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ANALYSIS OF PARTIAL REACTION STEPS WITH REPRESENTATIVE
SATURATED AND POLYUNSATURATED SUBSTRATES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

John Thomas Bernert, Jr., B.A., M.S.

* * * * *

The Ohio State University
1977

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<td>acyl-NHS</td>
<td>O-acyl ester of N-hydroxysuccinimide</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CoA</td>
<td>Coenzyme A</td>
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<td>CMC</td>
<td>Critical micelle concentration</td>
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<td>DTNB</td>
<td>5,5'-Dithiobis-(2-nitrobenzoic acid)</td>
<td></td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>ECL</td>
<td>Equivalent chain length</td>
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<tr>
<td>GLC</td>
<td>Gas-liquid chromatography</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<tr>
<td>IR</td>
<td>Infrared</td>
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<tr>
<td>k'</td>
<td>Apparent pseudo first-order rate constant</td>
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<tr>
<td>k''</td>
<td>Apparent Michaelis constant</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
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<tr>
<td>pTSA</td>
<td>para-Toluene sulfonic acid</td>
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<tr>
<td>RRT</td>
<td>Relative retention time</td>
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<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>Vmax'</td>
<td>Apparent maximum velocity</td>
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<td>v/s</td>
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INTRODUCTION

Fatty acid may be regarded as a generic term encompassing a variety of naturally occurring saturated and unsaturated long chain carboxylic acids. These compounds are important primarily as membrane constituents, energy storage forms and, in certain cases, as potential precursors of the prostaglandins. Although the diet may be an important source of fatty acids, and is in fact the only ultimate source of acids in the linoleate and linolenate families in mammals, many fatty acids may be totally synthesized in the animal through the integrated functioning of the cytoplasmic de novo fatty acid synthetase and the membrane bound desaturase and chain elongation systems. Through the activity of the latter two enzyme systems, in conjunction with partial \( \beta \)-oxidation (retroconversion) in the mitochondria, the animal may modify fatty acids obtained through the diet or synthesized de novo in order to meet its requirements.

Despite a central role of fatty acid chain elongation in lipid metabolism, relatively little is known about the process. It has been demonstrated that chain elongation occurring in the endoplasmic reticulum is malonyl-CoA dependent and involves a mechanism essentially identical to one cycle of the de novo synthetase, passing through \( \beta \)-keto, \( \beta \)-hydroxy and 2-trans intermediates, all two carbon atoms longer than the priming substrate. Chain elongation may also be effected by an acetyl-CoA dependent process in the mitochondria, although the biosynthetic significance of this path remains unclear. It has been suggested that
mitochondrial fatty acid chain elongation may pertain to energy metabolism rather than fatty acid chain extension *per se*.

The object of this investigation was to examine microsomal fatty acid chain elongation *in vitro* with representative saturated and polyunsaturated substrates in an attempt to answer two fundamental questions: (1) does chain elongation involve a single enzyme system operative with all substrates, or do separate substrate-specific systems exist; and (2) what factors contribute to the regulation of chain elongation? Due to the relatively complex nature of the overall chain elongation process, an analysis of individual reaction steps in the sequence seemed to offer the potential for a systematic evaluation of these questions. Consequently, studies were undertaken to develop reliable procedures for the analysis of these individual reaction steps which have been utilized in a further evaluation of the nature and regulation of the microsomal fatty acid chain elongation process.
CHAPTER I
LITERATURE REVIEW

Fatty Acid Biosynthetic Pathways

The elucidation of the processes by which fatty acids are biosynthesized has been a fairly recent development. One of the first indications that de novo synthesis did not simply involve reversal of the β-oxidation pathway as had previously been suspected was provided by studies demonstrating a requirement for malonyl-CoA in the reaction (1,2). It was further established that NADPH rather than NADH was the preferred cofactor (3,4). Subsequent detailed studies conducted primarily in the laboratories of Wakil (5) and of Lynen (6,7) have greatly contributed to an understanding of the nature and mechanism of de novo fatty acid biosynthesis in higher organisms. Although the major fatty acid synthetic activity was found to involve a soluble de novo process, a number of early reports described an additional particulate activity which was eventually subdivided into a malonyl-CoA dependent reaction in the microsomes and an acetyl-CoA dependent mitochondrial process (8-12). Both of the latter pathways were subsequently shown to involve a chain elongation mechanism rather than de novo synthesis.

Although the reversal of β-oxidation seemed to offer a logical explanation for the acetyl-CoA dependent mitochondrial pathway, this proposal suffered from an unfavorable equilibrium for the thiolase reaction and a consequent positive $\Delta G^0$ for the overall reaction.
sequence. However, the discovery by Langdon (13) of an NADPH-specific enoyl-CoA reductase in the mitochondrion led to a suggestion of the means by which this limitation might be circumvented. Inclusion of the NADPH-specific enoyl-CoA reductase in place of the flavin-linked fatty acyl-CoA dehydrogenase, in conjunction with the remaining β-oxidation enzymes, provides for a thermodynamically feasible synthetic route (14,15). While such a system has been shown to function *in vitro* (14), its significance for fatty acid chain elongation *in vivo* remains uncertain. Recently, Seubert and Podack (16) have proposed that the true function of this mitochondrial path may relate to energy metabolism rather than fatty acid chain extension. Recent reports have also suggested the existence of separate fatty acid elongation systems in the inner and outer membranes of beef heart and liver mitochondria (17-19) in addition to the putative β-oxidation reversal path. However, a certain degree of controversy continues concerning the significance of these systems in the cellular economy, due in part to their isolation from the closely related fatty acyl-CoA desaturases which are known to be strictly microsomal (20-22).

By contrast, considerable evidence has been accumulated suggesting that the malonyl-CoA dependent elongation process in the microsomes is an important means of modifying cellular fatty acid composition. Early studies by Abraham *et al* (23) and Lorch *et al* (24) established that rat liver microsomes, when incubated with NADPH and ATP, were capable of incorporating the labeled carbon from [2-14C] malonyl-CoA into fatty acids, but could not do so when the malonyl-CoA was replaced by either [1-14C] acetate or [1-14C] acetyl-CoA. They further established that contamination of the microsomal preparations by either *de novo* synthetase
activity or with mitochondrial enzymes could not account for these results. That this microsomal activity involved a malonyl-CoA dependent elongation of long chain fatty acids was first clearly demonstrated by Nugteren (25) and by Stoffel and Ach (11). The latter authors also found that a variety of saturated, monounsaturated and polyunsaturated fatty acids could be elongated as their CoA thioesters by this system in rat liver microsomes.

Subsequent studies by Nugteren (12) confirmed the microsomal location and broad substrate specificity of this system, and provided the first evidence that the following predicted reaction sequence was indeed involved in the chain elongation process:

\[
\begin{align*}
(1) & \quad R-C-(S)CoA + HOOC-CH_2-C-(S)CoA \rightarrow R-C-CH_2-C-(S)CoA + CoA + CO_2 \\
(2) & \quad R-\cdot CH_2-\cdot C-(S)CoA + NADPH + H^+ \rightarrow D(-) R-\cdot CH-\cdot CH_2-\cdot C-(S)CoA + NADP^+ \\
(3) & \quad D(-) R-\cdot CH-CH_2-\cdot C-(S)CoA \rightarrow R-C=C-C-(S)CoA + H_2O \\
(4) & \quad R-C-\cdot C=C-(S)CoA + NADPH + H^+ \rightarrow R-\cdot CH_2-CH_2-\cdot C-(S)CoA + NADP^+
\end{align*}
\]

Although the involvement of β-keto, β-hydroxy and 2-trans intermediates in the reaction sequence is reminiscent of the de novo synthetase, the microsomal system differs in both its primer specificity and in utilizing the CoA thioesters as intermediates rather than protein bound derivatives. Since CoA esters of the intermediates are active as substrates, it was possible for Nugteren to examine partial reactions in this system (12). It could thus be shown that in the myristic acid chain elongation sequence in rat liver microsomes, the conversion:

\[\beta\text{-keto}\text{palmitoyl-CoA} + 2 \text{NADPH} + 2 H^+ \rightarrow \text{palmitic acid} + H_2O + 2 \text{NADP}^+\]

occurred at a rate about ten times that of overall chain elongation. Conversion of DL β-hydroxypalmitoyl-CoA to palmitic acid (with NADPH) or to 2-trans-hexadecenoic acid (without NADPH), and the reduction of
2-trans-hexadecenoyl-CoA to palmitic acid, were also reported to occur much more rapidly than overall chain elongation. From these data it was possible to deduce that, at least in the myristic acid sequence, the initial condensation reaction was apparently rate-limiting overall (12).

Although rat liver has been the most common source, malonyl-CoA dependent fatty acid chain elongation has also been investigated in pigeon liver (26) in which the system appeared to be essentially the same. Chain elongation has also been shown to occur in microsomes from mammalian adrenals (27,28), adipose tissue (16,29), aorta (16) and kidney (30). Activity could not be detected in heart muscle, however, in which only the mitochondrial path was found (31,16). In addition, an active elongation system has been identified in the brain microsomes of rats (32-35), mice (36-38) and rabbits (39). Chain elongation in brain is of considerable importance due to the prominence of very long chain acids, particularly lignoceric and nervonic acid, in the sphingolipids of myelin. A relationship between fatty acid elongation and myelination is suggested both by the simultaneous onset of the two activities during early development in the mouse (36,40) and in the disruption of the long chain elongation system which has been found to accompany defective myelination in the "quaking" mouse mutant (36,38,41). There is no evidence that any of these systems differ materially in mechanism from the hepatic chain elongation process, but the substrate specificities may vary somewhat in different tissues.

**Hepatic Chain Elongation Substrate Specificity**

Although complicated by a lack of information concerning the number of enzymes involved in chain elongation, studies have been reported which provide some indication of the substrate specificity
for this reaction. There appears to be little doubt that one important factor is chain length, and it has been a common observation that rates of elongation tend to be higher with shorter chain substrates. For example, in rat liver microsomes the best saturated substrates are acids possessing 10-16 carbon atoms, while saturated acids of 18 or more carbons are very poorly elongated (12). In fact, stearate is probably metabolized almost exclusively to oleate in liver since this reaction proceeds rapidly in vitro, while the production of arachidic acid under the same conditions was found to be extremely slow (12,42). This same influence of chain length can be seen with unsaturated acids as well. When the number and position of the double bonds is constant so that a given proximal structure is maintained, the chain elongation rate may again be seen to decrease with increasing chain length. Thus it has been shown that under identical assay conditions, the elongation rate of palmitoleate is greater than that of oleate, while 6,9-16:2 is elongated more rapidly than 6,9-18:2, and 5,8,11-18:3 is much more rapidly elongated than is 5,8,11-20:3 (43).

In addition to chain length, the number of double bonds and their location in the potential substrate may exert a considerable influence on its ability to be elongated. Feeding studies by Schlenk et al (44,45) led them to propose that the proximal structure of the acid as dictated by double bond number and position is of considerable importance in determining its suitability for chain elongation. They found that 9,12-17:2 and 6,9,12-17:3 which possess structures identical to those of linoleate and linolenate respectively except for the absence of one methylene group in the distal portion of the chain were readily metabolized in vivo. By contrast, it was shown that 10,13-19:2 which
possess the terminal structure of linoleic acid but differs in its proximal configuration is not further elongated or desaturated in the rat. Sprecher et al (46) were also unable to detect any evidence of either desaturation or chain elongation when fat-deficient rats were fed 9,15-18:2, 12,15-18:2 or 7,13-20:2; all unnatural dienoic acids, two of which lack the normal divinyl methane pattern. It should be noted that these studies in intact rats required an initial uptake and activation of the acids to the CoA thioester prior to any further metabolism, and a structural influence on one or more of these preliminary processes cannot be completely discounted. Nevertheless, recovery from the tissues of both the administered acid and, in certain cases, partial degradation products was demonstrated, while longer chain and more highly unsaturated derivatives could not be detected (44,46).

When substrate chain length is constant, the influence of double bond number and position on the chain elongation rate may be more readily discerned. Within the octadecenoate series for example, a general trend toward increasing elongation activity with increasing substrate unsaturation can be demonstrated (43,47). Both 6,9,12-18:3 and 5,8,11-18:3 are very rapidly chain elongated by rat liver microsomes, while the rate with 6,9-18:2 is somewhat less and the elongation of oleate is rather slow (43). Again however, an influence of the substrates proximal structure must also be considered. While 6,9-18:2 is chain elongated at a moderately rapid rate, the elongation activities of 8,11-18:2 and 9,12-18:2 are only about 10% of that observed with the n-9 isomer. Similarly, the chain elongation of 5,8,11-20:3 was found to occur about five times as rapidly as that of 8,11,14-20:3 (43).
This positional effect has been clearly shown by Marcel and Holman (48) who examined an isomeric series of cis, cis methylene interrupted octadecadienes with the first double bond in positions 6 through 10. The 6,9 isomer was found to be the most active in this series, with a progressive decline in rate until a minimum was noted with linoleate; a rate moderately increased above that of linoleate was reported for the 10,13 isomer. It is interesting that in a study of the microsomal chain elongation activity of twelve saturated and unsaturated fatty acids, six of the eight actively elongated substrates possessed two or more double bonds with the first located in the 5 or 6 position (43). The four poorly converted substrates all had their first double bond in the 8 or 9 position. Palmitate and palmitoleate behaved exceptionally in this study, with both yielding rather high rates of chain elongation. However, with substrates possessing fewer than 18 carbon atoms, a chain length effect may predominate.

Podack et al (28) assayed the microsomal enoyl-CoA reductase reaction with a variety of short chain model substrates and found that 2-trans-hexenoyl-CoA was most rapidly reduced. The rate was not greatly lowered when substrates of 8 or 10 carbons were employed, but 2-trans-dodecenoyl-CoA and 2-trans-hexadecenoyl-CoA were relatively poorly converted and displayed considerable substrate inhibition which was attributed by the authors to an increasing interference of the alkyl residue with a hydrophobic region of the enzyme. Although 2-trans-hexenoyl-CoA is not a physiological substrate in this system, they proposed that it would possess the proximal configuration of a 6-cis unsaturated fatty acyl-CoA and that a preference for 6-unsaturated fatty acids in chain elongation might thus be attributed to this phenomenon.
However, the initial substrate in chain elongation is already extended at the carboxyl end by two carbons at the enoyl-CoA reductase step. Although the rate with 2-trans-octenoyl-CoA was also quite rapid and thus consistent with their proposal, a preference for 2-trans-hexenoyl-CoA in this reaction should, according to their interpretation, reflect an active elongation of 4-cis acids which has not been observed with acids of more than 16 carbon atoms (49,50). Also, while 2-trans-decenoyl-CoA was about as effective a substrate in this reaction as the 8 carbon derivative, fatty acids with their first double bond in the 8 position tend to be poorly elongated (43). Finally, the possibility that factors other than substrate specificity were involved in these results cannot be excluded since the inhibition by longer chain substrates occurred in the concentration range at which micelle formation would be expected to occur (51), while the shorter chain substrates which were not inhibitory would also be incapable of forming micelles.

Although currently available data do not permit a definitive evaluation of the effect of substrate structure on microsomal fatty acid chain elongation, it seems clear that the enzymes involved are capable of displaying a considerable degree of specificity which in turn may well contribute to the establishment of the fatty acid patterns characteristic of a given tissue. An understanding of the means by which this specificity is expressed, and the extent to which it is involved in regulating fatty acid chain elongation, awaits the availability of purified enzyme systems for further systematic analysis.

**Effect of Substrate Competition on Chain Elongation**

An additional means by which fatty acid metabolic paths might be regulated is through competitive substrate interactions. Studies conducted by Holman and his associates concerning the chain elongation of
linoleic acid (52) and of α- and γ-linolenic acids (53) suggested that a variety of saturated and unsaturated acids could influence these reactions in vitro. For example, all saturated fatty acids possessing 12-16 carbon atoms were effective inhibitors of linoleate elongation, giving greater than 50% inhibition relative to the control rate under the conditions employed. Pentadecanoic acid was found to be the best inhibitor of this series, with myristic acid nearly as effective. By contrast, saturated acids possessing fewer than 10 or more than 17 carbons were either ineffective or not inhibitory at all (52).

Among six polyunsaturated acids tested as potential inhibitors of linoleate chain elongation, the two most effective were α- and γ-linolenate. In fact, the 72% inhibition by α-linolenic acid was the greatest observed overall, while γ-linolenic acid was about as inhibitory as pentadecanoate. The converse was not apparently true, however, since the elongation of neither α- nor γ-linolenate was inhibited by linoleate. In fact, while the conversion of γ-linolenate was little affected, the elongation of α-linolenate was increased 80% when an equimolar amount of linoleate was included in the incubation (53). It was further demonstrated that the two triene isomers were mutually inhibitory - albeit mildly so - at higher concentrations, and that saturated acids influenced the elongation of γ-linolenate essentially the same as they did linoleate, while α-linolenate was not appreciably affected (53).

Although these results suggest that competitive substrate interactions can exert an influence on chain elongation activity in vitro, the marked variety of responses which may be elicited seems to defy any meaningful interpretation. One major problem with studies of this type involves the experimental design. When crude microsomal preparations are employed,
both substrate and the potential inhibitor may, after activation, serve as substrates for incorporation, desaturation and acyl-CoA hydrolase enzymes as well as for chain elongation. Consequently, it is necessary to assure that these competing reactions are not major determinants of the results obtained. In addition, the total amount of fatty acid present in the incubation as well as the fatty acid : microsomal protein concentration ratio may be expected to exert indirect effects on the reaction due to the detergency characteristics of both the fatty acid and its CoA derivative (51,54). Since these limitations are essentially impossible to completely circumvent at this time, the significance of competitive substrate interactions as measured in vitro in regulating fatty acid chain elongation in vivo remains uncertain and perhaps somewhat suspect.

The in vivo significance of reactions of this type may be investigated indirectly through an alternative approach employing competitive feeding experiments. Sprecher (55) has shown that while 6,9-18:2 and γ-linolenate exert strong mutual inhibition on their respective chain elongation reactions in vitro, such competition is not likely to be a major in vivo control point. If competitive substrate interactions were important in fatty acid metabolic regulation in the intact animal, one would expect a significant decline in the tissue arachidonate : 5,8,11-eicosatrienoate ratio as the level of 6,9-18:2 was increased. However, when a constant amount of γ-linolenate and variable amounts of 6,9-18:2 were fed to rats, no significant differences could be discerned in the hepatic levels of either arachidonate or 5,8,11-eicosatrienoate (56). While results obtained when arachidonate and 5,8,11-eicosatrienoate were fed competitively to rats suggest that competitive substrate interactions might be significantly involved at the level of incorporation into lipids
(56), it seems unlikely that substrate competition is of major consequence in the regulation of fatty acid chain elongation \textit{in vivo}.

\textbf{Other Potential Means of Regulating Chain Elongation}

Landriscina \textit{et al} (57-59) have suggested that the concentration of ATP may play an important role in the regulation of microsomal fatty acid chain elongation. Their studies have indicated that rat liver microsomes will incorporate label from [1,3-$^{14}$C] malonyl-CoA into fatty acids both in the presence and absence of ATP. However, in the absence of ATP the ratio of total to carboxyl $^{14}$C is greater than 7, and the predominant labeled acid is palmitic, while in the presence of ATP longer chain acids are recovered and the ratio of total to carboxyl $^{14}$C is greatly reduced. These results led them to propose that rat liver microsomes synthesize primarily palmitic acid by a \textit{de novo} mechanism in the absence of ATP, while in its presence the chain elongation of pre-existing acids predominates.

This phenomenon almost certainly reflects the requirement of ATP for activation of endogenous fatty acids prior to elongation. Nugteren (12) has shown that while CoA may be excluded from the incubation since the catalytic amounts required for chain elongation may be provided by concomitant malonyl-CoA deacylation, an absolute requirement for ATP exists. It has further been shown that this compound is obviated when the acyl-CoA derivative is employed (11,12,26). No evidence for a role of ATP in regulating chain elongation other than in fatty acid activation has yet been adduced. It would seem that since the activated form of the fatty acid is also a prerequisite for most of the synthetic and catabolic reactions in which it might participate, an important regulation of chain elongation by ATP is doubtful. It might be noted
that microsomal preincubation with ATP has been reported to affect the 6-desaturase (but not the 9-desaturase) to an extent beyond that attributable to CoA acylation alone (60). The significance of this observation remains somewhat uncertain, however, since the effect was only noted in twenty minute, low substrate - high protein incubations, and no effect could be demonstrated when initial velocity conditions were approximated (60).

The effect of a variety of hormones on fatty acid chain elongation has also been investigated, both with isolated particles in vitro and in cell cultures. In general, chain elongation activity does not appear to be responsive to hormonal regulation. Thus insulin, glucagon and epinephrine all had no effect on the elongation of linoleic or α-linolenic acid in isolated microsomes or in HTC cell cultures under the conditions employed (61,62). It has also been reported that dibutyryl-cAMP, at least in vitro, has no direct effect on chain elongation activity (59,60).

An indirect effect of cAMP might be expected, however, since this compound has been reported to inhibit acetyl-CoA carboxylase (63) and therefore would tend to deplete malonyl-CoA levels.

Faas et al (64) examined the effect of thyroxine in vitro and found that the addition of high concentrations of the hormone (10-100 μM) inhibited de novo fatty acid synthesis in rat liver supernatant. However, they could discern no consistent effect of thyroxine on the incorporation of label from [2-14C] malonyl-CoA into endogenous microsomal fatty acids, or on the chain elongation of exogenous palmitoyl-CoA. An augmentation of desaturation activity was detected, however, in confirmation of a previous report (65). In contrast, Landriscina et al (66) have recently reported that both triiodothyronine and thyroxine are capable of increasing de novo synthetase and microsomal chain elongation activity in rats,
while a propylthiouracil-induced hypothyroidism was associated with a pronounced diminution in activity. These authors suggested that the discrepancy between their results and those of Faas et al related to the experimental protocol since it was found necessary to administer the hormones to intact rats for greater than ten days before an effect could be demonstrated (66). However, Landriscina et al based their chain elongation assay solely on the incorporation of label from $[1,3-^{14}\text{C}]$ malonyl-CoA into endogenous microsomal fatty acids. Although an increase in this incorporation could be demonstrated in the treated animals, it was relatively small and always closely paralleled similar changes in the soluble de novo synthetase activity. Since it is well known that microsomal preparations tend to be persistently contaminated with de novo synthetic activity (35,57,67), their data do not completely exclude the possibility that these results primarily reflected an effect on this contaminating enzyme system rather than on chain elongation activity. It would seem that further studies are indicated before any reliable conclusion can be drawn concerning the effect of thyroid hormones on this process.

A number of studies have suggested that one potentially important means of regulating chain elongation activity in microsomes is through dietary influences. Elovson (68) has reported that while the fasting-induced repression of the hepatic fatty acid desaturase reaction in rats is more pronounced, chain elongation of palmitic acid is also depressed under the same conditions, with the maximum effect of fasting occurring within two days. Studies by Donaldson et al (31) based on the incorporation of label from either $[1-^{14}\text{C}]$ acetyl-CoA or $[1$ or $3-^{14}\text{C}]$ malonyl-CoA into endogenous fatty acids also demonstrated a marked
depression (83%) in the hepatic microsomal elongation reaction using starved rats, while the mitochondrial process was unaffected.

Sprecher (55) has studied dietary influences on microsomal chain elongation activity with specific fatty acid substrates and has shown that the elongation of palmitic acid, 6,9-octadecadienoic acid and 6,9,12-octadecatrienoic acid, when expressed on a specific activity basis, declined during fasting. Refeeding a normal diet to the fasted rats led to a restoration of chain elongation activity as monitored with the polyunsaturated substrates, and to an enhancement of activity with palmitic acid. In addition, chain elongation activity with the latter substrate was shown to be considerably increased when the fasted animals were refeed a fat-free diet, while elongation of the unsaturated acids was much less responsive. These results not only supported the involvement of a dietary component in the regulation of fatty acid chain elongation, but were also noted by the author to suggest the presence of more than one chain elongation system in rat liver microsomes.

Further support for this contention was provided by substrate competition studies among the three substrates (55). It was found that in the in vitro assay system employed, palmitic acid had no apparent influence on the chain elongation of 6,9-octadecadienoate, and the elongation of palmitic acid was only modestly depressed (20-30%) in the presence of saturating levels of the octadecadienoate. Conversely, saturating amounts of either 6,9-octadecadienoate or 6,9,12-octadecatrienoate exerted strong inhibition on the chain elongation of the other, consistent with the involvement of a common elongation system for these two acids which is different than that operative with palmitate.
The influence of starvation and refeeding on the hepatic microsomal elongation of palmitic acid has recently been confirmed by Nakagawa et al (69) who also have suggested that phospholipids may play an important role in the regulation of chain elongation (69-71). It was found that the addition of a sonicated dispersion of phosphatidylcholine to an in vitro system enhanced the elongation of palmitoyl-CoA by both normal and refeeding rat liver microsomes (69). In addition, the linear hydrolysis of phospholipids by treatment of microsomes with phospholipase C was reported to be paralleled by a concomitant linear decline in chain elongation activity (70), which was restored by the subsequent addition of phosphatidylcholine (69,70). The effect of phosphatidylcholine was suggested to involve, at least in part, an alleviation of product inhibition by the incorporation of stearic acid into phospholipids. Since no such effect of the phosphatidylcholine dispersions on the desaturation of palmitic acid was noted, these authors proposed that the addition of the lipid dispersion could lead to a preferential utilization of palmitoyl-CoA by the elongation rather than desaturation pathway (71).

At the time of this writing the factors controlling chain elongation remain somewhat obscure. As indicated above, a number of potential means of regulation have been investigated, but generally with negative or uncertain results, although dietary influences seem rather likely. In addition, the important question concerning the number of enzymes or enzyme systems involved in microsomal chain elongation remains unresolved. Since chain elongation is known to involve a multistep sequence of reactions, it would seem desirable to evaluate component reactions in the sequence employing various substrates in order to establish which reaction is...
rate limiting and whether regulation is indeed mediated primarily through that reaction. Such information might then be evaluated within the larger continuum of which chain elongation is a part.
CHAPTER II
MATERIALS AND METHODS

Reagents

CoA(SH), NADPH, dicyclohexylcarbodiimide, glutathione and bovine serum albumin containing less than 0.005% free fatty acids were purchased from the Sigma Chemical Company, St. Louis, Missouri. Malonyl-CoA and acetyl-CoA were obtained from P&L Biochemicals, Milwaukee, Wisconsin. Ethyl chloroformate was purchased from the Eastman Chemical Company, Rochester, New York; while triethylamine was a product of Matheson Coleman and Bell, Cincinnati, Ohio. Sodium deoxycholate was obtained from Difco Laboratories, Detroit, Michigan; and N-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoic acid) and N-hydroxysuccinimide were all products of the Aldrich Chemical Company, Milwaukee, Wisconsin. The fat-deficient diet was purchased from General Biochemicals, Chagrin Falls, Ohio. All solvents were reagent grade and were not further purified except where otherwise indicated.

Palmitic acid, methyl palmitate and 2-heptadecanone were purchased from Applied Science Laboratories, State College, Pennsylvania; while [1-14C] palmitic acid and [2-14C] malonyl-CoA were obtained from New England Nuclear, Boston, Massachusetts. [1-14C] 6,9-Octadecadienoic acid and [1-14C] 6,9,12-octadecatrienoic acid were gifts from Dr. Sprecher, and were prepared by total organic synthesis using procedures which have been described (72,73). [3-14C]-Labeled and unlabeled methyl esters of β-ketostearate, β-hydroxystearate, β-hydroxy-8,11-eicosadienoate,
2-trans-octadecenoate and 2-trans-8,11-eicosatrienoate were also gifts from Dr. Sprecher which were synthesized according to the methods of Stoffel and Pruss (74). All fatty acid substrates were purified as their methyl ester and converted to the free acid by saponification. While a KOH-ethanol medium was suitable for saponification in most cases, the α, β-unsaturated esters tend to form methoxy adducts under these conditions (75,76). Consequently, the aqueous tert-butanol saponification medium suggested by Davidoff and Korn (76) was employed with the latter esters.

**Analytical Equipment**

Ultraviolet (UV) spectra were determined on a Beckman Acta II spectrophotometer equipped with a Sargent-Welch model SRG recorder, while infrared (IR) analyses were run in carbon tetrachloride or chloroform solution using a Perkin-Elmer model 421 grating infrared spectrophotometer. Thin-layer chromatography (TLC) plates were prepared from a slurry of Silica gel G (Brinkman Instruments) and water, 1:2 (w/v). After allowing the plates to air dry at room temperature, they were activated at 100° for one hour and stored in a dessicator until needed. For TLC analysis of acyl-CoA esters, potassium oxalate-impregnated Silica gel H plates were prepared by the method of Ullman and Radin (77). Gas-liquid chromatography (GLC) was conducted with an F & M model 810 gas chromatograph equipped with a thermal conductivity detector and a cartridge fraction collector. The stainless steel columns, 10 feet long and 0.25 inch in diameter, were packed with 15% ethyleneglycol succinate on Gas-chrom P, 80-100 mesh. The helium flow rate was 60 ml/minute and the oven was usually maintained at 180-190°. All liquid scintillation determinations were made with a Packard Tri Carb liquid scintillation spectrometer, model 3380.
Preparation of Standards

Since saponification is unsuitable for the production of free \( \beta \)-keto acids from their esters due to the alkali-sensitivity of these compounds, \( \beta \)-ketostearic acid was produced from its methyl ester for use as a TLC standard by the acid cleavage procedure of Mitzi et al. (78). The product was purified by repeated recrystallization from acetone as described by the authors. 7,10-Nonadecadiene-2-one was prepared for use as a GLC standard by subjecting methyl \( \beta \)-keto-8,11-eicosadienoate to an overnight saponification. The reaction mixture was then acidified, extracted twice with ethyl ether, and the pooled ether extracts refluxed for one hour prior to purification by TLC in petroleum ether-ethyl ether-acetic acid (70:30:1, by volume) using authentic 2-heptadecanone as a standard. The unsaturated ketone product was eluted from the plate with ethyl ether, and its identity confirmed by its IR spectrum.

The acetate of methyl \( \beta \)-hydroxystearate was prepared for use as a GLC standard by dissolving 25 mg of methyl \( \beta \)-hydroxystearate and 8 mg of p-toluene sulfonic acid in 2 ml of acetic anhydride (75). After stirring at room temperature for 30 minutes, 5 ml of water was added and the mixture was extracted three times with petroleum ether. The pooled extracts were washed with water, then dried over Na\( _2 \)SO\( _4 \). Thin-layer chromatographic analysis of the resulting material with the same solvent system as described above resulted in a single component migrating ahead of a methyl \( \beta \)-hydroxystearate standard. An IR spectrum of this product in CC\( \text{l}_4 \) disclosed the absence of any absorption in the vicinity of 3500 cm\(^{-1} \), a strong absorption at 1230 cm\(^{-1} \) and a relative intensification of the carbonyl band at 1730 cm\(^{-1} \), consistent with the presence of methyl \( \beta \)-acetoxy stearate. The retention time of this
material on GLC relative to that of methyl stearate was approximately 4.8.

Synthesis of Acyl-CoA Derivatives

$[1-^{14}C]$ Palmitoyl-CoA, $[1-^{14}C]$ 6,9-octadecadienoyl-CoA and $[1-^{14}C]$ 6,9,12-octadecatrienoyl-CoA were all prepared from their hydroxysuccinimide esters. Synthesis of the hydroxysuccinimide esters was in turn based on the dicyclohexylcarbodiimide coupling procedure of Lapidot et al. (79). An equimolar amount (325 μmoles) of free fatty acid and N-hydroxysuccinimide was dissolved in about 10 ml of dry ethyl acetate which had been redistilled and stored over a molecular sieve. To this solution was added slowly with stirring a slight excess of dicyclohexylcarbodiimide dissolved in 2 ml of the same solvent. The reaction mixture, which became quite turbid within a few minutes, was maintained with constant agitation under N$_2$ at room temperature overnight. The dense precipitate of dicyclohexylurea was then removed by vacuum filtration, and the solvent was removed under a stream of nitrogen.

In the synthesis of the palmitate ester, the solid residue was dissolved with warming in absolute ethanol and crystallized at 0°. Following two successive recrystallizations under the same conditions, the product was dried under vacuum. The yield was 75%, and a melting point of 89-90° was obtained; lit (79) 90°. With the polyunsaturated esters, an oily residue was recovered following solvent removal. This material was dissolved in chloroform-methanol (2:1, v/v) and purified on Silica gel G plates using chloroform as the solvent. The product band was located through the use of a palmitoyl-NHS standard, and recovered by elution of the appropriate region of the chromatogram with ethyl ether. Yields of the polyunsaturated esters averaged about 50%.
Analysis of all three products by TLC with chloroform as the developing solvent disclosed a single spot with an \( R_f \) of about 0.3 when the plates were sprayed with a hydroxamate reagent (79). When visualization was conducted by sulfuric acid charring, a major spot at the same \( R_f \) was obtained, with a faint spot detectable at the origin as well. Elution of the major spot and redevelopment failed to eliminate this non-radioactive material. The radiochemical purity of all three esters in this system was > 97%.

The hydroxysuccinimide esters were then converted to the CoA derivatives essentially as described by Al-Arif and Blecher (80). To 40 \( \mu \)moles of CoA dissolved in 1 ml of water was added 42 mg of NaHCO\(_3\). A slight excess (ca. 48 \( \mu \)moles) of the appropriate hydroxysuccinimide ester dissolved in 4 ml of tetrahydrofuran (freshly distilled over LiAlH\(_4\)) was then added to the CoA solution. The resulting somewhat turbid suspension was subsequently clarified by the dropwise addition of water (about 1.6 ml). A clear, single-phase mixture was essential for acceptable yields, and the order of addition described above provided the best results in this regard. CoA acylation was permitted to proceed under \( \text{N}_2 \) with stirring at room temperature, with the reaction course monitored by assaying for the presence of free sulfhydryl groups by the Ellman procedure (81).

When the reaction was complete, the solution was concentrated under nitrogen and the CoA thioester precipitated by the addition of 5% HClO\(_4\). The solid product was recovered by centrifugation, and washed once with 0.8% HClO\(_4\), twice with (redistilled) acetone and twice with peroxide-free ethyl ether. After drying the powder under \( \text{N}_2 \), it was dissolved in 2-3 ml of water and precipitated with perchloric acid as before. After
washing the precipitate as described above, the final powder was carefully dried under a nitrogen stream and dissolved in 3 ml of aqueous 
HCl (pH 3). This solution, which was still somewhat contaminated by free fatty acid, was extracted three times with ethyl ether, then once with petroleum ether. After the final extraction, the mixture was centrifuged briefly and the clear infranatant recovered. Yields were generally 55-60% based on CoA. The final product was stored in solution under N₂ at -20°.

[3-¹⁴C] DL β-hydroxystearoyl-CoA and [3-¹⁴C] DL β-hydroxy-8,11-eicosadienoyl-CoA were also synthesized through their hydroxysuccinimide esters. Synthesis of these esters was conducted as described above except that dioxane-ethyl acetate (70:30, v/v) was substituted as solvent as employed by Kawanami (82) for the α-hydroxy acids. Following recrystallization from methanol, the hydroxysuccinimide ester of β-hydroxystearic acid (m.p. 101-102°) was recovered in 47% yield. The ester of 3-hydroxy-8,11-eicosadienoic acid was purified by TLC using chloroform-methanol (3:1, v/v) as solvent, and recovered in 45% yield following elution with ether-methanol (4:1). The purified hydroxysuccinimide esters of both β-hydroxy acids migrated as single spots on TLC with an Rf ~ 0.7 in the chloroform-methanol (3:1, v/v) system, and with an Rf ~ 0.15 in chloroform alone. Conversion of these compounds to the CoA derivatives was achieved in the manner described above.

Failure to synthesize enzymatically active CoA esters of 2-trans acids by the hydroxysuccinimide ester approach has been reported by Pullman (83), and this method was also found to be inadequate with the α, β-enoic acids used in this study; possibly because the rate of thioester formation is so slow that significant sulfide formation occurs by a
favored competing addition of the thiol group across the conjugated double bond. Consequently, the CoA thioesters of [3-\( ^{14}C \)\] 2-trans-octadecenoic acid and [3-\( ^{14}C \)\] 2-trans-8,11-eicosatrienoic acid were prepared by a modification of the mixed anhydride procedure of Goldman and Vagelos (84) as described below.

To 250 μmoles of the 2-trans acid dissolved in 2 ml of dry benzene was added 300 μmoles of (redistilled) triethylamine followed by 300 μmoles of ethylchloroformate. The reaction was maintained at room temperature with stirring under nitrogen, and the progress monitored by TLC on Silica gel G plates with the solvent system petroleum ether - ethyl ether - acetic acid (80:20:1, by volume). Following completion of the reaction (within one hour), the benzene as well as any remaining unreacted triethylamine and ethylchloroformate was removed under a stream of nitrogen and the residue dissolved in 5 ml of tetrahydrofuran (THF) which had been freshly distilled from LiAlH₄. The mixture was centrifuged to remove insoluble salts and the mixed anhydride quantitated by the hydroxamate procedure of Lapidot et al. (79), using succinic anhydride as the standard (85). Yields ranged from 90-100% of theoretical. Two ml of this solution containing a 2-3 fold molar excess of anhydride was then added to one ml of an aqueous solution of CoA which had been adjusted to pH 8, and maintained under N₂ with constant agitation. The use of this volume ratio of 2:1 (THF/H₂O) provided for an immediate clarification of the reaction mixture which was essential for maximum yields. The reaction was monitored by assays for free thiol as before, and was complete in within 3-5 minutes. The thioester was then precipitated with perchloric acid and purified as described above, with a final yield of about 70% based on CoA.
Due to interference by the 3-keto group (86), the above procedures could not be directly employed for the synthesis of [3-\textsuperscript{14}C] 3-ketostearoyl-CoA. Therefore, this derivative was prepared through the dioxolane as outlined in the following scheme by a modification of the procedure used by Al-Arif and Blecher (87) for the synthesis of 3-ketopalmitoyl-CoA.

\[
\begin{align*}
(1) \quad & \text{R-C-CH}_2\text{-C-OCH}_3 + \text{CH}_2\text{-CH}_2 \xrightarrow{\Delta \text{pTSA}} \text{R-C-CH}_2\text{-C-OCH}_3 \\
(2) \quad & \text{R-C-CH}_2\text{-C-OCH}_3 \xrightarrow{\text{KOH} 90\% \text{EtOH}} \text{R-C-CH}_2\text{-C-OH} \\
(3) \quad & \text{R-C-CH}_2\text{-C-OH} + \text{Cl-C-OEt} + \text{N(Et)}_3 \xrightarrow{\text{pH 8}} \text{R-C-CH}_2\text{-C-O-C-OEt} \\
(4) \quad & \text{R-C-CH}_2\text{-C-O-C-OEt} + \text{CoA(SH)} \xrightarrow{\text{acetone pTSA}} \text{R-C-CH}_2\text{-C-(S)CoA} \\
(5) \quad & \text{R-C-CH}_2\text{-C-(S)CoA} \xrightarrow{\Delta} \text{R-C-CH}_2\text{-C-(S)CoA}
\end{align*}
\]

To 3.8 mmoles of methyl 3-ketostearate dissolved in 20 ml of dry toluene was added 5.2 mmoles of (redistilled) ethylene glycol and 0.28 mmoles of p-toluene sulfonic acid (pTSA). The mixture was refluxed under a Dean-Stark trap with additional aliquots of ethylene glycol and pTSA added after the first few hours, until the reaction was essentially complete as judged by a FeCl\textsubscript{3} test for the presence of enol (88), and by the failure of any additional water to accumulate. After stopping the reaction, the pTSA was neutralized with KOH and the mixture was washed twice with water, centrifuged, and the clear, yellow organic phase dried over Na\textsubscript{2}SO\textsubscript{4}. 
This solution was then transferred to a round bottom flask and concentrated by distillation, with the remaining toluene removed under a nitrogen stream. Forty ml of 4% KOH in ethanol was added, and saponification allowed to proceed with stirring at room temperature overnight. The following morning, the ethanol solution was transferred to an ice bath, acidified to pH 1.5 with concentrated HCl, and immediately extracted three times with ethyl ether. The pooled ether extracts were thoroughly washed with water, then dried over Na$_2$SO$_4$. Solvent was then removed on a rotary evaporator and the solid residue dissolved in warm petroleum ether. Following two recrystallizations from the same solvent, the ethyleneketal of 8-ketostearic acid was dried under vacuum. A white powder with a melting point of 69-71° was recovered in about 50% yield based on methyl 8-ketostearate. An IR spectrum of this material disclosed the presence of a hydroxyl group and a single carbonyl band at 1710 cm$^{-1}$ as expected for the acid ketal; and no methyl ketone or residual methyl ester was detectable by TLC.

The ethyleneketal of 8-ketostearoyl-CoA was then synthesized from the above free acid by the mixed anhydride procedure described previously. Following recovery of the washed CoA thioester, it was dissolved in 1 ml of peroxide-free THF at 65°, and 1 ml of acetone containing 10 mg of pTSA was added. This mixture was then refluxed for about three hours to cleave the blocking group with the reaction course monitored spectrally. It is likely that catalytic amounts of water are required for the initial step in this reaction (89), and although the hydrate of pTSA was used, the addition of 1 or 2 drops of water to the mixture appeared to improve the reaction course without materially increasing thioester hydrolysis. After stopping the reaction, solvent was removed under N$_2$ and the residue
dissolved in 2 ml of water and adjusted to pH 4 with N KOH. The mixture was centrifuged, and the 6-ketostearoyl-CoA was precipitated from the clear solution with perchloric acid and washed with dilute perchloric acid, acetone and ether as before. The final precipitate was dissolved in water (pH 3) and extracted to clarity with ethyl ether. After a final centrifugation, the aqueous phase was recovered and residual ether was removed with nitrogen.

### Analysis of Acyl-CoA Derivatives

The UV absorption spectra of all the acyl-CoA esters was determined in order to monitor purity. Figure 1 (curve A) represents a typical absorption spectrum obtained in 0.1 M phosphate buffer, pH 7, with all of the compounds whose synthesis could be effected through the hydroxysuccinimide ester; i.e., palmitoyl-CoA, the two polyunsaturated thioesters and both β-hydroxyacyl-CoA derivatives. In each case, the ratio obtained by dividing the absorption at 232 nm by that at 259 nm was 0.53-0.55, which is characteristic of adenine-containing thioesters (90). Curve B in Figure 1 represents the UV spectrum obtained with the 2-trans-enoyl-CoA derivatives. In this case, the additional contribution of the conjugated double bond system in the region 220-240 nm may be readily discerned. When 3-ketostearoyl-CoA was scanned in the same buffer as employed above, a curve essentially identical to that in Figure 1 (curve A) was obtained. However, when this compound was scanned in 0.1 M Tris buffer, pH 8.0, which was 2.5 mM in MgSO₄, the spectrum represented in Figure 2 was obtained. The additional peak centered near 300 nm is due to the enolate ion which is favored by an alkaline pH and the presence of magnesium counterions (87,90). Since this absorption is not given by the corresponding ketal, the reaction course during ketal
Figure 1. Representative ultraviolet spectra of the acyl-CoA derivatives recorded in 0.1 M potassium phosphate buffer, pH 7; all except 2-trans-enoyl-CoA (A), 2-trans-enoyl-CoA (B).
Figure 2. Ultraviolet spectrum of [3-¹³C] ε-ketostearoyl-CoA recorded in 0.1 M Tris buffer, pH 8, which was 2.5 mM in MgSO₄.
cleavage could be followed in this system by the increase in extinction at 305 nm with time.

The acyl-CoA derivatives were also analyzed by TLC with the solvent system n-butanol-acetic acid-water, 5:2:3 (by volume). Since these compounds tend to streak during development, the potassium oxalate-impregnated Silica gel H plates described by Ullman and Radin (77) were employed. Under these conditions the thioesters migrated as discrete spots with an Rf of about 0.4, while free fatty acids remained close to the solvent front. The radiochemical purities of all eight acyl-CoA derivatives employed in this study as estimated by this procedure are summarized in Table I.

Finally, substrate purity was investigated by GLC following an interesterification from the CoA ester by refluxing in anhydrous HCl in methanol. In the case of β-hydroxystearoyl-CoA, the methyl ester was acetylated by the procedure described earlier prior to GLC. The unsuitability of the β-keto derivative for GLC precluded its direct analysis in this manner. Due to the close structural relationship between β-ketostearoyl-CoA and its ketal precursor, no effective means of separating the two was available. Since it was not possible to drive ketal removal to completion under the conditions available with this compound, it was necessary to determine the extent of unreacted dioxolane contaminating the final product. This was accomplished in an indirect manner by saponification of an aliquot of the thioester in ethanolic KOH under reflux conditions. It was assumed that the ketal acid would be stable to these conditions while the β-keto acid would be quantitatively decarboxylated to 2-heptadecanone (see Chapter III, section A). Since the acyl grouping was labeled in the 3 position, a radioactive thin-layer
<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific Activity</th>
<th>Radiochemical Purity (TLC)</th>
<th>232/259&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Methyl Ester Purity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-&lt;sup&gt;14&lt;/sup&gt;C] 16:0-CoA</td>
<td>0.57 Ci/mole</td>
<td>98.6%</td>
<td>0.55</td>
<td>99%</td>
</tr>
<tr>
<td>[1-&lt;sup&gt;14&lt;/sup&gt;C] 6,9-18:2-CoA</td>
<td>0.57</td>
<td>95.3</td>
<td>0.55</td>
<td>97.8</td>
</tr>
<tr>
<td>[1-&lt;sup&gt;15&lt;/sup&gt;C] 6,9,12-18:3-CoA</td>
<td>0.59</td>
<td>97</td>
<td>0.54</td>
<td>97.9</td>
</tr>
<tr>
<td>[3-&lt;sup&gt;14&lt;/sup&gt;C] β-OH-18:0-CoA</td>
<td>0.06</td>
<td>96</td>
<td>0.53</td>
<td>92&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>[3-&lt;sup&gt;14&lt;/sup&gt;C] β-OH-8,11-20:2-CoA</td>
<td>0.25</td>
<td>95.5</td>
<td>0.55</td>
<td>-</td>
</tr>
<tr>
<td>[3-&lt;sup&gt;14&lt;/sup&gt;C] 2-trans-18:1-CoA</td>
<td>0.16</td>
<td>96</td>
<td>-</td>
<td>98.3</td>
</tr>
<tr>
<td>[3-&lt;sup&gt;14&lt;/sup&gt;C] 2-trans-8,11-20:3-CoA</td>
<td>0.31</td>
<td>96.5</td>
<td>-</td>
<td>97.1</td>
</tr>
<tr>
<td>[3-&lt;sup&gt;14&lt;/sup&gt;C] δ-keto-18:0-CoA</td>
<td>0.04</td>
<td>97 [15.9]&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.55</td>
<td>-</td>
</tr>
</tbody>
</table>

a. UV absorption ratio (232 nm vs 259 nm) determined in 0.1 M phosphate buffer, pH 7.

b. Radiochemical purity (GLC) of the corresponding methyl ester derived by interesterification in anhydrous HCl in methanol.

c. The value reported is for the acetate derivative of the methyl ester (See text). Radioactivity not present in methyl 3-acetoxystearate could not be assigned to any specific region of the chromatogram, but represented a broad area of relatively low activity preceding the standard.

d. The value in brackets is the percent ketal contamination estimated by decarboxylation as described in the text.
analysis of the extracted residue could be used to determine the percentage contribution of the ethyleneketal and of \( \beta \)-ketostearic acid (as 2-heptadecanone). The results of these various analyses are also summarized in Table I.

**Preparation of Microsomes**

For most of these studies, male weanling Sprague-Dawley rats were maintained on a fat-deficient diet for at least six weeks prior to sacrifice. The "normal" animals which were occasionally employed were also male Sprague-Dawley rats weighing about 200-250 grams which had been maintained on a chow diet. After sacrificing the animals, the livers were quickly removed and homogenized in a Potter-Elvehjem vessel in 4 volumes of an ice-cold medium consisting of 0.25 M sucrose and 0.01 M potassium phosphate buffer, pH 7.4. Nuclei and cellular debris were removed by centrifuging the brei at 500 x g for ten minutes in a Sorvall RC-2B centrifuge. The resulting supernatant was then freed of mitochondria by centrifugation at 17,000 x g for ten minutes. Microsomes were recovered from the second supernatant by centrifugation at 100,000 x g for 60 minutes in a Beckman model L2-65B preparative ultracentrifuge. The microsomal pellet was resuspended in homogenizing medium and the protein concentration was determined by the procedure of Lowry *et al* (91) using bovine serum albumin as a standard. The protein concentration was adjusted to 25 mg/ml by addition of homogenizing medium, and the suspension used as such or lyophilized. When lyophilized preparations were employed, they were reconstituted to the same protein concentration with water, then further diluted as necessary with homogenizing medium. Lyophilized microsomes retained full activity in the reactions assayed in this study for at least six weeks when stored
Incubation and Assay Procedures

A. Condensation Reactions

All standard incubations were carried out for 2.5 minutes in serum bottles in a total volume of 1.5 ml at 37° in a metabolic shaker. Each vial contained 150 μmoles of potassium phosphate buffer, pH 7.4, 7.5 μmoles of glutathione, 0.3 μmoles of malonyl-CoA, 2.5 mg of microsomal protein and either 100 nmoles of [1-14C] 16:0-CoA or [1-14C] 6,9-18:2-CoA, or 140 nmoles of [1-14C] 6,9,12-18:3-CoA. In all cases except as indicated, bovine serum albumin (BSA) was included in a molar ratio of 1:2 (BSA/acyl-CoA) assuming a molecular weight of 66,000 for the albumin.

Reactions were initiated by the addition of microsomes, and terminated by adding 0.25 ml of 4 N NaOH and 5.0 ml of methanol to each bottle. The bottles were replaced in the metabolic shaker for one hour to saponify the lipids. A Folch extract (92) was then formed by addition of 0.25 ml of 9 N HCl, 10 ml of chloroform and 1 ml of water. Following centrifugation to accelerate phase separation, the top layer was removed and the bottom layer taken to dryness under a stream of N₂. The residue was taken up in a small volume of chloroform-methanol (2:1, v/v) containing appropriate standards and applied to a Silica gel G plate, which was then developed in a solvent system of petroleum ether (boiling range: 63-75°, permanganate purified), ethyl ether and acetic acid (75:25:1, by volume). This composition was determined to be optimal through the numerical analysis procedure of Turina et al (93). The quality of the separation was dependent on the nature of the petroleum ether used, and optimum results were obtained only with the purified solvent.
After development, components were located under UV light after spraying the plates with a 0.2% solution of 2',7'-dichlorofluorescein in ethanol and fractions were scraped into scintillation vials to which was added 10 ml of a dioxane-based cocktail prepared as described by Snyder (94). Radioactivity of the fractions was determined, and conversions were calculated as: \[ \left( \frac{\Sigma \text{cpm in product bands}}{\text{total cpm recovered}} \right) \times \text{nmoles substrate} = \text{nmoles of product formed} \]

B. The β-Hydroxyacyl-CoA Dehydrase Reaction.

These analyses were also conducted aerobically in serum vials in a total volume of 1.5 ml at 37° in a metabolic shaker. The vials contained 150 umoles of phosphate buffer, pH 7.4, 180 nmoles of substrate and 0.625 mg of microsomal protein. The incubation period was 1.5 minutes.

In all cases the reactions were initiated by the addition of microsomes, and terminated by adding 0.25 ml of 4 N NaOH and 5.0 ml of methanol. The reaction mixtures were then saponified, acidified and extracted as described above. After taking the bottom layer of the extract to dryness, the lipids were converted to methyl esters by refluxing in 5% anhydrous HCl in methanol. The acid methanol solution was then extracted three times with petroleum ether and the pooled extracts were washed with water, then dried over Na₂SO₄. The methyl ester solution was concentrated under reduced pressure, applied to Silica gel G plates and developed with standards in petroleum ether-ethyl ether-acetic acid (70:30:1, by volume). Procedures for locating the hydroxy and non-hydroxy methyl ester bands, and the calculation of reaction rates, were the same as described above for the condensation reaction.
C. The 2-trans-Enoyl-CoA Reductase Reaction

Conditions similar to those described for the dehydrase reaction were employed in these analyses, except that the protein concentration was 1.25 mg, 2.0 μmoles of NADPH were included in each vial and the bottles were flushed with N₂ for ten minutes prior to assay in order to repress desaturation. Substrate concentration varied but was generally about 90 μM, and a 0.5 minute incubation period was employed. Except as indicated, BSA was included in these reactions in the same manner as described for the condensation reaction.

The reactions were conducted, and methyl esters were formed, as described above for the dehydrase reaction. A combined TLC-GLC assay was then used in order to quantitate the amount of β-hydroxy acid produced by reversal of the dehydrase reaction, as well as the α, β-saturated product. The methyl esters were first applied to a Silica gel G plate and treated as described above except that only about 20% of the hydroxy and non-hydroxy methyl ester bands were scraped directly into scintillation vials. The remainder of the non-hydroxy methyl ester band was eluted with ethyl ether and the methyl esters then fractionated by radioactive GLC to separate unreacted substrate from the α, β-saturated product. Components were identified by the inclusion of suitable internal standards. The rate of the reductase reaction as well as that of dehydrase reversal was then determined essentially as described above by calculating the percent of radioactivity present in unreacted substrate, β-hydroxy fatty acid and in the α, β-saturated product.
D. Overall Chain Elongation and the \( \beta \)-Ketostearoyl-CoA Reductase Reaction

For analysis of overall chain elongation, the incubations were identical to those described for the condensation reaction except that 2 \( \mu \)moles/vial of NADPH were included and the bottles were flushed with \( \text{N}_2 \) for ten minutes prior to assay. Saponification, methyl ester formation and radioactive GLC analysis of the reaction was conducted as described above.

The \( \beta \)-ketostearoyl-CoA reductase assays were also similar to condensation analyses except that the fatty acyl-CoA and malonyl-CoA were replaced with \([3-^{14}\text{C}]\ \beta \)-ketostearoyl-CoA, and 2 \( \mu \)moles/vial of NADPH were included. Microsomal protein concentration was 1.25 mg, and an incubation period of one minute was employed. As with the other assays in which NADPH was present, the vials were flushed with \( \text{N}_2 \) before initiating the reaction. Reaction work-up and analysis was essentially as described for the condensation assay except that following TLC, a portion of the free fatty acid band (plus the corresponding methyl ester fraction) was eluted, methylated and analyzed by radioactive GLC in order to separate methyl stearate and methyl palmitate. Reaction rate calculations were then determined in a manner analogous to that described for the enoyl-CoA reductase reaction.

E. Analysis of Reaction Products as the CoA Esters

Analyses were conducted in this series exactly as previously described except that the reactions were killed by adding 6 ml of Dole's reagent (95) to the vial rather than by the addition of alkali. After cooling the vials in an ice bath, 2 ml of water and 4 ml of heptane were added and the mixture was then centrifuged. The bottom layer
recovered after centrifugation was washed once with heptane, then concentrated under N\textsubscript{2} at 55-60°. Aliquots of the concentrated solution were applied as a streak to potassium oxalate-impregnated Silica gel H plates and developed in n-butanol-acetic acid-water (5:2:3, by volume), or in chloroform-methanol-acetic acid-water (18:18:1:6, by volume). Further analysis of the isolated acyl-CoA band was conducted as described in Section E of Chapter III.
CHAPTER III

RESULTS

A. The Condensation Reaction

Based on an earlier analysis of microsomal fatty acid chain elongation activity with a variety of substrates (43), palmitic acid and 6,9-octadecadienoic acid which were both found to be excellent substrates for elongation by rat liver microsomes were chosen as representative saturated and unsaturated substrates for this study. 6,9,12-Octadecatrienoic acid which was the most rapidly elongated acid examined in that investigation was also included. In order to eliminate complexities introduced by an initial activation step, the coenzyme A esters were employed in all cases. Initial studies were then undertaken in order to determine appropriate conditions for the condensation reaction with these substrates.

Reaction Analysis

Due to the relatively labile nature of free β-keto acids, it was anticipated that some of this product of the condensation reaction would, under the conditions of analysis, be partially degraded to the corresponding methyl ketone. In the case of β-ketostearic acid this would be 2-heptadecanone. Figure 3 (A) represents a typical thin-layer chromatogram obtained following an incubation with [1-14C]16:0-CoA and malonyl-CoA in the absence of NADPH. In addition to unreacted fatty acid, two other prominent radioactive bands were detected; one migrating with the β-ketostearic acid standard, and...
Figure 3. Thin-layer chromatogram of the total reaction mixture following incubations with (A) \([1-^{14}C]\) 16:0-CoA and malonyl-CoA; or (B) 16:0-CoA and \([2-^{14}C]\) malonyl-CoA. Conditions of incubation and analysis were as stated in Chapter II. The standards employed were (1) \(\beta\)-ketostearic acid, (2) palmitic acid, (3) methyl \(\beta\)-ketostearate, (4) 2-heptadecanone, and (5) methyl palmitate.
another migrating with authentic 2-heptadecanone. Identical results were obtained when either [1-\textsuperscript{14}C] 6,9-18:2-CoA or [1-\textsuperscript{14}C] 6,9,12-18:3-CoA was substituted as primer. Although the extent of β-keto acid decarboxylation varied somewhat dependent upon the conditions of assay (e.g., time and temperature of saponification), the ratio of methyl ketone to β-keto acid remained fairly constant when assay conditions were carefully controlled. In addition to the above, a relatively small fraction of the label was invariably found to migrate ahead of the methyl ketone band. This fraction was subsequently found to consist of methyl esters which were apparently formed during the saponification step in methanolic KOH. It was rather surprising that methyl esters were recovered under saponification conditions, however Lewis (96) has recently reported a similar observation of base catalyzed methanolysis in aqueous methanolic KOH solutions leading to the recovery of small amounts of methyl esters. Due to this behavior, methyl palmitate was included as an additional TLC standard and the corresponding sample band was counted and included with the free fatty acid as unreacted substrate. It seems unlikely that any β-keto acid formed during the incubation was incorporated into microsomal lipids, but the possibility exists that methanolysis of the CoA ester of β-ketostearate could also result in methyl ester formation in this manner. Consequently, methyl β-ketostearate was included as a standard although a clean separation of this compound from the methyl ketone could not be achieved in this system, and no evidence was obtained that any significant amount of the methyl β-keto ester was actually formed.
In order to confirm the identity of the components separated by TLC, the band in Figure 3 (A) corresponding in R_f to the methyl ketone standard was scraped from the plate, eluted with ethyl ether and analyzed by radioactive GLC. Figure 4 demonstrates that the radioactive component recovered in this fashion following an incubation with [1-1^4C] 16:0-CoA had a retention time identical to that of authentic 2-heptadecanone included as an internal standard. A similar result following an incubation with [1-1^4C] 6,9-18:2-CoA, and using 7,10-nonadecadiene-2-one as an internal standard, is shown in Figure 5.

The identity of the compound migrating with the 8-keto acid standard during TLC was similarly established by a preliminary development and elution of the appropriate band with ethyl ether. Solvent was then removed and the residue refluxed for 3 hours in a solution of 4% KOH in ethanol. The mixture was then acidified, extracted and reanalyzed by TLC using the same solvent system as before. Results of this analysis following an incubation with [1-1^4C] 16:0-CoA as primer are given in Figure 6, and indicate that the radioactive component in the 8-keto acid band can be nearly quantitatively converted to the methyl ketone under conditions favorable to the decarboxylation of 8-keto acids, thus confirming the identity of the labeled compound in band 1 of the original chromatogram (Figure 3, A) as 8-ketostearic acid. The major radioactive product in Figure 6 was confirmed as being 2-heptadecanone by GLC as described above. A similar analysis established the identity of the component corresponding in R_f to band 1, Figure 3 (A) as 8-keto-8,11-eicosadienoic acid when [1-1^4C] 6,9-18:2-CoA was employed as primer. In addition to the methyl ketone, a small amount of radioactivity could be detected in the free fatty
Figure 4. Gas chromatogram of authentic 2-heptadecanone with the component eluted from region (4) of the chromatogram in Figure 3 (A).
Figure 5. Gas chromatogram of authentic 7,10-nonadecadiene-2-one with the component eluted from region (1) of a chromatogram corresponding to Figure 3 (A) following an incubation with $[1^{14}C] \, 6,9-18:2$CoA as primer.
Figure 6. Thin-layer chromatogram obtained after refluxing the component eluted from region (1) of Figure 3 (A) in ethanolic KOH for 3 hours, followed by acidification and ether extraction of the mixture. Conditions of analysis and the standards were the same as in Figure 3.
acid band in Figure 6. This probably represented [1-14C] palmitic acid produced by a competing "acid cleavage" hydrolysis which is the favored reaction with β-keto acids in concentrated alkali (97). The minimal yield of free fatty acid obtained under these conditions suggests that the acid cleavage side reaction was not a significant factor with the considerably milder saponification conditions employed after the enzymatic assays.

Elution of the free fatty acid and methyl ester bands (bands 2 and 5) of Figure 3 (A) followed by methylation and analysis by GLC indicated that essentially all of the radioactivity of these bands remained in palmitic acid when [1-14C] 16:0-CoA was the primer, with less than 1% of the total counts recovered in methyl stearate. In addition, no significant radioactivity could be detected in methyl 2-trans-octadecenoate during GLC indicating that in the absence of NADPH, the reaction was limited to the initial condensation step.

For final confirmation of the assay procedure, an analysis was conducted by incubating unlabeled 16:0-CoA with [2-14C]malonyl-CoA under the same conditions as employed above. Upon analysis of this reaction, the chromatogram depicted in Figure 3 (B) was obtained. In this case only two radioactive components were recovered possessing Rf values identical with β-ketostearic acid and 2-heptadecanone, indicating the incorporation of the methylene carbon of malonyl-CoA into these products. It should be noted, however, that in this case the ratio of radioactivity in the β-ketostearic acid to the 2-heptadecanone fractions was higher than previously found. Attempts to convert the presumed β-keto product to methyl ketone by the approach
described above were only partially successful. This result suggests that some other labeled component or components derived from malonyl-CoA and migrating with β-ketostearic acid are also produced under these reaction conditions. Many authors have noted the presence of de novo fatty acid synthetase activity in microsomes derived from rat liver (57,67) and brain (35), and such activity was detected in the preparations used in this study as well. Since it is known that fatty acid synthetase can form a variety of products from malonyl-CoA in the absence of NADPH, including β-hydroxy-β-methyl glutarate (98,99) and triacetic acid lactone (98-100), it is possible that part of the radioactivity recovered in the β-keto acid band in Figure 3 (B) represents products of the de novo enzyme complex. Nevertheless, it could be demonstrated that label from [2-14C] malonyl-CoA was incorporated into both β-ketostearic acid and 2-heptadecanone.

Since all of the quantitative assays in this study involved unlabeled malonyl-CoA and a radioactive fatty acyl-CoA, this presumably anomalous activity would have no affect on the condensation assay except to contribute to malonyl-CoA depletion during the course of the reaction.

Protein and Time Dependency

Protein and time dependency curves for the condensation reaction employing each of the three fatty acyl-CoA substrates are summarized in Figures 7 and 8. In all cases deviations from linearity were observed within the range examined, particularly with the more active polyunsaturated substrates. The non-linear response in specific activity which occurred with longer incubation periods (Figure 7) was
Figure 7. Time course of the condensation reaction conducted under the standard conditions of incubation with 2.5 mg of protein; 16:0-CoA (●), 6,9-18:2-CoA (○), 6,9,12-18:3-CoA (▲).
Figure 8. Protein dependency of the condensation reaction conducted under the standard conditions of incubation for 2.5 minutes; 16:0-CoA (○), 6,9-18:2-CoA (□), 6,9,12-18:3-CoA (△).
probably a reflection of substrate depletion due in part to competing reactions including deacylation (101-103) and incorporation into lipids (53). Since ATP was not included in the reaction, deacylation would produce an inactive free fatty acid substrate. On the other hand, the sharp decline in specific activity which occurred at higher protein levels (Figure 8) probably represented, at least in part, a reduced effective initial substrate concentration resulting from microsomal protein binding of these highly surface active substrates. Very similar protein dependency curves attributed to this phenomenon have been reported for microsomal acyl-CoA:phospholipid acyltransferase reactions (51,101). As a consequence of these results, time and protein values of 2.5 minutes and 2.5 mg were chosen for subsequent studies in order to maximize the conversion while maintaining reasonably linear reaction conditions.

**Substrate Dependency**

v/s Curves for the condensation reaction as a function of fatty acyl-CoA concentration are given in Figures 9-11. Kinetic constants could not be estimated from these data due to deviations from Michaelis-Menten behavior. Thus a Lineweaver-Burk plot of the v/s data for 16:0-CoA yields a biphasic result, while the v/s curves obtained with both 6,9-18:2-CoA and 6,9,12-18:3-CoA were somewhat sigmoidal. This result was more noticeable with 6,9,12-18:3-CoA (Figure 11). One factor contributing to this behavior is probably that of adsorption of the fatty acyl-CoA by both microsomal protein and BSA. A limitation of reaction velocity in phospholipid synthesis due to substrate adsorption by microsomal protein has been clearly shown to
Figure 9. Kinetics of condensation with [1-¹⁴C] 16:0-CoA at a fixed concentration (200 μM) of malonyl-CoA. Conditions of assay were as stated in Chapter II.
Figure 10. Kinetics of condensation with $[1^{-14}C]$ 6,9-18:2-CoA at a fixed concentration (200 μM) of malonyl-CoA. Conditions of assay were as stated in Chapter II.
Figure 11. Kinetics of condensation with [1-\(^{14}\)C] 6,9,12-18:3-CoA at a fixed concentration (200 \(\mu\)M) of malonyl-CoA. Conditions of assay were as stated in Chapter II.
occur with 16:0-CoA (104), and such binding has been tentatively implicated in sigmoidal v/s behavior with long chain acyl-CoA derivatives in other systems (105,106). It might be noted that 6,9,12-18:3-CoA also demonstrated the most pronounced deviation from linearity in the protein dependency curve (Figure 8), while 6,9-18:2-CoA was intermediate in this regard.

While binding of substrate by microsomal protein may have interfered with the condensation reaction, the presence of BSA in the reaction greatly facilitated condensation activity. In the analyses summarized in Figures 9-11, BSA was included as described in Chapter II. As can be seen in Figure 12 in comparison with Figures 9-11, the absence of BSA in the incubations is associated with a pronounced diminution in peak activity with all three substrates in conjunction with extensive substrate inhibition at relatively low concentrations of the saturated - but not polyunsaturated - acyl-CoA derivative. This effect may be more clearly seen in Figure 13 in which the data for 16:0-CoA and 6,9-18:2-CoA in the presence and absence of BSA are represented together in the same scale. Due to this marked enhancement of condensation activity by BSA, it was routinely included in the assay medium.

Malonyl-CoA saturation curves in the presence of a fixed concentration (66.7 μM) of fatty acyl-CoA are given in Figure 14. These curves could be linearized by double reciprocal plots as indicated in the inset to Figure 14 to yield apparent Km values of 47.5, 48.8 and 51.2 μM with 16:0-CoA, 6,9-18:2-CoA and 6,9,12-18:3-CoA respectively as substrate. It is interesting that these values are quite similar,
Figure 12. Kinetics of condensation with [1-14C] 16:0-CoA (●), [1-14C] 6,9-18:2-CoA (■) or [1-14C] 6,9,12-18:3-CoA (▲), and 200 μM malonyl-CoA in the absence of bovine serum albumin. Conditions of analysis, except for the omission of bovine serum albumin, were identical to those employed in Figure 9-11.
Figure 13. Summary of acyl-CoA condensation in the presence and absence of bovine serum albumin. 16:0-CoA with albumin (○); without albumin (●). 6,9-18:2-CoA with albumin (★); without albumin (▲).
Figure 14. Kinetics of condensation as a function of malonyl-CoA concentration. Incubations were conducted with 66.7 μM [1-14C] 16:0-CoA (○), [1-14C] 6,9-18:2-CoA (■), or [1-14C] 6,9,12-18:3-CoA (▲) in the presence of bovine serum albumin.
but it must be noted that these data were obtained at a single concentration of fatty acyl-CoA and thus represent only apparent $K_m$ values. The anomalous results of the fatty acyl-CoA saturation curves served to preclude the simultaneous variation of both substrate concentrations required to obtain true kinetic parameters from a bisubstrate reaction. As a consequence it has not yet been possible to evaluate the reaction in terms of its kinetic behavior.

**Sulfhydryl Inhibition**

Results obtained using sulfhydryl-directed reagents suggest the involvement of enzyme sulfhydryl groups in the overall condensation reaction. Figure 15 shows that the condensation reaction using 16:0-CoA as primer is rapidly - albeit only partially - inhibited in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), a reagent which interacts with free sulfhydryl groups by a disulfide exchange mechanism (81,107). That disulfide formation was involved in this inhibition is suggested by the reversal of inhibition achieved by the subsequent addition of either glutathione (GSH) or dithiothreitol. The former compound would be expected to compete with the enzyme-TNB complex in a second disulfide exchange, while dithiothreitol functions as a disulfide reductant (108). Failure to completely restore activity following addition of these reagents may indicate that the 2-fold molar excess of GSH and dithiothreitol which was employed relative to the DTNB concentration was not sufficient to completely reverse the inhibition. The decline in activity observed after partial reversal with either reagent is consistent with such an interpretation. However, the reason why only about 40% of the activity was rapidly lost
Figure 15. Inhibition of the condensation reaction by 2.5 mM DTNB and its partial reversal by dithiothreitol (DTT) or glutathione (GSH). The preincubation was initiated by mixing equal volumes of a microsomal suspension and a 5.0 mM solution of DTNB, and maintained at 37.5°. Aliquots containing 2.5 mg of protein were assayed as a function of time by the standard procedure (in the absence of GSH) using [1-14C] 16:0-CoA as primer. The control rate in the absence of DTNB was in 2.9 nmols product min⁻¹ mg⁻¹.
in the presence of DTNB after which no further inhibition occurred remains unclear. The gradual decline in activity following the addition of GSH or dithiothreitol suggests that the DTNB concentration was not limiting. This was also indicated qualitatively by the rapid intensification of the yellow color in the microsomal preparations when GSH or dithiothreitol was added, indicative of the further liberation of significant quantities of the thionitrobenzoate anion.

Controls run in the presence of dithiothreitol or GSH alone indicated that these reagents had no apparent effect on the condensation reaction under the conditions employed. Although failure to augment enzyme activity in the presence of a sulfhydryl protecting agent is not necessarily consistent with the essentiality of thiol groups in the reaction, the presence of both malonyl-CoA and fatty acyl-CoA in the reaction medium in conjunction with thioesterase activity directed towards both substrates may have contributed to a sufficient minimal level of free CoA sulfhydryl groups to maintain full enzyme activity.

In view of the apparent sensitivity of the condensation reaction to sulfhydryl inhibitors, studies were designed to investigate the apparent rate of inhibition of the reaction as monitored with different fatty acyl-CoA substrates. Due to limited inhibitor solubility, the extremely fast reaction and the potentially ready reversibility of the DTNB-mediated inhibition, this reagent did not appear to be an adequate choice for a detailed analysis of comparative inhibition rates. Consequently, the more soluble and irreversible alkylating reagent, N-ethylmaleimide (NEM), was investigated in this regard. In this series, a microsomal suspension was diluted 1:1 (v/v) with an
NEM solution in water to yield a final protein concentration of 12.5 mg/ml and an NEM concentration as indicated. The diluted suspension was immediately mixed and preincubated in the metabolic shaker at 37°. Aliquots of 0.2 ml were then analyzed as a function of preincubation time using the appropriate substrate and the standard incubation procedure. Controls were treated in the same manner except that the NEM solution was replaced with water. In all cases an excess of GSH was included in the reaction vials to scavange any unreacted NEM carried over with the microsomal aliquot, and NEM solutions were made immediately prior to their use in order to minimize the effect of spontaneous reagent hydrolysis (109).

The results of these determinations as summarized in Figure 16 indicate that under these conditions, the inhibition followed (pseudo) first-order kinetics with each substrate over the time interval examined. When the pseudo first-order rate constants derived from these data were replotted versus NEM concentration, the results in Figure 17 were obtained. As can be seen in this figure, the second-order rate constants so derived were quite similar when either polyunsaturated substrate was used, while differing markedly from the value obtained when the reaction was monitored with 16:0-CoA. The apparent second-order rate constant for NEM inhibition obtained through regression analysis of the data in Figure 17 was 2.43 L mol⁻¹ min⁻¹ when 16:0-CoA was employed as substrate, while values of 3.75 and 4.20 L mol⁻¹ min⁻¹ were obtained using 6,9-18:2-CoA and 6,9,12-18:3-CoA respectively as substrate. Statistical analysis (Student's t test) of the data used in generating the second-order plots indicates that
Figure 16. Inhibition of the condensation reaction by N-ethylmaleimide (NEM). The inhibition was conducted as described in the text and aliquots were assayed as a function of preincubation time by the standard procedure using (A) 16:0-CoA (66.7 μM), (B) 6,9-18:2-CoA (66.7 μM) or (C) 6,9,12-18:3-CoA (93.3 μM) as primer. NEM concentrations employed were 17.5 mM (●), 25 mM (○), 35 mM (▲), 50 mM (△), 60 mM (■) and 75 mM (●).
Figure 17. Plot of the apparent pseudo first-order rate constants for the inhibition of the condensation reaction derived from the data of Figure 16 as a function of inhibitor concentration; 16:0-CoA (●), 6,9-18:2-CoA (□), 6,9, 12-18:3-CoA (△).
the difference between the slope obtained with 16:0-CoA as substrate and those obtained using the polyunsaturated acyl-CoA substrates is highly significant ($p < .001$ in both cases), while the difference between the slopes obtained with 6,9-18:2-CoA and 6,9,12-18:3-CoA is not significant ($0.1 < p < 0.2$). These data indicate that the condensation reaction with 16:0-CoA involves at least one different enzyme than that which is involved with the polyunsaturated substrates. On the other hand, the results are consistent with - but do not confirm - a common condensation pathway for 6,9-18:2 and 6,9,12-18:3.

B. The 3-Hydroxyacyl-CoA Dehydrase Reaction

In order to further investigate intermediate reaction steps in the overall chain elongation sequence, palmitate and 6,9-octadecadienoate were chosen as representative saturated and unsaturated substrates and the corresponding 3-hydroxy derivatives (\([3^{-14}C]\) DL 3-hydroxystearyl-CoA and \([3^{-14}C]\) DL 3-hydroxy-8,11-eicosadienoyl-CoA, respectively) were examined. When 3-hydroxy-18:0-CoA was incubated with microsomes in the absence of reducing equivalents, after which the fatty acid extract was methylated and subjected to a thin-layer analysis as described in Chapter II, two radioactive bands were obtained; one migrating with an authentic methyl 3-hydroxystearate standard, and another with an $R_f$ identical to that of a non-hydroxy methyl ester. In order to establish the identity of the latter reaction product, the band was eluted and analyzed by GLC. Figure 18 demonstrates that most of this product had a retention time identical to that of authentic methyl 2-trans-octadecenoate which was included as an internal standard, while no label could be detected in the
Figure 18. Gas chromatogram of the non-hydroxy methyl esters derived from the dehydrase reaction using [3-\textsuperscript{14}C] \( \beta \)-hydroxy-stearoyl-CoA as substrate. Methyl 2-trans-octadecenoate was included as an internal standard.
overall chain elongation product. A similar analysis following an incubation with β-hydroxy-8,11-20:2-CoA indicated that the predominant product in this case was 2-trans-8,11-20:3 (Figure 19). In addition, a smaller peak of radioactivity was invariably detected on these chromatograms which possessed a retention time somewhat less than that of the main product. Although the amounts obtained were insufficient to characterize, this secondary peak has been tentatively identified as the Δ³ isomer based on its relative retention time. As indicated in Table II, the retention time relative to methyl stearate of the unidentified component obtained following an incubation with β-hydroxy-18:0-CoA was 1.25 which is quite close to the reported value for methyl 3-octadecenoate (75,76). The retention time relative to methyl 8,11-eicosadienoate estimated for the corresponding component recovered after an incubation with β-hydroxy-8,11-20:2-CoA was 1.23 (Table II).

Davidoff and Korn (76) have reported that Δ² methyl esters can add methanol across the conjugated double bond during saponification to form the 3-methoxy derivative. Subsequent, β, γ-elimination then leads to the formation of the Δ³ isomer as an artefact. However, while Barron and Mooney (75) confirmed the above results with Δ² methyl esters, they found that no Δ³ formation occurred when 2-trans compounds as either the free acid or the acyl-CoA ester were subjected to saponification conditions. No addition of methanol occurred with the free acid. Although the 3-methoxy derivative was formed when the enoyl-CoA was employed, no Δ³ acid was recovered which was attributed by the authors to the fact that thioester sulfur has little tendency
Figure 19. Gas chromatogram of the non-hydroxy methyl esters derived from the dehydrase reaction using [3-\(^{14}\)C] \(\beta\)-hydroxy-8,11-eicosadienoyl-CoA as substrate. Methyl 8,11-eicosadienoate and methyl 2-trans-8,11-eicosatrienoate were included as internal standards.
### TABLE 2

Evaluation of the Equivalent Chain Length (ECL) and Relative Retention Time (RRT)\(^a\) of the Unidentified Component Recovered from the \(\beta\)-Hydroxyacyl-CoA Dehydrase Reaction.

<table>
<thead>
<tr>
<th>Substrate Component</th>
<th>ECL</th>
<th>RRT</th>
<th>RRT(^b)</th>
<th>RRT(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl 18:0</td>
<td>18.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>(\beta)-OH-18:0-CoA(</td>
<td>X(_a)</td>
<td></td>
<td>18.95</td>
<td>1.25</td>
</tr>
<tr>
<td>Methyl 3-18:1</td>
<td>-</td>
<td>-</td>
<td>1.22</td>
<td>1.27</td>
</tr>
<tr>
<td>Methyl 2-trans-18:1</td>
<td>20.00</td>
<td>1.63</td>
<td>1.62</td>
<td>1.63</td>
</tr>
<tr>
<td>Methyl 8,11-20:2</td>
<td>21.45</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(\beta)-OH-8,11-20:2-CoA(</td>
<td>X_b)</td>
<td></td>
<td>22.55</td>
<td>1.23</td>
</tr>
<tr>
<td>Methyl 2-trans-8,11-20:3</td>
<td>23.80</td>
<td>1.61</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(\text{a. RRT is the retention time relative to the parent ester; i.e., methyl stearate or methyl 8,11-eicosadienoate.}\)

\(\text{b. reference 76}\)

\(\text{c. reference 75}\)
to participate in double bond formation so that electron delocalization would be more directed toward the carboxyl group than in the case of 0-acyl esters.

Therefore, if the only forms in which the 2-trans acids existed at the end of incubations in this study were free fatty acid or enoyl-CoA ester, no artefactual $\Delta^3$ formation would be expected to occur. On the other hand, a small amount of methyl ester formation during saponification was noted to occur in this study (cf. section A) despite the fact that base is a poor catalyst for alcohololysis.

Analysis of the acyl-CoA derivatives by acid-catalyzed interesterification to their methyl esters as reported in Chapter II clearly indicated that this reaction could occur in good yields with the 2-trans-enoyl-CoA thioesters under suitable conditions. In addition, the extent - if any - of incorporation of the 2-trans acids into microsomal lipids during these incubations is unknown. It is therefore impossible to determine to what extent the $\Delta^3$ isomer was a chemically produced artefact. However, regardless of the catalyst involved, formation of the $\Delta^3$ isomer would represent a further modification of the $\Delta^2$ derivative formed during the dehydrase reaction. No clear evidence for a direct $\beta$, $\gamma$-dehydration has been obtained with mammalian tissue (75,110), although such a reaction can occur in certain microorganisms (111). Even in the latter case however, the same enzyme was involved in both $\alpha$, $\beta$- and $\beta$, $\gamma$-dehydration. Therefore, the total ($\Delta^2 + \Delta^3$) radioactivity was utilized in calculating dehydrase reaction rates in this study.
Substrate Dependency

A typical v/s curve for the β-hydroxy-18:0-CoA dehydrase reaction is given in Figure 20 (curve A). Product formation rose with increasing substrate concentration to attain an initial plateau at a rate of about 52 nmols of substrate dehydrated min$^{-1}$ mg$^{-1}$ when the substrate concentration was 100-135 µM. However, when the substrate concentration was further increased, a secondary rise in activity occurred. This phenomenon has been repeatedly observed, both with the saturated substrate and with β-hydroxy-8,11-20:2-CoA (Figure 21). In these studies, a protein concentration of 0.625 mg per incubation was employed. Inspection of the v/s curve obtained with β-hydroxy-18:0-CoA as substrate (Figure 20, curve A) indicates that the secondary rise in activity occurred at a substrate to protein concentration ratio of about 310 (nmoles of acyl-CoA : mg microsomal protein). A similar ratio of about 290 was obtained with the polyunsaturated substrate. In addition, v/s curves with both substrates using 1.25 mg of protein showed the same behavior; i.e., a secondary rise in activity occurred, and the substrate concentration at which the secondary rise was first evidenced was also doubled yielding about the same acyl-CoA:protein ratio at the approximate inflection point.

Protein and Time Dependency

The time course for the dehydrase reaction was unremarkable (Figure 22) except that the reaction did not appear to be truly linear even during very short time periods. This may have related to the ease of reversal of this reaction as discussed below. However, atypical results were obtained in the protein dependence studies. When the
Figure 20. Kinetics of the dehydrase reaction using $[3^{14C}]$ \( \beta \)-OH-18:0-CoA as substrate with intact microsomes (A), or with the 100,000 x g supernatant (B) obtained after treating microsomes with deoxycholate-KCl.
Figure 21. Kinetics of the dehydrase reaction using [3-^{14}C] β-OH-8,11-20:2-CoA as substrate with intact microsomes.
Figure 22. Time course for the dehydrase reaction using 0.625 mg of microsomal protein and 120 μM [3-14C] β-OH-18:0-CoA as substrate.
β-hydroxy-18:0-CoA dehydrase reaction was assayed at a fixed substrate concentration (120 µM) and with variable amounts of protein, a non-linear curve resulted with an apparent activation at lower protein concentrations (Figure 23). The substrate to protein concentration ratio at the approximate point of activation in this curve was about 325 (nmol acyl-CoA:mg microsomal protein). Essentially identical results were obtained with respect to both time and protein when the poly-unsaturated substrate was used. These results suggest that the behavior observed in the v/s curves involved a critical substrate to microsomal protein concentration ratio which when surpassed led to a secondary increase in the dehydration rate.

Analysis of the Atypical Dehydrase Activity

Biophasic v/s curves of the form obtained in this reaction have been noted to occur in a variety of both soluble and particulate enzyme systems with a number of potential explanations advanced including: (a) allosteric enzymes possessing more than two substrate binding sites in which the relative magnitude of $K_{cat}$ of these sites first decreases, then increases as the enzyme is saturated (112), (b) enzymes acting on amphipathic substrates with differing activities above and below the substrates critical micelle concentration (113, 114) and (c) the presence of isozymes which differ markedly in $K_m$ (113,115). In addition, the possibility was considered that a substrate-induced "solubilization" of the enzyme leading to enhanced activity might be involved in this behavior. Such an explanation would be consistent with both the detergency characteristics of long
Figure 23. Dehydrase activity as a function of microsomal protein using 120 μM [3−14C] β-OH-18:0-CoA as substrate. The incubation period was 1.5 minutes.
chain acyl-CoA esters (51,116), and with the apparent critical sub-
strate: protein concentration ratio involved at the point of ac-
tivation. It was also suggested by preliminary studies indicating that
the dehydrase (unlike condensing activity) could be solubilized by
treating microsomes with a bile salt; and by a rise in activity ob-
served following microsome preincubation.

As depicted in Figure 24, when a microsomal suspension was pre-
incubated at 37.5°C and aliquots assayed for dehydrase activity as a
function of time, a gradual increase in activity to a maximum of
about 25% above the basal (non-incubated) rate was observed. A
possible explanation of this behavior is that a partial autolysis of
the particles may have occurred, liberating dehydrase activity. That
solubilization actually occurred under these conditions is suggested
by the results presented in Figure 25. In this experiment identical
microsomal preparations were incubated for 10 minutes at either
37.5°C or 4°C, then centrifuged at 100,000 × g for 60 minutes after
which supernatant activity was measured. The results show that a
small amount of soluble¹ activity was recovered from the 4°C prepar-
ation, while considerably more activity was present in the 37.5°C
supernatant, consistent with a partial solubilization of the dehy-
drase under conditions associated with enhanced activity.

1. The term "soluble" is used in this study to designate
activity remaining in the supernatant under the centrifugal
conditions described. A true soluble protein is not
necessarily implied.
Figure 24. The effect of preincubation time on the activity of the dehydrase reaction using 120 μM [3-\(^{14}\)C] β-OH-18:0-CoA as substrate. Assays were carried out with microsomal suspensions preincubated at 37.5° for the indicated time periods.
Figure 25. The effect of preincubation on the recovery of "soluble" dehydrase activity. Intact microsomes were preincubated for 10 minutes at the indicated temperature, then centrifuged and supernatant aliquots assayed using 120 μM [3-¹³C] ß-OH-18:0-CoA as substrate.
In order to further examine the influence of solubilization on dehydrase activity, the effect of sodium deoxycholate treatment of microsomal particles was investigated. Initial assays were conducted in order to determine optimum conditions for solubilization by this detergent. In this series, microsomes (10 mg protein/ml) were incubated with occasional mixing for ten minutes at 0-4° in a medium containing 0.5 M KCl, 0.1 M sucrose, 4 mM potassium phosphate buffer, pH 7.4, and various concentrations of sodium deoxycholate. At the end of the incubation period, the preparations were centrifuged at 100,000 x g for 60 minutes and supernatant aliquots containing 0.625 mg of protein were assayed for dehydrase activity. The results in Figure 26 indicate that while 0.5 M KCl alone resulted in approximately a 20% solubilization of both total protein and dehydrase activity, the recovery of soluble activity was enhanced as the concentration of deoxycholate was increased. As suggested by the close relationship in all cases between the percent total protein and percent dehydrase activity solubilized, no activation of the solubilized enzyme could be detected. This may also be seen in Figure 20 (curve B) in which a typical v/s curve determined with a soluble preparation is presented. If deoxycholate treatment activated the dehydrase one would expect to observe a maximum rate of dehydration exceeding that of the plateau region shown in curve A of Figure 20. In fact, however, the maximum specific activity with the soluble preparation was about the same as the plateau value. These results suggest that while dehydrase activity may be solubilized by detergent, the secondary activation of the dehydrase observed in Figure 20 (curve A) is unlikely to be due to a general detergency effect induced by high substrate concentrations.
Figure 26. The extent of solubilization of total microsomal protein (●) and dehydrase activity (■) following preincubation in various concentrations of sodium deoxycholate in 0.5 M KCl. Dehydrase assays were conducted with 0.625 mg of "soluble" protein and 120 μM [3-¹⁴C] β-OH-18:0-CoA under standard conditions.
A more detailed analysis of the effect of deoxycholate on dehydrase activity is summarized in Figure 27. Curve B was determined with the total microsomal preparation (uncentrifuged) after preincubation with deoxycholate. Since the presence of deoxycholate in the preparation was in itself sufficient to eliminate the secondary rise in activity, the kinetics of the dehydrase are most likely not those described by Teipel and Koshland (112), although one cannot exclude the possibility that the detergent disrupted enzyme subunit interactions. Following centrifugation, both soluble (curve A) and residual particulate activity (curve C) could be detected. However, the specific activity of the particulate enzyme was much reduced, and this activity represented only about 3% of the total activity recovered. These v/s curves (A and C) appear to be quite dissimilar, and a marked difference in their apparent kinetic constants is clear in Figure 28. The $K_m$ for the particulate enzyme estimated through regression analysis of the data in Figure 28 was 32.2 $\mu$M, and the $V_{max}$ was 16.4 nmole min$^{-1}$ mg$^{-1}$. The corresponding values for the non-particulate enzyme were 116 $\mu$M and 87.3 nmole min$^{-1}$ mg$^{-1}$, respectively. Such results would appear to suggest the existence of two separate enzymes as the reason for the atypical dehydrase behavior. However, the much higher $K_m$ for the soluble enzyme may have resulted from the concomitant solubilization of enzymes competing for the substrate which would tend to maintain the rather high $K_m$ observed with intact microsomes, while the residual particulate material may have been relatively depleted of this competitive activity. Similarly, since $V_{max}$ depends on enzyme concentration, the lower $V_{max}$ calculated from curve C
Figure 27. Kinetics of the dehydrase reaction following treatment of microsomes with deoxycholate. The microsomal preparation was preincubated at 6 mg protein ml⁻¹ with 0.9% sodium deoxycholate and 0.34 M KCl for ten minutes. Aliquots containing 0.625 mg of protein were assayed immediately with 120 μM 8-OH-18:0-CoA as substrate (Curve B). The preparation was then centrifuged at 100,000 x g for 60 minutes and aliquots of the soluble (Curve A) or particulate material (Curve C) assayed under the same conditions.
Figure 28. Double-reciprocal analysis of the data represented by curves A (□) and C (●) of Figure 27.
may simply have reflected the reduced specific activity of this material. In fact, the estimated ratios of specific activity and $V_{\text{max}}'$ (pellet/soluble) were not greatly dissimilar, being 0.26 and 0.19 respectively. Thus the data of Figures 27 and 28, while consistent with the presence of two enzymes acting on 3-hydroxy-18:0-CoA, do not necessarily confirm that proposal. In fact, such an explanation would not account for the loss of secondary activity in curve B of Figure 27. It appears more likely that the activity represented by curve C resulted from a small amount of residual dehydrase retained in the pellet under the conditions employed.

Since the presumed $\Delta^3$ isomer could always be detected following dehydrase reactions (Figures 18 and 19), the possibility was considered that this compound was at least partly produced enzymatically, and that this activity might then be responsible for the atypical kinetic behavior observed. In order to investigate this possibility, a $v/s$ analysis was made and the non-hydroxy methyl ester bands recovered following TLC were eluted and further fractionated by GLC. The results of this analysis are presented in Figure 29. The fact that $\Delta^3$ formation showed saturation behavior in this study is in fact more consistent with an enzymatic than chemical method of formation, since in the latter case one might expect the amount of the $\Delta^3$ isomer recovered to roughly parallel the amount of 2-trans-18:1 present. However, it is clear from the data in Figure 29 that the plateau region persists in the dehydrase $v/s$ curve even when the rate is expressed specifically in terms of 2-trans-18:1 production. Consequently, $\Delta^3$ formation cannot account for the overall $v/s$ behavior.
Figure 29. Kinetics of the dehydrase reaction conducted under standard conditions with 120 μM [3-14C] 8-OH-18:0-CoA as substrate, expressed as total non-hydroxy product (○), 2-trans-octadecenoate recovered ( ), or formation of the presumed Δ³ isomer (▲).
observed in this reaction. Although the dehydrase rate was customarily based on total \((\Delta^2 + \Delta^3)\) product recovery, this is probably valid since as noted earlier, whether the \(\Delta^3\) isomer is formed chemically or enzymatically by a \(\Delta^2 \rightarrow \Delta^3\) isomerase, it is still derived from the \(\Delta^2\) acid.

The involvement of a critical micelle concentration (CMC) effect in the dehydrase v/s behavior also appears to be somewhat doubtful. The CMC of \(\beta\)-hydroxyacyl-CoA derivatives has not been determined. However, Barden and Cleland (51) have found the CMC of a variety of saturated and unsaturated fatty acyl-CoA esters to be in the range of 2-10 \(\mu\)M under their conditions of analysis. While the CMC of the \(\beta\)-hydroxy derivatives might be expected to be somewhat higher, and while some microsomal binding of the acyl-CoA probably occurs lowering its effective concentration in the bulk phase (102), it seems unlikely that these effects could raise the observed CMC to the value (ca. 120 \(\mu\)M) required for the involvement of a micellar effect in dehydrase v/s behavior. Also, if a CMC effect were involved, the inclusion of BSA in the incubation might reasonably be expected to exert an influence on the reaction and thus on the v/s curve due to substrate binding. However, BSA was found to have no effect on the dehydrase reaction when it was included in the incubations under the same conditions as used in the condensation and enoyl-CoA reductase reactions, with a v/s curve essentially identical to that of Figure 20 (curve A) being obtained.

Although the data obtained through the use of deoxycholate do not appear to support a solubilization explanation for the dehydrase
v/s behavior, the use of a bile salt rather than substrate as detergent may have contributed to these results. In all cases when deoxycholate was used, it was also present during the enzyme assay and may have interfered either directly with the enzyme or indirectly through mixed micelle formation with the substrate. Thus while substrate induced solubilization of the enzyme does not appear to be a likely explanation, it cannot yet be completely discounted. In addition, the possibilities that β-hydroxyacyl-CoA derivatives activate the dehydrase by some type of membrane perturbation, or that two different enzymes are involved only one of which is solubilized by deoxycholate, cannot be eliminated at this time. Further studies with purified enzyme preparations may be required in order to conclusively establish the basis for the abnormal dehydrase v/s behavior observed in this work.

Reverse Dehydrase Activity

As indicated in Figure 30, when incubations were conducted under conditions identical to those employed in the above studies except that a 2-trans-enoyl-CoA was substituted for the β-hydroxyacyl-CoA substrate, a reverse (hydration) reaction took place. Nugteren (12) has previously reported that rat liver microsomes are able to convert 2-trans-hexadecenoic acid to β-hydroxyhexadecanoic acid. Both 2-trans-18:1-CoA and 2-trans-8,11-20:3-CoA were quite active in this reaction, with a somewhat more rapid hydration rate observed with the polyunsaturated substrate. Although not nearly as pronounced as in the dehydration reaction, both curves possess a point of discontinuity and appear to be slightly biphasic. Thus it seems likely that the same dehydrase enzyme(s) is involved in a simple reverse reaction
Figure 30. Kinetics of reverse dehydrase activity using [3-$^{14}$C] 2-trans-18:1-CoA (●) or [3-$^{14}$C] 2-trans-8,11-20:3-CoA (○) as substrate. Conditions of the assay were the same as used for the forward reaction.
with these substrates. Analysis of the non-hydroxy methyl ester band by GLC after an incubation with 2-trans-18:1-CoA indicated that only the hydration reaction was occurring in the absence of NADPH, with no radioactive stearate being detected. As expected, small amounts of the presumed Δ1 isomer could also be detected during GLC. That the product of this reaction was the β-hydroxy derivative was confirmed by elution of the appropriate TLC band, followed by acetylation and radioactive GLC with authentic methyl 3-acetoxystearate as an internal standard.

C. The 2-trans-Enoyl-CoA Reductase Reaction

Since Kass et al. (111) have reported that the thioester linkage of 2-trans-decenoyl-(N-acetylcysteamine) is unusually resistant to alkaline hydrolysis, it was important to investigate the effectiveness of the saponification procedures used in this study when the 2-trans-enoyl-CoA derivatives were present as either unreacted substrate or as products at the completion of incubations. This was accomplished by determining the recovery of added radioactivity in both the top (predominately aqueous) and bottom layers of the Folch extract following incubations with [3-14C] 2-trans-18:1-CoA. It was found that essentially all of the label was recovered in the bottom layer after either a normal (enoyl-CoA reductase) incubation, or a zero-time control assay carried through the usual work-up procedures (97 and 98%, respectively). Recovery was also checked with aliquots of 2-trans-18:1-CoA in 1.5 ml of water by first saponifying under the usual conditions, then acidifying and performing a Dole's extraction (95). In this case, radioactivity recovered in the combined heptane extracts was 93-95%. It is possible that the radioactivity not
recovered in the organic phase in these studies primarily resulted from sulfide formation with liberated CoA(SH) by thiol addition across the 2-trans bond since such addition is known to occur quite readily (117). Repeat analyses conducted in the presence of 10 mM HgBr₂ as a sulfhydryl trap were in agreement with this interpretation, since in this case radioactivity recovery in heptane was essentially 100%. Such results also suggest that the substrate was not itself contaminated with sulfide.

The results of these studies indicated that while a minor amount of labeled material may have been lost to the upper phase, the recovery of radioactivity in the predominately organic phase of the Folch extract following saponification was nearly quantitative. By contrast, Kass et al (111) reported that the extent of saponification was as low as 63% in their assays with 2-trans-decenoyl-(N-acetyl cysteamine), and that this result could lead to incorrect calculations of enzyme activity with this substrate. It is possible that the difference in extent of saponification observed between the study reported here and that of Kass et al related to the nature of the substrates involved, but this seems somewhat unlikely since the CoA moiety also is in essence an N-acylated cysteamine derivative. A more likely explanation might be based on differences in saponification conditions since Kass et al carried out their hydrolysis at 30°C for ten minutes. When they used 50°C for 15 minutes, 77% hydrolysis occurred. By contrast, all saponifications in this study were conducted at 37°C for one hour. It would appear that such conditions were adequate to provide for the essentially complete recovery of all substrate and products in these analyses.
Substrate Dependency

v/s Curves for the enoyl-CoA reductase reaction with 2-trans-18:1-CoA and 2-trans-8,11-20:3-CoA as substrate are given in Figure 31. In both cases, BSA was included in the reaction mixture. As noted in the preceding section, the microsomal preparations were also active in converting these substrates to their respective β-hydroxy derivatives. However, the use of a radiochemical assay technique in these studies permitted the simultaneous monitoring of both reductase and dehydrase activity through the combined TLC-GLC assay described in Chapter II. As may be seen in Figure 31, although enoyl-CoA reductase activity was quite high and predominated throughout the substrate concentration range examined in the presence of NADPH, small amounts of the corresponding β-hydroxy acids were always produced, and the rate of this hydration was essentially the same with either substrate.

The effect of NADPH concentration on the reductase reaction is presented in Figure 32. That the microsomal preparations were devoid of endogenous NADPH is apparent from the strict dependency on exogenous NADPH which was observed. Although the reduced coenzyme exerted a noticeable regulatory effect on the direction of the reaction at low concentrations, this effect was minimal at higher NADPH levels. In fact, the rise in enoyl-CoA reductase activity in the lower concentration region of these curves was much more pronounced than the decline in hydrase activity. Throughout the NADPH concentration range examined, the decline in hydration rate was less than 50% from the rate observed in the absence of reducing equivalents. Since in microsomal chain
Figure 31. Kinetics of the enoyl-CoA reductase reaction showing the simultaneous formation of stearic acid (●) and 8-OH-18:0 (■) when [3-14C] 2-trans-18:1-CoA was the substrate, and of 8,11-20:2 (○) and 8-OH-8,11-20:2 (▲) when [3-14C] 2-trans-8,11-20:3-CoA was the substrate. All incubations were carried out in the presence of NADPH with a substrate to BSA molar ratio of 2.
Figure 32. Kinetics of the enoyl-CoA reductase reaction as a function of NADPH concentration. Conditions of assay were as described in Chapter II, and the identity of the products is the same as in Figure 31.
elongation, β-hydroxyacyl-CoA is formed from the β-keto analogue at an earlier NADPH-dependent step, it is probably unlikely that this directional control by coenzyme concentration is of major physiological significance.

As with the condensation reaction, but in marked contrast to the dehydrase reaction, the inclusion of BSA in the enoyl-CoA reductase reaction was essential to obtain optimal activity. The curves in Figure 33 were obtained when this reaction was run in the absence of albumin. With both substrates, maximum reductase activity was much lower than that observed in the presence of BSA. Beyond the optimum substrate concentration of about 20 μM, the reduction of 2-trans-18:1-CoA was progressively inhibited, while activity towards the polyunsaturated derivative maintained a plateau rate throughout the remainder of the concentration range examined. This behavior is nearly identical to that observed with the corresponding fatty acyl-CoA substrates in the condensation reaction. As can be seen in Figure 33, once the reductase was saturated, both substrates were rapidly hydrated. Somewhat surprisingly, the rate increase with either substrate was nearly identical for this reaction despite the increasing difference in unconverted substrate concentration with the two substrates due to the inhibitory effect of 2-trans-18:1-CoA. This may have related to the slightly greater hydrase activity obtained with the polyunsaturated substrate (Figure 30). These studies thus further suggest that the dehydrase reaction, whether measured in a forward or reverse direction, is neither highly substrate specific nor BSA dependent.

Protein and Time Dependency

As indicated in Figure 34, the production of stearic acid from 2-trans-18:1-CoA (20 μM, without BSA) rose linearly through the protein concentration range. However, at the lowest protein concentration the
Figure 33. Kinetics of the enoyl-CoA reductase reaction in the absence of BSA. Identity of the products and conditions of assay were the same as in Figure 31 except for the exclusion of BSA.
Figure 34. Protein dependency of the enoyl-CoA reductase reaction assayed in the absence of BSA with 20 μM [3-14C] 2-trans-18:1-CoA (▲) showing the simultaneous production of 8-OH-18:0 (□) and stearic acid (●).
reverse reaction appeared to predominate during the short incubation time interval (0.5 min) employed. This potentially favored reverse reaction even in the presence of saturating levels of NADPH is also seen in the time course for the reaction (Figure 35) which was determined under the same conditions. During this reaction, hydration activity was somewhat greater initially, leading to slightly more β-hydroxy then α, β-saturated product at the end of the shortest time period examined (0.2 min). As expected, this process was reversed as time progressed so that after an initial rise in β-hydroxystearate production, its concentration fell to low levels while that of the α, β-saturated product continued to rise until the reaction was completed.

That hydration predominated during very short time intervals in this reaction in the absence of BSA is confirmed by the results summarized in Figure 36 in which 2-trans-8,11-20:3-CoA was used as substrate. Since substrate inhibition was not observed with the polyunsaturated derivative, a substrate saturating level could be used in this assay. Under these conditions, a definite predominance of β-hydroxy acid formation during very short time intervals with a subsequent reversal as the incubation progressed is clear. The influence of this reversal was minimized in the presence of BSA however, since in this case reductase activity was greatly facilitated (cf. Figure 31).

Although the results of Figure 35 in which a limiting amount of substrate was present suggest that the 2-trans-18:1-CoA substrate was only about 87% enzymatically active, this probably may be attributed for the most part to competing deacylation during the reaction. Since ATP was never included, deacylation would produce an inactive substrate. The persistence of low levels of β-hydroxystearate after one minute of
Figure 35. Time course of the enoyl-CoA reductase reaction assayed in the absence of BSA with 20 μM [3-14C] 2-trans-18:1-CoA (△) showing the simultaneous production of β-OH-18:0 (♦) and stearic acid (●).
Figure 36. Time course of the enoyl-CoA reductase reaction assayed in the absence of BSA with 48 μM [3-^{14}C] 2-trans-8,11-20:3-CoA (▲) showing the simultaneous production of β-OH-8,11-20:2 (•) and 8,11-20:2 (●).
incubation is in agreement with such an explanation. For the same reason, the product of the hydration reaction involving these substrates must either remain enzyme-bound, or be transferred to a CoA acceptor, since otherwise the reaction could not again be reversed during later time periods in the absence of an activating system.

D. The 8-Ketoacyl-CoA Reductase Reaction

In these studies of intermediate reaction steps only the condensation, dehydrase and enoyl-CoA reductase reactions have been considered in detail. This is because 8-ketoacyl-CoA reductase activity cannot be studied as an isolated reaction in intact microsomes; in the presence of NADPH the reaction will continue through the two remaining steps of chain elongation as well. Also, the synthesis of CoA esters of 8-keto acids has generally been limited to short chain acids. While the chemical synthesis of the CoA thioester of a long chain 8-keto acid (8-ketopalmitate) has been reported by Al-Arif and Blecher (87), the UV spectrum reported for this derivative suggested that the final product was not completely pure. For this study, [3-14C] 8-ketostearoyl-CoA was synthesized through the dioxolane essentially according to the above procedure (87), and its purity was estimated as described in Chapter II. The compound was then used to assay the overall reaction:

\[
\text{CH}_3-\text{(CH}_2\text{)}_{14}-\overset{O}{\text{C}}-\text{CH}_2-\overset{O}{\text{C}}-(\text{S})\text{CoA} + 2 \text{ NADPH} + 2 \text{ H}^+ \rightarrow \text{CH}_3-\text{(CH}_2\text{)}_{16}\text{-COOH} + \text{H}_2\text{O} + 2 \text{ NADP}^+
\]

A v/s curve constructed for this overall reaction is given in Figure 37. Although the analysis was complicated by both the limited purity and low specific activity of the substrate, it is apparent that the overall conversion to stearic acid occurred at least six times as
Figure 37. Kinetics of the β-ketostearoyl-CoA reductase reaction analyzed as described in Chapter II; 18:0 (●), 16:0 (◆).
rapidly as β-ketostearic acid was produced from 16:0-CoA and malonyl-CoA in the condensation reaction, thus indicating that the β-keto reductase step cannot be rate limiting in the overall chain elongation of palmitic acid. These findings confirm Nugteren's studies (12) which showed that the reduction of β-ketopalmitate was not rate-limiting in the microsomal chain elongation of myristic acid.

It is also apparent from Figure 37 that while the production of stearate predominated at all substrate concentrations employed, a partial conversion of β-ketostearoyl-CoA to palmitic acid could always be detected. While contamination of the microsomal preparation with thiolase might have contributed to this result, it should be noted that Nugteren (12) also has detected this reaction with rat liver microsomes in the presence of β-ketopalmitic acid; and Chang and Holman (118) have reported the chain shortening of a variety of fatty acyl-CoAs by rat liver microsomes in a process which they indicated could not be attributed to mitochondrial contamination. Whether the conversion of β-ketostearoyl-CoA to palmitic acid noted in this study resulted from a true microsomal reaction or a mitochondrial contaminant, it is apparent that significant conversions were only obtained at relatively high initial substrate concentrations. Since the concentration of β-ketoacyl-CoA during the condensation reaction would always be quite low, it is unlikely that this reaction seriously impaired condensation product recovery, although it may have contributed to a slight underestimation of condensing activity. It is also conceivable that the different conditions used for condensation assays may have further reduced the extent of this competitive reaction.
E. Assay of Reaction Products as the CoA Thioesters

It is generally accepted that the product of de novo fatty acid synthesis in higher organisms is the free fatty acid (98, 119, 120), while the product of chain elongation has been reported to be the CoA thioester (12). When chain elongation intermediates as the free acids are used as substrates, a CoA ester generating system is required (12) indicating that intermediate substrates also must be activated to the thioester prior to their further metabolism. However, although the coenzyme A ester is the required substrate in these reactions, it is not known whether intermediate products of partial reactions in the chain elongation sequence may be recovered as thioesters or only as free acids. The former possibility would be required if multiple enzymes rather than a single complex are involved in chain elongation, since an activating system is not required for overall chain elongation when the initial primer is present as the CoA ester. In order to investigate the nature of the product(s) of partial reactions in this study, incubations were terminated and analyzed by procedures designed to recover the labeled compounds in the CoA ester form.

The results of a typical thin-layer analysis of a reaction conducted and analyzed as described in Chapter II (Section E) are given in Figure 38. Most of the radioactivity was recovered in a band corresponding in Rf to that of an acyl-CoA standard. Essentially identical results were obtained in all cases since this system cannot separate fatty acyl-CoA derivatives differing only in the acyl group. The use of appropriate standards confirmed that the fatty acyl moiety of all substrates, including β-keto and β-hydroxy derivatives, whether as the methyl ester or the free acid, always migrated close to the solvent front in this system, and was well separated from the acyl-CoA band.
Figure 38. Representative thin-layer chromatogram obtained following a reaction analysis conducted under non-saponification conditions as described in Chapter II, section E. The standards employed were (1) palmitoyl-CoA and (2) palmitic acid.
When an overall chain elongation reaction using \[1^{14}C\] palmitoyl-CoA as primer was analyzed as described above, after which the material in the acyl-CoA band was saponified, acidified, extracted, converted to methyl esters and fractionated by GLC, the chromatogram represented in Figure 39 was obtained. Aside from unreacted substrate, the preponderance of recovered radioactivity was present in methyl stearate, indicating that the original acyl-CoA band was composed of a mixture of substrate and product. The estimated conversion derived from the data of Figure 39 is slightly higher than that obtained by the usual saponification conditions, consistent with relatively more deacylation and/or incorporation into lipids of substrate than product during the course of the reaction.

The condensation reaction was then analyzed by conducting the same reaction as above in the absence of NADPH. After scraping the acyl-CoA band from the plate, it was suspended in a solution of 4% KOH in 90% ethanol and vigorously stirred for four hours. The mixture was then freed of Silica gel and refluxed for an additional three hours. After cooling the mixture, it was extracted with ether and analyzed by TLC. Alkaline extraction conditions were used in an attempt to reduce contamination by unreacted palmitic acid. The results in Figure 40 indicate that only two major bands were recovered in this case, one migrating with the free fatty acid standard and representing residual unreacted substrate, and another migrating with the methyl ketone standard. Elution of the latter band and analysis by GLC as described earlier (Section A) confirmed that it was 2-heptadecanone. Since the methyl ketone would have been formed from \(\beta\)-ketostearic acid during saponification, these results confirm that the product of the condensation reaction also may be recovered as the CoA ester under these conditions.
Figure 39. Gas-liquid chromatogram of the methyl esters derived from the acyl-CoA region of Figure 38 following an overall chain elongation reaction with [1-\(^{14}\)C] 16:0-CoA in the presence of NADPH.
Figure 40. Thin-layer chromatogram of the saponification products of region (1) of Figure 38 following a condensation reaction with [1-$^{14}$C] 16:0-CoA as primer. The identity of the standards is the same as in Figure 3.
Similar incubations were conducted with \([3^{-14}C]\) \(\beta\)-hydroxystearoyl-CoA as substrate, both in the presence and absence of NADPH. After isolation of the acyl-CoA band and conversion of the recovered material to methyl esters as before, the extracts were analyzed by TLC. Essentially the same results were obtained in either case (Figure 41) in which both labeled methyl \(\beta\)-hydroxystearate and non-hydroxy methyl esters could be detected. The product band was then eluted and further fractionated by GLC.

As expected, when the reaction was conducted in the absence of NADPH, the product band consisted solely of methyl 2-trans-18:1 and a small amount of the presumed \(\Delta^3\) isomer. However, when the non-hydroxy methyl ester band resulting from an incubation in the presence of NADPH was analyzed, the gas-liquid chromatogram represented in Figure 42 was obtained. Most of the radioactivity (81%) was recovered in methyl stearate as would be anticipated. However, a significant portion of the label (18%) could be detected in methyl 2-trans-18:1, indicating its formation as the CoA ester during the reaction.

These data indicate that all of the intermediate (and overall) products of microsomal 16:0-CoA chain elongation may be recovered as the CoA thioester, with the possible exception of \(\beta\)-hydroxystearoyl-CoA which was not analyzed as a reaction product. The results of Figure 42 further suggest that 2-trans-18:1-CoA is produced as a distinct intermediate in the two step conversion of \(\beta\)-hydroxystearoyl-CoA to stearoyl-CoA. This would appear to be more consistent with the involvement of two separate enzymes in this reaction than with a single multi-enzyme complex as has recently been proposed (28). These data cannot confirm this point, however.
Figure 41. Thin-layer chromatogram of the methyl esters derived from the acyl-CoA region of Figure 38 following a dehydrase reaction conducted under standard conditions with [3-\(^{14}\)C] \(\beta\)-OH-18:0-CoA as substrate. The standards employed were (1) methyl \(\beta\)-hydroxystearate and (2) methyl 2-trans-octadecenoate.
Figure 42. Gas-liquid chromatogram of the methyl esters eluted from region (2) of Figure 41 following an incubation with [3-14C] β-OH-18:0-CoA conducted in the presence of NADPH. Methyl 2-trans-octadecenoate was included as an internal standard.
F. Comparison of Reaction Rates

Table III summarizes the mean rates of all the reactions measured with microsomes from rats raised on either chow or fat-free diets. Rates of overall chain elongation, condensation and enoyl-CoA reductase reactions were all measured using the standard incubation conditions described in Chapter II. Dehydrase reactions were determined using a substrate concentration of 120 µM which corresponds to the plateau region of the \( v/s \) curves obtained with these substrates. These conditions were selected so that product formation approximated zero-order conditions with respect to substrate in each case.

That subsequent intermediate reactions occur at much faster rates than condensation is obvious from the data in this Table. In the palmitic acid sequence with fat-deficient rats, the rates for dehydrase and enoyl-CoA reductase activities were respectively 17- and 33-fold higher than condensation when both condensation and reductase determinations were carried out in the presence of BSA. The corresponding increments in the 6,9-octadecadienoate pathway were 12- and 25-fold, respectively. It is interesting that the maximum dehydrase rates would be about the same as enoyl-CoA reductase activities in both cases if one assumes that all of the substrate could be bound by the enzyme, but that only 50% of the racemic mixture was enzymatically active. As may be noted in the Table, the increment in both intermediate activities relative to both condensation and overall chain elongation rates was considerably greater than the above when normal animals were utilized.

It is also apparent from these data that while chain elongation was greatly increased with all three substrates in the fat-deficient animals, the dietary effect on all partial reaction steps except condensation was
Table 3

Rates of Overall and Partial Reactions in the Microsomal Chain Elongation Pathway<sup>a</sup>

| Initial Substrate | 16:0-CoA | | | 6,9-18:2-CoA | | | 6,9,12-18:3-CoA | | |
|------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Dietary Condition | Normal | Fat-Free | [N/FF]<sup>b</sup> | Normal | Fat-Free | [N/FF] | Normal | Fat-Free | [N/FF] |
| Chain Elongation | 0.7 ± .01 | 2.9 ± .23 | [.25] | 2.8 ± .05 | 4.0 ± .10 | [.70] | 3.5 ± .08 | 6.0 ± .15 | [.59] |
| Condensation | 0.9 ± .03 | 3.0 ± .06 | [.30] | 3.0 ± .02 | 4.1 ± .04 | [.73] | 4.3 ± .09 | 6.8 ± .08 | [.63] |
| β-Hydroxy Acyl-CoA Dehydrase | 45 ± 1.3 | 50 ± 2.0 | [.92] | 46 ± 1.1 | 49 ± 1.6 | [.93] | - | - | - |
| 2-trans Enoyl-CoA Reductase | 100 ± 2.7 | 102 ± 2.5 | [.98] | 99 ± 1.0 | 104 ± 3.0 | [.95] | - | - | - |

<sup>a</sup> All results are expressed as nmols of product formed min<sup>-1</sup> (mg microsomal protein)<sup>-1</sup> ± SE. The number of determinations for each value is in parentheses. Each determination involved microsomes derived from two pooled livers, and was the mean of three separate assays.

<sup>b</sup> Ratio of specific activity with microsomes from rats raised on a normal versus a fat-free diet.
minimal. A Student's t test of the mean variation between normal and fat-free animals was highly significant (p<.001) for all three condensation reactions. This result might be compared with similar analyses of the data for overall chain elongation (p<.001 in all cases), and for both dehydrase and enoyl-CoA reductase activities (p>0.2 in each case). Thus while both chain elongation and condensation activities were significantly affected by diet, there was no significant dietary effect on any of the other reactions examined with either palmitate or 6,9-octadecadienoate intermediates. Since the values for each reaction obtained under the two dietary conditions were not intrinsically paired, and since the sample size varied in each case, the effect of diet on various paired reaction steps did not lend itself to statistical evaluation. That is, although both condensation and chain elongation rates were significantly elevated in the fat-deficient animal, it was not possible to test whether the extent of elevation was identical in both cases. However, it can be seen that good general agreement between the mean ratio of normal and fat-deficient activities was obtained for both overall chain elongation and condensation activity with all three substrates (column 3 under each substrate). Conversely, the mean ratios for the dehydrase and enoyl-CoA reductase reactions under the two dietary regimens do not agree well with those for overall chain elongation, and in all cases are close to unity. As noted above, diet did not significantly affect either of these two reactions. In addition, the effect of diet on condensation activity was much more pronounced with palmitic acid than with the unsaturated substrates, while the latter did not differ greatly from each other. These results strongly suggest that the dietary effect on chain elongation is mediated solely through condensation activity in agreement with a probable
regulatory role for this reaction, and provide further support for the existence of at least two separate condensation systems in rat liver microsomes as suggested earlier by the NEM inhibition studies.
CHAPTER IV

DISCUSSION

Nugteren (12) found that in the microsomal chain elongation of myristate to palmitate, the rates of the $\beta$-ketoacyl-CoA reductase, $\beta$-hydroxyacyl-CoA dehydrase and 2-trans-enoyl-CoA reductase reactions were all much greater than that of overall chain elongation. From these results he deduced that the rate-limiting step was probably the condensation reaction. In the work reported here it has been shown by direct measurement that the overall condensation reaction is rate limiting in the conversion of palmitate to stearate. Similarly, these studies have shown that the rate of overall chain elongation is the same as that of the condensation reaction for 6,9-18:2-CoA and 6,9,12-18:3-CoA with rat liver microsomes. Although the $\beta$-ketoacyl-CoA reductase reaction was not measured for these unsaturated substrates, the $\beta$-hydroxyacyl-CoA dehydrase and 2-trans-enoyl-CoA reductase reactions in the 6,9-18:2 sequence were found to proceed much more rapidly than the condensation step. These findings thus strongly suggest that the condensation step is also rate-limiting for the microsomal chain elongation of unsaturated fatty acids.

Results obtained in this study also support the existence of at least two chain elongation systems in rat liver microsomes, and are consistent with the involvement of only the condensation reaction in this regard. Multiple chain elongation systems have previously been suggested to exist in both rat liver and mouse brain microsomes, and the involvement of more than one overall chain elongation system for saturated fatty acid substrates
In the latter organ seems well established. Bourre et al (38) have provided evidence for the existence of at least two chain elongation systems in this tissue, one involved in the elongation of palmitic acid, and another functioning with longer chain substrates. The studies of Goldberg et al (36) suggest that the second system may in fact be further divided into one acting on stearic acid, and another elongating acids of twenty or more carbon atoms. Thus there appear to be at least three separate fatty acid elongation systems in mouse brain microsomes involved in the production of the very long chain saturated acids characteristic of this tissue.

A recent report by Sprecher (55) involving dietary manipulation and competitive substrate interactions suggested the existence of at least two chain elongating systems in rat liver microsomes: one extending palmitic acid, and another operative with long chain polyunsaturated substrates. The NEM inhibition rate studies of the condensation reaction reported here, in conjunction with the effect of dietary alteration on partial reaction steps, are in excellent agreement with those results and strongly suggest that this dichotomy involves - and may in fact be limited to - the initial condensation reaction. In fact, it is possible that two or more substrate-specific condensation systems contribute their respective β-keto products to a common set of enzymes to complete the overall chain elongation process. Such a proposal would imply that overall regulation of chain elongation is mediated primarily through the condensation reaction. It is interesting in this regard that D'Agnolo et al (121) have recently demonstrated the involvement of two condensing enzymes in E. coli fatty acid synthesis. The more recently discovered enzyme, designated β-ketoacyl acyl carrier protein synthetase II, appears
to be specific for the elongation of palmitoleic to cis-vaccenic acid, and may thus play a critical role in the regulation of unsaturated fatty acid composition in that organism.

The specific activity of reactions in the microsomal chain elongation process was also dependent on the conditions of assay. While the dehydrase reaction was independent of BSA under the conditions employed, the presence of albumin was found to exert a considerable effect on both the condensation and enoyl-CoA reductase reactions. In the absence of BSA, condensation activity was greatly reduced and substrate inhibition occurred with palmitoyl-CoA, but not with the polyunsaturated substrates. The enoyl-CoA reductase reaction displayed nearly identical results; i.e., the rate was severely reduced in the absence of BSA, and substrate inhibition occurred at a relatively low concentration of 2-trans-18:1-CoA, while no substrate inhibition could be discerned throughout the range examined when 2-trans-8,11-20:3-CoA was employed.

It is likely that the substrate inhibition observed in these two cases involved micelle formation. Substrate inhibition by long chain acyl-CoA derivatives has been a common observation in acyl-CoA transferase reactions of phospholipid biosynthesis (51,101,104), as well as in fatty acid desaturation (122,123) and microsomal fatty acid chain elongation (35,41). In all these cases the enzymes involved presumably act on substrate monomers and are inhibited by micelles (51). In both the condensation and enoyl-CoA reductase reactions, the substrate concentration at which inhibition was first noted in the absence of BSA was about 20 μM. Although this is well above the reported CMC for fatty acyl-CoA derivatives (51,124), it is important to consider the microsomal protein concentration in evaluating these data. The CMC is strictly a function of free acyl-CoA
concentration, and many authors have noted that microsomes are capable of binding fatty acyl-CoA thioesters thereby considerably reducing the concentration of free acyl-CoA in solution (51,101,102,104,106). Thus Lamb et al (102) found that when 40 nmoles of palmitoyl-CoA were incubated with 1.04 mg of microsomal protein, 90% of the acyl-CoA was bound to the microsomes and only 4 nmoles were free in solution. Since relatively high protein concentrations were used in the condensation and enoyl-CoA reductase reactions, the apparently elevated CMC value of 20 μM may well reflect this microsomal binding of substrate. However, it should be noted that the substrate concentration at which inhibition was first detected in the absence of BSA was about the same in both the condensation and enoyl-CoA reductase reactions, despite the fact that the former reaction involved twice as much microsomal protein. Whether the relative extent of binding of palmitoyl-CoA and 2-trans-18:1-CoA by microsomes could account for this result is unknown.

These considerations also fail to explain why no substrate inhibition of the condensation or enoyl-CoA reductase reactions was observed with 6,9-18:2-CoA or 2-trans-8,11-20:3-CoA, respectively. Gatt and Barenholz (125) have pointed out that enzymes acting on soluble amphipathic molecules only in the monomer form will show deviations from expected behavior if the CMC occurs in the substrate range examined. Thus enzymes susceptible to micelle inhibition will display biphasic v/s curves, having an ascending hyperbola, an inversion point and a descending portion (type III curve of Gatt and Barenholz). On the other hand, a v/s curve displaying a relatively sharp transition to a level plateau, then maintaining this (zero-order) rate as the substrate concentration is increased is suggestive of a monomer-specific enzyme which is neither active towards,
nor inhibited by micelles. Brockman (116) has noted that this zero-order behavior is consistent with the formation of a surface excess of acyl-CoA in the vicinity of the catalytic site of the enzyme. In both the condensation and enoyl-CoA reductase reactions in the absence of BSA, the v/s curves were of this latter form with the polyunsaturated acyl-CoA substrates.

With respect to the condensation reaction, these results might be explained if one assumes that the condensation system acting upon 16:0-CoA is susceptible to micellar inhibition by the substrate, while the system operative with the polyunsaturated substrates does not display a similar sensitivity. This explanation seems plausible to the extent that the results obtained in this study strongly suggest the existence of separate condensing systems for palmitic versus polyunsaturated acids. It is interesting that a similar dichotomy was observed in the desaturation reaction in which extensive substrate inhibition by stearoyl-CoA was noted in the absence of BSA, while no such inhibition by linoleoyl-CoA could be demonstrated (122). Two different enzymes are known to be involved in desaturating fatty acids at the 9- and 6-positions (126). However, this explanation would imply that rat liver microsomes contain two different enoyl-CoA reductases, one of which is inhibited by micelles of 2-trans-18:1-CoA while the other is insensitive to micelles of 2-trans-8,11-20:3-CoA, and no evidence supporting that contention has been obtained. The extensive studies in Seubert's laboratory also have provided no evidence that the microsome contains more than one enoyl-CoA reductase (16,28).

An alternative explanation might involve differences in the extent of formation or physical properties (e.g., size or aggregation number) of micelles formed from saturated versus polyunsaturated acyl-CoA.
derivatives. In this regard 2-trans-18:1-CoA would be expected to behave physically as a saturated fatty acid thioester due to the trans configuration. The unsaturated substrates might be expected to form micelles less readily, but the effects of unsaturation on the CMC of fatty acid salts is quite small (127,128), and no significant differences among the CMC values of a variety of saturated and unsaturated acyl-CoA esters was detected by Barden and Cleland (51). A slightly more likely possibility is that the micelles formed from 2-trans-18:1-CoA might differ from those produced from 2-trans-8,11-20:3-CoA and thus interact with a common reductase in different ways. This could involve, for example, differences in the "small to large" micelle transition (128) for the two substrates. However, in the absence of any data on the nature of acyl-CoA micelles, such an interpretation must remain highly speculative.

As was previously noted to occur with microsomal protein, the BSA-induced alleviation of substrate inhibition also probably involved a reversible acyl-CoA binding by the albumin (102,104,129) reducing the free acyl-CoA concentration at all substrate levels to a value below the CMC. This postulated effect of BSA has been advanced by many authors to explain results obtained with a variety of enzymes susceptible to micellar inhibition (34,51,101,104,130,131), and was originally proposed by Jeffcoat et al (122) to explain the elimination of substrate inhibition of the 9-desaturase by BSA. However, these same authors later suggested that the effect of BSA in their system may have been due solely to an inhibition of stearoyl-CoA hydrolase activity by the albumin (132).

This latter explanation does not seem compelling, and it is not likely that inhibition of acyl-CoA hydrolase activity by BSA can account for the results obtained in the study reported here. The ability of BSA
to reduce microsomal acyl-CoA hydrolase activity is well known (34,51,102, 104). However, an effect of substrate depletion on a competing reaction should be significant only when non-substrate saturating conditions are employed (104). Brophy and Vance (34) have shown that while BSA could completely inhibit stearoyl-CoA hydrolase activity in rat brain microsomes, this effect alone could not account for the augmentation in overall chain elongation which they observed with saturating levels of substrate in the presence of BSA. In addition, an inhibition of acyl-CoA hydrolase activity cannot explain the similar protective effect of BSA on the activities of the de novo synthetase (133,134), acetyl-CoA carboxylase (130) and citrate synthetase (135) in the presence of fatty acyl-CoAs, since in all these cases the thioester functions only as an inhibitor and not as a substrate. In fact, Enoch et al (123) found that a purified reconstituted stearoyl-CoA desaturase system was susceptible to substrate inhibition, that the substrate concentration at which inhibition was noted was a function of both the amount of lipid and enzyme protein present in the assay mixture, and that the inhibition could be alleviated by adding extra lipid to the assay. Thus it seems probable that both the 9-desaturase and microsomal chain elongation systems are susceptible to substrate micellar inhibition.

It is also possible that the desaturase and chain elongation systems respond somewhat differently to acyl-CoA binding proteins. Thus the BSA effect observed by Jeffcoat et al (122) on the stearoyl-CoA desaturase system involved only the apparent Km, and not Vmax'. Inclusion of BSA in their study alleviated substrate inhibition and caused saturation to be approached over a broader substrate concentration range, but the maximum conversion with 100 μM substrate at various BSA concentrations
was never greater than the activity at 10 μM in the absence of BSA (122). Conversely, in the condensation and enoyl-CoA reductase reactions as noted in Chapter III, BSA was found to both alleviate substrate inhibition and markedly accelerate the reaction. A very similar effect of BSA in both eliminating substrate inhibition and augmenting the Vmax' of stearoyl-CoA elongation has been demonstrated with rat brain microsomes (34).

In the analyses conducted in this study a constant acyl-CoA/BSA molar ratio was employed at all substrate concentrations. Obviously in this case the total amount of BSA present in the incubation was then a variable throughout the substrate concentration range. The increase in apparent Vmax well above the maximum rate obtained without BSA observed in these studies would suggest that the albumin was in some manner directly affecting the reaction, perhaps by serving as a model carrier, rather than by acting strictly through excess acyl-CoA adsorption. The ability of serum albumin as well as other soluble proteins to bind long chain acyl-CoA derivatives and thus contribute to the regulation of a (yeast) fatty acid synthetase system in vitro has been noted by Sumper (133), and by Sumper and Traüble (129). These authors have suggested that the function of the protein may be to serve as an acyl-CoA carrier to the membrane where a competition for the substrate by enzymes acting upon it may occur. Such a proposed role would appear to be quite consistent with the results obtained in this study. BSA might thus have served as a model carrier in these reaction, substituting for one or more of the naturally occurring acyl-CoA binding proteins (102,104,122, 130,131,136-138). It is interesting that under the conditions employed, BSA had no effect on the β-hydroxystearoyl-CoA dehydrase reaction, perhaps suggesting that a carrier is involved only in providing the
initial substrate and in removing the final reaction product of chain elongation.

Podack et al (139) have reported that rat liver microsomes possess acyl-CoA transferase activity as monitored with pantetheine as an acceptor. They have proposed that, for the chain elongation of a saturated fatty acid, a single acyl transferase functions to transfer malonyl-CoA and fatty acyl-CoA to the enzymes, and also catalyzes an exchange of each enzyme-bound intermediate with free CoA(SH). The latter reactions were proposed to explain why intermediate substrates as their CoA thioesters are active in chain elongation (12). In addition, a second transferase specific for polyunsaturated substrates has been postulated by these authors (139) although no evidence for its existence has yet been presented.

At this time there does not appear to be sufficient evidence available to either confirm or refute the involvement of an acyl transferase in microsomal chain elongation. If such activity is involved in this process, one might suggest that the differential substrate inhibition observed in this study between saturated and polyunsaturated substrates in both the condensation and enoyl-CoA reductase reactions involved specific acyl-CoA transferases rather than the condensing or reductase enzymes per se. However, this seems unlikely since in order to observe such an effect on the measured reaction rate, transferase activity would have to be (or become) rate-limiting. It is difficult to see how this enzyme could be rate-limiting for condensation but not for reductase (or dehydrase) activities under similar conditions, since the same transferase is purportedly involved in all these reactions (139). It might be noted that the inhibited reductase rate remained much greater
than the uninhibited condensation rate, and no inhibition could be
detected throughout the concentration range examined in the dehydrase
reaction, further suggesting that a common enzyme was not rate-limiting
in these reactions, although structural differences among the respective
substrates could conceivably have influenced these results.

Since condensation is much slower than all other partial reactions
of chain elongation, a common transferase clearly is unlikely to be rate-
limiting for the overall reaction. The dietary effect on chain elongation
which was mediated solely through the condensation reaction probably
cannot be attributed to an effect on the postulated common transferase
for the same reason. Thus whether or not the acyl-CoA thioester is the
immediate substrate in chain elongation, the results of this study remain
most consistent with a regulation mediated solely through multiple and
rate-limiting condensing enzymes.


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