ACTIVITY
(units/ml) x10³
0
1
2
3
0
5
10
15
20
µl FAS in assay

-NADPH, 2 mM, in 1 mM NaOH (50 μM)
-enzyme (0.04-0.12 units/ml)

All other details of the assay are as for the initial decalone reductase assay.

Fatty Acid Synthetase Purification. Purification of fatty acid synthetase according to the method of Kim, Unkefer, and Deal (1977) required about five days and yielded enzyme which was not as stable as desired. It was thought that the instability of the enzyme might be due to proteolysis during its purification. Therefore, the enzyme was purified in the presence of a protease inhibitor, phenylmethanesulfonylfluoride. This compound is not water soluble, so it was dissolved in isopropanol. A stock solution of 20 mM PMSF in isopropanol was prepared, yielding concentrations of 1 mM PMSF and 5% isopropanol throughout the enzyme purification. These substances stabilized the decalone reductase activity in the early stages of the purification. It also substantially decreased the amount of contaminating protein precipitated with fatty acid synthetase in the first ammonium sulfate fractionation step of the purification procedure. To determine whether these effects were due to PMSF, parallel purifications of fatty acid synthetase were performed in the presence of PMSF and isopropanol, or isopropanol alone. Isopropanol proved responsible for the effects noted above. So 5% isopropanol was incorporated into the homogenization buffer.

The initial method for purification of fatty acid synthetase included a DEAE-cellulose chromatography step which required about 18 hours to complete. By replacing this step with DEAE-cellulose pad chromatography, using stepwise rather than gradient elution, the time required for chromatography was reduced to three hours. Elimination of the calcium phosphate gel treatment further reduced the amount of time required for purification of the enzyme. These changes in the purification procedure decreased the time required for preparation of the enzyme to three days, without effect on overall yield or purity. The details of this improved purification procedure are presented below.

Improved Purification Procedure for Fatty Acid Synthetase. Unless otherwise indicated, centrifugation was performed in the Sorvall GSA rotor at 0° and 11,000 rpm for 20 minutes, or in the Sorvall SS-34 rotor at 0° and 18,000 rpm for 20 minutes. Step 1 and Step 2 were performed at

4°; Step 3 and Step 4 were performed at room temperature.

Step 1. Clarified centrifuged extract. Frozen pig liver (800 g) was cut into small pieces with a knife and was suspended in two volumes of cold homogenization buffer. This buffer contained 50 mM tris(hydroxymethyl)aminomethane (pH 7.4), 1 mM EDTA, and 5% (v/v) isopropanol. The suspension was homogenized for 30 seconds at low speed, 30 seconds at medium speed, and 60 seconds at high speed in a Waring commercial blendor. Homogenization at each speed was interrupted at the midpoint in time for 15 seconds; these interruptions were also performed when changing speeds. During homogenization, the temperature of the suspension typically increased from 0 to 6°. The homogenate was centrifuged in a GSA rotor for 40 minutes at 11,000 rpm and 0°. The supernatant was further centrifuged in polyallomer tubes in a Beckman Type 21 rotor at 0° for 150 minutes at 21,000 rpm. The dark red, faintly turbid supernatant from this centrifugation was designated the clarified centrifuged extract.

Step 2. Ammonium sulfate fractionation. The clarified centrifuged extract was brought to 0.25 saturation ammonium sulfate by adding saturated (4°) ammonium sulfate solution (previously adjusted to pH 7.4 at 23° and 0.1 saturation) with magnetic stirring. Stirring was continued for at least 15 minutes; the suspension was then centrifuged in a GSA rotor. The small amount of precipitated material was discarded, and the 0.25 saturated supernatant was brought to 0.36 saturation by adding more ammonium sulfate solution (saturated at 4°). Again, stirring was continued for at least 15 minutes. The suspension was then centrifuged in a GSA rotor. The pellets were collected and were suspended in homogenization buffer to a volume equal to that of the original clarified centrifuged extract. After the pellets dissolved, saturated ammonium sulfate solution was added with stirring to 0.36 saturation. After stirring at least 15 minutes, the suspension was centrifuged in a GSA rotor. The pellets from this centrifugation were resuspended in TED buffer (100 ml) and were then dialyzed overnight against a total of four liters of TED buffer, with one buffer change. Dialysis typically began 12 hours after homogenization of the liver. After dialysis, the dialyzate was centrifuged in an SS-34 rotor to remove denatured (precipitated) protein. The supernatant from this centrifugation was warmed to room

temperature.

Step 3. DEAE-cellulose pad chromatography. An 11.5 cm (d) x 3.0 cm (h) DEAE-cellulose pad was prepared in a Buchner funnel over Whatman No. 1 filter paper. Firm packing was achieved by preparing the pad under reduced pressure with the aid of an aspirator and a filtering flask. The pad was washed with TED buffer (one liter), the aspirator was detached, and the dialyzate supernatant from Step 2 was applied to the column. After the supernatant was absorbed by the pad, the aspirator was again attached. The pad was washed with TED buffer (one liter) and 50 mM KCl in TED buffer (one liter), drawn through the pad at a rate of about 50 ml per minute. The TED buffer wash fraction was extremely turbid. The KCl in TED buffer wash fraction was pale yellow-green and contained measurable catalase activity. Following these washes, fatty acid synthetase was eluted with 300 mM KCl in TED buffer. The bright yellow eluate was taken to 4° and was brought to 0.33 saturation with saturated (4°) ammonium sulfate solution. After stirring for 15 minutes, the suspension was centrifuged in a GSA rotor. The pellets from this centrifugation were combined and were suspended in a minimal volume (less than 20 ml) of PED buffer. The suspended pellets were dialyzed at 4° against PED buffer (500 ml) for at least two hours. The dialyzate was then centrifuged in an SS-34 rotor. The clear yellow-orange supernatant was warmed to room temperature.

Step 4. Sucrose density gradient centrifugation. Six linear sucrose density gradients (5 to 20%) containing PED buffer were prepared. The gradients (34 ml) were prepared in 1 x 3½ inch polyallomer tubes using an ISCO Model 570 gradient former. One-sixth volume of the dialyzate supernatant from Step 3 was applied to each gradient. The tubes were then placed in the buckets for a Beckman SW27 rotor and centrifuged for 18 hours at 27,000 rpm and 20° in a Beckman L3-50 ultracentrifuge. Ultracentrifugation typically began seven hours after the overnight dialysis at the end of Step 2. After centrifugation, the gradients were fractionated, using an ISCO Model UA-5 absorbance monitor to measure absorbance at 280 nm and an ISCO Model 183 gradient fractionator to fractionate. The fractionation rate was 1.5 ml per minute, and 1.5 ml fractions were collected. Fractions having significant FAS activity were combined and taken to 4°. Saturated ammonium sulfate solution was then added to

0.50 saturation with stirring. After stirring for at least 15 minutes, the suspension was centrifuged in an SS-34 rotor. The pellets were combined and were taken up in a minimal volume of PED buffer. The suspended pellets were dialyzed for at least two hours against PED buffer at 4°. If satisfactory removal of the 7 S contaminant(s) had been achieved, dialysis was continued overnight, and the purified enzyme was frozen at -30° for storage. If further removal of the contaminant was desired, ultracentrifugation of the dialyzate was repeated.

Comparison of the Original and Improved Purification Procedures for Fatty Acid Synthetase. Briefly, the changes introduced in the purification procedure for fatty acid synthetase were the following:

1. Isopropanol (5%) was incorporated in the homogenization buffer.
2. The first and second ammonium sulfate fractionations were changed from 0.2-0.33 and 0-0.33 saturation to 0.25-0.36 and 0-0.36 saturation, respectively.
3. DEAE-cellulose column chromatography with gradient elution was replaced by DEAE-cellulose pad chromatography with stepwise elution.
4. The calcium phosphate gel treatment step and the associated Sephadex G-25 desalting procedure were eliminated.

The effects of these changes in the purification procedure are detailed below.

Effects of changes in homogenization buffer and ammonium sulfate fractionations. Table 2 presents a summary of data from parallel, small scale purifications of fatty acid synthetase. The procedure of Kim, Unkefer, and Deal (1977), with and without 5% isopropanol in the homogenization buffer, was used. The same pig liver was used for both purifications. Essentially identical decalone reductase activity recoveries were obtained in the early fractions. It is informative to compare the 0.33 saturated supernatant fractions, which were discarded in the purification procedure. The supernatant fractions which were prepared with 5% isopropanol in the homogenization buffer contained about 2½ times more decalone reductase activity than the supernatant fractions prepared without isopropanol. Thus, fatty acid synthetase was more soluble in 0.33 saturated ammonium sulfate when 5% isopropanol was present. Other proteins were also more soluble under these conditions. This was demonstrated by the greater amount of protein in the 0.33 saturated supernatants and by the smaller pellets obtained from the 0.33 saturated

Table 2. Comparison of Preparations of Fatty Acid Synthetase Carried Out With and Without Isopropanol (5%) in the Homogenization Buffer[a]

Homogenization Buffer Without Isopropanol				Homogenization Buffer With Isopropanol		
Total Volume (ml)	Total DR[b] Activity (units)	Yield (%)	SAMPLE	Total Volume (ml)	Total DR Activity (units)	Yield (%)
210	194	100	GSA-centrifuged homogenate	200	168	100
155	130	67	clarified centrifuged extract	145	116	69
179	123	63	0.2 saturated supernatant	168	108	64
200	12	(6)[c]	1st 0.33 saturated supernatant	198	30	(18)
200	3	(1)	2nd 0.33 saturated supernatant	198	8	(4)

[a]The purifications were performed by the method of Kim, Unkefer, and Deal (1977) with the exceptions of starting with 100 g pig liver and, for one purification, adding 5% isopropanol to the homogenization buffer.

[b]DR is an abbreviation for decalone reductase.

[c]These values are in parentheses because these fractions are discarded.

suspensions when isopropanol was present (protein content was measured by both the tannin turbidimetric method and by relative intensity of banding on native polyacrylamide gels).

To minimize the loss of decalone reductase activity during the ammonium sulfate fractionations with isopropanol present, the upper cut was raised from 0.33 to 0.36 saturation. The lower cut was raised from 0.2 to 0.25 saturation because fatty acid synthetase did not precipitate at 0.25 saturation, regardless of the presence or absence of isopropanol.

Effects of changes in DEAE-cellulose chromatography. A comparison of the original and improved procedures for DEAE-cellulose chromatography (on the dialyzates obtained at the end of Step 2 in the purification procedures) is presented in Table 3. The dialyzate supernatant obtained using the original procedure of Kim, Unkefer, and Deal (1977) was turbid dark green and contained 7400 mg protein. That obtained using the improved procedure was also turbid, but was a much lighter golden-tan color and contained only 420 mg protein from the same amount of liver.

Table 3. Comparison of Yields and Degrees of Purity of Fatty Acid Synthetase Fractionated on DEAE-cellulose by Gradient Elution from a Column or by Stepwise Elution from a Batch-scale Pad.

COLUMN CHROMATOGRAPHY[a]					PAD CHROMATOGRAPHY[b]			
Total Volume (ml)	Total DR[c] Activity (units)	Total Protein (mg)	Yield (%)	SAMPLE	Total Volume (ml)	Total DR Activity (units)	Total Protein (mg)	Yield (%)
130	236	7400	100	Step 2 Dialyzate[d]	129	652	820	100
1000	34	-[e]	14	FAS eluted from DEAE-cellulose	960	508	-[e]	78

[a] Columns were prepared and eluted by the method of Kim, Unkefer, and Deal (1977).

[b] Pads were prepared and eluted by the method given in Results.

[c] DR is an abbreviation for decalone reductase.

[d] Enzyme for column chromatography was obtained from 800 g pig liver by the method of Kim, Unkefer, and Deal (1977); enzyme for pad chromatography was prepared from 1600 g liver by the procedure in the Results.

[e] Protein concentration was not determined for these fractions.

Also, its specific activity was twenty-five times that of the dialyzate supernatant at the corresponding point in the original purification procedure. The difference in the purity of these dialyzate fractions is due to a solubilizing effect of 5% isopropanol upon many contaminating proteins which precipitated with fatty acid synthetase in the 0.33 saturated ammonium sulfate suspensions in the original procedure.

The amount of protein in the dialyzate fraction from the original procedure was so great that about half the protein did not bind to the DEAE-cellulose column. This apparently accounts for most of the activity lost during DEAE-cellulose chromatography in the original procedure; the remainder was apparently lost due to irreversible binding to, or denaturation on, the column. The amount of protein in the dialyzate obtained using the present procedure is sufficiently low that the quantity of DEAE-cellulose used was reduced to one-third of that used in the original procedure. The 300 cm^3 of DEAE-cellulose in the pad is sufficient to bind all the fatty acid synthetase in the dialyzate. Therefore, loss of activity during pad chromatography is apparently due only to

irreversible binding or denaturation of the enzyme on the column.

One major purpose of the DEAE-cellulose chromatography step was to remove catalase, since it is similar in size to FAS and cannot be readily separated from FAS by ultracentrifugation. Both procedures were largely successful in removing catalase; however, FAS purified by either procedure has some catalase activity.

An entire day was required for column chromatography in the original procedure; pad chromatography in the present procedure requires three hours.

Yield and Purity of the Purified Enzyme. Both purification procedures yielded between 15 and 30% of the fatty acid synthetase in the clarified centrifuged extract as pure enzyme. Lower total yield and lower percent yield of enzyme was obtained from livers which had been stored for long period of time. Also, less enzyme was recovered from the DEAE-cellulose pad in Step 3 when more than the minimum amount of ion-exchanger necessary for binding of the enzyme was used.

The specific activity of the enzyme purified by the present procedure is comparable to that of the enzyme purified by the original procedure (typically 1.6 units per milligram protein, based on the decalone reductase assay). The contaminating proteins in the purified enzyme, however, were distinctly different for the two procedures. Since the present procedure did not use the calcium phosphate gel treatment step, a small quantity of hemoprotein H-450 was present in the purified enzyme. This hemoprotein, with its visible absorption maxima, gave the FAS purified by the present procedure its pale yellow color.

Fatty acid synthetase purified by the original procedure, although presumably free of hemoprotein H-450, had several other measurable enzymic activities. Relatively high levels of pyruvate kinase, malate dehydrogenase, and glutamate dehydrogenase activities were found, as well as lesser phosphoglyceromutase and catalase activities. Only catalase was found in FAS purified by the present procedure. All these contaminating activities were located primarily in the first 0.33 saturated supernatant of Step 2 in the original purification procedure for fatty acid synthetase. Apparently, in the present

purification procedure, even more of these enzymes are eliminated during the ammonium sulfate fractionations; that is, they are more soluble in 0.36 saturated ammonium sulfate with isopropanol present than in 0.33 saturated ammonium sulfate.

Stability of the Purified Enzyme. The overall FAS activity was extremely sensitive to air-oxidation. When the enzyme was stored at 4° in PED buffer, 70% of the overall activity was lost after 24 days, and 95% was lost after 45 days. Over the same 45 day period less than 5% of the decalone reductase activity was lost. Rejuvenation of the air-oxidized enzyme was attempted by the addition of reducing agent at several different concentrations (Figure 2). Dithiothreitol was far superior to 2-mercaptoethanol in rejuvenating the enzyme. Enzyme stored in high concentrations of reducing agent (20-50 mM), however, lost all activity within 24 hours. Dithiothreitol at lower concentration (3 mM) reactivated the enzyme at a slower rate, but it also allowed the increase in overall activity to be retained for a much longer period of time. Therefore, overnight dialysis of the enzyme against PED buffer was tested; it was perhaps the most effective way of restoring overall activity to air-oxidized enzyme.

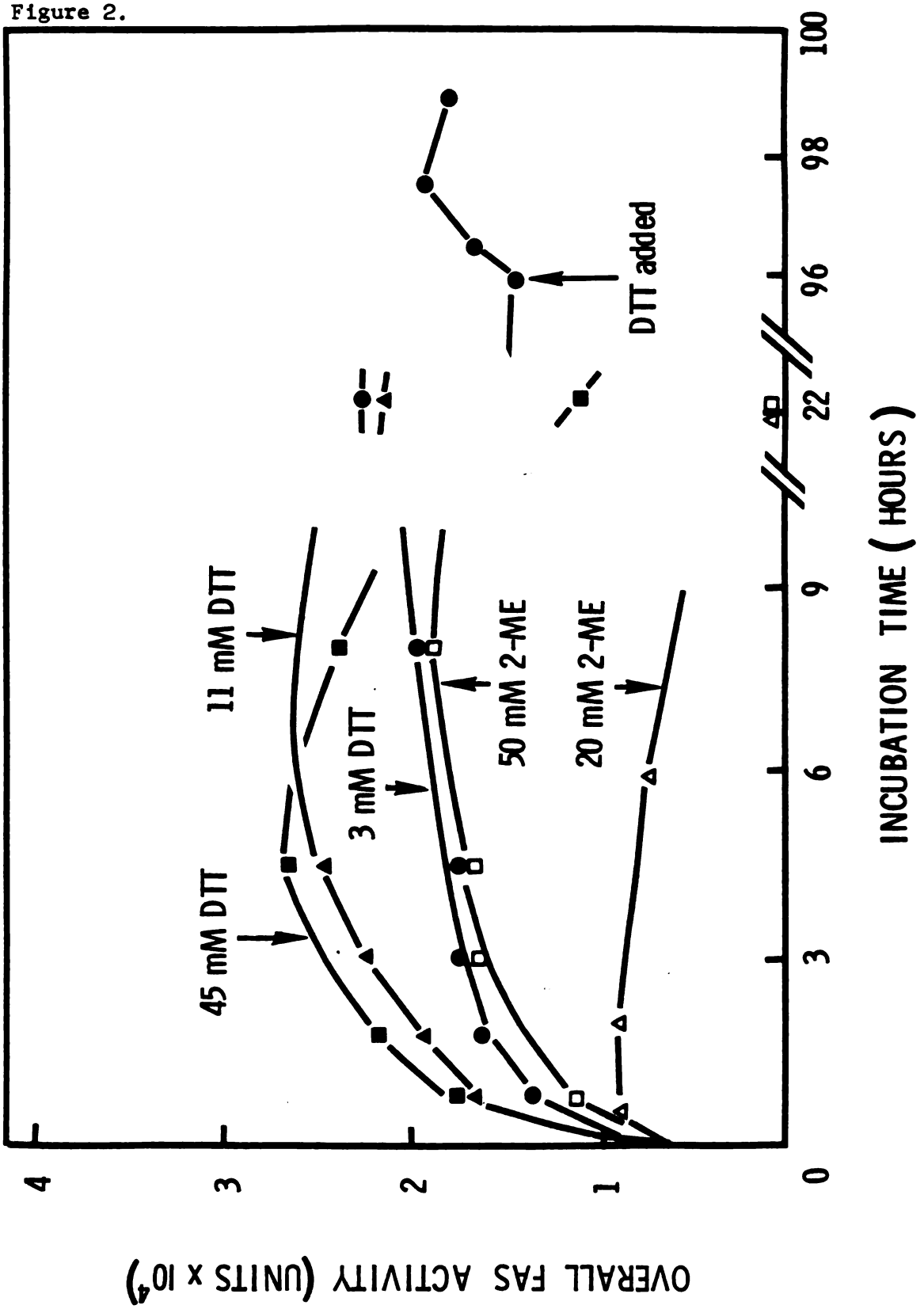
The most effective means of preventing air-oxidation of the enzyme was to store the enzyme frozen at -30° in PED buffer. Less than 20% of the overall activity was lost after the enzyme had been frozen for three months. Most of the lost overall activity could be recovered by overnight dialysis against PED buffer. The remaining activity was apparently lost as irreversibly denatured enzyme, which was present in the thawed solution as a fine flocculent precipitate.

DISCUSSION

The initial decalone reductase assay would be the assay of choice for crude preparations of FAS. Unfortunately, trans-1-decalone precipitated in the presence of these fraction because of their high salt and protein concentrations. By increasing the ethanol concentration in the assay from 5% to 10% and decreasing trans-1-decalone concentration from 100 mM to 50 mM, the assay can now be

Figure 2. Air-oxidation of Fatty Acid Synthetase and Its Reversal by Reducing Agents. An aliquot of the freshly purified enzyme had 3.8×10^{-4} units of overall activity. After storing the enzyme at 4° for 23 days, it was dialyzed overnight against PED buffer. The enzyme was then stored at 4° an additional 20 days, at which time an aliquot of equal volume had 0.6×10^{-4} units of overall activity and 1.7×10^{-2} units of decalone reductase activity. Reducing agent was then added to aliquots of the enzyme: dithiothreitol to 3 (●), 11 (▲), or 45 (■) mM, or 2-mercaptoethanol to 20 (Δ) or 50 (□) mM. The sample containing 3 mM dithiothreitol was taken to 13 mM dithiothreitol after 96 hours. The decalone reductase activities of the aliquots were constant throughout the experiment.

Figure 2.


OVERALL FAS ACTIVITY (UNITS x 10⁴)
INCUBATION TIME (HOURS)
45 mM DTT
11 mM DTT
3 mM DTT
50 mM 2-ME
20 mM 2-ME
DTT added

used at any step in the purification of fatty acid synthetase. The total decalone reductase activity in the assay should be above 0.015 units, however, as below this level the specific activity of the enzyme is a function of its concentration. The cause is apparently a partial inactivation of the enzyme by trans-1-decalone, although dissociation of the enzyme into subunits with lesser (or without) decalone reductase activity is also possible.

The initial procedure for the purification of fatty acid synthetase yielded enzyme of good purity, but required much time and yielded enzyme which was not as stable as desired. Using the present purification procedure, the time required for purification of the enzyme has been reduced from five days to three days. Most of the reduction in time comes from using a DEAE-cellulose pad and stepwise elution in place of gradient elution from a column. Some time is also saved by eliminating the calcium phosphate gel treatment step. This step can be incorporated for removal of the hemoprotein H-450 when desired.

Comparable yields and specific activities of FAS are obtained using either procedure. The number of contaminating enzymic activities is apparently much less, however, in FAS purified by the present procedure. Storing FAS either frozen in PED buffer or in dialysis at 4 ^{o}against PED buffer minimizes air-oxidation of the enzyme and subsequent loss of overall activity. Freezing is preferable for long-term storage.

As a final note, the fractional saturations with ammonium sulfate in Step 2 of the present purification procedure assume that isopropanol does not affect the solubility of ammonium sulfate.

CHAPTER 3

Inactivation of Fatty Acid Synthetase in the Presence of Various Pellet Fractions from Pig Liver

ABSTRACT

A search for activators and inactivators of fatty acid synthetase in pig liver fractions has led to the discovery of inactivating capacities in the 6,000 x g, 30,000 x g, and 124,000 x g pellet fractions from the crude homogenate. The 124,000 x g pellet fraction has the greatest inactivating capacity per gram wet weight.

The rate of inactivation of fatty acid synthetase in the presence of suspension of these pellet fractions is dependent upon a number of factors. The rate of inactivation is increased at higher temperature and at higher concentration of pellet fraction. The rate of inactivation is also increased when air-oxidized enzyme is used. The presence of 1 mM dithiothreitol or 200 μM NADPH decreases the rate of inactivation. Continued addition of reducing agent to pellet fraction suspensions during incubation with fatty acid synthetase completely prevents the inactivation. The inactivation as measured by loss of overall FAS activity follows first-order kinetics.

A preliminary study was performed to determine whether the inactivating capacities of the various pellet fractions could be extracted from the particulate material. The 124,000 x g pellet fraction released most of its inactivating capacity in soluble form immediately upon resuspension in TE buffer at room temperature; the other pellet fractions released only a small portion of their inactivating capacities, even after extended periods of incubation at 37°. Release of inactivating capacity from the pellet fractions was accompanied by a small but reproducible decline in pH of the suspensions which was proportional to the amount of inactivating capacity in the unincubated suspension supernatants.

The mechanism of inactivation of fatty acid synthetase in pellet fraction suspension was investigated using several techniques. In pellet suspensions containing FAS and incubated at 37°, overall FAS activity was lost more rapidly than decalone reductase activity. Analysis of these suspensions by sucrose density gradient centrifugation revealed that the native FAS peak decreased in magnitude as the extent of inactivation increased. Native polyacrylamide gel electrophoresis of these suspensions also demonstrated the loss of the native form of the enzyme upon inactivation. These observations suggest that a nonspecific aggregation of FAS to a very high molecular weight material occurs shortly following inactivation. Since inactivation of FAS causes loss of the native form of the enzyme from native polyacrylamide gels, and native forms of other proteins extracted from the pellet fractions during incubation are not changed, the inactivating capacity may be specific for FAS.

At 4°, isopropoanol (5%) decreased the rate of loss of decalone reductase activity in crude extracts of pig liver, and also decreased the rate of loss of overall activity of the purified enzyme in TE buffer. At 37°, however, both FAS in TE buffer and FAS in pellet fraction suspensions lost overall activity more rapidly in the presence of 5% isopropanol. At 4°, sodium octanoate (0.5%) decreased the rate of overall activity loss by the purified enzyme in TE buffer. At 37°, the rate of overall activity loss by the pure enzyme in TE buffer containing 0.1% octanoate was greater than that in TE buffer; however, the rate of inactivation of FAS in pellet suspensions containing 0.1% octanoate was less than in suspensions without octanoate. These observations suggest that the inactivation of FAS by pellet fraction suspensions cannot be expressed solely in terms of nonspecific changes in the structure of the enzyme.

INTRODUCTION

Much work has been done on the isolation and characterization of fatty acid synthetase from a variety of sources. Although much is now known about many of its physical and chemical properties, little is known about short-term control of the enzyme by factors other than its substrates and products. Consequently, studies were undertaken to determine whether activators or inactivators of the enzyme are present in the liver tissue from which the enzyme is isolated.

It has long been known that the microsomal fraction of liver from most species tested inactivates fatty acid synthetase. The enzyme is unstable in liver fractions prior to the removal of the microsomal fraction. This is the initial report of an inactivating capacity in the 6,000 x g (10 min), 30,000 x g (15 min), and 124,000 x g (70 min) pellet fractions obtained from pig liver homogenates. This chapter describes the preparation of several inactivating pellet fractions and a partial characterization of the inactivation process.

MATERIALS AND METHODS

Reagents. Caprylic (octanoic) acid and dithiothreitol were obtained from Sigma Chemical Co. Malonyl-CoA, acetyl-CoA, and NADPH were products of P-L Biochemicals. Trans-1-decalone was obtained from Aldrich Chemical Co. Acrylamide and N,N'-methylenebis(acrylamide) were products of Bethesda Research Laboratories, and N,N,N',N'-tetramethylethylenediamine of Canalco. All other reagents were of analytical reagent grade.

Livers. Pig livers were obtained from Peet Packing Co. (Chesaning, MI) within two hours of slaughter and were frozen on dry ice. The livers were stored at -30°.

Fatty Acid Synthetase. Fatty acid synthetase was prepared as described in Chapter 2.

Assays for Fatty Acid Synthetase. The decalone reductase activity and overall FAS activity assays as described in Chapter 2 were used.

Polyacrylamide Gel Electrophoresis. A modification of the method of Clark and Switzer (1977), as described in Chapter 2, was used to perform native polyacrylamide gel electrophoresis.

pH Measurement. Values of pH were determined at room temperature using a Radiometer PHM-4c meter equipped with a GK2321C electrode.

Sucrose Denisty Gradient Centrifugation. Linear 5-20% sucrose gradients were prepared by the procedure of Martin and Ames (1961). The 5.1 ml gradients, which contained TED buffer, were formed in ½ x 2 inch cellulose nitrate tubes. After sample application, the tubes were centrifuged in a Beckman SW50.1 rotor at 20° and between 10,000 and 47,000 rpm for between 15 and 150 minutes. After centrifugation, the gradients were examined visually and then the absorbance profile at 280 nm was determined. Fractionation was performed using an ISCO Model 183 density gradient fractionator and an ISCO Model UA-5 absorbance monitor. During fractionation the flow rate was 0.37 ml per minute, and 0.185 ml fractions were collected.

RESULTS

The aim of this research was to determine whether activators or inactivators of fatty acid synthetase were present in pig liver. So various fractions were prepared from pig liver homogenates by differential centrifugation. Initial tests for inactivation were performed at 4°, but inactivation was very slow. Subsequent tests were conducted at 37° to obtain faster inactivation.

Preparation of Liver Fractions. Pig liver was cut into small pieces and suspended in two volumes of TE buffer. The suspension was homogenized for two minutes in a Waring blendor and then separated into several fractions by differential centrifugation by one of two methods. In method I (summarized in Figure 3), which was used primarily to obtain extract fractions, the homogenate was centrifuged at 11,000 rpm in a Sorvall GSA rotor at 0° for 40 minutes. The turbid supernatant from this centrifugation was called centrifuged extract. Upon centrifugation of the centrifuged extract at 21,000 rpm in a Beckman Type 21 rotor at 0° for 150 minutes, the microsomal pellet fraction was

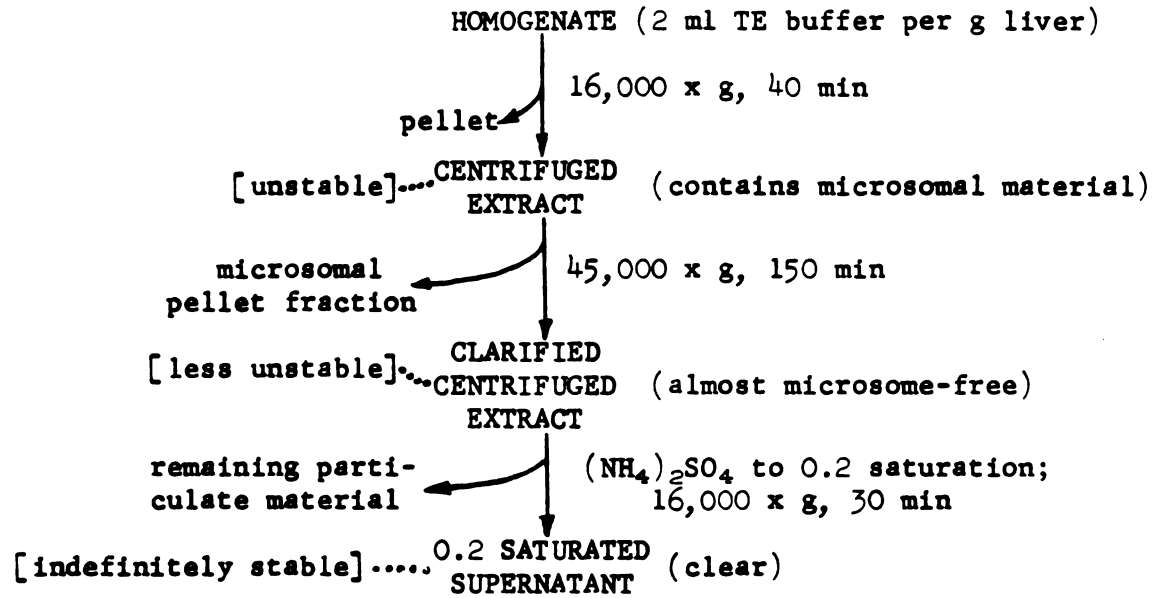


Figure 3. Preparation of Supernatant Fractions from Pig Liver Homogenate and the Stability of the Decalone Reductase Activities in the Fractions. The conclusion is that an inactivator of fatty acid synthetase is present in the particulate fraction of pig liver.

obtained. The faintly turbid supernatant from this centrifugation was called the clarified centrifuged extract.

In Method II (summarized in Figure 4), which was used to obtain pellet fractions, three such fractions were obtained from the liver homogenate. In this method, the homogenate was first centrifuged at 6,700 rpm in a Sorvall GSA rotor (6,000 x g) at 0° for 10 minutes. The nuclear and cell debris pellet fraction was obtained from this centrifugation. The supernatant from the 6,000 x g centrifugation was then centrifuged at 18,000 rpm in a Sorvall SS-34 rotor (30,000 x g) at 0° for 15 minutes. The pellet obtained was called the "premicrosomal" pellet fraction. The turbid supernatant from this centrifugation was then centrifuged at 0° in either a Beckman Type 21 rotor at 21,000 rpm (44,000 x g) for 150 minutes or a Beckman Type 42.1 rotor at 40,000 rpm (124,000 x g) for 70 minutes. The pellet obtained was the microsomal pellet fraction.

Each of the pellet fractions was further purified by washing at 4° with TE buffer. Washing consisted of resuspension of the pellet fractions followed by centrifugation. The pellet fractions were resuspended

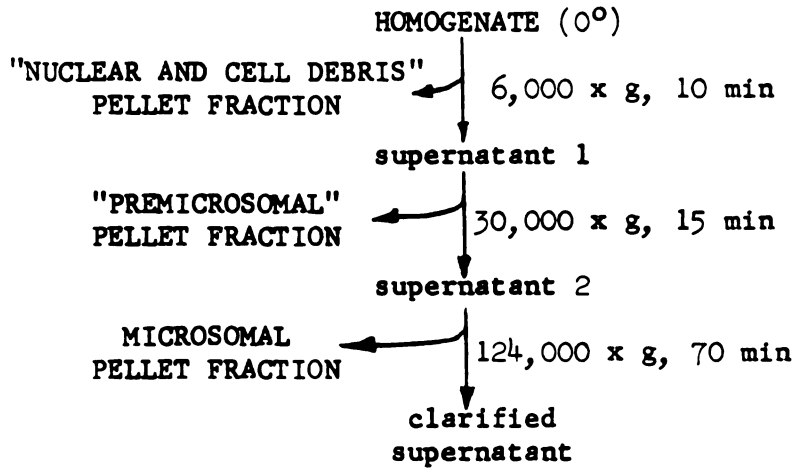


Figure 4. Preparation of Pellet Fractions from Pig Liver Homogenate by Differential Centrifugation. All pellet fractions were washed before use to remove any remaining soluble material from the homogenate. Washing consisted of resuspending the pellets and repeated centrifugation.

in a volume of buffer equal to the volume of suspension from which they originally came. Shaking was used to resuspend the fractions obtained by centrifugation in GSA rotors. Fractions obtained by centrifugation in Beckman rotors were resuspended by a single 30 second burst of homogenization in a glass Waring blendor. The pellet fractions (resuspended by shaking or by homogenization) were then centrifuged under conditions identical to those used to initially obtain the particular fractions. Washing was continued until soluble proteins present in the crude homogenate had been removed (as demonstrated by native polyacrylamide gel electrophoresis. Three or four washes were typically required. Some protein was extracted from the pellet fractions during washing. These proteins gave banding patterns in native polyacrylamide gels which were characteristic for each pellet fraction (Figure 5).

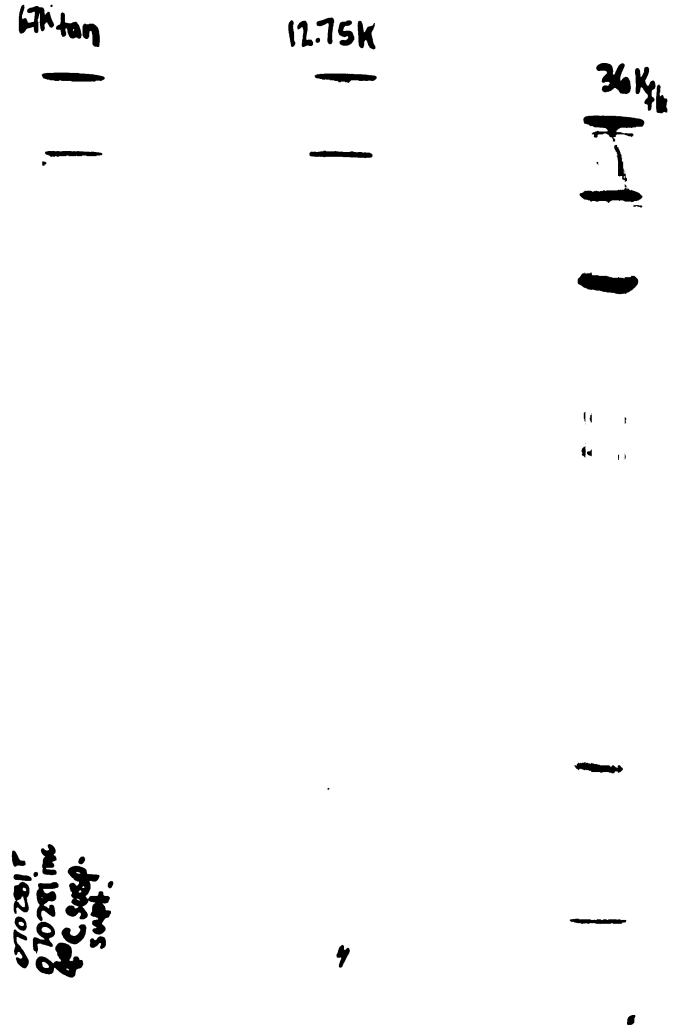


Figure 5. Native polyacrylamide Gel Electrophoresis Banding Patterns of Solubilized Proteins of Purified Pellet Fractions. After the pellets were washed, they were resuspended in ten volumes of water. The suspensions were centrifuged at 0° and 40,000 rpm for 70 minutes in a Beckman 42.1 rotor. Native polyacrylamide gel electrophoresis was performed on the supernatants using the procedure given in Chapter 2, yielding the banding patterns shown for the nuclear and cell debris (6.7K tan), pre-microsomal (12.75K), and microsomal ($36K_{floc}$) pellet fractions.

Tests for Inactivation of the Decalone Reductase Activity of Fatty Acid Synthetase by the Supernatant Fractions. Endogenous decalone reductase activity was measured as a test for inactivation of the decalone reductase activity of FAS by the centrifuged extract and the clarified centrifuged extract fractions. There was a slow loss of activity in both extracts at 4° (Figure 6 and Figure 7). Half of the activity was lost in two days in the centrifuged extract and in three days in the clarified centrifuged extract.

The clarified centrifuged extract was faintly turbid. To determine whether the turbid material was responsible for the inactivation a clear supernatant was obtained from the clarified centrifuged extract by taking the extract to 0.2 saturation with ammonium sulfate and centrifuging at 11,000 rpm in a Sorvall GSA rotor at 0° for 25 minutes. The decalone reductase activity in this supernatant was stable for more than ten days (Figure 8).

Taking the clarified centrifuged extract to 0.2 saturation with ammonium sulfate is part of the standard procedure for the purification of FAS. Tests for inactivation of fractions in the purification procedure for FAS beyond the 0.2 saturation ammonium sulfate fractionation were negative; the fractions were stable indefinitely. Hence, the turbid material seems responsible for the inactivation of FAS.

Tests for Inactivation of Overall FAS Activity by Pellet Fractions.

In order to determine the factors upon which the rate of inactivation of overall FAS activity in pellet fraction suspensions might depend, a standard procedure for inactivation experiments using pellet fractions was defined. This procedure was as follows: a suspension of one gram pellet fraction in ten milliliters TE buffer was prepared. The pellet fraction was suspended using a 15 ml Dounce homogenizer with a "large" clearance pestle. Fatty acid synthetase in PED buffer was then added to an aliquot of the suspension to a concentration of 0.16 units (overall activity) per milliliter of FAS-containing suspension. Between one and two volumes of FAS in PED buffer for each ten volumes of pellet fraction suspension was typically required. After measuring the overall activity of a 20 µl aliquot of the FAS-containing suspension, the suspension was placed in a water bath at 37° to incubate. Aliquots were removed at regular intervals

Figure 6. Decalone Reductase Activity in the Centrifuged Extract of Pig Liver: Effect of 5% Isopropanol. The initial decalone reductase activities in the centrifuged extracts of pig liver prepared by homogenization in buffer with (o) and without (•) 5% isopropanol were 1.29 and 0.90 units per ml, respectively.

Figure 6.

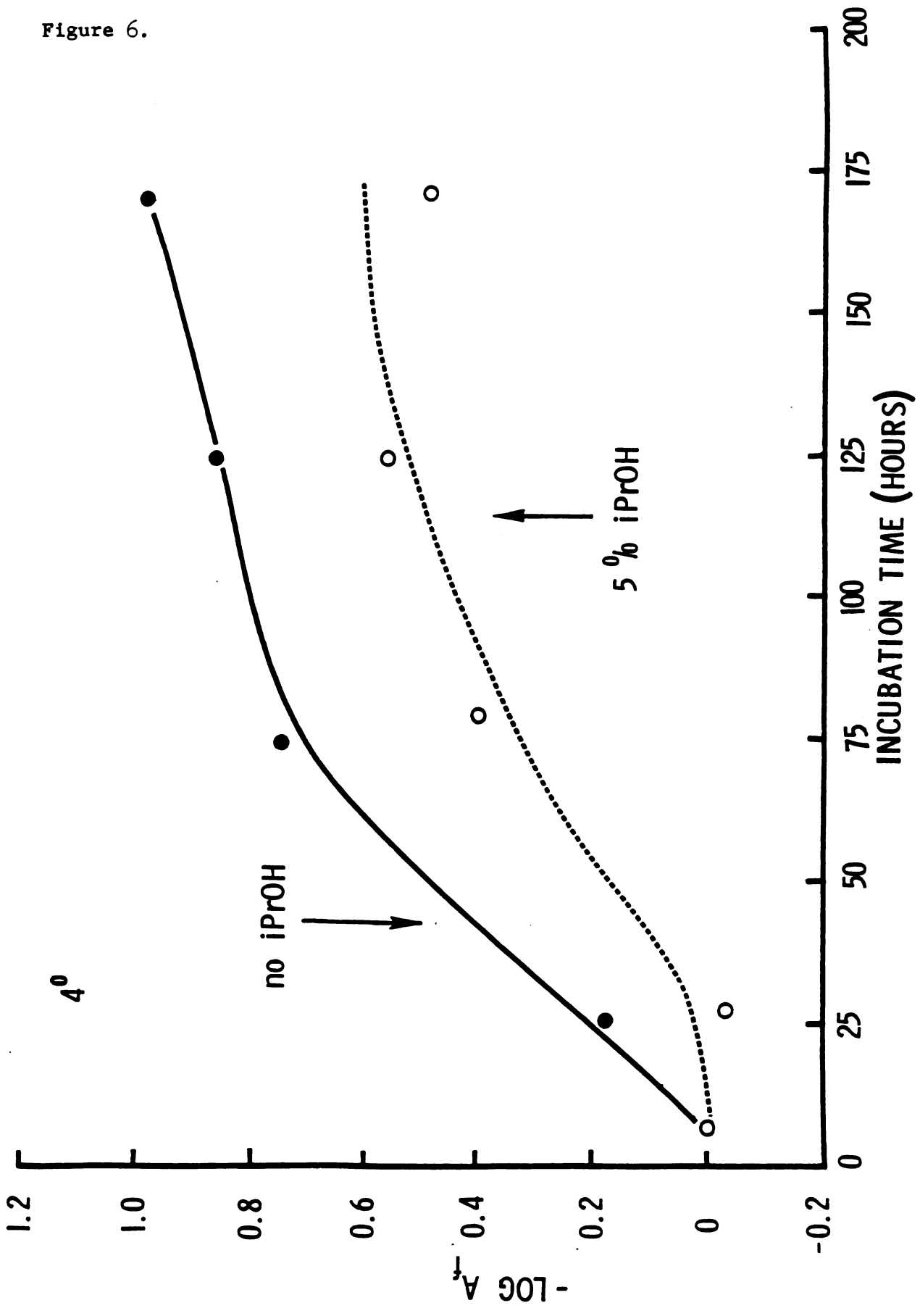
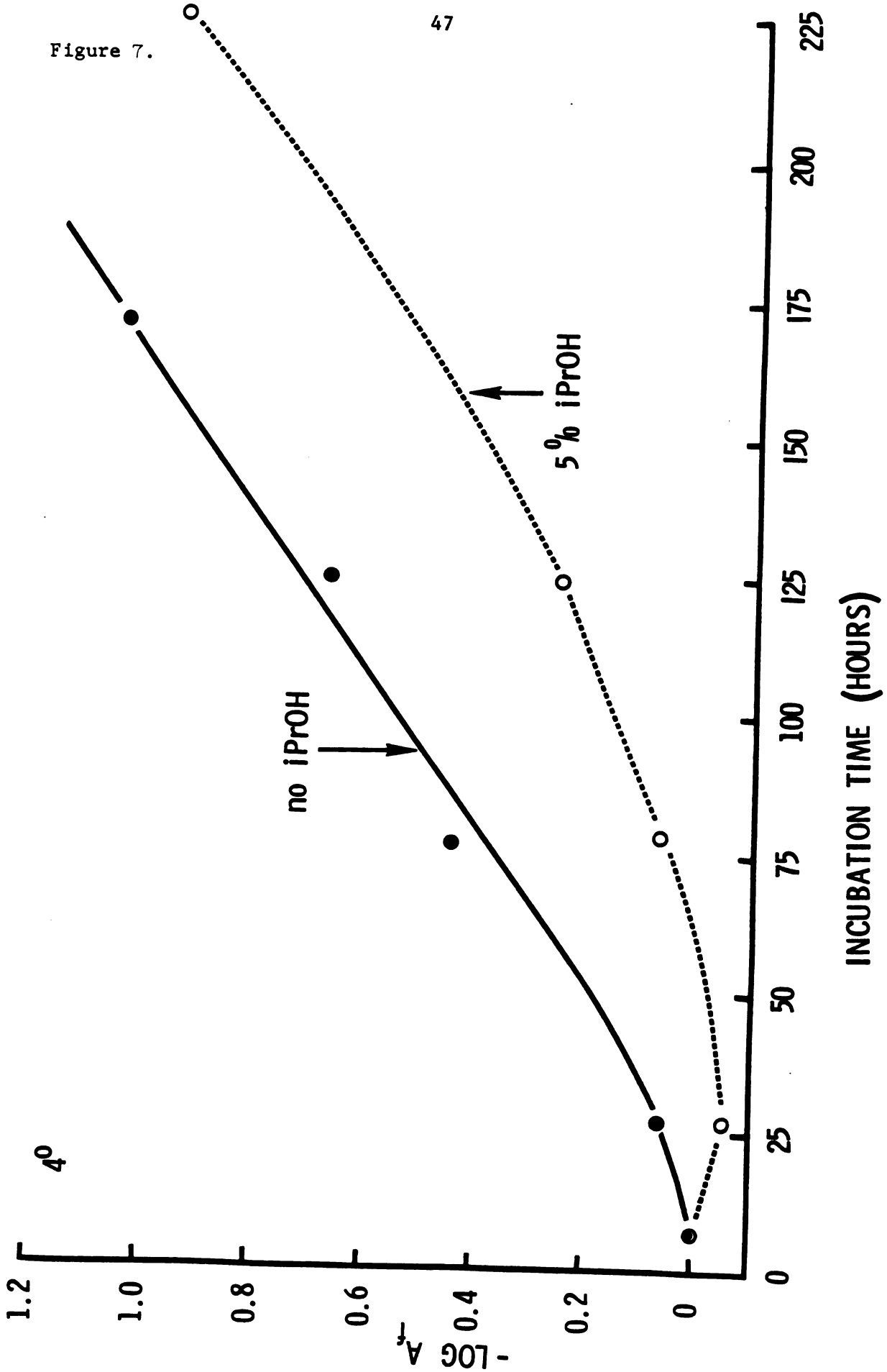

4°
no iPrOH
5 % iPrOH
- LOG A_t
INCUBATION TIME (HOURS)
1.2
1.0
0.8
0.6
0.4
0.2
0
-0.2
0
25
50
75
100
125
150
175
200

Figure 7. Decalone Reductase Activity in the Clarified Centrifuged Extract of Pig Liver: Effect of 5% Isopropanol. The initial decalone reductase activities for clarified centrifuged extracts prepared from pig liver, with (o) and without (•) 5% isopropanol in the homogenization buffer, were 1.20 and 1.36 units per ml, respectively.

Figure 7.


4°
-LOG A_t
1.2
1.0
0.8
0.6
0.4
0.2
0
0
25
50
75
100
125
150
175
200
225
INCUBATION TIME (HOURS)
no iPrOH
5% iPrOH

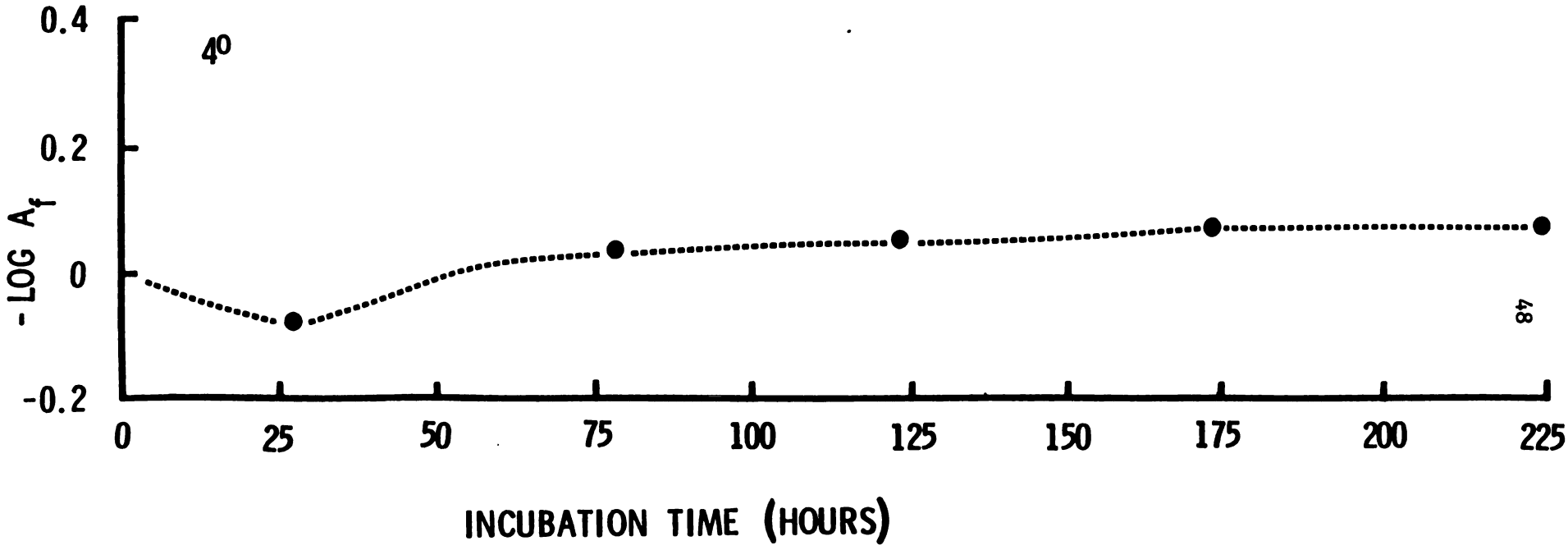


Figure 8. Decalone Reductase Activity in the 0.2 Saturated Ammonium Sulfate Supernatant from Clarified Centrifuged Extract of Pig Liver. The initial decalone reductase activity of the supernatant was 0.97 units per ml.

and assayed for overall activity.

The first inactivation tests on the pellet fraction suspensions were temperature dependence studies. Overall FAS activity was measured in all tests.

Effect of temperature on the inactivation rate. Washed pellet fractions were suspended at 4° in TE buffer at a concentration of 0.1 gram pellet per milliter suspension using Potter-Elvehjem homogenizer. Pellet fraction suspension (325 µl) was then mixed with FAS in PED buffer (50µl, 0.13 units). The mixture was incubated at 4° and assayed intermittently (Figure 9). The most rapid loss of activity was in the mixture containing microsomal pellet fraction. The loss of activity at 4° was very slow, however. Pellet fraction suspension containing FAS and incubated at 37° had relative inactivating capacities similar to those at 4°, but about seventeen-fold greater (Figure 10). For this reason, inactivation studies using pellet fraction suspensions were performed at 37°.

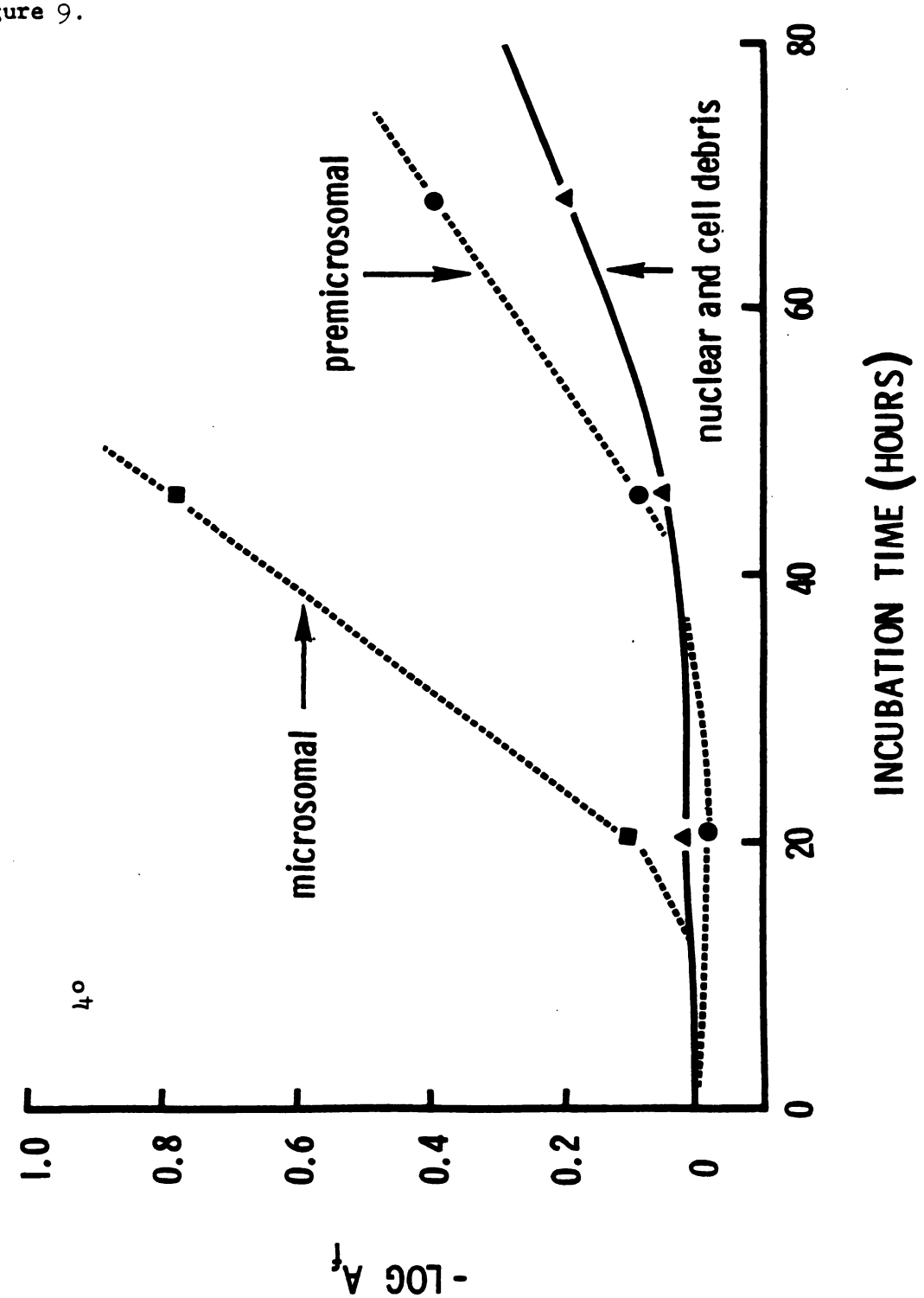
Effect of pellet concentration on the inactivation rate. The rate of inactivation of FAS in pellet fraction suspensions is directly proportional to the concentration of pellet fraction. Studies at several concentrations of pellet fraction demonstrated the inverse proportionality between pellet fraction concentration and the half-life of overall FAS activity (Figure 11).

Effect of air-oxidation of the enzyme on inactivation rate. The rate of inactivation of FAS in pellet fraction suspensions was greatly affected by the relative oxidation state of the enzyme. Purified FAS stored at 4° eventually loses a considerable portion of its overall activity, but only much later loses any of its decalone reductase activity (Figure 2). The loss of overall activity was apparently caused by a reversible air-oxidation of the enzyme, as the overall activity could be restored by the addition of, or dialysis against, a reducing agent. Consequently, accurate elucidation of the effects of potential inactivators on FAS requires a knowledge of the relative oxidation state of the enzyme, particularly when the pellet fraction suspensions contain a reducing agent. The most reliable and easily interpretable results were obtained with fresh enzyme in its fully reduced, most active state. Generally, therefore, tests were conducted with enzyme which had been dialyzed overnight against PED buffer.

Figure 9. Inactivation of Fatty Acid Synthetase by Pellet Fraction Suspensions at 4^{o}. The suspensions were prepared and incubated under standard conditions with these exceptions: incubation was at 4^{o} and each suspension contained 2 mM dithiothreitol. The reducing agent temporarily stabilizes the overall activity (see text). Half-lives of overall FAS activity in the nuclear and cell debris, premicrosomal, and microsomal pellet fraction suspensions were 85, 63, and 28 hours, respectively. The inactivating capacity of a fraction is ultimately related to the enzyme activity as a function of time. The overall activity decreases with time during inactivation experiments, and the process is first order or pseudo- first order with respect to enzyme; i.e., a straight line is obtained when the logarithm of the fraction of the initial enzyme activity remaining at a given time ($\log A_f$) is plotted as a function of time. Such a curve has a negative slope since enzyme activity decreases with time.

The graph of $+\log A_f$ versus time gives lines with slopes whose absolute magnitudes are proportional to inactivating capacity (i. e., the greater the absolute magnitude of the slope, the greater the inactivating capacity). But since all slopes are negative, such graphs do not give a simple, direct proportionality to inactivating capacity. Graphs of $-\log A_f$ versus time do. For this reason, the data in this thesis are plotted as $-\log A_f$ versus time, and the slopes of these plots are directly proportional to relative inactivating capacity.

Figure 9.


-LOG A_t
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
INCUBATION TIME (HOURS)
nuclear and cell debris
premicrosomal
microsomal
4°

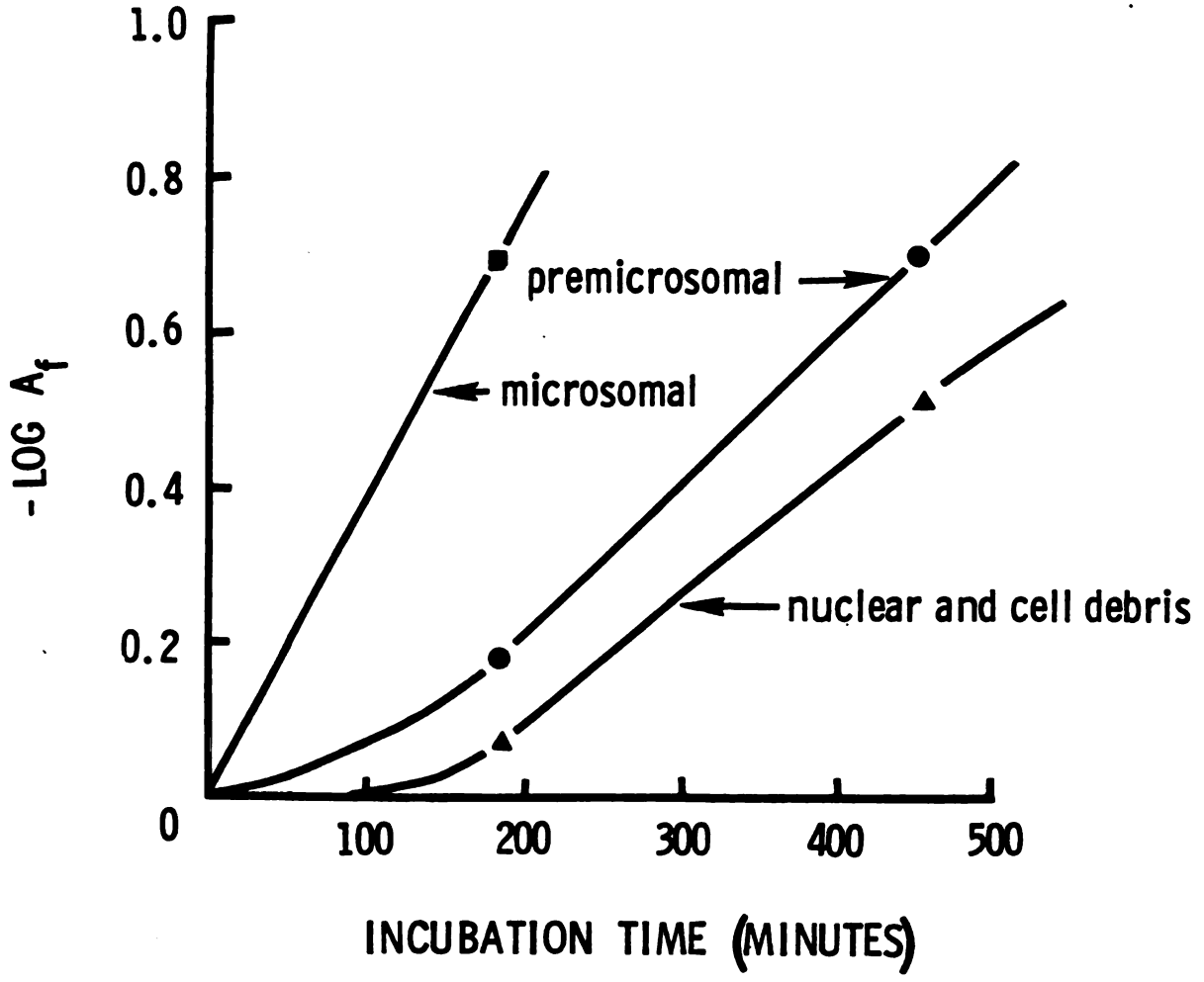


Figure 10. Inactivation of Fatty Acid Synthetase by Pellet Fraction Suspensions at 37°. The suspensions were prepared and incubated under standard conditions with the exception of the suspensions containing 2 mM dithiothreitol. The reducing agent temporarily stabilizes the overall activity (see text). Half-lives of overall FAS activity in the nuclear and cell debris (▲), premicrosomal (●), and microsomal (■) pellet fraction suspensions were 320, 245, and 76 minutes, respectively. The slope of each curve is proportional to the inactivating capacity of the corresponding suspension (see Figure 9 legend for further details.

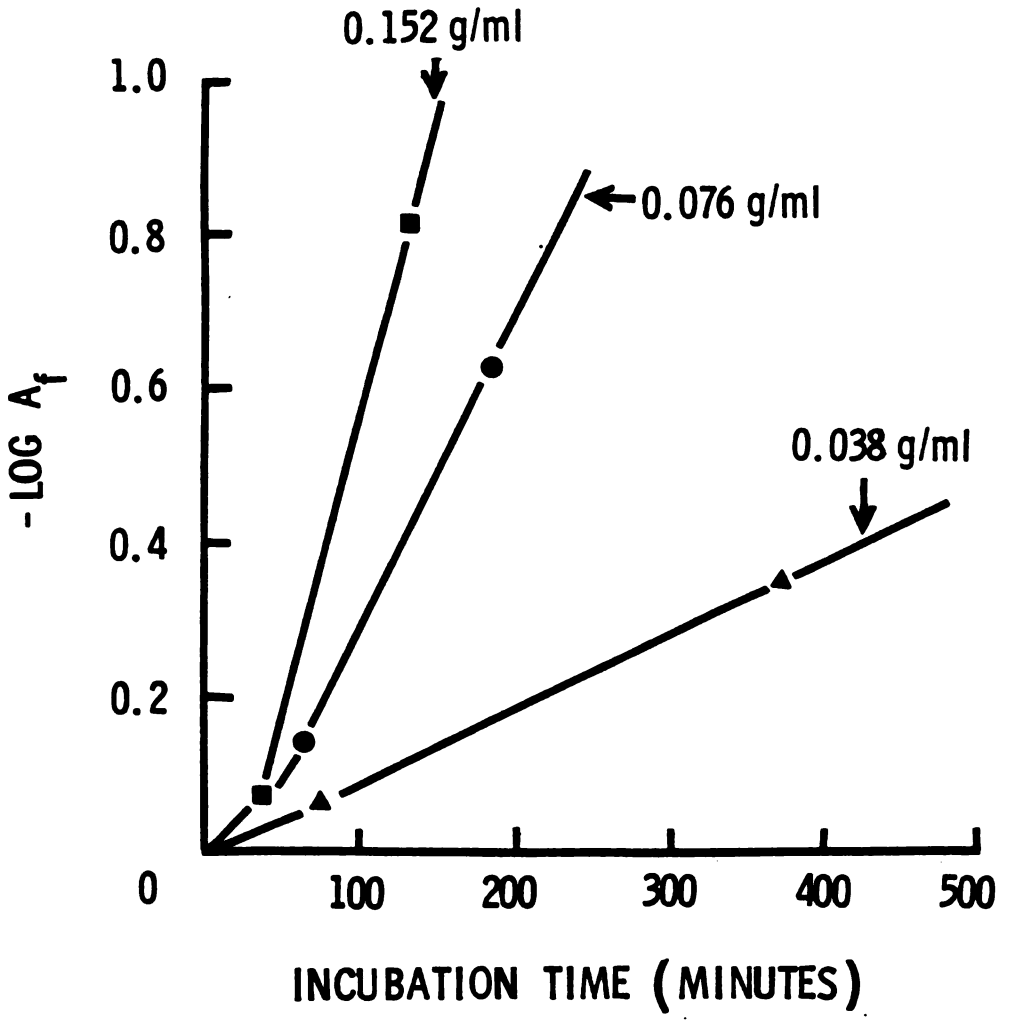


Figure 11. Inactivation of Fatty Acid Synthetase by Various Concentrations of Pellet Fraction. Premicrosomal pellet fraction and fatty acid synthetase were combined and analyzed under standard conditions with one exception: pellet fraction concentrations were 0.038 (▲), 0.076 (●), and 0.152 (■) grams per ml suspension. The relative specific inactivating capacities (the slope of the plot of -log A_f versus time divided by the pellet fraction concentration) for these suspensions were 0.026, 0.049, and 0.051 min·ml/g, respectively. So at least at higher pellet fraction concentration, inactivating capacity is proportional to the slope of curve (see Figure 9 legend for further details.

Effect of reducing agent on the inactivation rate. When 1 mM dithiothreitol was added to pellet fraction suspensions containing FAS prior to incubation, the inactivation rate was decreased (inactivation was prevented) for a time. Nuclear and cell debris, premicrosomal, and microsomal pellet fraction suspensions containing FAS and 1 mM dithiothreitol had inactivation half-lives for FAS of 90, 245, and 325 minutes, respectively. Addition of dithiorthreitol to FAS-containing pellet fraction suspensions at a rate of 1 mM/hr prevented the inactivation (Figure 12). Thus, the redox environment in the incubation mixture significantly affects the inactivation rate.

Effect of NADPH, malonyl-CoA, and acetyl-CoA on the inactivation rate. Incubation of FAS in pellet fraction suspensions containing a saturating level of NADPH (200 μM) reduced the rate of inactivation of the enzyme (Figure 13). Half-lives of inactivation for FAS in the premicrosomal and microsomal pellet fraction suspensions were 88 and 62 minutes in the absence of NADPH, and 167 and 88 minutes in the presence of NADPH, respectively. Addition of 50 μM acetyl-CoA and 50 μM malonyl-CoA to FAS-containing pellet fraction suspensions slightly destabilized the enzyme (Figure 13). Half-lives of inactivation for FAS in the premicrosomal and microsomal pellet fraction suspensions in the presence of acetyl-CoA and malonyl-CoA were 77 and 52 minutes, respectively.

The Inactivation of Overall FAS Activity by Pellet Fraction Suspensions Follows First-Order Kinetics. The inactivation of FAS in pellet fraction suspensions containing no dithiothreitol follows first order kinetics (Figure 11). Therefore, the relative inactivating capacities of the suspensions can be determined from the slope of a plot of the logarithm of the fraction of the initial overall FAS activity in the suspension (logAf) as a function of time. Plotting -logAf versus time results in the slope of the curve being directly proportional to the amount of inactivating capacity present (see Figure 9 legend).

As previously shown (Figure 9 and Figure 10), dithiothreitol at an initial concentration as low as 0.2 mM in pellet fraction suspensions containing FAS has a stabilizing effect on the enzyme. The duration of the stabilization is proportional to the amount of dithiothreitol in the suspensions. After the period of stabilization, inactivation of the enzyme again follows first-order kinetics . Consequently, relative

Figure 12. Periodic Addition of Dithiothreitol Prevents Inactivation of Fatty Acid Synthetase by Pellet Fraction Suspensions. Microsomal pellet fraction suspensions were prepared and analyzed under standard conditions with the following exceptions: both suspensions contained 2mM dithiothreitol at the start of incubation, and one suspension had aliquots of 100 mM dithiothreitol added during incubation. The dithiothreitol concentration in this suspension was increased by 1 mM at 30, 65, 95, 125, 160, and 188 minutes after incubation was begun, and by 2 mM at 220, 330, and 370 minutes after incubation was begun. The stability of the enzyme in the absence of pellet fraction is shown for comparison.

Figure 12.

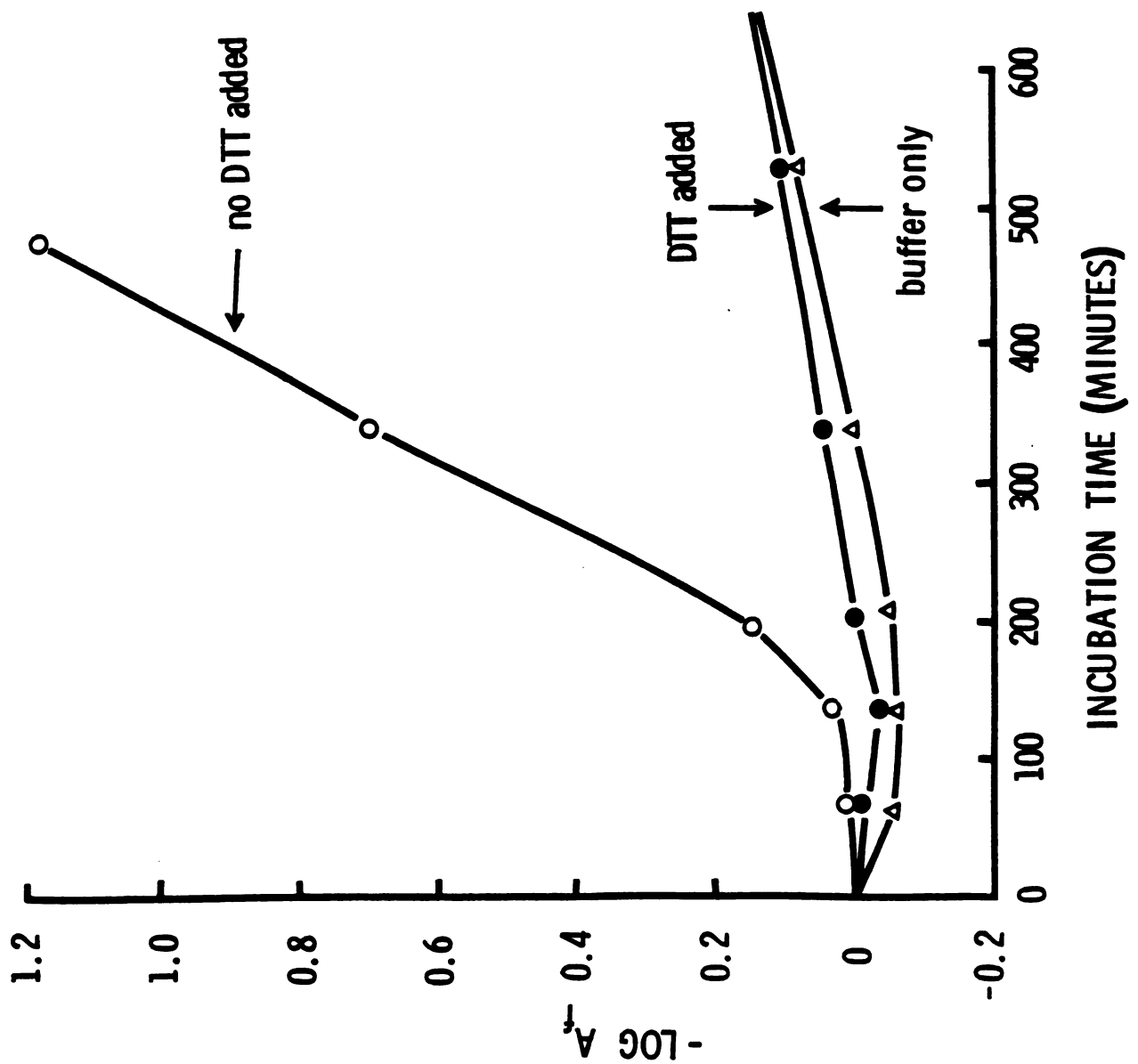
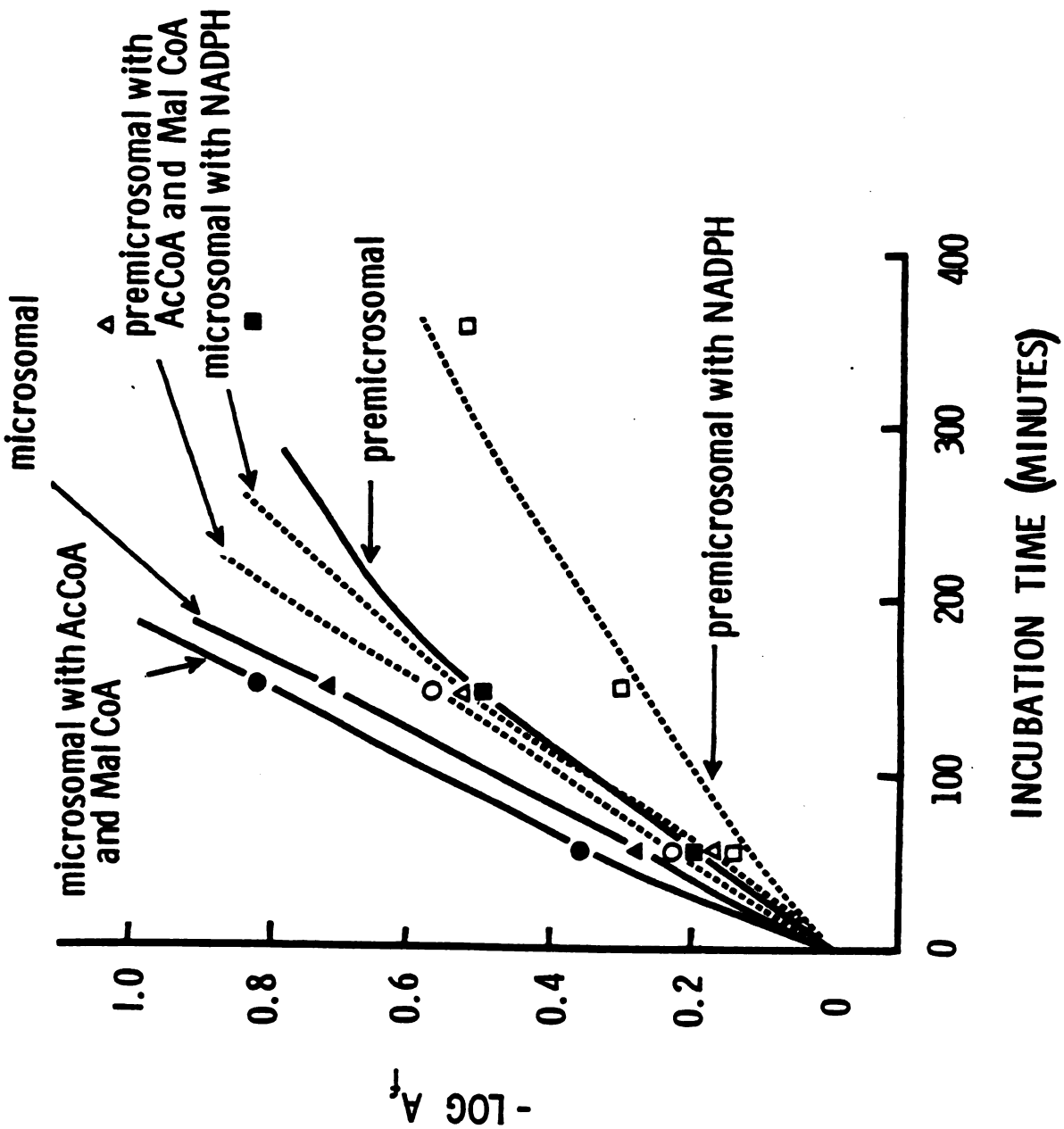

no DTT added
DTT added
buffer only
-LOG A_f
1.2
1.0
0.8
0.6
0.4
0.2
0
-0.2
0
100
200
300
400
500
600
INCUBATION TIME (MINUTES)

Figure 13. Substrate and Cofactor Effects on the Inactivation of Fatty Acid Synthetase by Pellet Fraction Suspensions. Microsomal and premicrosomal pellet fraction suspensions were prepared and analyzed under standard conditions with the exception of adding either 200 μM NADPH or 50 μM acetyl-CoA and 50 μM malonyl-CoA to the suspensions. The standard microsomal (▲) and premicrosomal (Δ) pellet fraction suspensions inactivated half the initial overall FAS activity in 68 and 88 minutes, respectively. Microsomal (●) and premicrosomal (o) pellet fraction suspensions containing acetyl-CoA and malonyl-CoA inactivated half the initial activity in 52 and 77 minutes, respectively. Microsomal (■) and premicrosomal (□) pellet fraction suspensions containing NADPH inactivated half the initial activity in 88 and 167 minutes, respectively.

Figure 13.


microsomal
premicrosomal with AcCoA and Mal CoA
microsomal with NADPH
premicrosomal
premicrosomal with NADPH
microsomal with AcCoA and Mal CoA
1.0
0.8
0.6
0.4
0.2
0
0
100
200
300
400
INCUBATION TIME (MINUTES)
- LOG A_f

inactivating capacities of suspensions containing reducing agent can be determined from the slope of the plot of -logAf versus time in the region where inactivation has begun.

One unit of inactivating capacity is defined as that amount of inactivating capacity required to produce the loss of one unit of overall FAS activity in one minute at 37° in the inactivation assay. One unit of overall FAS activity is as defined in Chapter 2; that is, one unit of overall FAS activity is the amount of FAS catalyzing the oxidation of 1 μmole NADPH per minute in the standard overall FAS activity assay. Extraction in Aqueous Solution of the Inactivating Capacity from Pellet Fractions and the Requirement of Intact Membranous Pellet Fraction for the Inactivation of FAS. The long range goal is the isolation and identification of the inactivating componenets in the pellet fractions. The first step toward this goal was to determine whether the inactivating capacity could be extracted and separated from the pellet fractions. Thus, the questions were (a) is intact pellet fraction membrane required for inactivation, and (b) if not, can the inactivating component be extracted from the pellet fraction?

To determine whether the inactivating capacity required intact pellet fraction, the pellet fractions were resuspended at room temperature in TE buffer containing 2 mM dithiothreitol in standard proportion. The suspensions were then divided into three portions. One portion was incubated with FAS and analyzed under standard conditions. Another portion was centrifuged at 40,000 rpm for 70 minutes in a Beckman Type 42.1 rotor at 0°. Fatty acid synthetase was then added to the supernatant to a concentration of 0.13 units (overall activity) per ml supernatant. The FAS-containing supernatant was then incubated at 37° and assayed intermittently for overall FAS activity. The third portion was incubated at 37° for 20 hours and then centrifuged at 0° for 70 minutes in a Beckman Type 42.1 rotor at 40,000 rpm. Fatty acid synthetase was added to the supernatant to a level of 0.13 units (overall activity) per ml supernatant and then incubated at 37°. The FAS-containing supernatant was assayed intermittently for overall FAS activity.

The results from these inactivation tests are given in Table 4.

Table 4. Rate of Solubilization of Inactivating Capacities from Pellet Fraction Suspensions at 37°.

pellet fraction	Inactivating Capacities (units/ml)x10^4		
	suspension[a]	supernatant of unincubated suspension[b]	supernatant of incubated suspension[c]
nuclear and cell debris	3.8	0.9	1.7
premicrosomal	5.8	1.3	2.4
microsomal	7.9	7.8	7.4

[a]Pellet fractions were suspended in TE buffer containing 2 mM dithiothreitol. Suspensions contained 1 g pellet fraction/ 10 ml suspension.

[b]Supernatants were obtained by centrifugation of suspensions at 40,000 rpm for 70 minutes at 0° in a Beckman 42.1 rotor.

[c]The suspension was incubated at 37° for 20 hours.

The inactivating capacity of the microsomal pellet fraction was released in soluble form immediately after resuspension at room temperature. Only about one-fourth of the inactivating capacities of the other pellet fractions was released upon resuspension of the pellet fractions, and less than half of the inactivating capacity was released even after incubation at 37° for 20 hours. For this reason, the inactivating capacity of the microsomal pellet fraction seems to be different from the inactivating capacities of the other pellet fractions.

The pH values of the pellet fraction suspensions for these inactivation tests were measured. A decline in pH, which was proportional to the inactivating capacity of the unincubated suspension supernatants, was observed. The pH values for FAS-containing suspensions of the nuclear and cell debris, premicrosomal, and microsomal pellet fractions were 7.52, 7.52, and 7.47, respectively. The pH of a solution of FAS in buffer alone was 7.53. Thus, the pH is lowered about 0.01 for each 10^{-4} unit of inactivating capacity per ml unincubated suspension supernatant. The significance of this phenomenon is not yet known.

Studies on the Mechanism of Inactivation of FAS by Pellet Fraction Suspensions. To determine whether a conformational change occurred

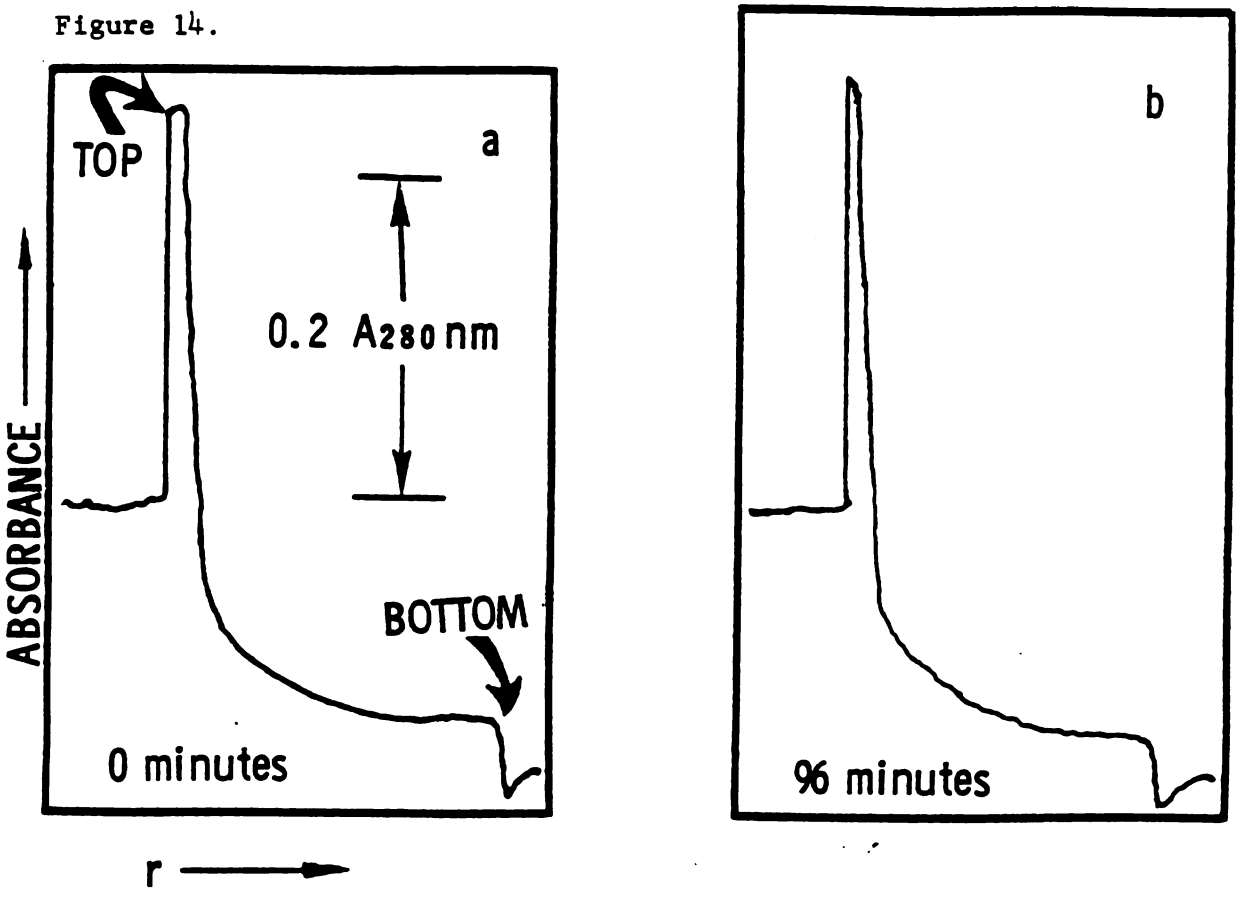
in fatty acid synthetase upon its inactivation by pellet fraction suspension, sucrose density gradient centrifugation was performed on pellet fraction suspensions containing FAS which had been incubated at 37° for various periods of time. As the overall FAS activity was inactivated, the magnitude of the native FAS peak in the sucrose density gradients, as measured by absorbance at 280 nm, decreased (Figure 14). At no time during the inactivation process was a peak other than that for native FAS observed in the sucrose density gradients. Consequently, in terms of gross physical structure, inactivation results in an aggregation of FAS to very high molecular weight material. The aggregation is apparently nonspecific, as no new discrete peaks appear in the sedimentation patterns obtained from sucrose density gradient centrifugation of the FAS-containing pellet fraction suspensions.

A similar conclusion was drawn from comparable experiments using native polyacrylamide gel electrophoreisis instead of sucrose density gradient centrifugation. The native FAS band decreased in intensity as the enzyme was inactivated, and no other bands appeared in the polyacrylamide gels (Figure 15).

Native polyacrylamide gel electrophoresis performed on FAS-containing pellet fraction suspensions also suggested that the inactivation by the microsomal pellet fraction may be specific for FAS. Fatty acid synthetase, 0% or 98% inactivated by microsomal pellet fraction suspension, was clarified by centrifugation at 18,000 rpm in a Sorvall SS-rotor at 0° for 20 minutes, and the supernatant was subjected to native PAGE. The native FAS bands decreased markedly in intensity upon inactivation of the enzyme, while bands from contaminating proteins (solubilized during resuspension of the pellet fraction; see Figure 5) remained at about the same intensity, apparently unchanged by incubation with the inactivating capacity. Also, after 98% inactivation, there was a substantial amount of native FAS still present, indicating that aggregation follows inactivation. The relative rates at which decalone reductase activity and overall FAS activity were lost during incubation of FAS with pellet fraction suspensions were measured. Assuming that the aggregated enzyme has neither partial nor overall FAS activity, then if aggregation is concommitant with

Figure 14. Inactivation of Fatty Acid Synthetase and Loss of the Native FAS Peak During Sucrose Density Gradient Sedimentation Velocity Analysis. Microsomal pellet fraction was suspended and incubated with fatty acid synthetase under standard conditions for 0, 96, 220, or 380 minutes. After these incubation periods, 0, 52, 74, and 93%, respectively, of the overall FAS activity was inactivated. After incubation, the suspensions (150 µl) were applied to sucrose density gradients (see Methods). Centrifugation was initially at 5,000 rpm (for 5 minutes) in order to gently transport the particulate material into the gradients. Centrifugation was then continued for 20 minutes at 30,000 rpm. The elution profiles at 280 nm, obtained during fractionation of the gradients, are for (a) no incubation, (b) 96 minutes incubation, (c) 220 minutes incubation, and (d) 380 minutes incubation. Similar profiles were obtained when incubating fatty acid synthetase with premicrosomal pellet fraction.

Figure 14.


TOP
a
0.2 A280 nm
ABSORBANCE
BOTTOM
0 minutes
r
b
96 minutes
c
220 minutes
d
380 minutes

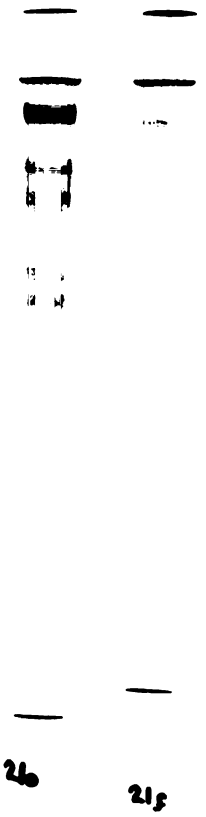


Figure 15. Native Polyacrylamide Gel Electrophoresis Banding Patterns of Microsomal Pellet Fraction Suspension Containing Fatty Acid Synthetase before and after Inactivation of the Enzyme. The pellet fraction suspension was prepared and incubated under standard conditions with one exception: the suspensions contained 1 mM dithiothreitol. Overall FAS activity was 0 (21o) or 98% (21f) inactivated when polyacrylamide gel electrophoresis was performed on the clarified suspensions (centrifuged at 0° and 18,000 rpm for 20 minutes in a Sorvall SS-34 rotor). The procedure for PAGE presented in Chapter 2 was used.

FAS inactivation, overall and decalone reductase activities should be lost at proportionate rates. Experimentally, the decalone reductase activity was lost less rapidly than the overall activity of the enzyme (Table 5).

Table 5. Relative Rates of Decalone Reductase and Overall Fatty Acid Synthetase Activity Loss During Inactivation of Fatty Acid Synthetase by Microsomal Pellet Fraction Suspensions at 37°.

Incubation time (minutes)	0	30	94	160	230	310
Fraction of initial overall activity remaining	1.00	1.03	0.83	0.56	0.30	0.24
Fraction of initial decalone reductase activity remaining	1.00	0.73	0.78	0.69	0.72	0.54

Model Compounds for the Inactivating Capacity of the Pellet Fractions. Two model compounds for the inactivating capacity in the pellet fractions were examined to see how closely they mimicked the inactivation of FAS by pellet fraction suspensions. These model compounds were isopropanol and sodium octanoate; how they were chosen and their effects upon FAS are examined below.

Inactivation of overall FAS activity by isopropanol. Isopropanol (5%) was examined as a result of studies performed during the purification of fatty acid synthetase. In those experiments, isopropanol was used as a solvent for phenylmethanesulfonylfluoride, a widely used protease inhibitor. The goal of these experiments was to determine whether a more stable FAS preparation might be obtained by preparation of the enzyme in the presence of a protease inhibitor.

A control purification of the enzyme was performed using isopropanol without the phenylmethanesulfonylfluoride. This 5% isopropanol control was found to significantly stabilize the enzyme at early stages in the purification (Figure 6 and Figure 7). The half-lives of decalone reductase activity in the centrifuged extract and clarified centrifuged extract at 4° with 5% isopropanol were 90 and 130 hours, and without isopropanol were 45 and 60 hours, respectively. Isopropanol also had a

stabilizing effect on the "natural" inactivation (air-oxidation) of the overall activity of the pure enzyme in TED buffer at 4^{o} (Figure 16).

The stabilization of overall FAS activity in the isopropanol containing buffer and extracts at 4^{o} is apparently caused by isopropanol lowering the dielectric constant of the solution. Changes in the dielectric constant can cause changes in the stable conformations of proteins by altering hydrogen, electrostatic, and hydrophobic bonding of the proteins. Hence, it appears that the most stable conformation(s) for FAS in the presence of 5% isopropanol at 4^{o} is different from that in its absence, and that the conformation of FAS induced in 5% isopropanol is less sensitive to both inactivation and air-oxidation.

At 37^{o}, isopropanol inactivated the pure enzyme in TED buffer (Figure 17). The half-lives of overall FAS activity in the presence of 2% and 5% isopropanol in TED buffer (0.13 units/ml) were 300 and 46 minutes, respectively. Both native PAGE and density gradient sedimentation velocity analysis of the 5% isopropanol-containing FAS solution at 37^{o} demonstrated the loss of the native form of the enzyme during or closely following inactivation. Therefore, the most stable conformer(s) of FAS in 5% isopropanol at 37^{o} is apparently either inactive or much more susceptible to air-oxidation than the enzyme in the absence of isopropanol, as overall FAS activity was lost rapidly and the enzyme aggregated nonspecifically to high molecular weight material.

The aggregation of FAS was also observed when FAS was inactivated by the pellet fraction suspensions. In the presence of both pellet fraction suspension and 5% isopropanol at 37^{o} the inactivation rate of overall FAS activity was greater than that observed using either pellet fraction suspension or 5% isopropanol alone (Figure 18). Hence, 5% isopropanol and the pellet fraction suspensions, separately or combined, produced the same overall effects on FAS; the enzyme was inactivated and aggregated nonspecifically. However, isopropanol

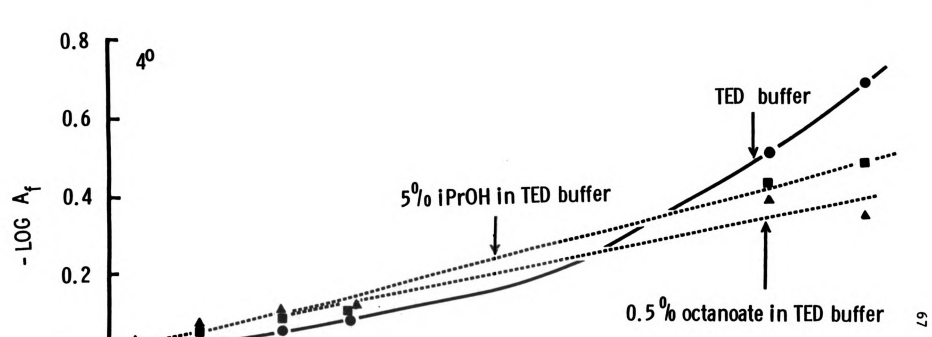


Figure 16. Stability of Fatty Acid Synthetase at 4° in Either TED Buffer, TED Buffer Containing 0.5% Octanoate, or TED Buffer Containing 5% Isopropanol. Purified fatty acid synthetase (0.062 units of overall activity) was added to 360 µl of either TED buffer (●), TED buffer containing 0.5% octanoate (▲), or TED buffer containing 5% isopropanol (■). The samples were stored at 4° and were assayed intermittently for overall FAS activity.

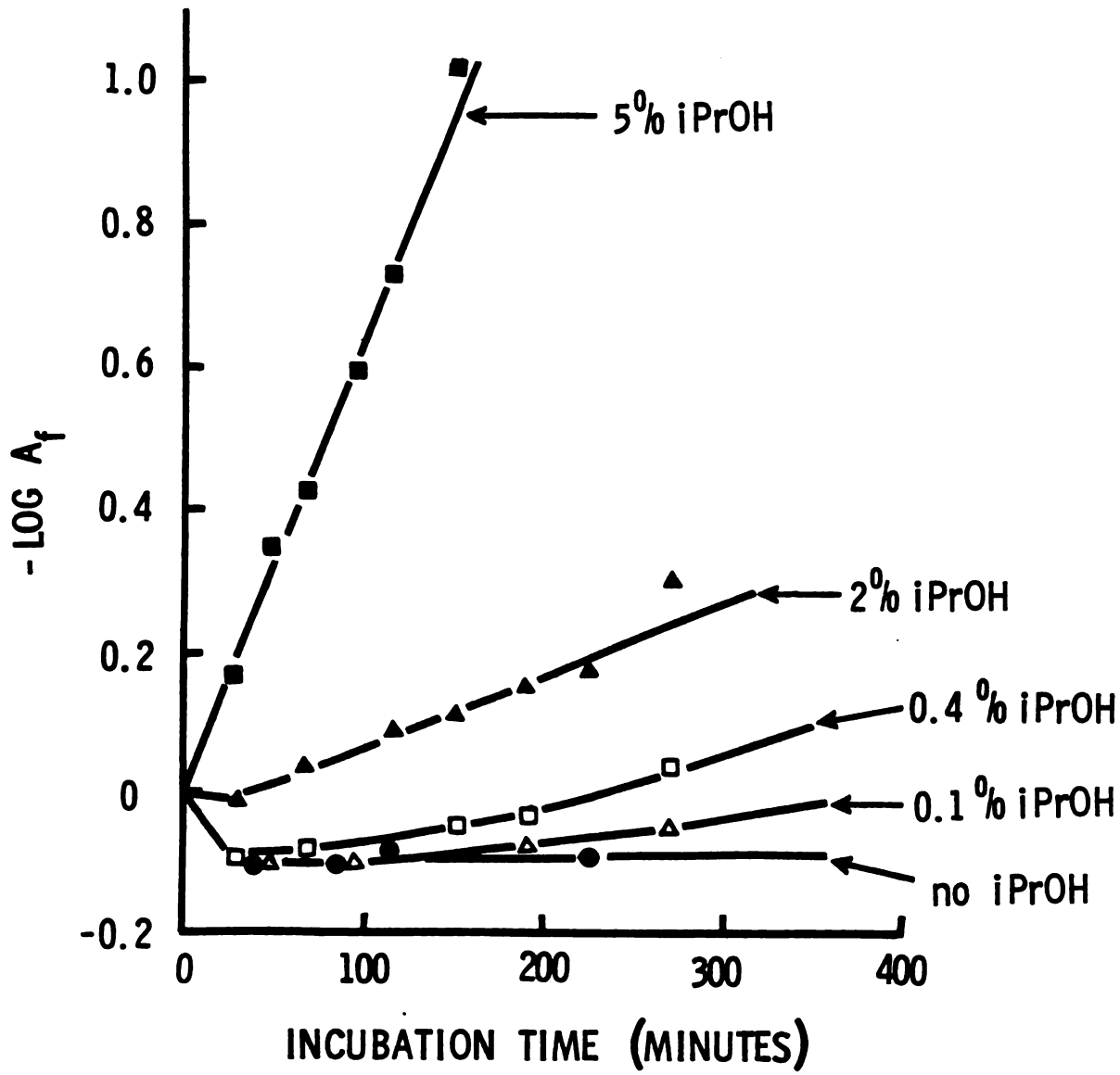


Figure 17. Inactivation of Fatty Acid Synthetase in TED Buffer by Isopropanol at Various Concentrations. Purified fatty acid synthetase (15 µl, 0.029 units of overall activity) was added to TED buffer (300 µl) containing 0 (•), 0.1 (Δ), 0.4 (□), 2.0 (▲), or 5 % (■) isopropanol. The samples were incubated at 37° and assayed intermittently for overall fatty acid synthetase activity.

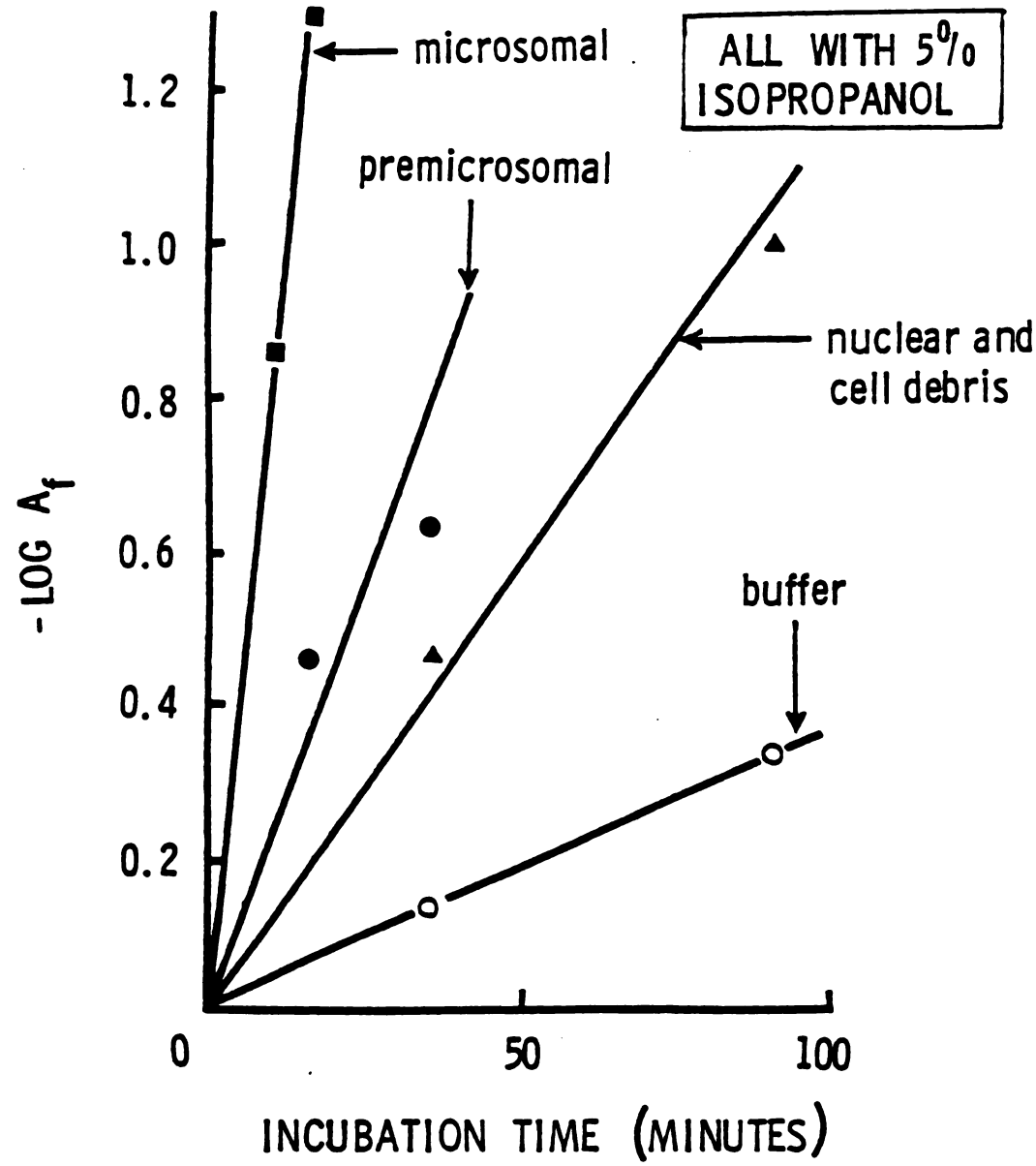


Figure 18. Inactivation of Fatty Acid Synthetase at 37° by Pellet Fraction Suspensions in TED Buffer Containing 5% Isopropanol. Suspensions were prepared and analyzed under standard conditions with the exception of adding 5% isopropanol to all suspensions (and buffer). The half-lives of overall FAS activity in the nuclear and cell debris (▲), premicrosomal (●), and microsomal (■) pellet fraction suspensions were 25, 13, and 3 minutes, respectively. Inactivation of enzyme in TE buffer containing 5% isopropanol is shown for comparison.

is distinctly different from the inactivating capacity in the pellet fraction suspensions, in that at 4° isopropanol stabilizes the enzyme, while the pellet fractions inactivate it.

Inactivating of overall FAS activity by octanoate. The pellet fractions consist of various membrane-enclosed organelles and thus have a relatively high lipid content. Therefore it seemed that one possible mechanism for the inactivation of FAS in pellet fractions might involve a detergent-like denaturation of FAS by fatty acids either on the surface of the membranes, or released from them. For this reason, a representative fatty acid was tested for its inactivating capability. Octanoate was chosen because it is very soluble and, unlike palmitate, has little tendency to form micelles even at relatively high concentration. The hydrocarbon portion of octanoate, although short enough to minimize micelle formation, was long enough to be expected to penetrate the hydrophobic regions of the enzyme.

At 4°, octanoate (0.5%) had a stabilizing effect on FAS; it slowed the "natural" inactivation (air-oxidation) of the pure enzyme in TED buffer (0.13 units overall FAS activity/ ml; Figure 16). When the incubation was carried out at 37°, however, overall FAS activity was inactivated in octanoate-containing TED buffer (Figure 19). At 0.1 and 0.5% octanoate, the half-lives of overall FAS activity were 415 and 55 minutes, respectively. Sucrose density gradient sedimentation velocity analysis of the inactivation of FAS in solutions containing octanoate revealed that a peak with a higher sedimentation coefficient than that of native FAS was formed during inactivation of the enzyme (Figure 20). This faster sedimenting peak had neither decalone reductase nor overall FAS activity. It was an intermediate in the denaturation of the enzyme as indicated by its relatively small size and by the appearance of pellets at the bottoms of tubes to which enzyme that was inactivated to large extents had been applied.

These observations suggest that at 4° octanoate did not significantly alter the conformation of FAS. The stabilization of the enzyme may be due to octanoate forming a monolayer at the interface between the solution and air, thereby slowing the diffusion of oxygen into the solution, and so slowing air-oxidation of the enzyme. At 37°, the octanoate has clearly affected enzyme conformation, apparently yielding

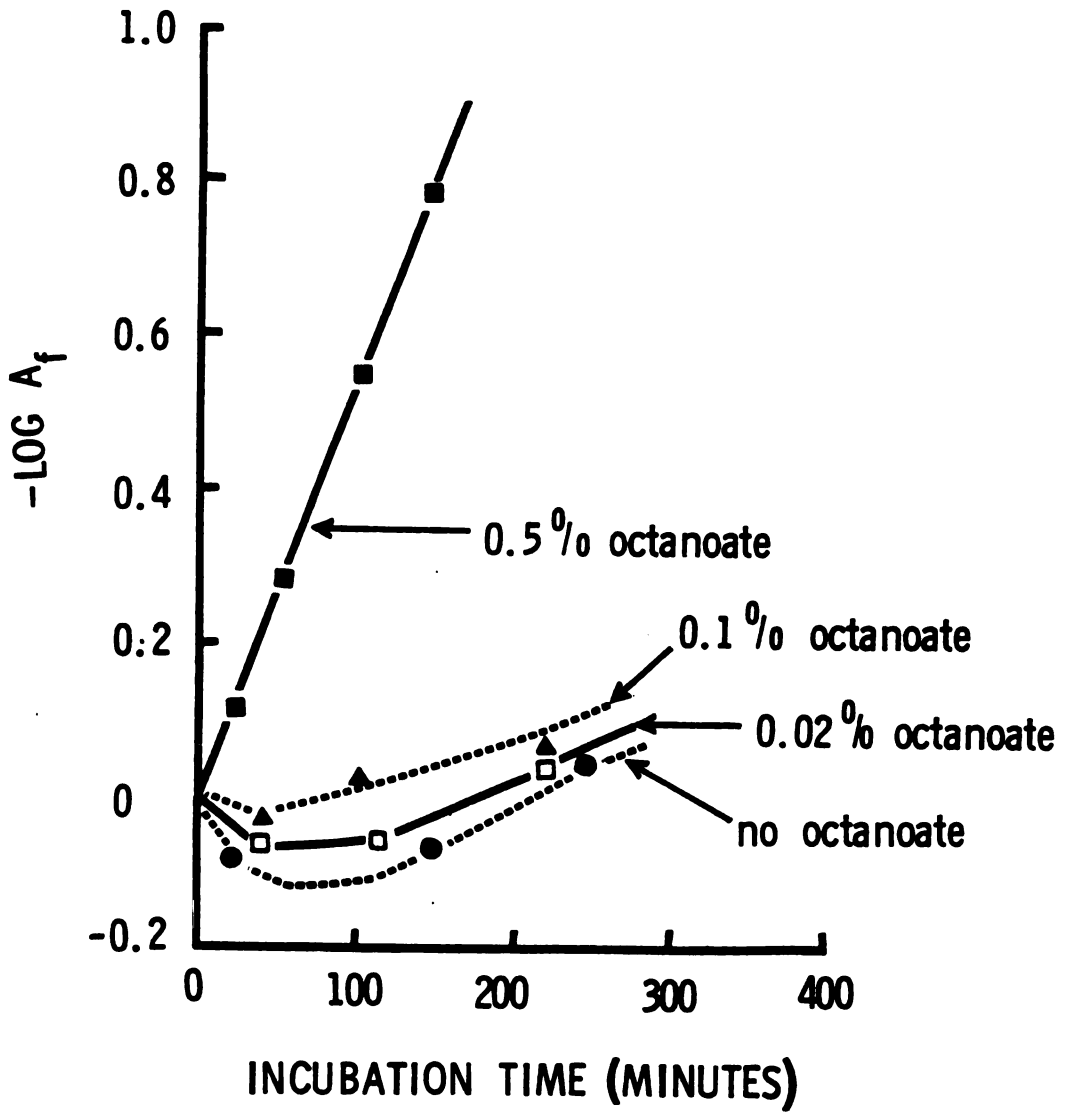
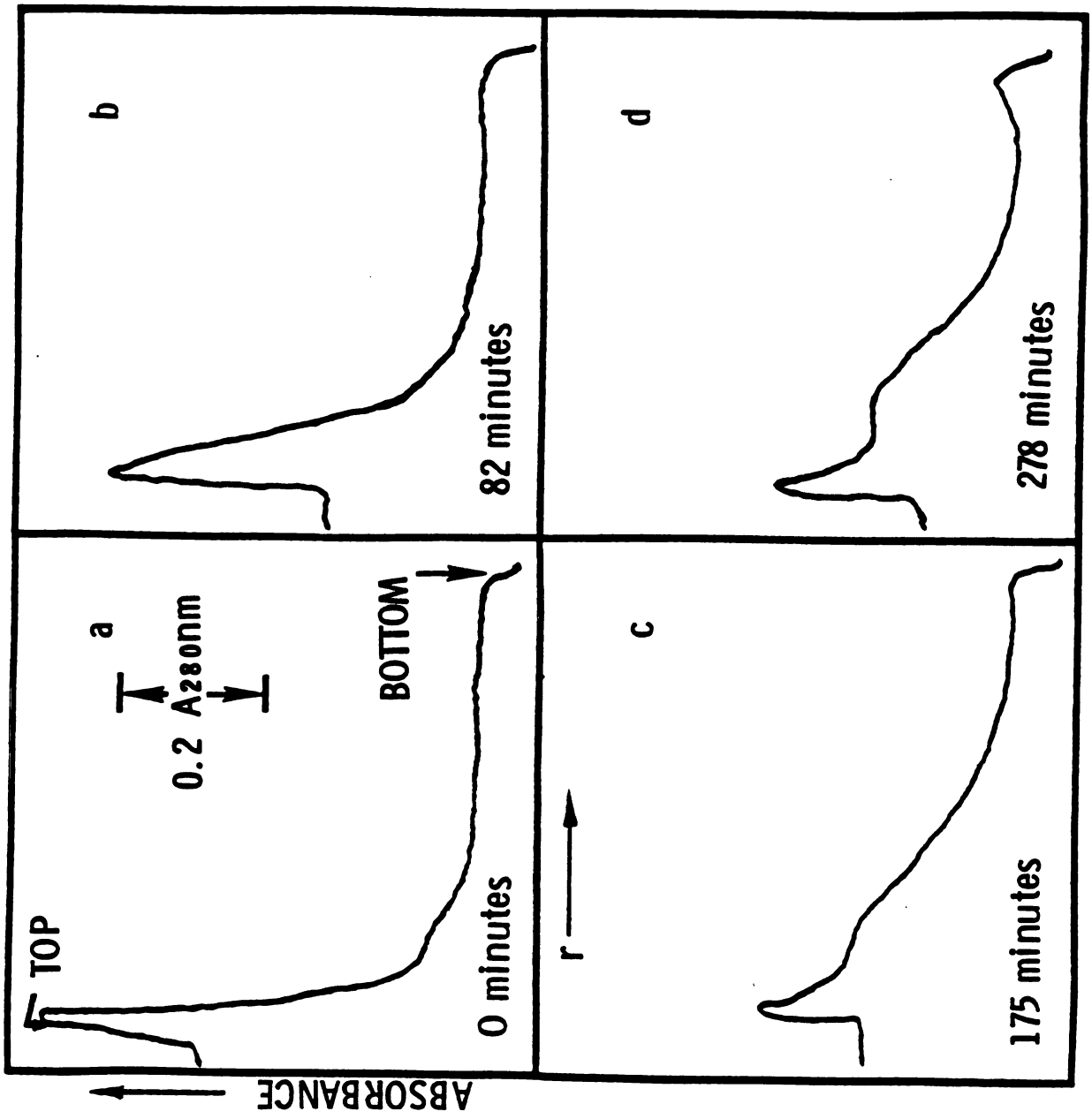


Figure 19. Inactivation of Fatty Acid Synthetase at 37° in TED Buffer by Octanoate at Various Concentrations. Purified fatty acid synthetase (20 µl, 0.03 units of overall activity) was added to TED buffer (250 µl) containing 0 (•), 0.02 (□), 0.1 (▲), or 0.5% (■) sodium octanoate. The samples were incubated at 37° and were assayed intermittently for overall FAS activity.

Figure 20. Sucrose Density Gradient Sedimentation Velocity Analysis of the Inactivation of Fatty Acid Synthetase at 37° in TED Buffer By 0.5% Octanoate. Purified fatty acid synthetase (0.5 ml, 0.74 units of overall activity) was combined with TED buffer containing 0.5% octanoate (1.50 ml). Aliquots (0.32 ml) were applied to sucrose density gradients prepared by the procedure given in Methods after incubation at 37° for 0 (a), 82 (b), 175 (c), and 278 minutes (d). Approximately 0, 50, 70, and 90% , respectively, of the overall FAS activity was lost after these incubation periods. Ultrcentrifugation was at 30,000 rpm for 15 minutes at 23°. The elution profile at 280 nm obtained upon fractionation of each gradient is shown. The faster sedimenting shoulder on the native FAS peak exhibited neither decalone reductase nor overall FAS activity.

Figure 20.


ABSORBANCE
TOP
a
0.2 A280nm
BOTTOM
0 minutes
b
82 minutes
c
r
175 minutes
d
278 minutes

a confirmation of the enzyme which eventually aggregates to high molecular weight material.

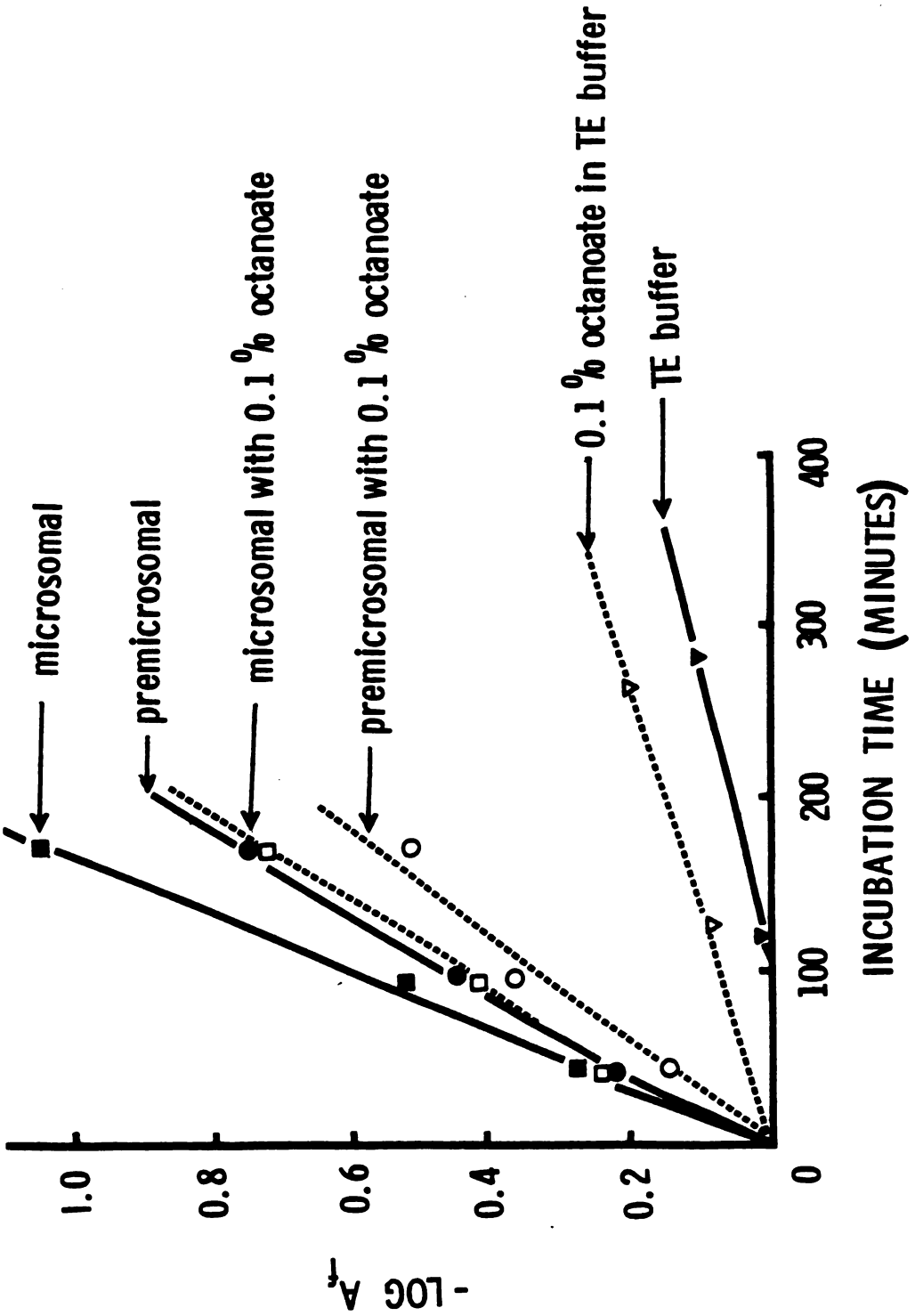
In the presence of pellet fraction suspension containing 0.1% octanoate, the octanoate protected the enzyme from inactivation to a small extent (Figure 21). Fatty acid synthetase in premicrosomal and microsomal pellet fraction suspensions containing 0.1% octanoate (0.13 units overall FAS activity/ ml) had half-lives of overall FAS activity of 92 and 68 minutes; in the absence of octanoate, half-lives were 68 and 49 minutes, respectively. The decreased rate of overall FAS activity loss in pellet fraction suspensions containing octanoate suggests that the conformational change(s) induced by octanoate, although slightly destabilizing to FAS, protects the enzyme to some extent from interaction with the inactivating capacity of the pellet fraction; that is, octanoate seems to block the site(s) of interaction between the pellet fraction and the enzyme.

DISCUSSION

These studies provide a preliminary characterization of the inactivation of fatty acid synthetase by pellet fractions obtained from pig liver homogenates. The microsomal pellet fraction is distinctly different from the other pellet fractions with respect to inactivating capacity. Microsomal pellet fraction suspensions have 40% and 110% more inactivating capacity (per gram) than the premicrosomal and nuclear and cell debris pellet fractions, respectively. Also, almost all the inactivating capacity of the microsomal pellet fraction is soluble after resuspension of the fraction at room temperature. Only about one-fourth of the inactivating capacities of the premicrosomal and nuclear and cell debris pellet fractions is soluble after resuspension at room temperature, and only one-half is soluble after incubation of the suspensions at 37° for 20 hours.

The rate and extent of solubilization of the inactivating capacity in the various pellet fractions may be explained most easily by either or both of the following possibilities ; (a) the inactivating capacity of the microsomal pellet fraction is chemically different

Figure 21. Inactivation of Fatty Acid Synthetase at 37° by Pellet Fraction Suspensions in TE Buffer or in TE Buffer Containing 0.1% Octanoate. Suspensions were prepared and analyzed under standard conditions with the exception of adding 0.1% octanoate to one of a parallel series of samples. Half-lives of overall FAS activity in the standard microsomal (■) and premicrosomal (●) pellet fraction suspensions were 49 and 68 minutes, respectively. Half-lives in the microsomal (□) and premicrosomal (○) pellet fraction suspensions containing 0.1% octanoate were 69 and 92 minutes, respectively. The inactivation rates for enzyme in TE buffer or in TE buffer containing 0.1% octanoate are shown for comparison.


microsomal
premicrosomal
microsomal with 0.1 % octanoate
premicrosomal with 0.1 % octanoate
0.1 % octanoate in TE buffer
TE buffer
1.0
0.8
0.6
0.4
0.2
0
100
200
300
400
INCUBATION TIME (MINUTES)
- LOG A_t

from that of the other two pellet fractions, or (b) the inactivating capacities are compartmented differently in the various pellet fractions.

The pellet fractions were comprised of different cellular materials as indicated by analysis using native PAGE and sucrose density gradient sedimentation velocity experiments. The nuclear and cell debris pellet fraction contained relatively few solubilizable proteins, and most of these proteins had smilar relative mobilities on native polyacrylamide gels. The premicrosomal and microsomal pellet fractions had identical banding patterns on native polyacrylamide gels, except the microsomal pattern had a very strong band (at a relative mobility of 0.12 with respect to bromphenol blue) corresponding to the protein ferritin. Sucrose density gradient sedimentation velocity analysis of these two pellet fractions revealed that some of the premicrosomal material sedimented at the same, slow rate as the microsomal material. It was also shown that freezing of the premicrosomal pellet fraction suspension causes the formation of a considerable amount of particulate material which sediments at the same rate as the microsomal pellet fraction. These observations suggest that the manner in which the inactivating capacities are compartmented is responsible for the different rate and extent of solubilization of the inactivating capacities.

The preceding statement is also supported by the similar effect of the various factors tested upon the rate of inactivation of FAS, regardless of the type of pellet fraction used (e.g., dithiothreitol prevented inactivation of FAS by any of the pellet fractions, and NADPH slowed inactivation by any of the pellet fractions).

CHAPTER 4

Further Purification and Characterization of an Inactivating Factor for Fatty Acid Synthetase

ABSTRACT

The previous chapter reported the discovery of several particulate fractions from pig liver which were capable of inactivating fatty acid synthetase. The process of inactivation in pellet fraction suspensions was partially characterized, and it was shown that the inactivating capacity could be extracted. This chapter describes further purification and characterization of the inactivating factor from the microsomal pellet fraction. The steps in the further purification of the inactivating capacity involve: incubation of the microsomal pellet fraction in water at 20° to release the factor in soluble form, ultrafiltration to separate high molecular weight compounds, DEAE-cellulose chromatography, and Sephadex G-10 gel filtration. The inactivating factor is a small substance (molecular weight less than 750 daltons) and is of net neutral or slightly negative charge at physiological pH. It is stable at 100° in aqueous solution for at least five minutes, and it contains no detectable free sulfhydryl or disulfide groups. The elution profile of the inactivating capacity from Sephadex G-10 suggests that it is composed of several distinct compounds, presumably of similar physical properties since they copurify through so many steps in the purification. Iron may be a component of the inactivating factor.

Several factors affecting the inactivation of overall FAS activity by crude preparations of the inactivating factor (pellet fraction suspensions) were described in Chapter 3. The effects of these factors, and some additional ones, were examined using the further purified inactivating factor. The inactivation process still follows first order kinetics. Addition of 1 mM dithiothreitol at any time during the inactivation process temporarily prevents, but does not reverse, inactivation of FAS. Addition of dithiothreitol at a rate of 4 mM/hr prevents inactivation of the enzyme indefinitely. The rate of inactivation is significantly reduced by 20 mM EDTA and

and by 200 μM NADPH; the stabilizing effect of NADPH is increased when 50 μM acetyl-CoA and 50 μM malonyl-CoA are also present. The rate of inactivation is very slightly reduced by 50 μM acetyl-CoA, by 50 μM malonyl-CoA, and by 40 μM palmitate.

The mechanism of inactivation is not yet known. Because EDTA decreased the rate of inactivation of FAS by the inactivating factor, suggesting the presence of a multivalent cation, and because ferritin (about 1.5 mg/ml) inactivates FAS, 1 mM ferric ion was investigated as a model compound for the inactivating factor. Ferric ion was an unsatisfactory model compound for the inactivating factor because the kinetics of inactivation were different for the two substances. In high concentrations of dithiothreitol (2-4 mM) inactivation of FAS by ferric ion occurred extremely rapidly after a brief period of time during which the overall FAS activity was stable; inactivation of FAS by the inactivating factor was prevented for a period of time, and then occurred at a rate similar to that observed when dithiothreitol was present only at a very low concentration (0.2 mM).

INTRODUCTION

An inactivating capacity for fatty acid synthetase, derived from the particulate fraction of pig liver, was reported in the previous chapter. A procedure for preparation of inactivating pellet fractions and a partial characterization of the inactivation process were given. It was also discovered that the inactivating capacity from the microsomal pellet fraction could be easily extracted in soluble form. This chapter describes the further purification of the inactivating capacity from the microsomal pellet fraction and some further observations concerning the process of inactivation.

MATERIALS AND METHODS

Reagents. Malonyl-CoA, CoA, and NADPH were products of P-L Biochemicals. Dithiothreitol was a product of Eastman-Kodak, and 2-mercaptoethanol of Mallinckrodt. Palmitic acid,5,5'-dithiobis(2-nitrobenzoic acid), and sodium nitroprusside were products of Sigma Chemical Co. Dowex 1 (OH^-form, 200-400 mesh) and Dowex 50W-X8 (H^+ form, 50-100 mesh) were obtained from Dow Chemical Co. CM-cellulose (standard, 0.67 meq/gm) and DEAE-cellulose (standard, 0.91 meq/gm) were products of Schleicher and Schuell, and Sephadex G-10 and Sephadex G-25 (coarse) of Pharmacia Fine Chemicals. Acetyl-CoA was prepared by a modification of the method of Simon and Shemin (1953), using continuous diethyl ether extraction of the acidified reaction mixture for four hours to remove reaction by-products. All other reagents were of analytical reagent grade.

Livers. Pig livers were obtained from Peet Packing Co. (Chesaning, MI) within two hours of slaughter and were frozen on dry ice. The livers were stored at -30°.

Fatty Acid Synthetase. Fatty acid synthetase was prepared from pig liver by the procedure given in Chapter 2.

Assay for the Inactivation Factor. The inactivating factor was assayed by measuring the rate of overall FAS activity loss in the presence of the inactivating factor. The assay for overall FAS

activity is given in Chapter 2. The incubation mixture was fatty acid synthetase in PED buffer and the appropriately diluted sample to be tested for inactivating factor, combined on ice. Sufficient FAS was added to the incubation mixture to give an absorbance change of 0.05 per minute when 20 µl of incubation mixture was assayed. The incubation mixtures were then taken to 37°, and at periodic intervals, samples (20 µl) of the incubation mixture were removed and assayed for overall FAS activity. One unit of inactivating factor was defined as the amount of inactivating factor required to produce the loss of one unit of overall FAS activity in one minute at 37° in the inactivation assay.

Measurement of pH and Conductivity. Values of pH were determined at room temperature with a Radiometer PHM4c pH meter equipped with a GK2321C electrode. Conductivity was measured with a Radiometer CDM2e conductivity meter equipped with CDC114 conductivity cell (cell constant 0.56 cm).

Absorption Spectra. Ultraviolet absorption spectra were obtained with a Beckman DB spectrophotometer attached to a Sargent Model SRL recorder.

Sulfhydryl and Disulfide Analysis. Sulfhydryl content of samples was determined by denaturing any protein present with sodium dodecyl sulfate and then reacting the sulfhydryl groups with DTNB (Habeeb, 1971). Sample was diluted to 1.0 ml with a solution containing 2% sodium dodecyl sulfate, 80 mM sodium phosphate, pH 8.0, and 0.5 mg/ml EDTA. To 0.5 ml of the solution was added 0.02 ml DTNB solution (40 mg DTNB in 10 ml of 0.1 M sodium phosphate buffer, pH 8.0). The color was developed for 15 minutes and the absorbance at 410 nm determined against the remaining 0.5 ml of sodium dodecyl sulfate-treated sample, to which DTNB was not added. A reagent blank is subtracted from the apparent absorbance to give the net absorbance. For calculation of sulfhydryl content, the net absorbance is employed with a molar extinction coefficient of 13,600 $m^{-1}cm^{-1}$.

Disulfide content was determined by the reduction of disulfide to free sulfhydryl in 8M urea followed by the reaction of the sulfhydryl with DTNB (Cavallini, Graziani, and Dupre, 1966). Test tubes, 10

x 75 mm, with marks at 0.6 and 1.2 ml, were used. The following material was added to the tubes in the order shown; 0.29 grams solid urea, 0.02 ml of 0.1 M EDTA, .1-.2 ml sample, 0.2 ml of 2.5% sodium borohydride, water up to the 0.6 ml mark, and a drop of n-octyl alcohol as an antifoaming agent. The tubes were shaken at 38° to dissolve the urea and were then kept at this temperature for 30 minutes. At the end of the temperature treatment, 0.1 ml of 1M KH_2PO_4 containing 0.2 M HCl was added. The wall of the test tube was carefully wetted with the solution in the tube in order to destroy traced of $NaBH_4$ adhering to it. Five minutes after the addition of the KH_2PO_4-HCl solution, the destruction of $NaBH_4$ was completed by adding 0.4 ml acetone. The mouth of the tube was closed with Parafilm and the tube inverted several times to bring the solution in contact with the wall. Nitrogen was bubbled slowly through the solution for five minutes. The DTNB solution (0.1 ml of 0.01 M DTNB in water) was then added to the solution, and the solution brought to 1.2 ml with water. Nitrogen was again bubbled through the solution for 2 minutes. The tube was then stoppered and allowed to stand at room temperature for 15 minutes. The absorbance at 412 nm was then determined against water in a Gilford 2000 spectrophotometer. The absorbance of blank solutions, which contained all the reactants except the sample, were subtracted from the sample absorbances. A standard curve was constructed from absorbances. for samples which contained 0.25-40 nmoles dithiothreitol. Concentrations of disulfides were determined from this standard curve.

RESULTS

Chapter 3 has described initial studies on the inactivating capacities from pig liver. The inactivating capacity from the microsomal pellet fraction was shown to be extracted upon resuspension in TE buffer at room temperature. Inactivating capacities of the other pellet fractions were only partially extracted, even after incubation at 37° for 20 hours. Also, the total inactivating capacity

of the microsomal pellet fraction was greater than that of the other pellet fractions. For these reasons, the microsomal inactivating capacity was chosen for further purification and characterization.

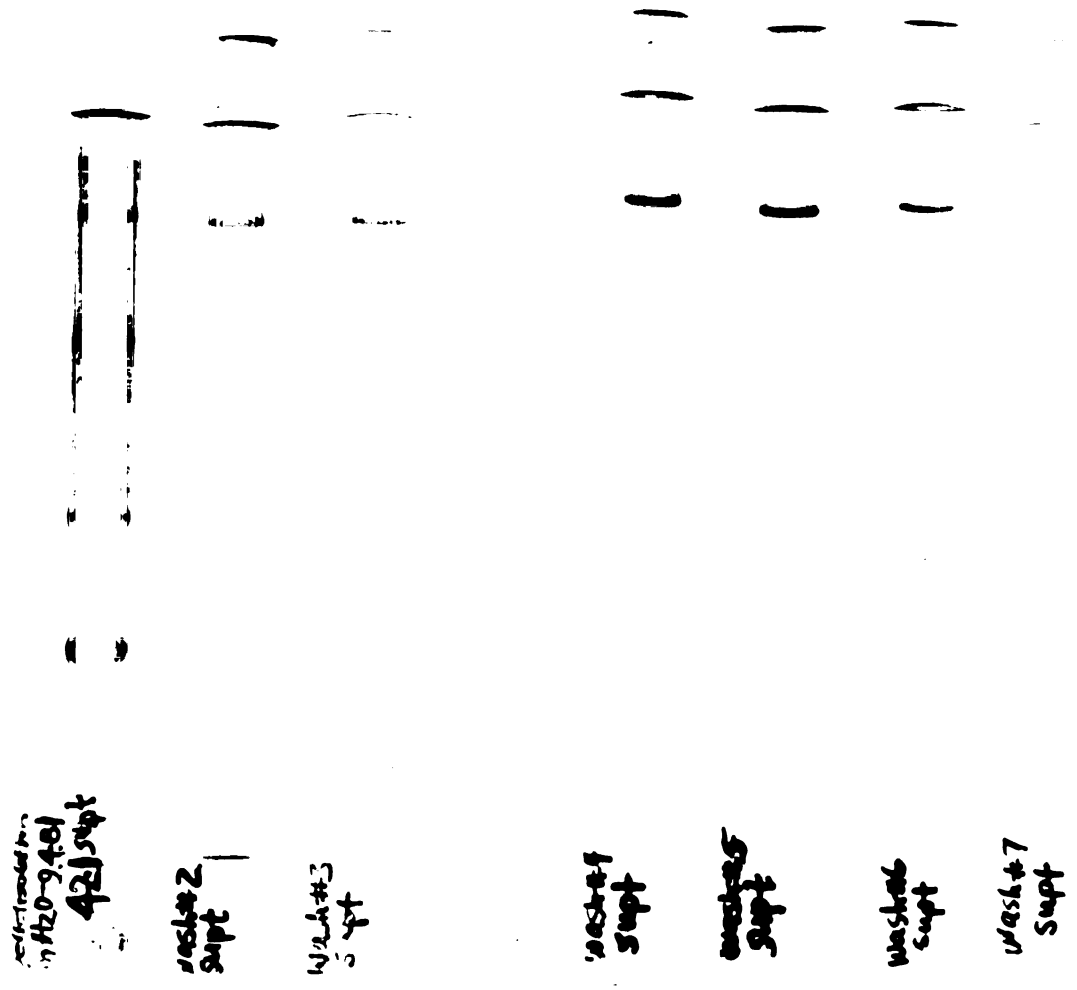
Development of Washing and Extraction Procedures. The first step in purification of the inactivating factor was the purification of the microsomal pellet fraction. This was accomplished in Chapter 3 by repeated washing of the microsomal pellet fraction in TE buffer at 4°. This buffer, of moderate ionic strength, apparently immediately extracts the inactivating factor from microsomal pellet fractions at 20° (Table 4). Hence it is expected that some fraction of the inactivating capacity would be released from the pellet fraction during washing with TE buffer, even at 4°.

So water at 0° was tested as a washing solution for the microsomal pellet fraction. The amount of inactivating capacity in the wash supernatants decreased with each washing. The first, second, and third wash supernatants had inactivating capacities of 1.3×10^{-5}, 5.1×10^{-6}, and 1.5×10^{-6} units/ml, respectively. The inactivating capacity decreased to very low levels (the apparent inactivating capacity of water is about 10^{-6} units/ml) as the remaining soluble components of the homogenate were removed (Figure 22). Thus, the inactivating capacity in the wash supernatants is apparently derived from the soluble components, not the pellet fraction. This is consistent with the discovery of inactivating capacity in the clarified centrifuged extract of pig liver homogenate which was reported in Chapter 3. Therefore, very little inactivating capacity seems to be released by the microsomal pellet fraction during washing with ice-cold water.

The washed microsomal pellet fractions were extracted by suspension in water for two hours at 20°. The amount of inactivating factor extracted was equal to the inactivating capacity in the pellet fraction suspension. The extract, which was separated from the extracted pellet fraction by centrifugation, was of sufficiently low ionic strength to be applied to ion exchange columns (see below). Also, the absence of added buffer in the extract reduced the amount of ionic substances eluted from Sephadex columns with the inactivating

Figure 22. Purification of Microsomal Pellet Fraction by Washing with Water at 0°. Pig liver was homogenized in water at 0° and the homogenate was centrifuged at 18,000 rpm for 13 minutes at 0° in a Sorvall SS-34 rotor. The supernatant was centrifuged again in the SS-34 rotor under identical conditions. The supernatant from the second centrifugation was then centrifuged at 0° for 60 minutes at 42,000 rpm in a Beckman 42.1 rotor. The supernatant from this centrifugation is the first wash supernatant (pH 6.34, conductivity 2.15 mmho). The pellets from this centrifugation were taken up to 20 ml with water at 0° and were resuspended using a Potter-Elvehjem homogenizer. The suspension was taken up to 150 ml with more water at 0° and was then centrifuged once again in the Beckman 42.1 rotor as described above. The supernatant from this centrifugation is the second wash supernatant (pH 6.62, conductivity 320 μmho). Further wash supernatants were obtained by repeating the resuspension and centrifugation steps.

Figure 22.



factor (see below). It is useful to minimize ionic strength for another reason as well; fatty acid synthetase is inactivated at high ionic strength (Table 6).

Table 6. Spontaneous Inactivation of Fatty Acid Synthetase in Solutions of TE Buffer of Various Ionic Strengths.

Fold normal TE buffer concentration[a]	1	5	9	13	17
Half-life of overall FAS activity (min)	900	510	335	265	218

[a]Normal TE buffer contains 50 mM tris(hydroxymethyl)aminomethane, pH 7.4, and 1 mM EDTA. Incubation was carried out at 37°. The inactivation rate is not proportional to TE buffer concentration. A more highly inactivating solution gives a shorter half-life for overall FAS activity.

Preliminary Characterization of the Inactivating Factor and the Development of Separations Based on Molecular Size and on Net Charge. The goals of (1) development of a purification procedure for the inactivating factor, and (2) characterization of the inactivating factor, were closely interrelated. Studies in either area yielded information useful in the other. For example, examination of prospective purification steps based on the size of the inactivating factor (gel filtration, dialysis, ultrafiltration) yielded some knowledge of its size. Similarly, examination of separations based on the net charge properties of the inactivating factor (Dowex and DEAE- and CM-cellulose chromatography) yielded information about its net charge.

Early experiments were undertaken to determine the size of the inactivating factor. The initial test was based on gel filtration. Microsomal pellet fraction suspension in TE buffer (1 g pellet fraction/ 10 ml suspension) was centrifuged at 23° in a Beckman 42.1 rotor for 70 minutes at 40,000 rpm. The clear yellow supernatant from this centrifugation (20 ml) was applied to a Sephadex G-25 column (coarse, 2.2 x 50 cm) and eluted with TE buffer; 0.031 of 0.034 units of inactivating capacity (90%) was eluted in the low molecular weight region. Therefore, the molecular weight of the inactivating factor was less than the exclusion limit of the gel (5,000 daltons).

The next test of size for the inactivating factor consisted of dialyzing the microsomal pellet fraction in aqueous suspension (4 g pellet fraction in 16 ml water) against 500 ml water at 20° for two hours. This was followed by overnight dialysis at 4° against the same 500 ml water, to allow diffusion of low molecular weight components (less than 14,000 daltons) across the dialysis membrane to reach equilibrium. A considerable amount of inactivating capacity was found outside the dialysis casing, substantiating the small size of the inactivating factor as determined by gel filtration.

Subsequently, ultrafiltration was used to separate the solubilized inactivating factor from the microsomal pellet fraction and proteins. Ultrafiltration was performed using an Amicon PM-30 ultrafiltration membrane. Molecules of molecular weight more than 30,000 daltons were retained, while the inactivating factor passed through (as greater than 80% of the inactivating capacity applied).

Tests for separation of the inactivating factor from contaminants by gel filtration on Sephadex G-10 columns also provided evidence that the inactivating factor was very small, since most of the inactivating capacity (greater than 80% of that applied) was eluted in the low molecular weight region (see below). Hence, the molecular weight of the inactivating factor is less than 750 daltons (the exclusion limit of the gel).

Tests for separation of the inactivating factor from contaminants by ion exchange chromatography showed that the inactivating factor has neutral or slightly negative net charge near neutral pH. Ultrafiltered extract of microsomal pellet fraction suspensions was applied to columns (0.6 x 16 cm) of Dowex 50W-X8 (H^+ form), Dowex 50W-X8 (Na^+ form), and Dowex 1 (OH^- form), and the columns were eluted with water (Table 7). The eluate from the Dowex 50W-X8 (Na^+ form) column contained all the applied inactivating capacity, and apparently more. So the inactivating factor is apparently chemically altered when passing through this column, and is not bound. The eluate from the Dowex 50W-X8 (H^+ form) column contained about 10% of the applied inactivating capacity. Again, the inactivating factor appears to have been chemically modified, this time the modification decreased the effectiveness of the inactivating factor in inactivating fatty acid synthetase. The eluate from the Dowex 1 (OH^- form) column had essentially no inactivating capacity. Since the inactivating factor was bound by this column, it apparently has a net

negative charge.

Table 7. Binding of Partially Purified Inactivating Factor to Dowex Resins.

Resin used[a]	Total Inactivating Capacity of Applicate (units) x 10^3	Total Inactivating Capacity of Eluate (units) x 10^3
Dowex 50W-X8 (Na^+ form)	1.5	4.0
Dowex 50W-X8 (H^+ form)	8.2	0.82
Dowex 1 (OH^- form)	8.2	<0.1

[a]Inactivating factor taken through Step 2 in its purification procedure was applied to 0.6 x 16 cm columns of the resins. The columns were eluted with water at 23°. The eluate was taken to pH 7.4 with 6M NaOH or 6M HCl prior to assaying for the inactivating factor.

To confirm the net charge on the inactivating factor, DEAE-cellulose and CM-cellulose chromatography were performed on the extracted, ultrafiltered inactivating capacity from the microsomal pellet fraction. Binding of the inactivating capacity to CM-cellulose could not be demonstrated. At sufficiently low ionic strength, however, the inactivating capacity was bound quantitatively to DEAE-cellulose. When eluted with a 0 to 0.25 M NaCl gradient, the inactivating capacity peaked at 50 mM NaCl (Table 8), again suggesting a slightly negative net charge on the inactivating factor. Since the binding of a nonionic surfactant, Triton X-100, to DEAE-cellulose has been demonstrated (unpublished results from

Table 8. Inactivating Capacities of Fractions Obtained from DEAE-cellulose Chromatography During Purification of the Inactivating Factor.

Fraction number[a]	37	38	39	40	43
Inactivating capacity (units) x 10^3	0.56	1.09	0.44	0.17	0.44

[a]Fractions 38 and 43 correspond to the peaks at 3.3 and 6.1 mmho conductivity, respectively, in Figure 24. The first peak has ten times the absorbance of the second at 254 nm.

this laboratory), the binding of the inactivating factor to DEAE-cellulose may be due either to a negative charge, or to nonelectrostatic interaction between the inactivating factor and DEAE-cellulose.

The studies just described led to the following standard purification procedure for inactivating factor from microsomal pellet fraction. This procedure is summarized in Figure 23.

Standard Procedure for the Partial Purification of Inactivating Factor from the Microsomal Pellet Fraction of Pig Liver. Distilled deionized water was used throughout.

Step 1. Microsomal pellet fraction isolation. Frozen pig liver (800 g) was cut into small pieces and homogenized for 90 seconds with 1600 ml water at 4° in a Waring blendor. The homogenate was centrifuged at 6,700 rpm for 10 minutes in Sorvall GSA rotors at 0°. This centrifugation removed larger organelles, connective tissue, and whole cells. The supernatant was centrifuged at 18,000 rpm for 15 minutes in Sorvall SS-34 rotors at 0°. The supernatant from this centrifugation was centrifuged again under identical conditions to complete removal of mitochondria, lysosomes, and peroxisomes. The supernatant from the repeat centrifugation was then centrifuged in a Beckman Type 21 rotor at 21,000 rpm and at 0° for 150 minutes. The microsomal pellet fraction was obtained from this centrifugation. The pellet fraction was washed by resuspending the pellets in ice-cold water to a volume of 150 ml using a "large" clearance Dounce homogenizer, and then centrifuging the suspension at 40,000 rpm in a Beckman Type 42.1 rotor at 0° for 70 minutes. Three washes were completed before continuing to the next step.

Step 2. Extraction of the inactivating capacity and separation from high molecular weight contaminants. Four grams of washed microsomal pellet fraction were suspended in thirty-six milliliters of water by Dounce homogenization at room temperature. After incubation at room temperature for two hours, the suspension was centrifuged in a Beckman Type 42.1 rotor at 20° for 70 minutes at 40,000 rpm. The clear yellow supernatant (36 ml) was applied to an Amicon Model 52 pump with a PM30 ultrafiltration membrane and was filtered at 15 psi. The pale yellow filtrate (32 ml) was collected. The solution retained in the filtration apparatus was golden yellow and turbid.

Step 3. DEAE-cellulose chromatography. A DEAE-cellulose column (1.25 x 12 cm) was prepared and equilibrated with TE buffer. The column was

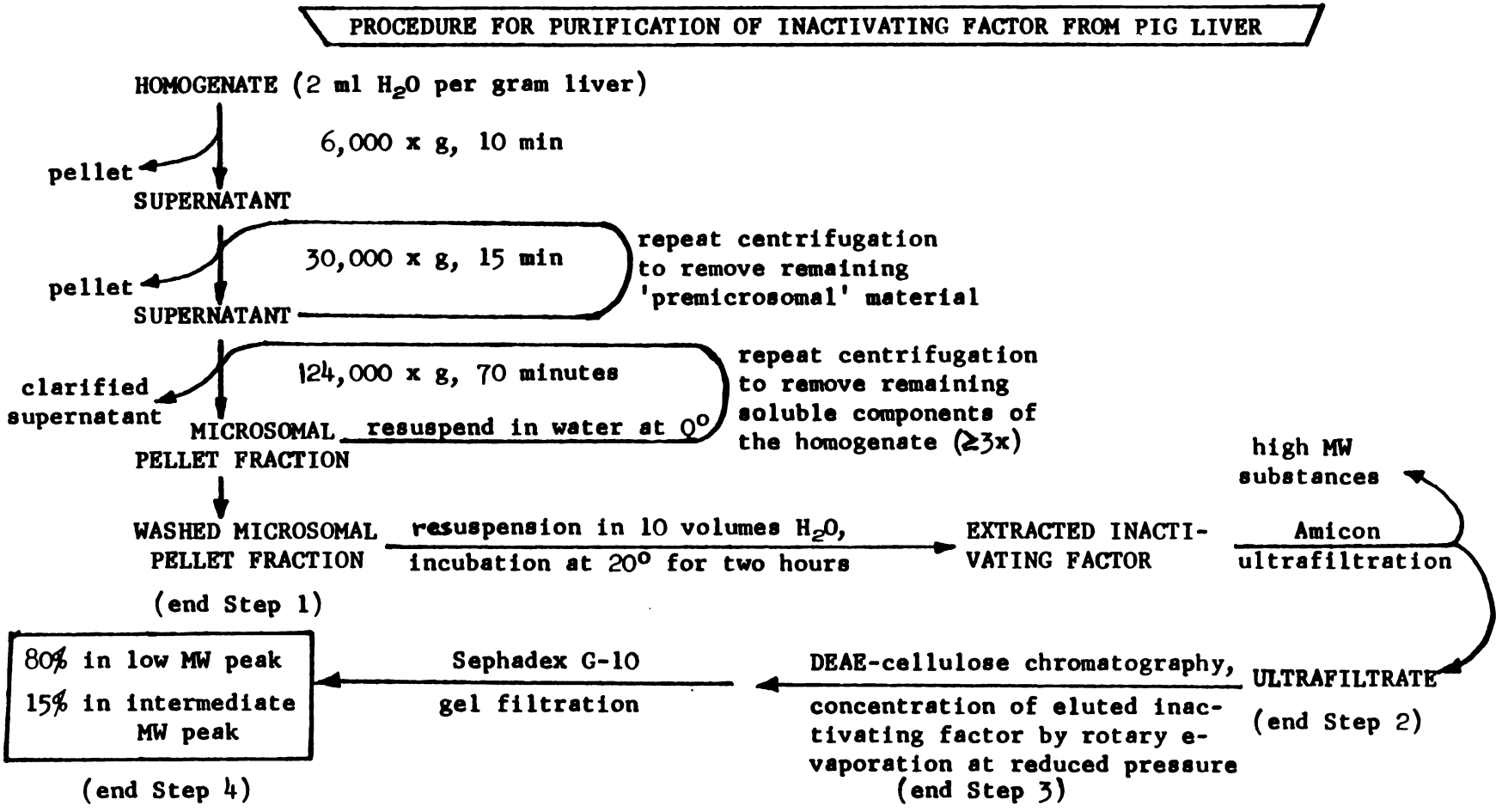


Figure 23. Flow Chart for the Purification of Microsomal Inactivating Factor.

then washed with water until the conductivity of the eluate was less than 5 μmho. The conductivity of the ultrafiltrate from the previous step was adjusted to less than 15 μmho and was then applied to the column, after which the column was washed with 30 ml water. The column was eluted with a linear 0 to 0.25 M NaCl gradient (104 ml, 52 ml each reservoir). A characteristic elution profile at 254 nm was obtained (Figure 24). Twenty drop fractions (about 0.8 ml) were collected using an ISCO Model 328 fraction collector. The peak of inactivating capacity eluted at 3 mmho conductivity (about 50 mM NaCl). Fractions with inactivating capacity were combined and concentrated by rotary evaporation under vacuum at 40° to a volume of less than 2 ml.

Step 4. Sephadex G-10 gel filtration. The concentrated inactivating factor from the DEAE-cellulose chromatography was applied to a Sephadex G-10 column (1.25 x 99 cm) and was eluted with water at a flow rate of 6.6 ml per hour. Twenty drop fractions (about 0.7 ml) were collected. A typical elution profile at 254 nm is shown in Figure 25. More than 80% of the inactivating capacity eluted in the NaCl (low molecular weight) peak.

Additional Properties of the Inactivating Factor. The studies described at the beginning of the Results indicated that the inactivating factor is small (less than 750 daltons) and has a net neutral or slightly negative charge near neutral pH. Some additional tests were performed on the inactivating factor in order to further elucidate its physical and chemical structure. The results of these tests are presented below.

Stability in aqueous solution at 100°. Inactivating factor extracted from the microsomal pellet fraction, but not yet ultrafiltered, was taken to 100° in a boiling water bath and was allowed to incubate for five minutes. After incubation, inactivating capacities of both unincubated and incubated extracts were determined. No inactivating capacity was lost as a result of the incubation.

Sulfhydryl and disulfide content. All tests for sulfhydryl groups and disulfide bonds in the inactivating factor were negative. The inactivating factor for these experiments was obtained by performing Sephadex G-25 gel filtration on the extracted inactivating capacity from the microsomal pellet fraction. High and low molecular weight eluates from the column were combined separately and were concentrated to levels twenty times those in the applied samples. When assayed for free sulfhydryl

Figure 24. DEAE-cellulose Chromatography Elution Profile at 254 nm for Partial Purification of Inactivating Factor. The applied sample (107 ml) was obtained by dialyzing microsomal pellet fraction against water for a day at room temperature. The solution outside the tubing was then applied to the column and eluted at room temperature by the procedure given in the Results. Inactivating capacity peaked at 3.3 mmho conductivity. The inactivating capacities of several fractions are given as points on the figure.

Figure 24.

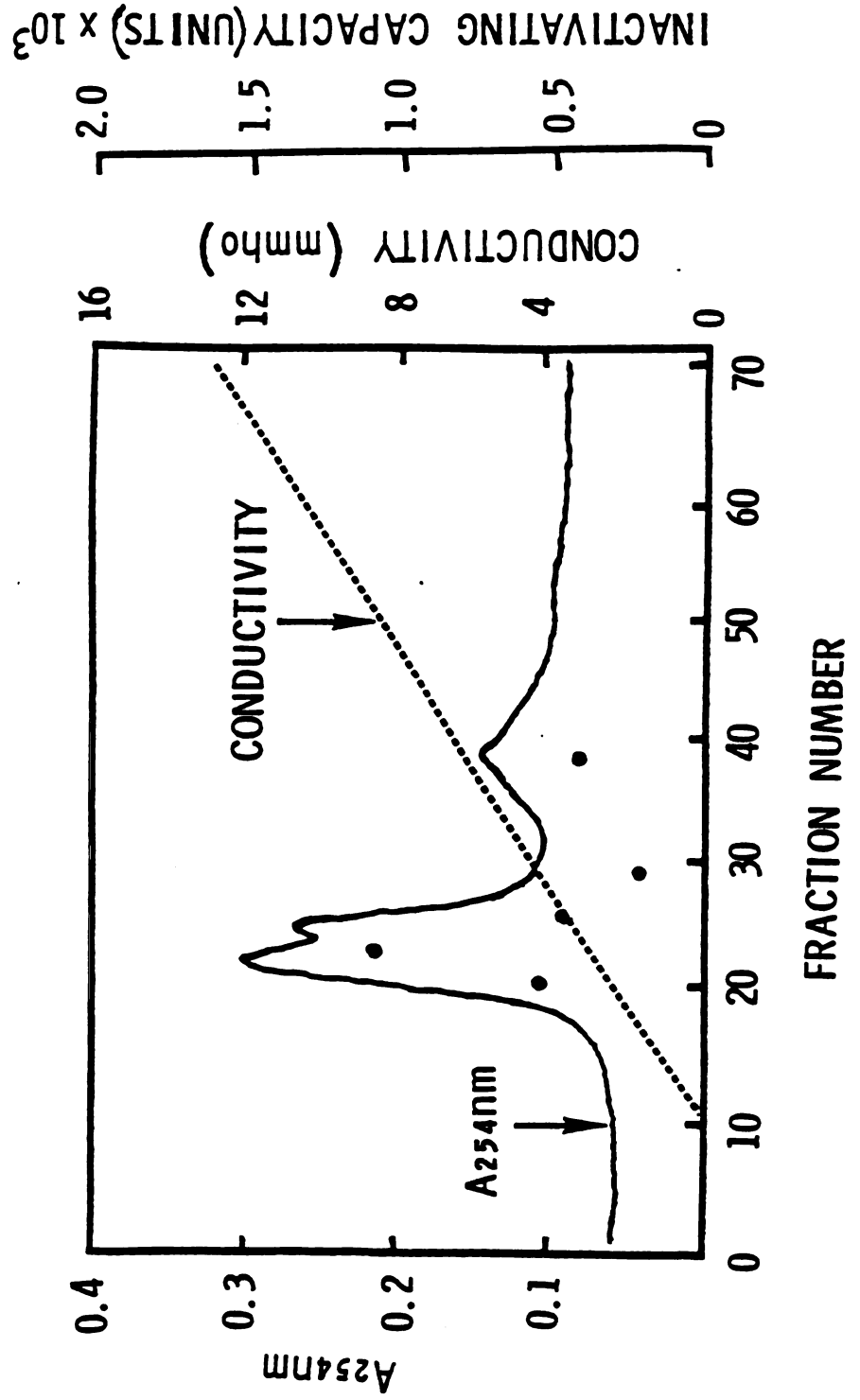
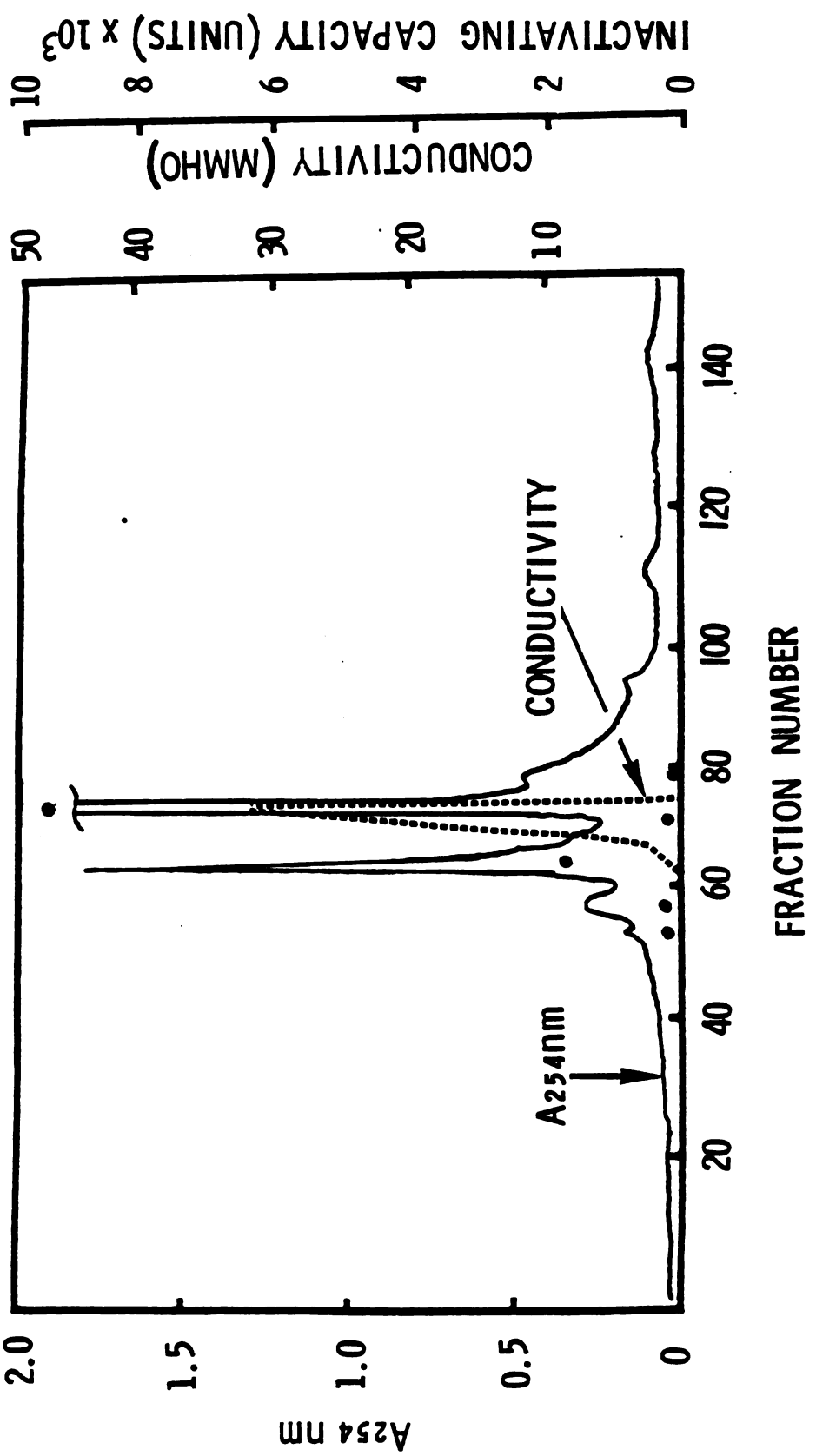

A254nm
0.4
0.3
0.2
0.1
0
CONDUCTIVITY (mmho)
16
12
8
4
0
INACTIVATING CAPACITY (UNITS) x 10³
2.0
1.5
1.0
0.5
0
CONDUCTIVITY
A254nm
0
10
20
30
40
50
60
70
FRACTION NUMBER

Figure 25. Sephadex G-10 Gel Filtration Elution Profile at 254 nm for Partial Purification of Inactivating Factor. The applied sample was a concentrated composite of the inactivating fractions from several DEAE-cellulose chromatography columns. The column was eluted with water at room temperature according to the procedure given in Results. The inactivating capacities of several fractions are indicated by the points.


A254 nm
2.0
1.5
1.0
0.5
0
20
40
60
80
100
120
140
FRACTION NUMBER
A254nm
CONDUCTIVITY
50
40
30
20
10
CONDUCTIVITY (MMHO)
10
8
6
4
2
0
INACTIVATING CAPACITY (UNITS) x 10³

and disulfide content, less than 10 μM free sulfhydryl and 20 μM disulfide were found in the low molecular weight concentrate. The protein-containing high moelecular weight concentrate had 200 μM disulfide and less than 20 μM free sulfhydryl.

Heterogeneity:several copurifying compounds. The inactivating factor purified using the standard procedure apparently contains at least two major components of different molecular weight (Table 9). Most of the

Table 9. Inactivating Capacities of Fractions Obtained from Sephadex G-10 Gel Filtration During Purification of the Inactivating Factor.

Fraction[a]	Conductivity (mmho)	Inactivating Capacity (units) x 10^3
54 (leading edge of high MW peak)	0.15	0.31
57 (high MW peak)	0.10	0.27
63 (intermediate MW region)	0.61	1.59
68 (leading edge of low MW peak)	6.45	0.34
73 (low MW peak)	24.0	>10
79 (trailing edge of low MW peak)	0.01	0.15

[a]The fractions are from the gel filtration whose elution profile is shown in Figure 25. The portions of the elution profile from which the fractions were taken are indicated in parentheses.

inactivating factor eluted in the low molecular weight (NaCl) peak (80%), but an additional 15% eluted in the fractionation range for the gel (the 'intermediate' molecular weight range). The remaining inactivating capacity was spread throughout the eluted fractions. The inactivating capacities had associated with them an absorbance at 226 nm, 270 nm, or both (Figure 26).

Extractibility in several organic solvents. The extractibility of inactivating factor in several organic solvents was determined. Addition of hydrochloric acid to 0.15 M to the inactivating factor did not affect its absorption spectrum for at least 24 hours, so the extractions were performed on inactivating factor (with associated absorption maxima at 226 nm and 270 nm) in 0.15 M HCl. Extraction with either cyclohexane or methylene chloride gave similar results; the material with absorption maximum at 226 nm was not extracted, and that with absorption maximum at 270 nm was

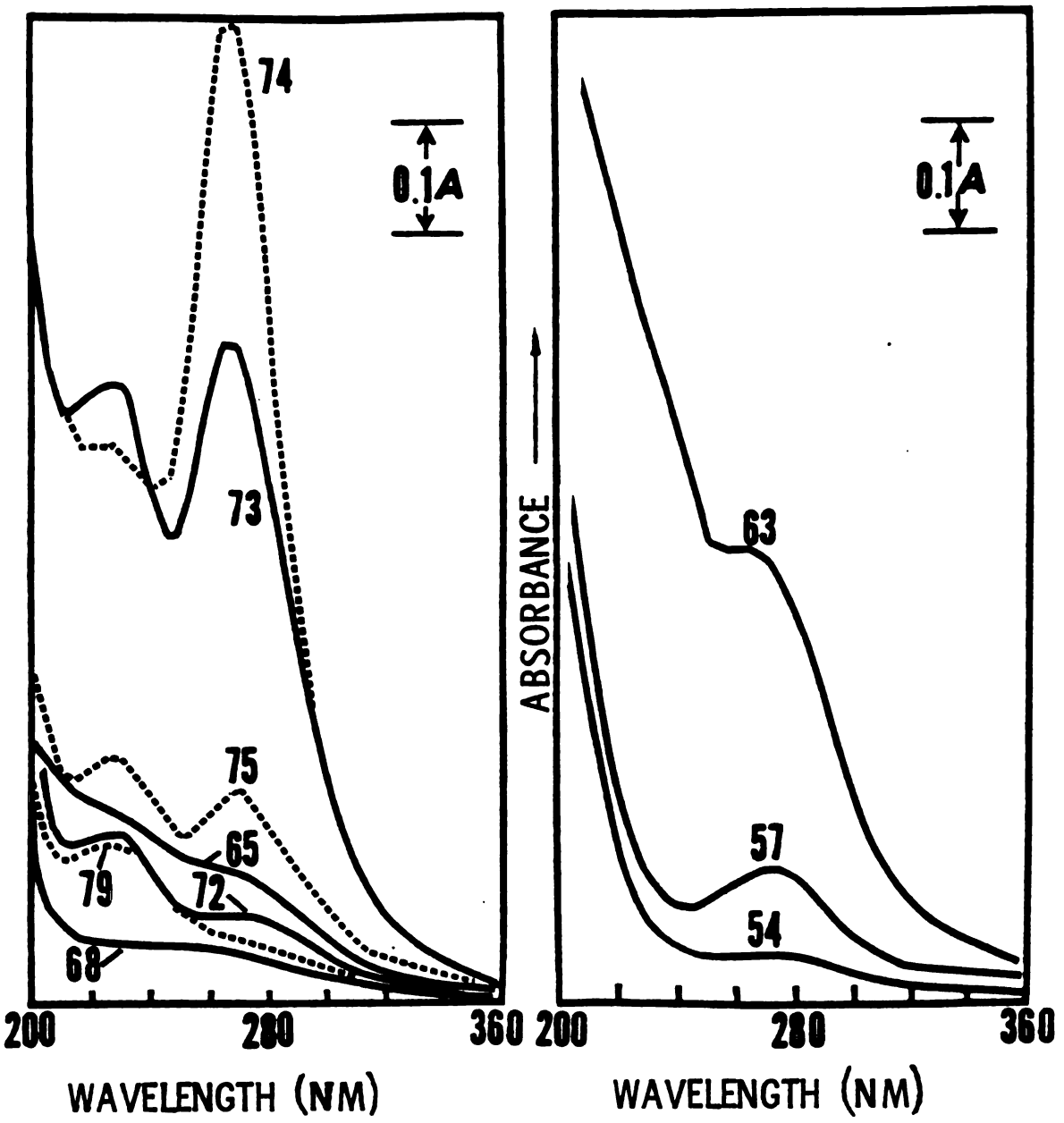


Figure 26. Ultraviolet Absorption Spectra of Fractions from Sephadex G-10 Gel Filtration for Partial Purification of Inactivating Factor. These fractions are from the same gel filtration column described by Table 9 and Figure 25. Fraction 73 contained the greatest amount of inactivating capacity. Absorption spectra of fractions 54, 57, and 63 were measured at thirteen-fold dilution; those of the other fractions were measured at thirty-one -fold dilution. Absorption spectra were obtained using a Beckman DB spectrophotometer.

only partially extracted. When taken to dryness by rotary evaporation under reduced pressure, the extracts yielded oily orange solids. Extraction with diethyl ether gave a turbid colorless solution. Ultraviolet analysis of the extract was not possible because of the very high background absorbance of the remaining ether.

Complexed ferric ion. The presence of ferric ion was consistent with several observations made during the purification and characterization of the inactivating factor. The inactivating factor gave a translucent orange, noncrystalline solid when taken to dryness by rotary evaporation. It gave no absorption maxima in the visible absorption spectrum. Also, at several steps in the purification of the inactivating factor, orange precipitates occasionally formed; formation of the precipitate was associated with the loss of inactivating capacity. The redissolved precipitates gave ultraviolet-visible absorption spectra without maxima, with absorption increasing monotonically with decreasing wavelength. These absorption characteristics are consistent with those for ferric ion.

The inactivation of fatty acid synthetase by the inactivating factor is inhibited by EDTA (see below); however, EDTA did not eliminate the inactivation of the enzyme. Also, DEAE-cellulose will not bind a cationic metal ion. Thus, if a metal ion is present in the inactivating factor, it must be in a complexed form which has a net neutral or negative charge. The behavior of the inactivating factor on Dowex resins can be easily explained if the inactivating factor has a complexed metal ion. On Dowex 1, the inactivating factor would be degraded to metal ion, which binds to the column, and free ligand, which is released and eluted from the column. On Dowex 50W-X8 (H^+ form), the inactivating factor would be degraded in part, so that a small amount of free metal ion might elute from the column, and so account for the small inactivating capacity observed in the eluate. On Dowex 50W-X8 (Na^+ form), the ligands might be more completely stripped, or the complex changed in some other manner, so that the free metal ion would be eluted quantitatively from the column. The effect of ferric ion on fatty acid synthetase will be presented shortly.

Further Characterization of the Inactivation Process. Tests of the inactivation process using the further purified inactivating factor gave results similar to those obtained in Chapter 3 using the inactivating capacity in microsomal pellet fraction suspensions. Continuous addition of

dithiothreitol to the incubation mixture at a rate of 4 mM per hour was sufficient to almost halt the inactivation of fatty acid synthetase, even in extremely high concentrations of inactivating factor (Table 10). Addition of 1 mM dithiothreitol was sufficient to halt, but not reverse, the

Table 10. Periodic Addition of Dithiothreitol to Incubation Mixtures Prevents Inactivation of Fatty Acid Synthetase by Inactivating Factor[a].

Incubation time (minutes)		0	38	78	152	250
Fraction of overall FAS activity remaining	no DTT added	1.00	0.03	0	0	0
	DTT added[b]	1.00	0.91	0.90	0.76	0.60

[a]The sample was the concentrated inactivating capacity obtained at the end of Step 3 in the purification of the inactivating factor.

[b]Dithiothreitol concentration in this sample was increased by 1 mM every 15 minutes with a 100 mM stock solution of dithiothreitol. The incubation was carried out at 37°.

inactivation process. The rate of inactivation of the enzyme was also significantly reduced in the presence of 20 mM EDTA, 200 μM NADPH, and especially 200 μM NADPH with 50 μM acetyl-CoA and 50 μM malonyl-CoA. Malonyl-CoA, acetyl-CoA, or palmitate alone only slightly stabilized the enzyme (Table 11).

Table 11. Effectors of the Inactivation of Fatty Acid Synthetase by Inactivating Factor at 37°.

Additional component in the incubation mixture[a]	None	Palmitate (40 μM)	AcCoA (50 μM)	MalCoA (50 μM)	NADPH (200 μM)	EDTA (20 mM)	MalCoA (50 μM), AcCoA (50 μM), NADPH (200 μM)
Relative inactivating capacity	1.0	0.68	0.74	0.88	0.43	0.34	0.21

[a]Incubation mixture contained fatty acid synthetase and Amicon ultrafiltrate from the purification of the inactivating factor (latter at two-thirds its initial concentration).

Inactivation of Fatty Acid Synthetase by Ferric Ion Differs Greatly from That by Inactivating Factor. Several observations (see previously) were consistent with the presence of iron in the inactivating factor. Also, ferritin, which is present in large amounts in the soluble extract from microsomal pellet fractions, inactivates fatty acid synthetase. Freshly purified, fully reduced ferritin at about 1.5 and 8 mg per ml inactivated half the overall enzyme activity in a standard incubation mixture containing 1 mM dithiothreitol in 750 and 350 minutes, respectively. This suggested that ferritin might be the source of the inactivating factor; ferrous ion might be released from ferritin, oxidize, and then react with fatty acid synthetase. The capacity of ferric ion to oxidize free sulfhydryl groups at concentrations as low as 0.1 mM is well established. Thus, the inactivation of fatty acid synthetase by ferric ion would be expected to closely model that by inactivating factor if an oxidative modification of the enzyme is a part of the inactivation process. For this reason, ferric ion was tested as a candidate for the inactivating factor. Ferric ion was obtained from ferric chloride brought to neutral pH with 1 M NaOH solution.

Inactivation of fatty acid synthetase by ferric ion (1 mM) occurred at a rate comparable to the inactivation of the enzyme by inactivating factor when using the extracted material at Step 2 in the purification procedure as the source of inactivating factor (Figure 27). The kinetics of inactivation were the same using either ferric ion or inactivating factor when the dithiothreitol concentration was low (less than 0.2 mM). At higher dithiothreitol concentrations, however, dithiothreitol protected the enzyme from inactivation by inactivating factor, but accelerated greatly the inactivation of the enzyme by ferric ion. For this reason, ferric ion proved to be a poor model for the inactivating factor.

DISCUSSION

These studies provide a partial characterization of the inactivating factor derived from the microsomal pellet fraction of pig liver. The data show that the inactivating factor is a low molecular weight substance (less than 750 daltons) of net neutral or slightly negative net charge. It is stable in aqueous solution at 100° for at least 5 minutes

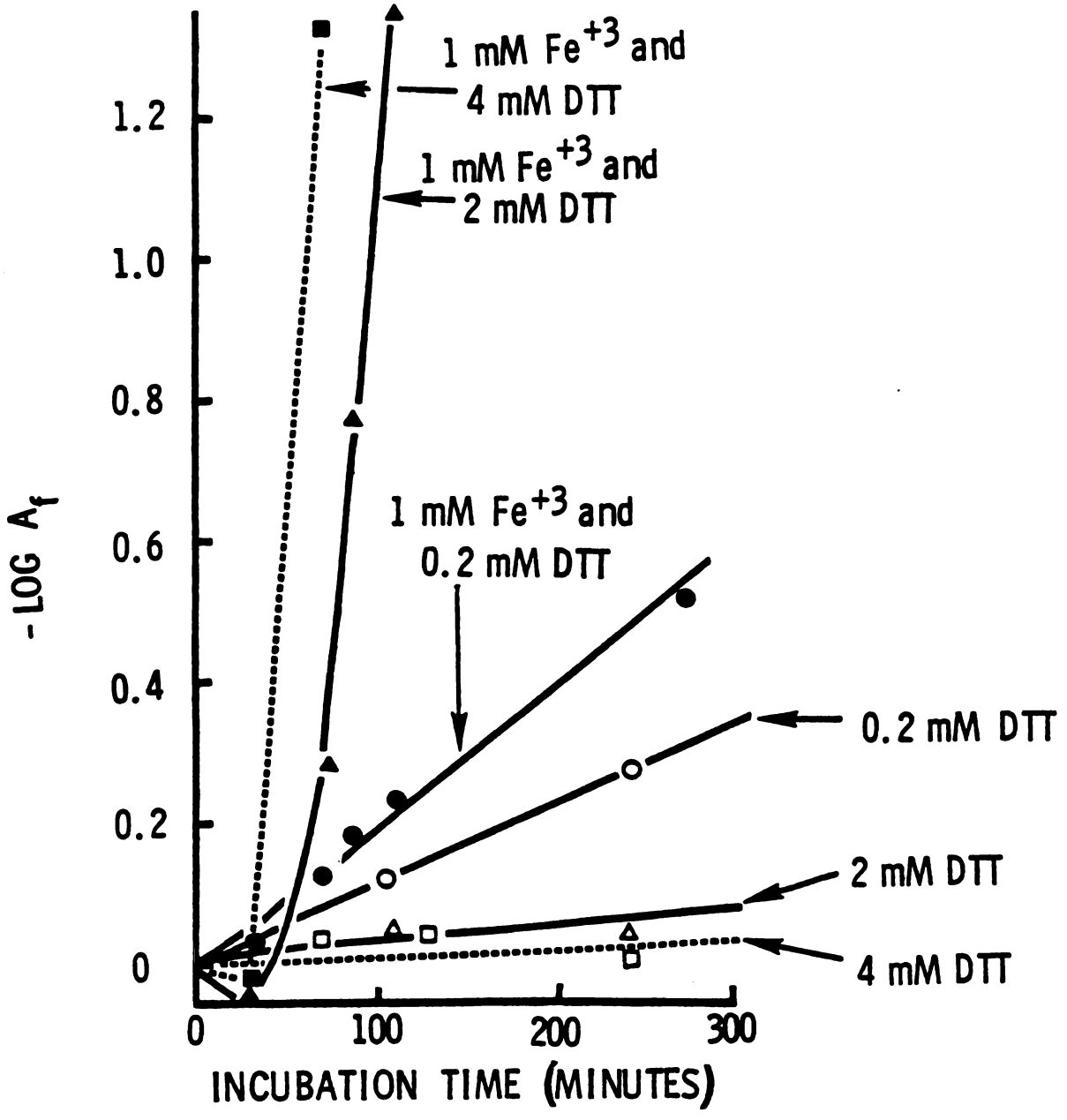


Figure 27. Inactivation of Fatty Acid Synthetase by 1 mM Fe^{+3} in TE Buffer Containing Various Amounts of Dithiothreitol. Fatty acid synthetase and Fe^{+3} were combined in a standard incubation mixture containing either 0.2 (●), 2 (▲), or 4 mM (■) dithiothreitol (DTT). The effect of TE buffer containing 0.2 (o), 2 (△), or 4 (□) mM dithiothreitol upon overall FAS activity is shown for comparison.

and apparently contains no free sulfhydryl nor disulfide groups. Its elution profile from Sephadex G-10 indicates that it may be comprised of a series of closely related compounds. Its visible absorption spectrum, with absorption monotonically increasing as wavelength decreases, is consistent with that of ferric ion. The ultraviolet absorption spectrum, which exhibits peaks at 226 nm and at 270 nm in varying proportion, suggests the presence of one or more organic compounds. Since the chemical structure of the inactivating factor has not yet been elucidated, further studies in this area are indicated.

Oxidation of fatty acid synthetase as a step in its inactivation seems likely since dithiothreitol, a reducing agent, has an inhibitory effect on the inactivation. Because EDTA also has a (lesser) inhibitory effect on the inactivation, and because ferritin is a major component in the soluble extract from microsomal pellet fraction, ferric ion was suggested as a candidate for the inactivating factor. Tests revealed that ferric ion was not the inactivating factor. The data also showed that it was not a good model compound for the inactivating factor; at high dithiothreitol concentrations the inactivation of fatty acid synthetase by inactivating factor was inhibited, while inactivation of the enzyme by ferric ion was greatly accelerated.

Although ferric ion was eliminated as the inactivating factor, oxidation was not eliminated as a mechanism for the inactivation. Detergent-like denaturation of fatty acid synthetase by the inactivating factor is very unlikely because an intermediate conformer was formed during incubation of the enzyme with octanoate, and no such intermediate was observed during incubation of the enzyme with inactivating factor. The total mass of the inactivating factor is sufficiently small (considerably less than 0.1% of the mass of the microsomal pellet fraction, although not exactly known) that a nonspecific effect on hydrogen or electrostatic bonding is also unlikely (e. g., 1% isopropanol was needed to achieve inactivation of the enzyme at a rate far enough above that in buffer alone to be detected). Consequently, the best mechanism for the inactivation involves an oxidation, or other covalent modification, of the enzyme which results in the enzyme having an increased tendency to aggregate. Further studies are needed to elucidate the exact mechanism.

CHAPTER 5

ISOELECTRIC FOCUSING IN THIRTY-FIVE MINUTES

ABSTRACT

Certain unstable macromolecules require rapid analytical techniques for fractionation and analysis. This chapter describes procedures which greatly shorten the time required for isoelectric focusing in density gradients. Focusing times as short as thirty-five minutes were obtained for hemoglobin; the shortest time previously reported was two hours. Key characteristics of the present system are: (1) a short solution column (50 mm); (2) incorporation of dilute KCl (0.03%) in the gradient column, the gel plug soak solution, and both reservoir solutions; (3) a uniform initial ampholyte concentration in the density gradient, at the lowest concentration consistent with the formation of a linear pH gradient (0.50%); and (4) incorporation of sucrose in the gel plug. In addition, this chapter also describes various conditions to further maximize the linearity of the pH gradient, though at the expense of longer focusing times. Standard polyacrylamide gel electrophoresis apparatus is used except for the electrofocusing tubes, which are specially prepared from standard glass tubing.

INTRODUCTION

Isoelectric focusing of macromolecules in density gradients has several major advantages over isoelectric focusing in polyacrylamide gels. These include: (1) rapid and quantitative recovery of sample for further analysis, (2) easier determination of pH values at each point in the tube, and (3) faster electrofocusing for comparable column heights. For these reasons, there has been an interest in development and refinement of faster and better techniques for isoelectric focusing in density gradient solutions (for references to reviews, see Behnke et al., 1975).

Massey and Deal (1973) described an apparatus and techniques for rapid isoelectric focusing. The key feature of this procedure was the use of a short (110 mm) density gradient column formed over an agar plug. Technical difficulties with the agar plug were overcome by replacing it with a polyacrylamide gel plug (Behnke et al., 1975). In addition, it was demonstrated that multiple tubes could be easily run simultaneously. This procedure for isoelectric focusing was a great improvement over that using the commercially available apparatus, which required up to 48 hours for focusing, but did not give focusing times short enough to accommodate certain unstable samples. For this reason, the factors affecting focusing time, as well as those affecting the accuracy and reliability of isoelectric point determinations, were systematically studied.

A number of factors affecting focusing time and linearity of the pH gradient have been identified. Under conditions where focusing time is minimized, the focusing time of hemoglobin has been reduced by a factor of almost four, to about 30 minutes. Procedures which yield greater resolution and maximal pH gradient linearity at the expense of longer focusing times have also been developed.

MATERIALS AND METHODS

Ethanolamine (95%) was obtained from Aldrich Chemical Co. Ampholyte (Biolyte 3/10) was purchased from BIORAD; it had a pH range of 3 to 10 and a concentration (w/v) of 40% solids. Bovine hemoglobin was obtained from Sigma Chemical Co. Ammonium persulfate, N,N'-methylenebis(acrylamide), and acrylamide were products of EM Laboratories. Canalco

supplied N,N,N',N'-tetramethylethylenediamine. All other reagents were of analytical reagent grade.

A standard polyacrylamide gel electrophoresis apparatus was modified by replacing the grommets holding the PAGE tubes in the upper reservoir with grommets to hold 10 mm (o. d.) glass tubing. Two sizes of electrofocusing tubes were used, a 10 x 90 mm tube and a 10 x 150 mm tube. Each was made from 10 mm (o. d.) glass tubing; a slight bulb was blown at the bottom of each tube as previously described (Behnke et al., 1975). The shorter tube was used for the ultimate test of minimum focusing time. The longer tube was used in selecting optimum electrofocusing conditions with respect to other variables because focusing time differences were greater, and deviations from linearity in the pH gradient were easier to detect, in the longer column.

In preparing for an experiment, the electrofocusing tubes were provided with a polyacrylamide gel plug in the bottom to support the density gradient solution. The bottoms of the tubes were tightly sealed with Parafilm and the tubes were filled to 3 mm above the tops of the bulbs with aqueous gel plug solution. This solution contained 7.5% acrylamide, 0.18% N,N'-methylenebis(acrylamide), and 25% sucrose. After addition of the gel plug solution, a layer of water was applied. Upon polymerization of the gel plug solution, the water overlayer was removed and the tubes were filled with, and immersed in, a gel plug soak solution for a minimum of seven hours. The soak solution contained 3% acetic acid, 0.03% KCl, and 25% sucrose. Soaking periods of longer than seven hours did not enhance or diminish performance of the plugs.

Just prior to use, the soak solution was removed, the electrofocusing tube was rinsed with distilled water, and the tube was blotted dry with absorbent tissue. In the short focusing tube, a 2.50 ml (50 mm height) density gradient containing 5 to 12% sucrose was formed. In addition to sucrose, the gradient contained 0.50% ampholyte, 0.03% KCl, and 50 to 200 μg protein, all distributed uniformly throughout the gradient. When the long focusing tube was used, a 5.6 ml (112 mm height) 5 to 20% linear sucrose gradient with 0.86% ampholyte was formed; all other solution components were as for the short tubes.

The lower electrophoresis chamber was filled with 3% (v/v) acetic

acid solution containing 0.03% KCl. The electrofocusing tubes containing the gradients were then moistened near their tops with glycerol and were inserted into the empty upper electrophoresis chamber. Upper reservoir solution, 3% (v/v) ethanolamine containing 0.03% KCl, was carefully layered over each gradient until each tube was filled, and the upper chamber was then filled. The positive lead of the power supply was attached to the lower reservoir and the negative lead was attached to the upper reservoir. Electrofocusing was begun at 95 volts for the short tubes or at 200 volts for the long tubes, yielding an initial current of about three milliamperes per tube. Using this procedure, hemoglobin focused within thirty-five minutes in the short tube.

After electrofocusing, the tubes were removed and mounted vertically on a stand; the upper reservoir solution was then removed from the tubes with a Pasteur pipet. The top of a tube was stoppered with a cork through which the barrel of an 18 gauge needle extended; the needle was attached to plastic tubing leading to the inlet of an ISCO Model UA-5 spectrophotometric flow cell with an attached fraction collector. Another 18 gauge needle, attached by plastic tubing to a syringe pump which contained 50% sucrose, was inserted upward through the gel plug until its tip was just above the gel plug. This operation was carried out with the pump on slowly so that no air bubbles were introduced at the bottom of the density gradient. The 50% sucrose was pumped at a rate of 0.37 ml per minute by an ISCO Model 183 density gradient fractionator. Fifteen second (0.09 ml) fractions were collected for the short tube; thirty second fractions were collected for the long tube. The pH values of the fractions were determined using a Radiometer PHM4c pH meter equipped with a GK2321C combination microelectrode.

Focusing times were determined using hemoglobin as the test sample since its color allowed visual monitoring of the focusing process. Sharp protein boundaries were formed near the extremes of each column initially; they migrated toward each other and ultimately coalesced into a single, focused boundary. This process was described in detail in a previous paper from this laboratory (Behnke, et al., 1975).

RESULTS

The major goal of this study was to determine the conditions which would minimize the focusing time for macromolecules subjected to iso-

electric focusing in sucrose density gradients. However, because of a sharp decline in pH in the lower third of the sucrose density gradients using the previous method (Behnke et al., 1975), it was first necessary to improve the linearity of the pH gradient. For these studies, as well as for studies on the effects of various factors on focusing time, the original, longer (112 mm, 5.6 ml) density gradient column was used. This allowed greater sensitivity in detecting differences in both focusing times and shapes of pH gradients. Unless otherwise indicated, the experiments described below were performed with the longer (5.6 ml) gradients.

Sucrose in the Gel Plug: Effect on pH Gradient Linearity. Working on the assumption that convection caused the sharp decline in pH in the bottom third of the density gradient, 25% sucrose was incorporated in the gel plug. This greatly improved the linearity of the pH gradient in the bottom portion of the density gradient, but at the expense of a slightly increased focusing time (Figure 28). Nevertheless, the improvement in linearity of the pH gradient was judged more important than the increase in focusing time, so inclusion of sucrose in the gel plug was adopted as part of the standard procedure.

Acetic Acid as the Lower Reservoir Electrolyte: Effect on pH Gradient Linearity. Since the pH gradient was still steeper than desired at the bottom of the density gradient, a weaker acid was substituted for the strong acid generally used in the acid reservoir solution. The use of acetic acid instead of sulfuric acid substantially improved the linearity of the pH gradient by raising the pH at the bottom of the density gradient about two units (Figure 29). Hence, acetic acid (3%) was incorporated in the standard procedure.

Uniform Initial Ampholyte Distribution: Effect on pH Gradient Linearity. In the previous electrofocusing system (Behnke et al., 1975), an ampholyte concentration gradient was used to complement the sucrose gradient, thereby providing a greater initial density gradient. Comparison of this system with one having a uniform initial ampholyte concentration showed the latter system to yield a more linear pH gradient (Figure 30). So uniform initial ampholyte concentration is a part of the standard electrofocusing procedure.

Figure 28. Effect of Adding Sucrose to the Gel Plug Supporting the Sucrose Density Gradient in Isoelectric Focusing. Isoelectric focusing was performed by the method of Behnke et al. (1975) (——), or with the single modification of incorporating 25% sucrose in the gel plug (-----). Focusing times for hemoglobin in these two systems were 115 and 126 minutes, respectively.

Figure 28.

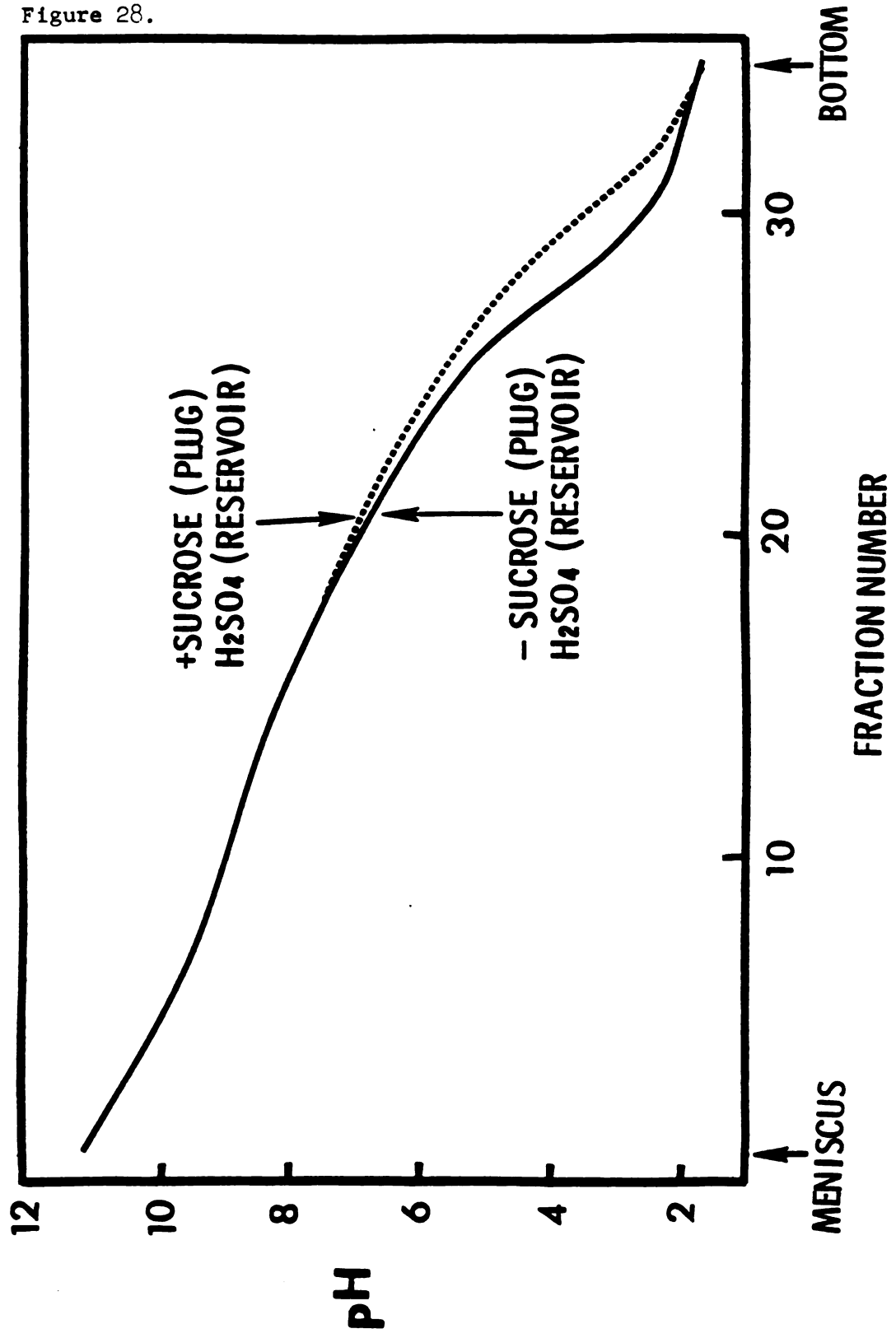

pH
12
10
8
6
4
2
MENISCUS
BOTTOM
10
20
30
FRACTION NUMBER
+SUCROSE (PLUG)
H2SO4 (RESERVOIR)
– SUCROSE (PLUG)
H2SO4 (RESERVOIR)

Figure 29. Effect of Substituting Acetic Acid for Sulfuric Acid in the Lower Reservoir Solution for Isoelectric Focusing. Isoelectric focusing was performed by the method of Behnke et al. (1975) with these modifications: the gel plugs in the electrofocusing tubes contained 25% sucrose, and the lower reservoir solution was either 3% sulfuric acid (——) or 3% acetic acid (-----). Focusing times for hemoglobin in these two experiments were 126 and 150 minutes, respectively.

Figure 29.

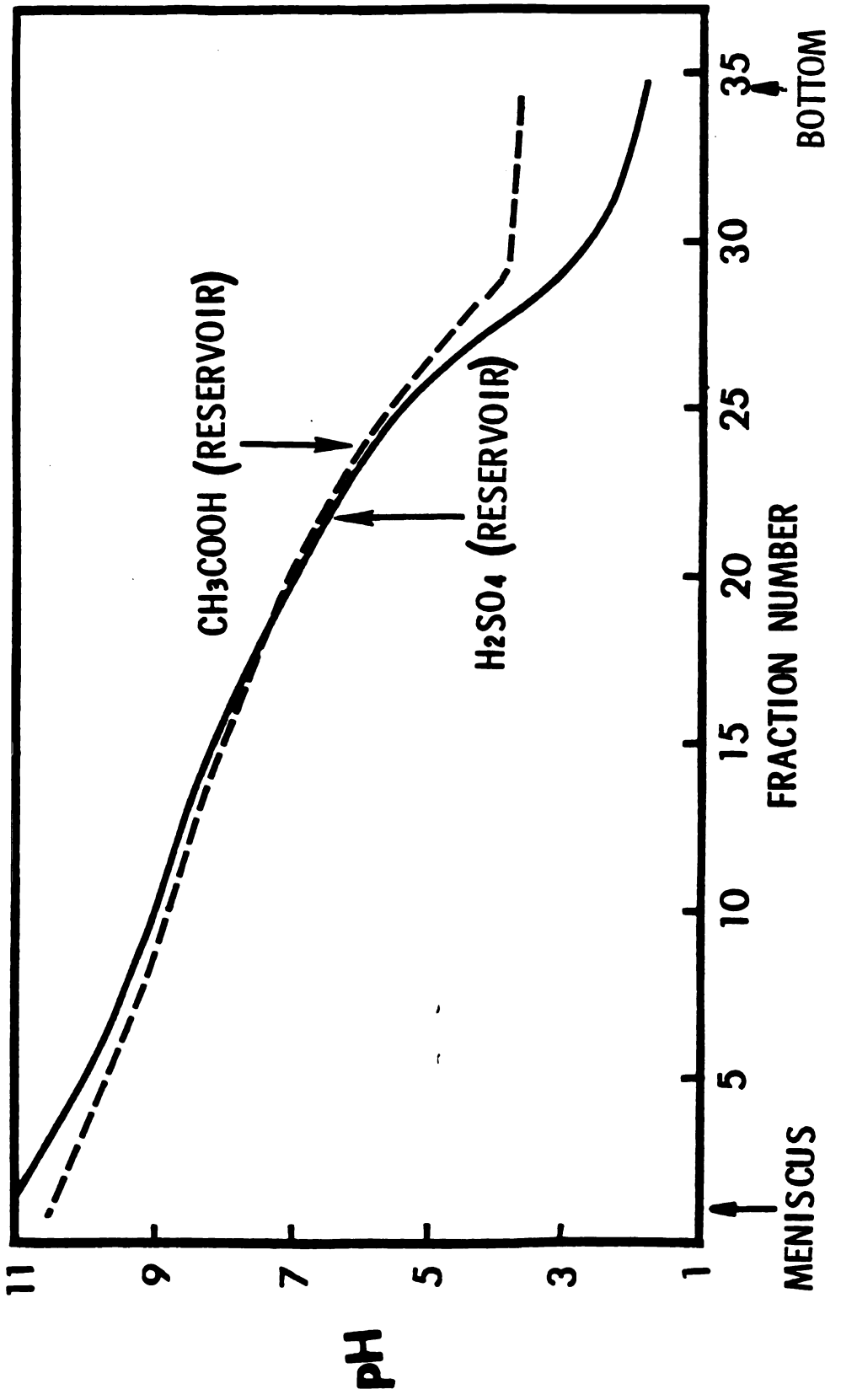
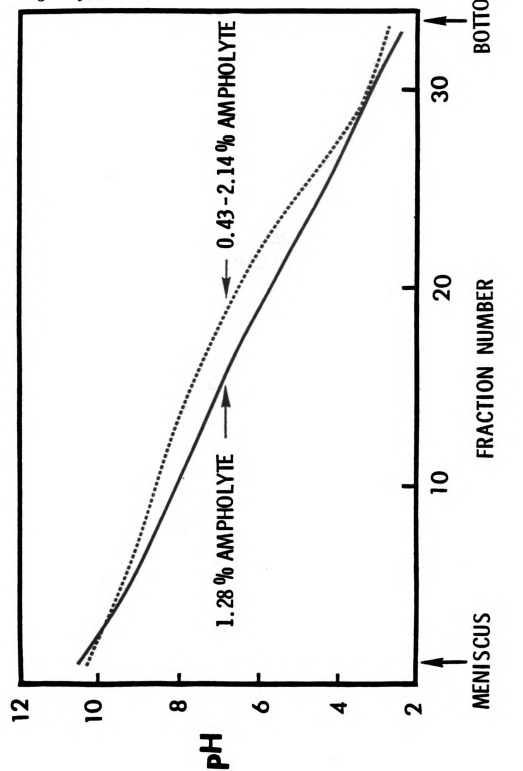

pH
11
9
7
5
3
1
MENISCUS
5
10
15
20
25
30
35
BOTTOM
FRACTION NUMBER
CH3COOH (RESERVOIR)
H2SO4 (RESERVOIR)

Figure 30. Effect of Initial Ampholyte Distribution upon pH Gradient Linearity in Isoelectric Focusing. Isoelectric focusing was performed according to the standard 112 mm gradient column height procedure described in the Materials and Methods, except the ampholyte was present in the sucrose density gradient either as a gradient of 0.43 to 2.14% ampholyte (-----) or at a uniform concentration of 1.28% (——). These ampholyte distributions and concentrations are those used in the method of Behnke et al. (1975) and 1.5 times that used in the present 112 mm procedure, respectively. Focusing times for hemoglobin in these experiments were 132 and 126 minutes, respectively.

Figure 30.


12
10
8
6
4
2
pH
0.43-2.14 % AMPHOLYTE
1.28 % AMPHOLYTE
MENISCUS
10
20
30
FRACTION NUMBER
BOTTOM

Decrease of Focusing Time by Decreasing Ampholyte Concentration. As shown in Figure 31, the time required for electrofocusing decreased as ampholyte concentration decreased. However, as ampholyte concentration was decreased, the pH gradient became nonlinear and the magnitude of the slope of the pH gradient increased. Presumably this is a reflection of the loss of ampholyte buffering capacity at the extremes of the sucrose gradient, where the gradient is in direct contact with acid and base. For the long and short columns, ampholyte concentrations of 0.86 and 0.50%, respectively, were chosen as the best compromise between focusing time on the one hand and linearity and slope of the pH gradient on the other.

Decrease in Focusing Time by Including 0.03% KCl in the Gel Plug Soak Solution, Density Gradient, and Reservoir Solutions. The presence of a low concentration of electrolyte in the density gradient and in the reservoir solutions significantly shortened the electrofocusing time (Table 12). The optimal concentration of KCl is about 0.03%. When no

Table 12. Effect of KCl (0.03%) in Various Components of the Isoelectric Focusing System upon Hemoglobin Focusing Time.

Components to which KCl was added[a]	Focusing time (minutes)
none	122
gel plug	122
gradient	103
gradient and gel plug	103
gradient, gel plug, and reservoirs	100

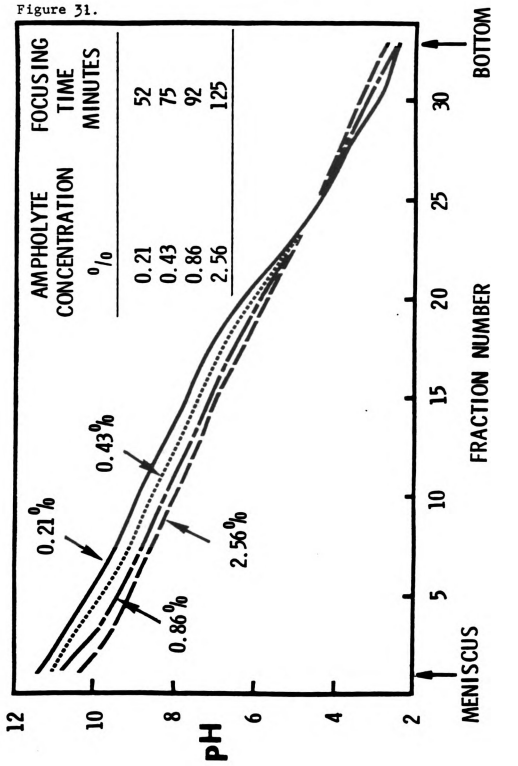
[a]The standard 112 mm column height procedure for isoelectric focusing was used with the exception of adding KCl only to the solutions indicated.

electrolyte is added, focusing time is increased by about 20%. If the sample to be focused contains electrolytes, best results are obtained with nonfocusable electrolyte equivalent to about 5 mM (0.035%) KCl. Higher concentrations cause significant heating.

Decrease in Focusing Time by Acid Soaking the Gel Plug. In the previous electrofocusing system (Behnke et al., 1975), soaking the gel plugs in lower reservoir solution was required for good performance. If the gel plugs were not acid-soaked, focusing times were increased by 20 to 25%. In the present procedure, soaking of the gel plugs in lower reservoir solution containing 25% sucrose decreases focusing time about

Figure 31. Effect of Ampholyte Concentration upon Focusing Time and pH Gradient Linearity in Isoelectric Focusing. Isoelectric focusing was performed using the standard 112 mm gradient column height procedure described in the Materials and Methods, except ampholyte concentrations in the gradients were as indicated. Focusing times for hemoglobin in these gradients are shown in the Figure. The pH gradients for experiments with ampholyte concentrations of 0.43 and 0.86% overlap below pH 4.

Figure 31.


AMPHOLYTE CONCENTRATION %
FOCUSING TIME MINUTES
0.21 52
0.43 75
0.86 92
2.56 125
0.21%
0.43%
0.86%
2.56%
pH
12
10
8
6
4
2
MENISCUS
5
10
15
20
25
30
BOTTOM
FRACTION NUMBER

five percent. The different soaking effects in the two procedures are due to the incorporation of conducting electrolyte in the gradient and to the uniform initial ampholyte distribution in the gradient.

Decrease in Focusing Time by Decreasing the Gradient Column Height. With the improvements made in pH gradient linearity, it was possible to shorten the gradient column height. This is the most effective way of decreasing focusing time. Figure 32 shows the effect of decreasing the column height from 112 mm in the previous procedure (Behnke et al., 1975) to 50 mm in the present procedure. The time required for focusing in the shorter column is about one-third that in the longer column. The pH gradient, although not as linear as in the longer column, is still satisfactory.

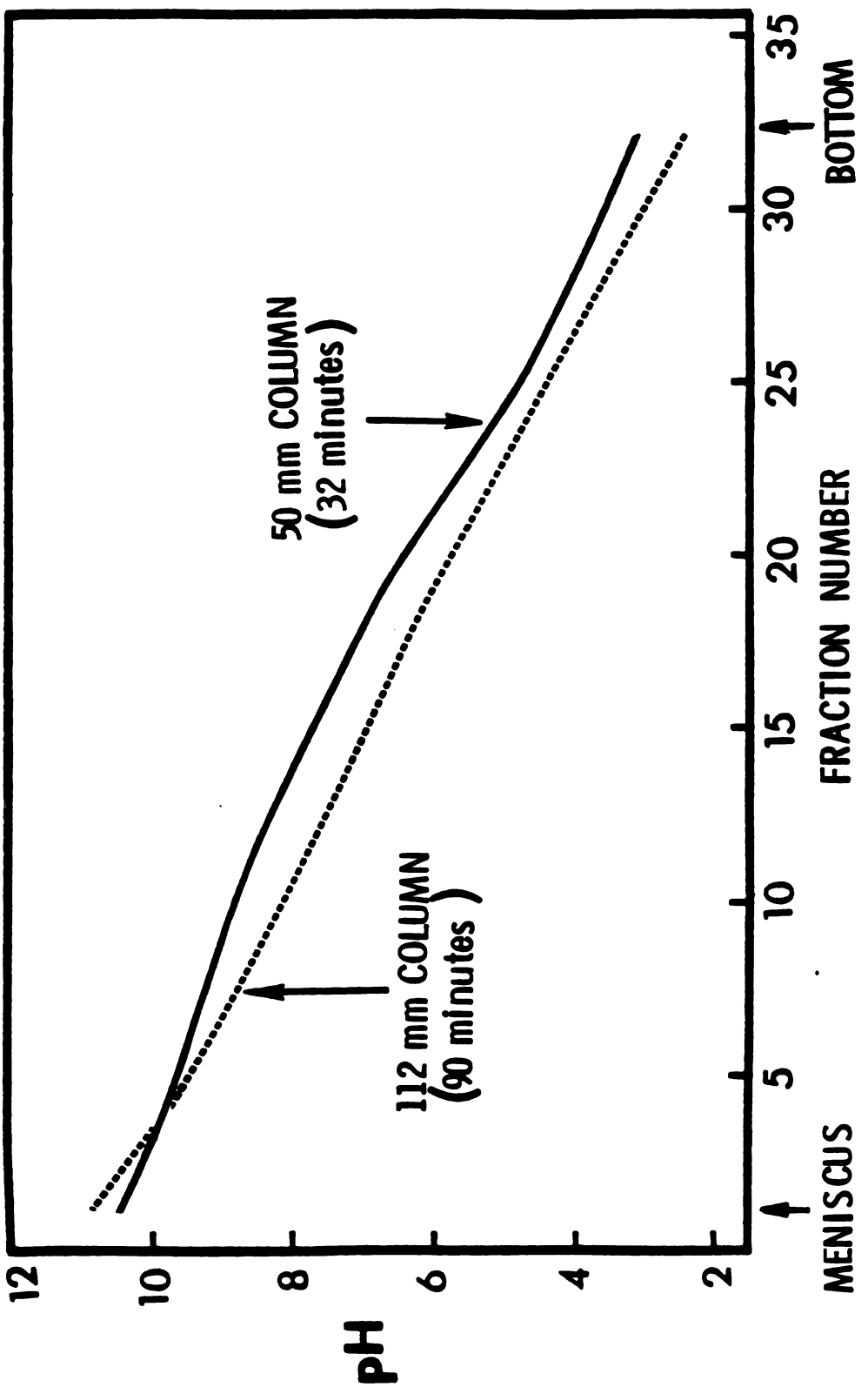
DISCUSSION

Two major advances are provided by the present method of isoelectric focusing in sucrose density gradients: (1) the time required for electrofocusing has been reduced to its limit, to as little as thirty-two minutes for hemoglobin, and (2) the linearity of the pH gradient has been greatly improved, yielding more accurate and reliable isoelectric point values. Figure 33 summarizes the differences between the initial and improved electrofocusing procedures. Figure 34 summarizes the changes made in the initial procedure during the development of the current procedure and the effects on focusing time and pH gradient linearity of those changes.

Many factors affected both focusing time and pH gradient linearity, so changing one factor at a time was required in determining the effects of each factor on the electrofocusing system. The following factors always produced relatively large effects: (1) reducing the ampholyte concentration, which always decreased focusing time, (2) reducing the gradient column height, which always reduced focusing time, and (3) incorporation of sucrose in the gel plug, which always yielded more linear pH gradients. The magnitude of the effects of the following factors often varied, depending on the other conditions of the experiment: (1) the presence and concentration of KCl, (2) the acid used in the lower reservoir solution, (3) the initial ampholyte distribution, and (4) the soaking of gel plugs prior to isoelectric focusing.

Figure 32. Effect of Decreased Column Height on Focusing Time and pH Gradient Linearity in Isoelectric Focusing. Isoelectric focusing was performed using the standard 50 mm and 112 mm gradient column height procedures as described in Materials and Methods except: the ampholyte concentration was 0.50% in both columns, and the fractions collected had volumes of 0.042 and 0.093 ml for the 50 mm (——) and the 112 mm (-----) columns, respectively. Focusing times for hemoglobin in the 50 mm and 112 mm columns were 32 and 90 minutes, respectively.

Figure 32.


50 mm COLUMN
(32 minutes)
112 mm COLUMN
(90 minutes)
pH
12
10
8
6
4
2
MENISCUS
5
10
15
20
25
30
35
BOTTOM
FRACTION NUMBER

INITIAL PROCEDURE (FOCUSING TIME 115 MINUTES FOR HEMOGLOBIN):

- -3% SULFURIC ACID IN LOWER RESERVOIR, 3% ETHANOLAMINE IN UPPER RESERVOIR.
- -POLYACRYLAMIDE GEL PLUG IN ELECTROFOCUSING TUBE (TO SUPPORT SUCROSE DENSITY GRADIENT), SOAKED OVERNIGHT IN LOWER RESERVOIR SOLUTION.
- -112 mm SUCROSE DENSITY GRADIENT COLUMN (5-20%) CONTAINING 0.43-2.14% AMPHOLYTE GRADIENT.
- -200 VOLTS.

IMPROVED (PRESENT) PROCEDURE (FOCUSING TIME 32 MINUTES FOR HEMOGLOBIN):

- -3% ACETIC ACID IN LOWER RESERVOIR, 3% ETHANOLAMINE IN UPPER RESERVOIR, BOTH RESERVOIRS ALSO CONTAINING 0.03% KCl.
- -POLYACRYLAMIDE GEL PLUG IN ELECTROFOCUSING TUBE (TO SUPPORT SUCROSE DENSITY GRADIENT), PLUG CONTAINING 25% SUCROSE, SOAKED AT LEAST SIX HOURS IN LOWER RESERVOIR SOLUTION CONTAINING 25% SUCROSE.
- -50 mm SUCROSE DENSITY GRADIENT COLUMN (5-12%) CONTAINING 0.50% AMPHOLYTE AND 0.03% KCl.
- -95 VOLTS.

Figure 33. Components of Initial and Improved Isoelectric Focusing Procedures. The initial procedure is that of Behnke et al. (1975). The improved procedure is that presented in the Materials and Methods.

The 50 mm gradient column height electrofocusing procedure represents the best combination of the factors studied for obtaining minimal focusing time with good pH gradient linearity. The 112 mm gradient column height procedure represents the best combination of the factors studied for obtaining maximal pH gradient linearity and maximal resolution with satisfactory focusing time.

The reduction in time to reach equilibrium during isoelectric focusing which was achieved by shortening the gradient column height is not as pronounced as might be expected based on sedimentation equilibrium theory, where the time to reach equilibrium is proportional to the square of the solution column height. The difference in time reduction in isoelectric focusing and sedimentation is due to the fact that in sedimentation equilibrium experiments, the centrifugal field may be maintained as solution column height is decreased; in isoelectric focusing experiments, the electrical field must be reduced as the gradient column height is decreased. Maintaining the electric field strength as the gradient column height is reduced in isoelectric focusing results in heating due to the lower resistance and higher current flow across the shorter column.

CHANGE	EFFECT ON FOCUSING TIME	EFFECT ON pH GRADIENT
ADDING SUCROSE TO THE GEL PLUG	+10%	LINEARITY IMPROVED
ACETIC ACID REPLACING SULFURIC ACID	+20%	MINIMUM pH RAISED
ADDING KCl TO GRADIENT AND RESERVOIRS	-20%	MINIMUM pH LOWERED
INITIALLY DISTRIBUTING AMPHOLYTE UNIFORMLY RATHER THAN AS A GRADIENT	- 5%	LINEAR (pH 10.5 to 2.5)
REDUCING AMPHOLYTE CONCENTRATION	-10%	"
REDUCING COLUMN HEIGHT	-65%	~LINEAR (pH 10.5 to 3.0)

Figure 34. Summary of the Steps in the Development of an Improved Procedure for Isoelectric Focusing in Sucrose Density Gradients. Presented are the changes made in the initial procedure for isoelectric focusing (Behnke et al., 1975) and their effects upon focusing time and pH gradient linearity.

CHAPTER 6

Purification and Partial Characterization of Pig Liver Ferritin

ABSTRACT

A yellow material copurified with fatty acid synthetase when using the procedure of Kim, Unkefer, and Deal (1977) to purify fatty acid synthetase. A portion of this material was removed at several steps in the purification, but some remained with the enzyme even after sucrose density gradient centrifugation. This material has been shown to be the iron storage protein, ferritin.

Since ferritin copurifies with fatty acid synthetase, it was of interest to determine whether there was any interaction between them. Also, ferritin is the major protein extracted in water from the microsomal pellet fraction of pig liver described in Chapter 3 and Chapter 4. So it was also of interest to see whether ferritin inactivates fatty acid synthetase. Consequently, a purification procedure for ferritin was developed.

The purification procedure for pig liver ferritin is a modification of the method of Kim, Unkefer, and Deal (1977) for the purification of fatty acid synthetase. It includes ammonium sulfate fractionation of the centrifuged crude extract, DEAE-cellulose pad chromatography, and sucrose density gradient centrifugation. An extremely pure preparation of the protein is obtained: the banding pattern of the purified ferritin on native polyacrylamide gels is practically identical to that of commercially prepared horse spleen ferritin.

The subunit molecular weight of pig liver ferritin is about 20,000 daltons based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric point of the protein is 5.5 in 6 M urea and 5.8 in 2 M urea. The sedimentation coefficient of the protein on sucrose density gradients is independent of concentration. In sucrose density gradient sedimentation velocity experiments, the peak for protein as measured by the tannin turbidimetric method and the peak for iron as measured by absorbance at 480 nm were not coincident; the iron peak was further down the gradient than the protein peak. The solubility of ferritin

depends strongly upon its oxidation state: in TE buffer containing 0.33 saturated ammonium sulfate, ferritin will air-oxidize and precipitate over a period of weeks; in TE buffer containing 10 mM dithiothreitol, 0.60 saturated ammonium sulfate is required to precipitate the protein.

Native polyacrylamide gel electrophoresis showed that the native conformation of fatty acid synthetase was not changed by incubation with ferritin. However, inactivation of fatty acid synthetase by ferritin was demonstrated.

INTRODUCTION

A yellow-colored substance copurified with fatty acid synthetase during the latter's purification by the method of Kim, Unkefer, and Deal (1977). This substance has been identified as the iron storage protein, ferritin. Ferritin is comprised of an approximately spherical hollow protein shell containing up to 4500 atoms of iron in the form of an oxyhydroxidephosphate. It is also the major protein extracted from the microsomal pellet fraction from pig liver; this fraction was shown in Chapter 3 and Chapter 4 to inactivate fatty acid synthetase. So it was of interest to determine whether there was any interaction between fatty acid synthetase and purified ferritin. This chapter describes a purification procedure for pig liver ferritin and the results of tests for interaction between it and fatty acid synthetase. Some properties of the purified ferritin are also given.

MATERIALS AND METHODS

Reagents. Dithiothreitol was a product of Eastman-Kodak. DEAE-cellulose (standard, 0.91 meq/g) was obtained from Schleicher and Schuell. Coomassie Brilliant Blue R, horse spleen ferritin, gum arabic, 2-mercaptoethanol, sodium dodecyl sulfate, and tris(hydroxymethyl)aminomethane were purchased from Sigma Chemical Co. Ampholyte (Biolyte 3/10) was obtained from BIORAD; it had a pH range of 3 to 10 and a concentration (w/v) of 40% solids. N,N,N',N'-tetramethylethylenediamine was a product of Canalco. Ammonium persulfate, N,N'-methylenebis(acrylamide), and acrylamide were obtained from EM Laboratories. Phenol (88%, liquified) was a product of Mallinckrodt. Tannic acid was purchased from Baker Chemical Co. All other reagents were of analytical reagent grade.

Absorbance Measurements. Iron content of ferritin was estimated by measuring absorbance at 480 nm using a Gilford Model 2000 absorbance recorder with water as the reference.

Protein Determinations. Protein concentrations were determined by the tannin turbidimetric method described in Chapter 2.

Native Polyacrylamide Gel Electrophoresis. Native polyacrylamide gel

electrophoresis was performed using the modified method of Clark and Switzer (1977) as described in Chapter 2.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis was performed using a modification of the method of Clark and Switzer (1977) as described below. Distilled deionized water was used throughout.

The stock reagents for the procedure were the following:

1. SDS gel buffer: 7.80 g $NaH_2PO_4 \cdot H_2O$, 20.44 g Na_2HPO_4, 2.00 g sodium dodecyl sulfate, and water to two liters.
2. BIS-AC solution: 22.2 g acrylamide, 0.50 g N,N'-methylene-bis(acrylamide), and water to 100 ml. This solution is filtered with Whatman No. 1 filter paper and is stored at 4°.
3. Persulfate solution: 15 mg ammonium persulfate per ml water; this solution is prepared just before use.
4. SDS stock solution: 1.00 g sodium dodecyl sulfate in 10 ml water.
5. Dye solution: 0.2 mg bromphenol blue in 10 ml 50% (v/v) glycerol in water.
6. Staining solution: 120 mg Coomassie Brilliant Blue R is dissolved in 125 ml isopropanol; 50 ml glacial acetic acid and 325 ml water are then added.
7. Destaining solution 1: 500 ml isopropanol and 200ml glacial acetic acid to two liters with water.
8. Destaining solution 2: 300 ml 95% ethanol and 280 ml glacial acetic acid to four liters with water.
9. Protein standard solution: In these experiments, this solution contained each of the following proteins at 1 mg/ml: Bovine serum albumin, yeast hexokinase, yeast enolase, and rabbit muscle glycerol-3-phosphate dehydrogenase-triose phosphate isomerase; the molecular weights of which are 68,000, 51,000, 44,000, 34,000, and 26,500, respectively.

The electrophoretic procedure is as follows:
Gel tubes of dimensions 7 mm (o. d.) x 13 cm were sealed at one end with Parafilm. The tubes were then mounted vertically and the aqueous gel solution was prepared on ice. The gel solution contained 5.00 ml SDS gel buffer, 4.50 ml BIS-AC solution, 0.5 ml persulfate solution, and 12 µl TEMED for each six tubes. Immediately after preparing the gel solution, it was added to the gel tubes (1.8 ml per tube) using a Pasteur pipet. A thin layer of water was then carefully added to the top

of the gel solution in each tube. Polymerization was allowed to occur for at least one hour.

While the gel solution was polymerizing, the protein samples were prepared by adding (for each sample in its separate tube) protein (10-50 μl of a 1-2 mg/ml solution), SDS stock solution (5 μl), and 2-mercaptoethanol (5 μl). The samples were then heated in a boiling water bath for ten minutes. After cooling, dye solution (50 μl) was added to each sample.

After polymerization of the gel solution in the gel tubes, the water overlayers were removed. The tubes were then placed in the upper electrophoresis chamber with the unfilled end of the tube upward. Both reservoirs were filled with SDS gel buffer and any air bubbles at either end of the gel tubes were removed. The boiled samples were then applied to the gels using an Eppendorf pipet; each tube received one sample, applied just above the gel to minimize mixing with the reservoir solution. The positive lead from the power supply was attached to the lower reservoir and the negative lead to the upper reservoir. Electrophoresis was then performed at 90 volts until the dye front reached the bottom of the tubes. The power supply was turned off and the gel tubes were removed.

The gels were removed from the tubes by gently rimming the lower end of the tube with a 22 gauge needle supplied with a stream of water from a faucet. The gels were cut at the front edge of the tracking dye and were then placed in 16 x 150 mm screw-top test tubes which contained staining solution. Staining was allowed to occur overnight. The staining solution was then decanted from the gels and was replaced with destaining solution 1. Destaining in this solution was allowed to occur overnight. The solution was then decanted and replaced with destaining solution 2. Destaining with this solution was continued until the gels were adequately destained.

Isoelectric Focusing. Isoelectric focusing was performed using the standard 112 mm gradient height procedure described in Chapter 5 except the gel plugs contained 30% sucrose and the system contained no KCl. Also, for isoelectric focusing in 2 M urea, the sucrose density gradient contained 2 M urea and 750 μg ferritin protein, the upper reservoir contained 2 M urea in 1.8% ethanolamine, and the lower reser-

voir contained 2 M urea in 1.8% sulfuric acid. For isoelectric focusing in 6 M urea, the sucrose density gradient contained 6 M urea and 350 μg ferritin protein, the upper reservoir contained 6 M urea in 1.2% ethanolamine, and the lower reservoir contained 6 M urea in 1.2% sulfuric acid. Duplicate gradients were prepared for each sample; one gradient was fractionated after two hours at 200 volts and the other tube was fractionated after four hours at 200 volts. The position of ferritin in the gradients was determined both by measuring the absorbance of the fractions at 280 nm and by performing native polyacrylamide gel electrophoresis on the fractions.

Sucrose Density Gradient Centrifugation and Sedimentation Velocity Analysis. An ISCO Model 570 gradient former was used to form 36 ml linear 10 to 25% sucrose density gradients buffered with TED buffer in 1 x 3½ inch polyallomer tubes. For purification of ferritin, centrifugation was at 20° for four hours at 25,000 rpm in a Beckman SW27 rotor. After centrifugation, fractions (1.5 ml) were collected using an ISCO Model 183 gradient fractionator with a flow rate of 3 ml/min. An ISCO Model UA-5 absorbance monitor with a 10 mm pathlength flow cell was used to detect absorbance at 280 nm during fraction.

For analysis of the relative protein-to-iron ratio in purified ferritin, the above procedure was used except 20 mg ferritin protein in 2 ml PED buffer was applied to the gradient, centrifugation was performed at 27,000 rpm for three hours, and the gradient was fractionated into 1.00 ml fractions.

For sucrose density gradient sedimentation velocity analysis of ferritin, 5.3 ml linear 5 to 20% sucrose density gradients containing PED buffer were prepared in ½ x 2 inch cellulose nitrate tubes. Purified ferritin (0.15 ml) at a concentration between 0.1 and 10 mg protein per milliliter was applied to the gradients. Centrifugation was performed at 47,000 rpm for 60 minutes in a Beckman SW50.1 rotor at 25°. The gradients were then fractionated, monitoring the absorbance at 280 nm with an ISCO Model UA-5 absorbance monitor. Fractionation was performed at 0.75 ml per minute, and 0.25 ml fractions were collected.

RESULTS

Pig liver ferritin was initially isolated as a byproduct in the purification of fatty acid synthetase by the method of Kim, Unkefer, and Deal (1977). The initial steps in the purification of ferritin described below are therefore modified from the procedure for the purification of fatty acid synthetase.

Ammonium sulfate fractionation. The method of Kim, Unkefer, and Deal (1977) for the purification of fatty acid synthetase was followed through the first dialysis step with these modifications: the first ammonium sulfate fractionation was changed from 0.2 to 0.33 saturation to 0.30 to 0.40 saturation, and the second ammonium sulfate fractionation was taken to 0.40 saturation rather than 0.33 saturation. These changes reflect the fact that ferritin is slightly more soluble than fatty acid synthetase in ammonium sulfate solution at this stage in its purification.

DEAE-cellulose chromatography. The dialyzate obtained following the ammonium sulfate fractionations was centrifuged at 20° for 30 minutes at 18,000 rpm in a Sorvall SS-34 rotor to remove a soft-packing precipitate. The supernatant was then applied to a 16 (d) x 4 cm (h) DEAE-cellulose pad which had been formed over a piece of Whatman No. 1 filter paper in a Buchner funnel. After the applied sample was absorbed, the pad was washed with one liter of TED buffer and one liter 50 mM KCl in TED buffer; the eluates were discarded. Ferritin was then eluted as a bright orange solution using 1.5 liters of 300 mM KCl in TED buffer. The eluted ferritin was cooled to 6° and ammonium sulfate was added to 0.30 saturation. After stirring for 15 minutes, the suspension was centrifuged at 11,000 rpm for 25 minutes at 0° in a Sorvall GSA rotor. The supernatant was brought to 0.50 saturation ammonium sulfate with stirring to precipitate the ferritin. The suspension was centrifuged at 11,000 rpm for 25 minutes at 0° in a Sorvall GSA rotor. The pellets were collected and suspended in a minimal volume of TED buffer (less than 20 ml). The ferritin suspension was then dialyzed against one liter of TED buffer at 4° until the ferritin was solubilized.

Sucrose density gradient centrifugation. The dialyzate from the DEAE-cellulose chromatography step was centrifuged in a Sorvall SS-34 rotor at 16,000 rpm for 20 minutes at 20°. The supernatant was divided into

six equal portions and was applied to six SW27 gradients. Centrifugation was performed as indicated in Methods. Following fractionation of the gradients, the bottom two-thirds of the gradients were combined, taken to 4°, and brought to 0.50 saturation with saturated ammonium sulfate solution. After stirring for 15 minutes, the suspension was centrifuged in a Sorvall SS-34 rotor at 0° for 20 minutes at 16,000 rpm. The pellets were collected, suspended in a minimal volume of TED buffer, and dialyzed against TED buffer until the ferritin dissolved. Once solubilized, the ferritin is ready for a second SW27 centrifugation. Density gradient centrifugation is repeated until native polyacrylamide gel electrophoresis no longer reveals contaminants. The contaminants are visible after the first SW27 centrifugation as a slower moving shoulder in the gradient fractionation profile at 280 nm (Figure 35).

Characteristics of the Protein: Identity. The purified protein was identified as ferritin by comparison of its electrophoretic behavior with that of commercial horse spleen ferritin. In our native polyacrylamide gel system, both produced bands with relative mobilities of 0.04 and 0.12. The band with higher relative mobility was always present in greater quantity (Figure 36).

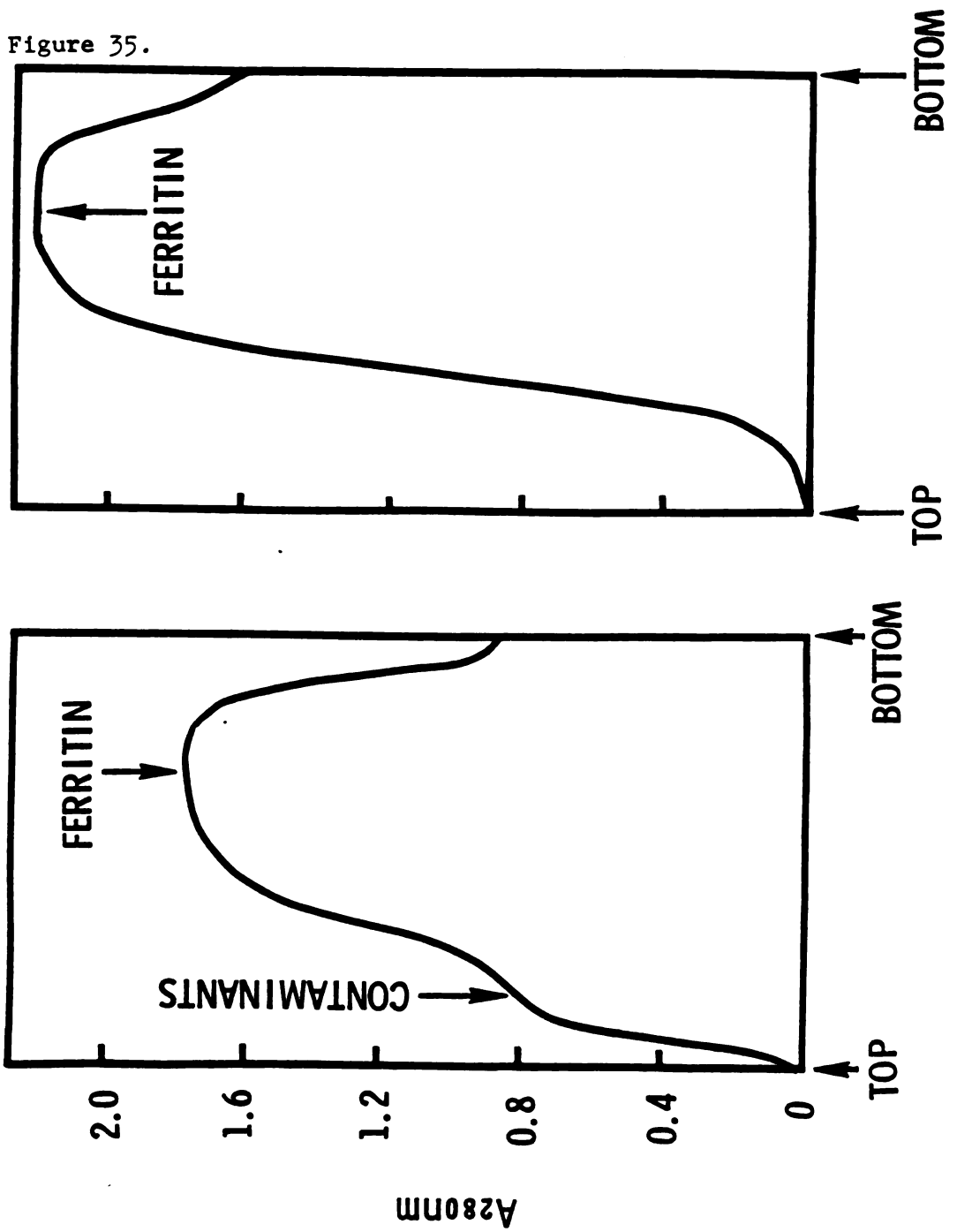
Subunit molecular weight. The molecular weight of pig liver ferritin subunits was about 20,000 daltons, based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When ferritin was incubated with 2-mercaptoethanol and sodium dodecyl sulfate, the solution changed in color from orange to rose to colorless. When the sample was applied to the gels and subjected to electrophoresis, an orange material (presumably an iron oxyhydroxide) remained at the top of each gel; the ferritin subunits, which migrated into the gel, were colorless.

Isoelectric point. Ferritin precipitated when it was subjected to isoelectric focusing in the absence of urea. Although some of the protein precipitated in 2 M urea, a preliminary value of 5.8 was obtained for the isoelectric point of ferritin. In 6 M urea, the protein remained soluble, and a value of 5.5 was determined for its isoelectric point.

Nonuniform iron distribution. The preparation of pig liver ferritin was polydisperse as determined by sucrose density gradient sedimentation velocity analysis; that is, the protein shells of ferritin enclosed

Figure 35. Sucrose Density Gradient Sedimentation Velocity Analysis of Partially and Fully Purified Ferritin. Centrifugation was performed using the procedure given in Methods. The contaminants are visible as a shoulder on the slower sedimenting side of the ferritin peak (a). This shoulder is absent from the absorption profile at 280 nm for the gradient with fully purified ferritin (b).

Figure 35.


A280nm
2.0
1.6
1.2
0.8
0.4
0
TOP
BOTTOM
CONTAMINANTS
FERRITIN
TOP
BOTTOM
FERRITIN

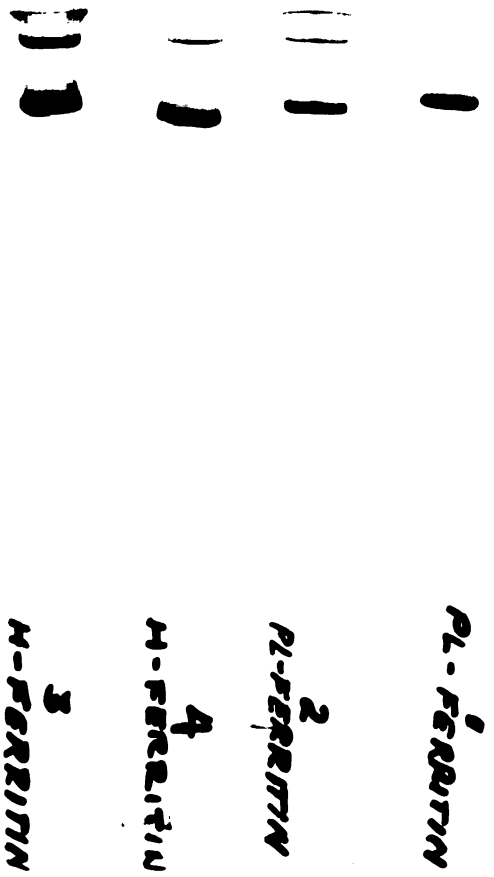


Figure 36. Native Polyacrylamide Gel Electrophoresis Banding Patterns of Purified Pig Liver Ferritin and Commercial Horse Spleen Ferritin. Polyacrylamide gel electrophoresis was performed using the standard procedure described in Chapter 2. Pig liver ferritin (PL-ferritin) and horse spleen ferritin (H-ferritin) were both applied at two different concentrations.

variable amounts of iron. Protein and iron concentrations were determined throughout a sucrose density gradient (see Methods). The maximum protein concentration, as measured using the tannin turbidimetric method, was located several fractions from the maximum iron concentration, as measured by absorbance at 480 nm; the iron peak was further down the gradient (Figure 37). Hence, ferritin molecules on the slower sedimenting shoulder of the protein peak contained less iron than those molecules on the faster sedimenting shoulder of the protein peak.

Oxidation state-dependent solubility. The solubility of pig liver ferritin was strongly dependent on its oxidation state. Most of the ferritin in the clarified centrifuged extract precipitated at 0.40 saturation ammonium sulfate. After overnight exposure to 1 mM dithiothreitol during the purification, only a little of the ferritin was precipitated at 0.40 saturation, and 0.50 saturation was needed for complete precipitation. If ferritin in the same solution was taken to 0.33 saturation and stored at 4° for several weeks, however, it precipitated, presumably due to air-oxidation. In TE buffer containing 10 mM dithiothreitol, 0.60 saturation ammonium sulfate was needed to precipitate the ferritin.

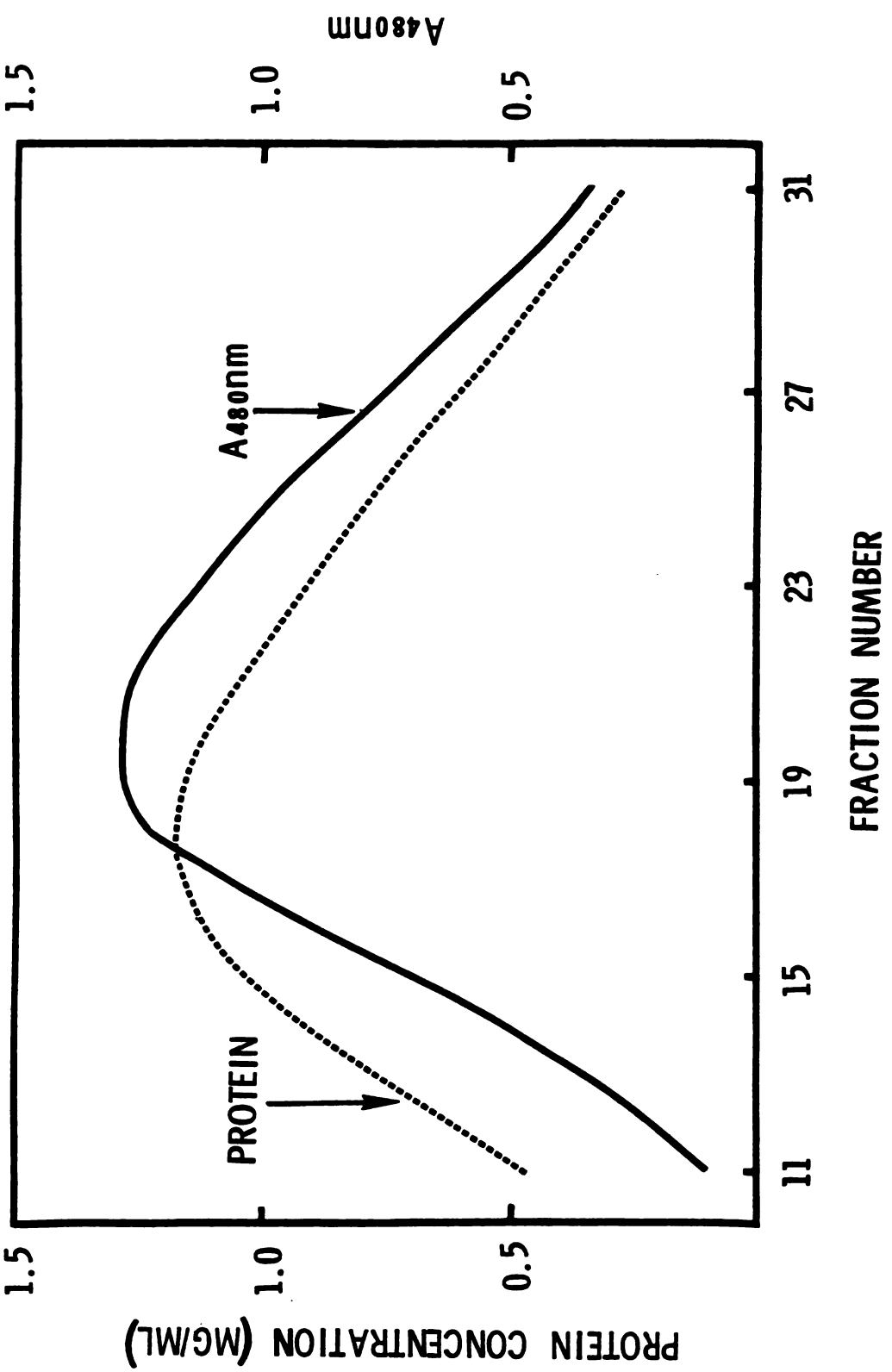
Concentration independent sedimentation coefficient. Despite the extremely oxidation dependent solubility of ferritin, the sedimentation coefficient of the soluble protein was essentially constant. Ferritin subjected to sucrose density gradient sedimentation velocity analysis at concentrations between 0.1 and 10 mg protein per milliliter had invariant sedimentation rates (Figure 38). Hence a concentration dependent association of ferritin seems unlikely.

Interaction of Fatty Acid Synthetase with Ferritin. Interaction between ferritin and fatty acid synthetase was tested by two methods. In the first method, ferritin and fatty acid synthetase were mixed to give protein concentrations of 7 mg/ml and 0.6 mg/ml, respectively, in TE buffer containing 100 mM dithiothreitol. After incubation at 23° for 26 hours, native polyacrylamide gel electrophoresis was performed. The relative mobilities of ferritin and fatty acid synthetase were not altered, and no new bands were detected.

In the other method, ferritin and fatty acid synthetase were combined and incubated at 37° in TED buffer. The concentration of fatty acid

Figure 37. Sucrose Density Gradient Sedimentation Velocity Analysis of Ferritin: Heterogeneity of the Protein with Respect to Iron Content. Centrifugation was performed as described in Methods. Protein concentration was determined using the tannin turbidometric method. Iron concentration was estimated using the absorbance at 480 nm. Only fractions 11 through 31 (obtained upon fractionation of the gradient) were analyzed.

Figure 37.


A480nm
1.5
1.0
0.5
A480nm
PROTEIN
PROTEIN CONCENTRATION (MG/ML)
1.5
1.0
0.5
11
15
19
23
27
31
FRACTION NUMBER

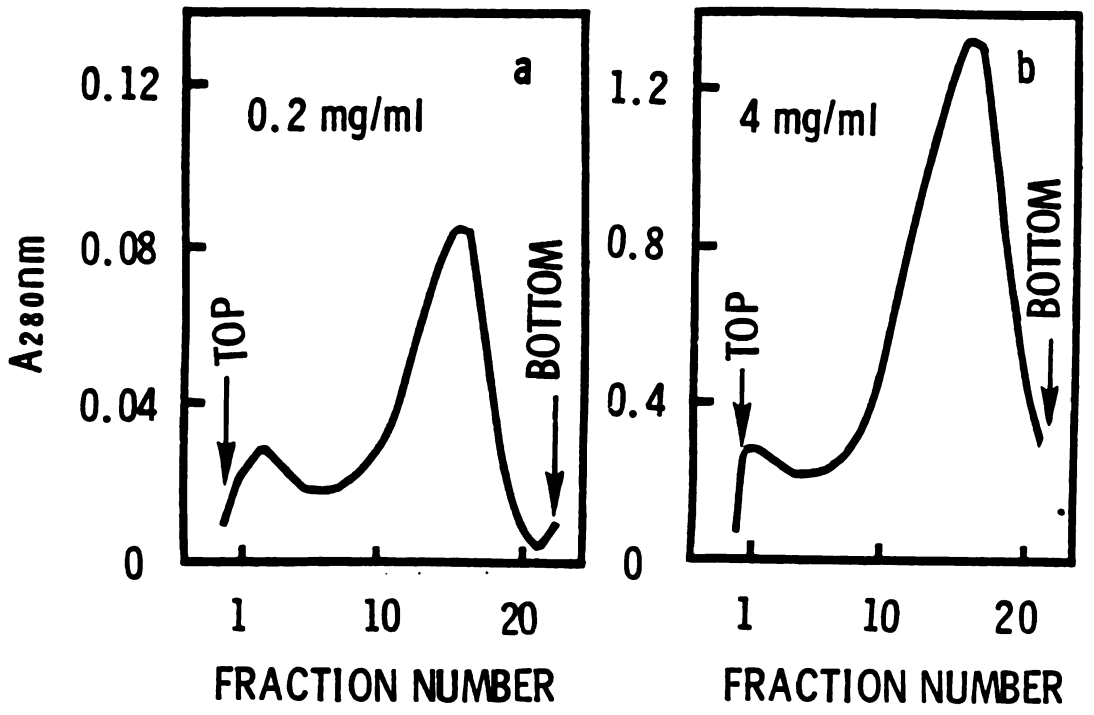


Figure 38. Sucrose Density Gradient Sedimentation Velocity Analysis of Ferritin at Two Concentrations. Centrifugation was performed using the procedure given in Methods. Ferritin in TED buffer (0.15 ml) at either 0.2 mg/ml (a) or at 4.0 mg/ml (b) was applied. Absorbance at 280 nm was measured during fractionation of the gradients.

synthetase was sufficient to yield an initial absorbance change of 0.05 per minute when 10 μl of incubation mixture was assayed for overall FAS activity. Two different ferritin concentrations were tested. Half-lives of overall FAS activity in incubation mixtures with 0.8 mg/ml and 4 mg/ml ferritin were 750 and 350 minutes, respectively, after taking into account the initial stabilization of overall FAS activity by dithiothreitol.

DISCUSSION

A procedure for the purification of ferritin has been presented. The purified protein is practically identical to the commercially prepared horse spleen ferritin in banding pattern on native polyacrylamide gels. The most interesting observations about the protein are its oxidation-state dependent solubility in ammonium sulfate solutions and its inactivation of fatty acid synthetase.

Ferritin in a more highly oxidized state has been shown to be less soluble in ammonium sulfate solutions. One explanation for this phenomenon is a change in conformation of the protein when its exposed sulfhydryl groups are oxidized. As was demonstrated by sucrose density gradient sedimentation velocity analysis and native polyacrylamide gel electrophoresis, this conformational change does not detectably alter the sedimentation or electrophoretic behavior of the protein. So, apparently, oxidation causes movement of nonpolar amino acid residues to the outer surface of the shell or movement of polar residues to the interior of the shell, or both.

Inactivation of fatty acid synthetase by ferritin can be explained as a change in the oxidation state of the enzyme. When fatty acid synthetase was incubated in high concentration of reducing agent (e. g., 45 mM dithiothreitol or 50 mM 2-mercaptoethanol), overall FAS activity initially increased, but then declined rapidly until it was lost completely (Figure 2). When fatty acid synthetase was incubated in TE buffer, air-oxidation of the enzyme occurred, resulting in inactivation of the enzyme. So there is apparently a narrow range of redox environments in which fatty acid synthetase is stable. Ferritin may alter the environment sufficiently to destabilize (inactivate) the enzyme.

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