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POLYUNSATURATED FATTY ACIDS.

The Ohio State University,
Ph.D., 1978
Chemistry, biological

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SUBSTRATE SPECIFICITY STUDIES ON THE
MALONYL-CoA DEPENDENT CHAIN ELONGATION
OF POLYUNSATURATED FATTY ACIDS

DISSERTATION

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

By
Stephen A. Ludwig, B.A., M.S.

* * * * * * *

The Ohio State University
1978

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ABSTRACTS

associated intermediary conversions of octadecadienoic acids, Fed.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
</tbody>
</table>

## CHAPTERS

I LITERATURE REVIEW                                                   3
II MATERIALS AND METHODS                                               36
III MALONYL-CoA DEPENDENT CHAIN ELONGATION

The substrate specificity and rates of conversion for an isomeric series of octadecadienoic acids and selected \(\beta\)-hydroxy and 2-trans analogues as determined with rat liver and rat testicular microsomes

Results                                                                65
Discussion                                                             92

The substrate specificity and proposed binding site of the microsomal chain elongation enzyme

Results                                                                101
Discussion                                                             108

IV SUMMARY                                                              132

BIBLIOGRAPHY                                                           135


### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Known rates of chain elongation expressed as percent of fastest chain elongated fatty acid</td>
<td>28</td>
</tr>
<tr>
<td>2.</td>
<td>Percent purity, specific activity, and R₁₈:₀ of the chemically synthesized [1-¹⁴C]-octadecadienoic acids</td>
<td>43</td>
</tr>
<tr>
<td>3.</td>
<td>Percent purity and specific activity of miscellaneous chemically synthesized [1-¹⁴C] fatty acids</td>
<td>44</td>
</tr>
<tr>
<td>5.</td>
<td>Rates of chain elongation, condensation, dehydration, and reduction for an isomeric series of octadecadienoic acids and associated β-hydroxy and 2-trans intermediates</td>
<td>89</td>
</tr>
<tr>
<td>6.</td>
<td>Comparison of the rates of chain elongation, condensation, dehydration, and reduction for rat liver microsomes and rat testicular microsomes</td>
<td>91</td>
</tr>
<tr>
<td>7.</td>
<td>Comparative rates of chain elongation for fatty acids with identical proximal and dissimilar distal moieties</td>
<td>105</td>
</tr>
<tr>
<td>8.</td>
<td>Comparative rates of chain elongation for fatty acids with identical distal and dissimilar proximal moieties</td>
<td>106</td>
</tr>
<tr>
<td>9.</td>
<td>Comparative rates of chain elongation for fatty acids as a function of double bonds and position</td>
<td>107</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Simplified schematic diagram of the similarities between de novo synthesis, chain elongation, and β-oxidation</td>
<td>4</td>
</tr>
<tr>
<td>2.</td>
<td>The linolenate, linoleate, palmitoleate, and oleate pathways of polyunsaturated fatty acid metabolism</td>
<td>8</td>
</tr>
<tr>
<td>3.</td>
<td>The reactions of microsomal chain elongation</td>
<td>13</td>
</tr>
<tr>
<td>4.</td>
<td>Assay of the condensation reaction</td>
<td>59</td>
</tr>
<tr>
<td>5.</td>
<td>Assay of the dehydration reaction</td>
<td>61</td>
</tr>
<tr>
<td>6.</td>
<td>Assay of the 2-trans reduction reaction</td>
<td>63</td>
</tr>
<tr>
<td>7.</td>
<td>Rates of activation to the CoA derivative for an isomeric series of [1-14C] octadecadienoic acids</td>
<td>66</td>
</tr>
<tr>
<td>8.</td>
<td>Rates of chain elongation and condensation for an isomeric series of [1-14C] octadecadienoic acids</td>
<td>68</td>
</tr>
<tr>
<td>9.</td>
<td>Time dependent rates of chain elongation for [1-14C] 7,10-octadecadienoic acid</td>
<td>69</td>
</tr>
<tr>
<td>10.</td>
<td>Protein dependent rates of chain elongation for [1-14C] 7,10-octadecadienoic acid</td>
<td>70</td>
</tr>
<tr>
<td>11.</td>
<td>Kinetics of [1-14C] 7,10-octadecadienoic acid chain elongation as a function of the malonyl-CoA and NADPH concentration</td>
<td>71</td>
</tr>
<tr>
<td>12.</td>
<td>Kinetics of [1-14C] 7,10-octadecadienoic acid chain elongation as a function of the substrate concentration</td>
<td>72</td>
</tr>
<tr>
<td>13.</td>
<td>Time dependent rates of condensation for [1-14C] 7,10-octadecadienoic acid</td>
<td>74</td>
</tr>
<tr>
<td>14.</td>
<td>Protein dependent rates of condensation for [1-14C] 7,10-octadecadienoic acid</td>
<td>75</td>
</tr>
<tr>
<td>15.</td>
<td>Kinetics of [1-14C] 7,10-octadecadienoic acid condensation as a function of the substrate concentration</td>
<td>76</td>
</tr>
</tbody>
</table>
16. Time dependent rates of dehydration and reduction for [3-14C] β-hydroxy-9,12-eicosadienoyl-CoA........................................ 78
17. Protein dependent rates of dehydration and reduction for [3-14C] β-hydroxy-9,12-eicosadienoyl-CoA........................................ 79
18. Kinetics of [3-14C] β-hydroxy-9,12-eicosadienoyl-CoA dehydration and reduction as a function of the NADPH concentration................................. 80
20. Time dependent rates of reduction for [3-14C] 2-trans-9,12-eicosatrienoyl-CoA...................................................... 83
21. Protein dependent rates of reduction for [3-14C] 2-trans-9,12-eicosatrienoyl-CoA...................................................... 84
22. Kinetics of [3-14C] 2-trans-9,12-eicosatrienoyl-CoA reduction as a function of the NADPH concentration................................. 85
23. Time dependent rates of hydration for [3-14C] 2-trans-9,12-eicosatrienoyl-CoA with and without NADPH........................................ 86
24. Time dependent rates of hydration for [3-14C] 2-trans-9,12-eicosatrienoyl-CoA with and without NADPH and NADP............... 87
25. Kinetics of [3-14C] 2-trans-9,12-eicosatrienoyl-CoA reduction as a function of the substrate concentration................................. 88
27. Rates of chain elongation for [1-14C] 7,10- fatty acids of increasing chain length.......................................................... 104
28. Possible model of the substrate-binding site involving the interaction of the fatty acyl moiety with a planar or unrestricted region on the surface of the chain elongation enzyme........................................ 117
29. Proposed model of the substrate-binding site involving the interaction of the fatty acyl moiety in a cleft of the chain elongation enzyme........................................ 119
30. Selected features of the fatty acyl moiety and the cleft involved with binding in the proposed model........................................ 122
31. Possible binding energies of 7,10-octadecadienoyl-CoA............................................................. 124
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>ACP</td>
<td>Acyl carrier protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>2',7'-DCF</td>
<td>2',7'-dichlorofluorescein</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EE</td>
<td>Ethyl ether</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavine adenine dinucleotide</td>
</tr>
<tr>
<td>GLC</td>
<td>Gas liquid chromatography</td>
</tr>
<tr>
<td>Km</td>
<td>Apparent Michaelis constant</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide, oxidized</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced</td>
</tr>
<tr>
<td>PE</td>
<td>Petroleum ether</td>
</tr>
<tr>
<td>dimethyl POPOP</td>
<td>1,4-bis-(2-(4-Methyl-5-phenyloxazolyl)benzene</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-Diphenyloxazole</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>Vmax</td>
<td>Apparent maximum velocity</td>
</tr>
<tr>
<td>v/s</td>
<td>Velocity as a function of the substrate concentration</td>
</tr>
<tr>
<td>7,12-18:2-CoA</td>
<td>7,10-octadecadienoyl-CoA</td>
</tr>
<tr>
<td>β-hydroxy-9,12-20:2-CoA</td>
<td>β-hydroxy-9,12-eicosadienoyl-CoA</td>
</tr>
<tr>
<td>2-trans-9,12-20:3-CoA</td>
<td>2-trans-9,12-eicosatrienoyl-CoA</td>
</tr>
</tbody>
</table>
INTRODUCTION

Fatty acids may be defined as monocarboxylic, aliphatic acids, either saturated or unsaturated, and containing a sufficient number of methylene groups to render the molecule lipid soluble. Although this definition is governed by organic chemistry and suggests that practically an infinite variety of fatty acids can exist, biological systems define how many are actually found. This is the result of careful control mechanisms of living organisms which are responsible for the determination of chain length and degree of unsaturation. Thus, in-de novo synthesis palmitate is usually formed, although a variety of shorter or longer chain fatty acids could in theory be synthesized. In addition, the further metabolism of endogenous fatty acids from de novo synthesis or exogenous fatty acids from the diet is carefully controlled to produce only those particular fatty acids which are needed for eventual metabolic energy production, as membrane constituents, or in the synthesis of biologically active metabolites, e.g. prostaglandins.

Of the three reactions controlling polyunsaturated fatty acid metabolism—desaturation, chain elongation, and retroconversion, these studies focus largely on chain elongation, and more specifically on chain elongation which occurs in the microsomal fraction of certain cells. It has been shown that this reaction involves the condensation of a preexisting fatty acyl-CoA with malonyl-CoA forming the β-keto fatty acyl-CoA. The β-keto fatty acyl-CoA is then reduced to the
β-hydroxy, dehydrated to the 2-trans, and again reduced giving a fatty acid containing two additional methylene groups. Although chain elongation is well characterized as to the reaction steps involved in the sequence and the cofactors which are necessary for those reactions to occur, a number of fundamental questions remain as to the specificity of the individual reactions, the relationship of the individual enzymes to one another, and the factors in the fatty acid molecule which are responsible for recognition by the initial condensation enzyme.

It was the intention of this study to examine microsomal chain elongation using an isomeric series of octadecadienoic acids and selected β-hydroxy and 2-trans analogues of the parent acids in an attempt to answer some of these questions. In addition, other acids having specific chain lengths, degrees of unsaturation, and positions of unsaturation were used to further define the nature of the binding site in microsomal chain elongation.
CHAPTER I
LITERATURE REVIEW
PATHWAYS OF FATTY ACID METABOLISM

It is now clearly recognized that fatty acid metabolism can be divided into three main categories--de novo synthesis, β-oxidation, and the polyunsaturated pathways, with the last category further subdivided into desaturation, chain elongation, and retroconversion type reactions. In the early exploration of the biosynthetic and biodegradative pathways, the initial compositional studies did not provide much information on how or where fatty acids were metabolized. It was not until the introduction of carbon-14 labeling and certain chromatographic techniques, especially gas liquid chromatography, that great strides in the elucidation of the different pathways have been made. However, the initial observation by Knoop of the two carbon fragment, i.e. acetate, being involved in β-oxidation has been shown to be the unifying feature of the other pathways involving chain lengthening or chain shortening. This suggested that the intermediates in de novo synthesis, β-oxidation, and chain elongation might all be similar, and indeed it was eventually shown that all three reactions involved the β-keto fatty acid, β-hydroxy fatty acid, and α,β-enoyl fatty acid intermediates as indicated in Figure 1. However, the similarities stopped here in that the cofactors used and cellular location of the four reactions were all different and thus did not simply represent alternations in the direction of a single, universal pathway as had been originally proposed (1).
Figure 1. Simplified schematic diagram of the similarities between de novo synthesis and chain elongation (clockwise direction), and \(\beta\)-oxidation (counterclockwise direction).
The reactions of β-oxidation

Fatty acid oxidation is exclusively a mitochondrial event (2,3). It involves the reversal of reactions in Figure 1 after the introduction of the particular fatty acid, as its carnitine derivative, into the mitochondrial compartment (4), and its subsequent acylation to intramitochondrial coenzyme A (5). The intermediates and short chain fatty acids which appear during cycles of acetate removal remain acylated to CoASH (6). They appear only in minor concentrations and suggest that once β-oxidation is initiated by the organism the pathway is committed to their final destruction. The first enzyme, fatty acyl-CoA dehydrogenase (fatty acyl-CoA:FAD oxidoreductase) catalyzes the removal of two hydrogens to form the 2-trans intermediate and requires FAD as coenzyme. This enzyme shows a substrate specificity in terms of chain length (7) and will be discussed in more detail, as will the substrate specificities of other fatty acid metabolizing enzymes, in another section. Enoyl hydratase (L-β-hydroxyacyl-CoA hydratase) catalyzes the stereospecific addition of water to produce the L-β-hydroxy intermediate while β-hydroxyacyl-CoA dehydrogenase (L-β-hydroxyacyl-CoA:NAD oxidoreductase) oxidizes it to the β-keto stage. The final step involving the removal of acetyl-CoA strongly favors cleavage and is catalyzed by β-ketothiolase (acetyl-CoA:acetyl-CoA C-acetyl-transferase). The cycle is repeated with shorter and shorter fatty acids until oxidation is complete.

The reactions of de novo synthesis

De novo synthesis, on the other hand, was eventually recognized as being distinct from β-oxidation by two important observations--cellular
location and cofactor requirements. Whereas \( \beta \)-oxidation occurred solely in the mitochondria (3), de novo synthesis was shown by a number of investigators to occur in the soluble extracts of tissues from higher organisms (8,9). In addition, Wakil et al. (10) were able to demonstrate different cofactor requirements for fatty acid synthesis. However, it was not until the discovery of acetyl-CoA carboxylase (10,11) that the actual route of de novo synthesis became apparent. Thus malonyl-CoA serves as the acetate donor in Figure 1 and primer amounts of acetyl-CoA are required to initiate the sequence of reactions (12) in the forward direction to produce fatty acids typically with sixteen carbons. The reactions are identical to those of \( \beta \)-oxidation except for direction and formation of \( \beta \)-3-hydroxy fatty acids rather than \( L \)-isomers.

The mitochondria, typical of its independent nature, has also demonstrated the ability to synthesize fatty acids. Wakil and coworkers (3,13) have shown that mitochondrial systems supplemented with NADH, NADPH, and ATP were capable of incorporating acetyl-CoA into fatty acids. Further elucidation of the system revealed that both de novo synthesis (13), similar to those reactions which occur in the soluble portion of the cell, and chain elongation of preformed fatty acids (14) were occurring. Both reactions are of questionable significance, but the later will be discussed in more detail in that it is a chain elongation type reaction.

Polyunsaturated fatty acid metabolism

Depending upon its needs as determined by food intake and energy expenditures, the organism has been shown to be capable of synthesizing and degrading the common storage fat, palmitate. In addition to these
two pathways, animals can further metabolize fatty acids originating from either de novo synthesis or dietary sources by the introduction of double bonds and additional methylene groups with reactions which take place in the microsomal fraction of various tissues (15,16). Klenk (17) and Mead (18) have further established that linolenate, linoleate, oleate, and palmitoleate each serve as the initial unsaturated precursor in the different and independent families of polyunsaturated fatty acids as depicted in Figure 2. Unlike de novo synthesis or β-oxidation, in which acetate groups are added or removed sequentially, the metabolism of the precursor fatty acids to the final incorporated product occurs by alternating desaturation and chain elongation reactions with specific enzymes, and thus does not permit the crossover of metabolites from one pathway to another (17). Double bonds are introduced only in a divinyl rhythm by enzymes which specifically produce desaturations at positions 9-, 6-, 5-, and 4- after the appropriate chain elongation reaction. Chain elongation, on the other hand, occurs by the condensation of malonyl-CoA with preexisting fatty acids and the subsequent reduction, dehydration, and reduction reactions as illustrated in Figure 3 (19). In addition to desaturation and chain elongation reactions, partial degradation, rather than complete β-oxidation, of polyunsaturated fatty acids can also occur. These retroconversion reactions take place in the mitochondrial fraction of the cell and involve either the loss of an acetate group or the loss of an acetate group and a double bond (20-22). The cell is thus capable of manipulating precursor fatty acids into a wide spectrum of polyunsaturated
**POLYUNSATURATED FATTY ACID PATHWAYS**

**FATTY ACID FAMILY**

**Δ9 DESATURATION**

**CHAIN ELONGATION**

**Δ6 DESATURATION**

**CHAIN ELONGATION**

**Δ5 DESATURATION**

**Δ4 DESATURATION**

**LINOLENATE (ω-3)**


**LINOLEATE (ω-6)**


**PALMITOLEATE (ω-7)**

16:0 → 16:1 → 16:2 → 18:2 → 18:3 → 20:3 → 20:4

**OLEATE (ω-9)**

16:0 → 18:0 → 18:1 → 18:2 → 20:2 → 20:3 → 22:3

---

Figure 2. The linolenate, linoleate, palmitoleate, and oleate pathways of polyunsaturated fatty acid metabolism. Chain elongation and desaturation (Δ9, Δ6, Δ5) occur as indicated; the method of formation for the Δ4 double bond remains uncertain. From Klenk (17) and Mead (18)
fatty acids which are then utilized in the synthesis of complex lipids and prostaglandins.

How the cell actually controls fatty acid composition has been actively investigated. Numerous investigators have measured many of the individual reactions, either desaturation, chain elongation, or retroconversion, in the different polyunsaturated fatty acid pathways in an attempt to correlate reaction rates with either the amount of precursor fatty acid or product fatty acid actually found in the tissue. In a heroic effort by Bernert and Sprecher (23) the rate of chain elongation or desaturation of all individual fatty acids of the linoleate, oleate, and palmitoleate pathways were measured with rat liver microsomes. Their results indicated both a correlation and lack of correlation between in vitro reaction rates and in vivo fatty acid composition, and suggests that other controls, i.e. competitive interactions, retroconversion, and complex lipid incorporation specificities, must intervene in controlling fatty acid composition.

The desaturation reaction

Although the precise mechanism of cis double bond formation by animal desaturases has not been entirely elucidated, both the cofactor requirements and the fact that double bonds are introduced by a Δ9, Δ6, and Δ5 desaturase have been known for some time. The reaction is strictly aerobic and requires reducing equivalents in the form of NADH or NADPH (15,24,25). The actual substrate for desaturation is the CoA derivative (26). In the desaturation of stearate to oleate by Δ9 desaturase, a flow of electrons from NADH (27,28) to cytochrome b5 (29,30) via NADH-cytochrome b5 reductase (31) has been ascertained, and this is
followed by the eventual reduction of the actual desaturase enzyme (28, 29,32,33). The enzyme is thus capable of activating molecular oxygen for the final desaturation of the substrate fatty acid (29,34). The dehydrogenation is strictly stereospecific such that the enzyme removes only the D-hydrogen atoms on adjacent carbons to produce the cis double bond (35-37). It has been proposed that a conformational change occurs in the enzyme while it is binding the substrate and positions the two D-hydrogen atoms by rotation about carbons 9-10 for the eventual cis elimination (38). It also implies that a very specific substrate-enzyme binding site complex must be formed. Numerous substrate specificity studies have substantiated the limited binding by desaturases (38-42).

In addition to the studies on the mechanism of desaturation, the individual components of the desaturation reaction, cytochrome b5 (43), cytochrome b5-NADH reductase (44), and the terminal stearyl-CoA desaturase enzyme (45), have been isolated. Although there is convincing evidence to indicate that the enzymes responsible for the introduction of cis double bonds in positions 6- and 5- are different from the Δ9 desaturase, it is assumed that they all function by a similar mechanism. The method of introducing a cis double bond in position 4- has yet to be determined.

The chain elongation reaction

Perhaps the earliest indication that microsomes were involved in the chain elongation of fatty acids was provided by Abraham and Chaikoff (46-48) during their studies on the factors controlling de novo fatty acid synthesis. They had observed an increase in the incorporation
of radioactivity from \(^{14}\text{C}\) acetate when the microsomal fraction of rat liver was added to a system containing supernatant, ATP, NADPH, HCO\(_3\)\(^-\), and Mg\(^{++}\) (46). If malonyl-CoA, on the other hand, was substituted for acetyl-CoA in a system consisting of supernatant plus microsomes, an even greater incorporation was observed (47). In fact, microsomes incubated alone seemed equally capable of incorporating malonyl-CoA (47).

With the realization that the predominant fatty acid being synthesized from malonyl-CoA by the supernatant was palmitate and that the predominant fatty acid being synthesized from malonyl-CoA by the microsomal fraction was stearate (43), microsomal chain elongation came into being.

Although the studies by Abraham and Chaikoff indicated an absolute requirement by the microsomal fraction for malonyl-CoA, ATP, NADPH, and Mg\(^{++}\) in the synthesis of stearate and suggested that endogenous palmitate served as its precursor, it was not until the initial studies by Nugteren (49) and those of Stoffel and Ach (16), that the full potential of microsomal chain elongation was realized. Nugteren confirmed the malonyl-CoA dependent chain elongation in microsomes using \([1-{\text{14}}\text{C}]\) 6,9-octadecadienoate, as well as palmitate and linoleate, as substrates, and was able to prove that chain elongation occurred by the addition of acetate groups to the precursor fatty acids. Using a variety of saturated, monoenoic, and polyenoic fatty acid substrates, Stoffel and Ach (16) were able to show that the rate of chain elongation varied with chain length, the degree of unsaturation, and position of unsaturation of the substrate fatty acids.
Nugteren (19) was instrumental in confirming that microsomal chain elongation proceeded through the intermediates as illustrated in Figure 3. Its similarity to Figure 1 is quickly recognized. The rates of the overall chain elongation reaction, and the individual rates of the initial condensation reaction, β-keto reduction, β-hydroxy dehydration, and α-β-enoyl reduction were successfully measured using rat liver microsomes and the appropriate substrates. The results indicated that the initial condensation reaction of myristoyl-CoA with malonyl-CoA was rate limiting and that the conversion of β-ketopalmitate to palmitate, β-hydroxy palmitate to 2-trans palmitate, and the final reduction of 2-trans palmitate to palmitate all proceeded at a rate greater than the initial reaction. An absolute requirement for malonyl-CoA was shown and the results clearly indicate that microsomal chain elongation was not occurring due to contamination by other cellular fractions. In addition, a variety of saturated and unsaturated fatty acids of different chain lengths were tested for their ability to be chain elongated and the results suggest that features within the acyl portion of the fatty acid substrate may be important in determining the rate. However, because of the incubation conditions used in measuring the rates of chain elongation for these different fatty acids were not respectful of enzyme kinetics, their significance is doubtful.

Using three key substrates, 16:0-CoA, 6,9-18:2-CoA, and 6,9,12-18:3-CoA, Bernert and Sprecher (50) were able to show that the rate of the initial condensation reaction with malonyl-CoA and the overall rate of chain elongation for enzymatic conversions performed with rat liver microsomes were identical. In addition, the β-hydroxy and 2-trans
Figure 3. The reactions of microsomal chain elongation. (R is equivalent to long chain saturated or unsaturated fatty acid tail.) From Nugteren (19).
intermediates of palmitic acid and 6,9-18:2 chain elongation were synthesized and used as their CoA derivatives to confirm the rate limiting nature of the condensation reaction. It had also been previously shown that the rates of in vitro chain elongation for 16:0, 6,9-18:2, and 6,9,12-18:3 all declined during the fasting of an animal (51). However, refeeding a lipogenic fat free diet increased the rate of chain elongation for 16:0 many fold greater than nonfasted controls, but only returned the rate of chain elongation for 6,9-18:2 and 6,9,12-18:5 back to normal. These results along with competitive substrate inhibition studies suggested that at least two chain elongating enzymes existed, perhaps one for saturated and the other for unsaturated fatty acids, and this observation has been confirmed by N-ethylmaleimide inhibition studies (50).

Although rat liver has been the most commonly investigated tissue for microsomal chain elongation studies, considerable advances in characterizing chain elongating systems have been made by Podack, et al. (52) using beef adrenal cortex. In an attempt to remove interfering malonyl-CoA decarboxylase activity, an eight fold increase in chain elongation activity, as measured by the incorporation of radioactivity from [1,3-14C] malonyl-CoA into the petroleum ether soluble phase, and a four fold decrease in malonyl-CoA decarboxylase activity was observed. In addition, the specific activities of the terminal enoyl-CoA reductase enzyme and the overall chain elongation activity remained essentially constant during the rather limited purification scheme, and suggested that the enoyl-CoA reductase is an integral part of the microsomal chain elongating system. The importance of the integrity of the complete system and its general insoluble character is
exemplified by the fact that a variety of organic solvents, detergents, and sonication failed to solubilize the system. However, this does not exclude the possibility that one or more of the individual enzymes could be solubilized and purified providing that only that particular individual enzyme involved was being measured. The cofactor requirements for chain elongation were identical to those as previously described (19). Although the substrate specificity of the overall chain elongation process was not investigated, the authors were successful in demonstrating the pseudo substrate specificity and inhibitory characteristics of the terminal enoyl-CoA reductase enzyme (52). An apparent maximum rate of reduction was observed with 1-hexenoyl-CoA, and considerably less activity with crotonoyl-CoA and fatty enoyl-CoA's greater in chain length than 1-decenoyl-CoA. This chain length specificity of the enoyl-CoA reductase for 2-trans hexenoyl-CoA, as measured both by the rate of reduction and $K_m$ value, suggested that the distal saturated methylene groups interfere with the binding of the substrate with the enzyme. The apparent substrate inhibition of the enoyl-CoA reductase by 2-trans dodecenoyl-CoA and 2-trans hexadecenoyl-CoA are suggested as supporting evidence for this interference. However, it must be noted that micellar formation of fatty acyl-CoA's occurs in the concentration range used (53), and the apparent substrate inhibition might only reflect a sudden change in the availability of the substrate. The authors conclude that the optimal activity with 2-trans hexenoyl-CoA is undoubtedly of no physiological significance in that long chain and polyunsaturated fatty acids are preferentially chain
elongated by the microsomal system, but rather reflects the similarity of the 2-trans hexenoyl-CoA to the proximal portion of the rapidly chain elongated 6,9,12-octadecatrienoyl-CoA (52).

Whether the substrate during microsomal chain elongation remains covalently bound to the enzyme or not has received only limited attention. Although Nugteren's original study indicated that fatty acyl-CoA's served as the immediate substrates for chain elongation (19), the work did not imply that enzyme bound intermediates were not being formed (54). In a further characterization of the mechanism of malonyl-CoA dependent chain elongation with rat liver microsomes, Podack, *et. al.* (52,54) suggested that a common transferase is responsible for catalyzing the exchange of malonyl and acyl groups from their CoA derivatives to the condensing enzyme, the exchange of the intermediates of chain elongation from one enzyme to another, and the final exchange of the chain elongated fatty acid from the enoyl-CoA reductase back to free CoA. Although the authors suggest that the iodoacetamide inhibition characteristics support a serine hydroxyl at the active site of the transferase enzyme (54), only the purification and further characterization of all enzymes involved in microsomal chain elongation will allow for declarations as to the type of mechanisms involved.

Microsomal chain elongation activity has been found in a variety of tissues other than liver and adrenal cortex (55-58) and is of particular importance in the brain where large concentrations of long chain fatty acids, such as 24:0 and 15-24:1, are found in the sphingolipids of myelin (59). The cofactor requirements for chain elongation are similar to those of liver (60-62) and the incorporation of malonyl-CoA into
fatty acids by the microsomal fraction was first shown to be the highest during the period of most active myelinogenesis (63). The relationship between the increase in microsomal chain elongation activity and the simultaneous onset of neonatal myelination is also supported by the increase in rate of chain elongation for palmitate, stearate, arachidate, and behenylate (64,65). However, the rate of increase and time of onset suggested that separate systems existed for the chain elongation of palmitoyl-CoA, stearoyl-CoA, and the two fatty acids, arachidyl-CoA and behenyl-CoA (64). The presence of different chain elongating enzymes for palmitate and stearate had originally been demonstrated with brain microsomes derived from a Quaking mutant mouse strain (66-68), which is characterized by defective myelination of the central nervous system. The pattern of chain elongation activity with microsomes obtained from the Quaking mutant confirmed the existence of the third enzyme responsible for the chain elongation of both arachidyl-CoA and behenyl-CoA (64). The apparent enzymatic defect present in the Quaking mutant has not been determined, but would be informative in elucidating the mechanism and control of chain elongation.

The chain elongating capabilities of the mitochondria have been further investigated by a number of groups (69-71) in that a simple reversal of the β-oxidation sequence would offer a logical explanation for the acetyl-CoA dependent reaction. However, the β-keto thiolase strongly favors cleavage and the resulting negative ΔG° for β-oxidation suggests that a simple reversal is not feasible. The unfavorable thermodynamics could, however, be circumvented by the addition of a reaction or reactions which would result in a total negative ΔG° for the
sequence. With a substitution of the FAD dependent fatty acyl-CoA dehydrogenase by a NADH (NADPH) dependent enoyl-CoA reductase, the equilibrium is shifted in favor of the synthetic process (69-71). Seubert et al. (70) were first to experimentally demonstrate the reversal of β-oxidation and since then both the acetyl-CoA dependent mitochondrial chain elongating system and the key enoyl-CoA reductase enzyme have been characterized and partially purified (72,73). The chain elongation of the shorter, saturated fatty acids (octanoate, decanoate, and dodecanoate) seems to be favored over the longer and unsaturated fatty acids (73). The terminal enoyl-CoA reductase enzyme isolated from mitochondria has been shown by pH profiles, substrate specificity studies, and solubility characteristics to be different from the microsomal enoyl-CoA reductase (72). Although the physiological significance of mitochondrial chain elongation remains speculative, the absolute requirements for NADPH by the terminal enoyl-CoA reductase suggests that it might be involved in the transfer of reducing equivalents from NADPH to the respiratory chain (67) or of reducing equivalents from the cytosol into the mitochondrial compartment (74).
THE SUBSTRATE SPECIFICITY OF ENZYMES
UTILIZING FATTY ACIDS

Although an initial impression of a fatty acid molecule could suggest that the acyl moiety, once it is sufficiently extended, might appear similar in biological systems irrespective of its chain length, degree of unsaturation, and position of double bonds, this rather naive approach is quickly diminished by examination of the molecular structures as projected by Vandenheuvel (75). These alterations in the geometrical shape of the fatty acid molecule which occur by the addition of cis double bonds and their positioning in the acyl chain suggest that they might play an important role not only in maintaining a certain conformational nature of the membrane, but also in affecting substrate-enzyme interactions as reflected in alterations of the enzymatic rates or Km. This in fact has been the situation and is still being actively investigated.

The substrate specificities of both microsomal chain elongation and desaturation will be discussed in detail as to the preferences of the enzymes for particular chain lengths, positions of double bonds, and number of double bonds. In addition, a brief review of a variety of other enzymatic conversions which utilize fatty acids and whose substrate specificities have been studied will also be presented.

In examining the substrate specificities of the individual enzymes of fatty acid synthesis in E. coli, a specificity for acetyl-CoA as compared to long chain fatty acyl-CoA's has been observed for the acetyl-CoA-ACP transacylase (76). The termination of chain elongation has been attributed to the β-ketoacyl-ACP synthetase, in that the ACP
derivatives of fatty acids shorter than palmitate were readily condensed with malonyl-CoA, but hexadecanoyl-ACP was completely inactive (77). The β-ketoacyl-ACP reductase, on the other hand, appears to have a broad specificity as to the chain length of the substrate and its degree of unsaturation (78,79). The discovery of two β-hydroxyacyl-ACP dehydrogenases explained the initial observation of a bimodal substrate specificity for this enzyme and the equal abundance of both saturated and unsaturated fatty acids in E. coli (80). Two enoyl-ACP reductases are also present (81). The NADPH enoyl-ACP reductase is most active with short chain 2-trans fatty acids, whereas the NADH dependent enzyme has a broader chain length specificity. The termination of chain lengthening can perhaps be attributed to the palmitoyl-ACP thioesterase in that minimal activity was observed with saturated acyl-CoA's shorter than dodecanoyl-CoA and maximal activity with palmitoyl-CoA, palmitoleyl-CoA, and cis-vacceny-CoA (82). Unfortunately, the extensive substrate specificity studies that have been performed with the E. coli enzymes have not been done with either yeast or liver fatty acid synthetase. It must also be recognized that although definite substrate specificities have been observed, the type of interaction that apparently is conveyed by the acyl moiety is still speculative.

The enzymes of β-oxidation also exhibit substrate specificities. Two fatty acyl-CoA dehydrogenases have been found (83,84). The one isolated from pig liver is most active with fatty acyl-CoA's of intermediate chain length (C₈ to C₁₂) and is only partially active with butyryl-CoA (83). On the other hand, a butyryl-CoA specific dehydrogenase has been discovered in monkey liver. Obviously the two enzyme
activities complement each other. Watterson and Hill (85) were first to demonstrate with a purified enoyl-CoA hydratase isolated from bovine liver that although the enzyme acts on the CoA derivatives of all 2-trans acids containing 4 to 16 carbons, the Vmax for this series decreases with increasing chain length. The β-hydroxyacyl-CoA dehydrogenase was shown to be active on all β-hydroxyacyl-CoA derivatives tested from C4 to C12, but did show higher rates for β-hydroxy octanoyl-CoA (86). Finally, Middleton (87) has found not only an acetoacyl-CoA specific β-ketothiolase in the cytosol and mitochondria of numerous tissues, but also a β-ketothiolase which is active on longer chain β-keto fatty acids such as 3-ketodecanoyl-CoA. Again, the type of interaction that is apparently conveyed by the acyl moieties of different chain lengths is still unknown.

An enzyme similar to the α,β-enoyl-CoA reductase of de novo synthesis and microsomal chain elongation, but involving the reduction of a 4-cis rather than a 2-trans double bond, has been found in the mitochondria and is associated with the partial β-oxidation of polyunsaturated fatty acids known as retroconversion (22). Besides the prerequisite 4-cis double bond which the 4-enoyl-CoA reductase reduces, a specific number of double bonds in the fatty acyl moiety have been shown to be necessary for recognition by the enzyme. All acids with fewer double bonds than 4,7,10,13-22:4, i.e. 4,7,10-22:3, 4,7-22:2, and 4-22:1, were not reduced and the authors concluded that recognition is probably conferred by the complete semicircle of the fatty acyl tail. In addition, although 4,7,10,13-20:4 was retroconverted, 5,8,11,14-21:4 was not, thus illustrating that recognition occurs from the carbonyl rather than the methyl end (22).
A number of different investigators have examined the substrate specificity of long chain acyl-CoA synthetases (88-90). Using a series of fatty acids from decanoate to stearate, Pande (90) has found separate peaks of activity for laurate and palmitate, which suggested two enzymes. Also, the introduction of a double bond in the 9- position of stearate increased the rate of acyl-CoA formation two fold. However, Suzue and Marcel (91) demonstrated only one peak of activity at laurate with a similar series of fatty acids but concur that the enzyme might have a binding site specific for a substrate with a Δ9 double bond (89). This is further substantiated by an isomeric series of octadecadienoic acids with double bonds in the 6,9- through 10,13- positions which shows the lowest Km for 9,12-18:2 (92). Except for 6,9-18:2, the Vmax's were essentially equivalent (92).

A variety of long chain saturated and unsaturated fatty acids have been used as substrates in acyl-CoA:phospholipid acyltransferase studies. The discussion is complicated by the different enzyme sources used and by the fact that esterification can occur at both the 1- and 2- positions of different lysophospholipid accepting molecules. Nonetheless, obvious substrate specificities were observed. For instance, the acyl transferase responsible for esterifying 1-acylglycero-3-phosphorylcholine in the 2- position is different from the acyl transferase acting on 1-acyl-glycero-3-phosphate in that the former showed high rates with both C18 and C20 polyunsaturated fatty acids, whereas the latter was active only with C18 polyunsaturated fatty acids (93). An isomeric series of 18:1 fatty acids were used as substrates in demonstrating the substrate specificity of acyl-CoA:1-acylglycero-3-phosphorylcholine acyltransferase (94),
Interestingly, the monoene most commonly found esterified at the 2-position, i.e. 9-18:1, was the preferred substrate. In addition, this group has investigated the substrate specificity of this same enzyme with an isomeric series of octadecadieonoyle-CoA's (95). Both rat and pig liver microsomes show high rates of esterification for acids when their double bonds were located nine or more carbon atoms from the carbonyl group. Lower rates were noted for acids whose double bonds were closer to the carbonyl group, with the exception of 5,8-18:2. The apparent correlation between the substrate showing the high rates of esterification and those acids commonly found at position 2- in lecithin isolated from rat liver was noted by the authors (95). The 7,10-isomer was essentially inactive towards esterification. It should again be recognized that only a random sampling of acyltransferase specificity has been included.

Microsomal Chain Elongation

During prior investigations of microsomal chain elongation a variety of different fatty acid substrates and incubation conditions have been used. The substrates have varied from short chain, saturated fatty acids to long chain, polyunsaturated fatty acids, either as the salt or CoA derivative, and the incubation conditions have varied from concentrations that probably were not substrate saturating to incubation times that probably caused denaturation of the enzymatic systems well before termination. Hence, in any discussion of microsomal chain elongation rates, these factors must be remembered and have permitted only a limited panorama of the actual specificity.
Stoffel and Ach (16) were perhaps the first investigators to demonstrate that differences in the rate of chain elongation could be anticipated with variations in chain length and the degree of unsaturation. Using 90 minute incubation periods and rat liver microsomes, they found low rates of chain elongation for both 16:0 and 18:0, and for monounsaturated fatty acids with double bonds in positions 9- and 11-. However, the introduction of a second double bond at position 12- of 9-18:1 increased the rate many fold. According to their results, the total number of double bonds and their positions obviously greatly influences the overall rate, in that 6,9,12-18:3 was converted the fastest of all acids tested, whereas 8,11,14-18:3 was perfectly inert. On the other hand, 8,11,14-20:3 was converted faster than 8,11,14-18:3, indicating a chain length factor when additional double bonds were added. This is substantiated by the fact that 5,8,11,14-20:4 was converted faster than the other 8,11,14- fatty acids.

Nugteren's studies (19) would appear to confirm many of the results of Stoffel and Ach (16). However, relatively rapid rates of chain elongation were reported for short chain, saturated fatty acids, such as 16:0, but the low rate of 18:0 chain elongation is in agreement. In addition, although Stoffel and Ach (16) found low rates of conversion for monoenoic acids, which appear to be confirmed by the negligible rates of chain elongation for 9-18:1 and 9-trans 18:1, an equal mixture of 7-16:1 and 9-16:1 was converted at rates equalling the fastest chain elongated fatty acid (19). In that chain elongation was measured by the incorporation of radioactivity from [1,3-14C] malonyl-CoA, it is difficult to determine which particular fatty acid was actually chain elongated. Increases in rate are also observed when additional
double bonds are added distally to the carbonyl end, as in the series 9-18:1, 9,12-18:2, and 9,12,15-18:3. As reported by Stoffel and Ach (16), movement of the double bonds proximally to the carbonyl carbon increases the ability of the fatty acid to be chain elongated, since 6,9,12-18:3 was the most rapidly converted acid. Its rate was approximately twice that of its 9,12,15-18:3 isomer.

In a limited investigation on the influence of chain length of saturated fatty acids, Guchhait et al. (53), using pigeon liver microsomes, again observed a general increase in the ability of an acid to be chain elongated with an increase in chain length for acids 8:0 through 16:0. Unfortunately 18:0 was not tested.

During the determination of rates of conversion for those fatty acids of the linoleate, oleate, and palmitoleate pathways by Bernert and Sprecher (23), a variety of unsaturated fatty acids were tested for their ability to be chain elongated and generally confirm the findings that the chain length, the number of double bonds, and their positions are important in determining the ability of a fatty acid to be chain elongated. For example, 9-16:1 was chain elongated at a moderate rate while 9-18:1 was converted at a very slow rate. A similar decrease is observed when two additional methylene groups are added distally to 5,8,11-18:3 to give 5,8,11-20:3. A limited number of dienes reconfirms the generally observed fast rate of chain elongation for 6,9-18:2, but 9,12-18:2, with an extremely slow rate of conversion, is in contradiction to a moderate rate as observed by other investigators (16,19). An equally slow rate with 8,11-18:2 was also reported. The rates with trienoic acids indicate that both 5,8,11-18:3 and 6,9,12-18:3 are
rapidly chain elongated, but that 7,10,13-20:3 (96) and 8,11,14-20:3 are poor substrates for chain elongation. Since a large decrease in rate is observed with 5,8,11-20:3 as compared to 5,8,11-18:3, perhaps the slower rates for 7,10,13-20:3 and 8,11,14-20:3 are caused not necessarily by the number and position of the double bonds, but rather by chain length.

In an earlier work by Christiansen et al. (97), the rates of chain elongation for 9-18:1, 9,12-18:2, 9,12,15-18:3 and 6,9,12-18:3 were measured and comparison of the rates indicates that addition of double bonds distally to the double bond at the 9- position increases the rate of chain elongation. Thus 9,12-18:2 was chain elongated faster than 9-18:1, and 9,12,15-18:3 faster than 9,12-18:2. Interestingly, movement of the 9,12,15- sequence of γ-linolenic acid to 6,9,12- of α-linolenic acid more than tripled the rate. Whether the increase in rate with the increase in the number of double bonds as observed with 9-18:1 isomers would also occur with 6-18:1 isomers remains speculative.

Using a limited series of 18:2 positional isomers, Marcell and Holman (98) have extended their specificity studies by reporting that there was a steady decrease in the rates of chain elongation as the two double bonds were moved from the 6,9- positions to the 9,12- positions of octadecadienoic acid, and that an apparent upward trend was initiated with 10,13-18:2. Unfortunately, no other octadecadienoic acid isomers were investigated.

An apparent reversal in the decrease in the rates of chain elongation with an increase in chain length was observed with brain
microsomes as compared to liver microsomes. Whereas Nugteren (19) reported an extremely low rate of chain elongation for 18:0 in relation to 16:0, Bourre et al. (62) indicated that 18:0 was chain elongated faster than 16:0, and 24:0 faster than 18:0. However, Goldberg et al. (64) reported that there is a general decrease in rate in the series 20:0, 18:0 and 16:0, and this has been confirmed, at least with 18:0 and 16:0, by Brophy and Vance (61). The variation in the rate of chain elongation for palmitate might, however, also reflect contamination by fatty acid synthetase from the cytosol.

A limited picture of the substrate specificity of microsomal chain elongation can nonetheless be established using the reported enzymatic rates. Table 1 lists by investigator those fatty acids which are comparable and are listed as percent of the most actively chain elongated fatty acid. As already mentioned, the short chain saturated fatty acids are readily chain elongated. However, excluding brain microsomes, stearate appears to be a poor substrate for chain elongation. No particular pattern for monoenoic acids can be formulated. The rates of chain elongation for polyunsaturated fatty acids indicate a general lack of agreement, except for 6,9-18:2 and 6,9,12-18:3. Both of these isomers with their first double bonds in the 6- position are universally reported as being excellent substrates for chain elongation. It would also appear that movement of the double bonds distally from the 6- position decreases the ability of the fatty acid to be chain elongated, whereas movement proximally maintains its acceptability. As with the monoenoic acids, no particular correlation with chain length is readily recognizable. This would suggest that a systematic isomeric
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<th>17.1</th>
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Percent of ester link elongated fatty acid

Known rates of chain elongation expressed as

Table I
series of perhaps octadecadienoic acids would show a profile of chain elongation with the maximum rate observed with the 6,9- isomer. The ability of the other isomers to be chain elongated would also have to remain speculative at this point.

Although the substrate specificity of the individual enzymes for both de novo synthesis and β-oxidation have been well characterized, the equivalent conversions in microsomal chain elongation have yet to be thoroughly explored. Nugteren, however, did demonstrate that 8-keto palmitoyl-CoA, β-hydroxypalmitoyl-CoA, and 2-trans hexadecenoyl-CoA, the intermediates of myristoyl-CoA chain elongation were converted (19), which would be anticipated in that this acid is readily chain elongated, In addition, the β-hydroxy and 2-trans intermediates of both palmitic and 6,9-18:2 have been synthesized and their rates determined (50). Both rates of dehydration and reduction for the two β-hydroxy fatty acids and the two 2-trans fatty acids were essentially equivalent, implying that a broad specificity for both enzymes exists. However, Podack et al. (52) using short chain 2-trans fatty acids clearly indicated a chain length specificity for enoyl-CoA reductase from beef adrenal cortex. Both crotonyl-CoA and acids longer than 2-trans decenoyl-CoA were reduced at minimal rates, while 2-trans hexenoyl-CoA was the preferred substrate. Although the authors recognized the nonphysiological nature of an acid of this chain length, they speculated that it is equivalent to the proximal portion of 6,9-18:2 and 6,9,12-18:3 acid before the appearance of the kink produced by the double bond in the 6- position (52). Thus they anticipated that α,β-enoyl-CoA reductase would show a marked substrate specificity with long chain 2-trans fatty acids.
Microsomal desaturation

Of all the reactions involving long chain saturated and unsaturated fatty acids, perhaps the desaturases have been the most thoroughly explored in the context of substrate specificity and have resulted in the actual conception of substrate binding sites. In that the formation of double bonds at the 9-, 6-, and 5- positions is commonly considered to be performed by three different enzymes, the known substrate specificities will be discussed sequentially for the individual desaturases.

A definite chain length specificity has been reported for the $\Delta 9$ desaturase derived from a number of different sources (38,39). The common feature of every chain length profile is the sharp specificity for 18:0 and that considerable activity is lost with a shorter and especially a longer chain length. In a number of the systems investigated, a second peak of activity appears with 14:0 and these two peaks of activity suggest that two separate $\Delta 9$ desaturases may be present (38). An identical profile has been reported for a partially purified $\Delta 9$ desaturase from rat liver (99). In addition, the investigators explored the ability of the enzyme to desaturate an isomeric series of methyl octadecanoic acids (38). Only those methyl isomers with the methyl group situated near the carbonyl and terminal end were desaturated. The authors concluded that a very restricted cleft, essentially the size of the preferred stearate substrate, was involved in binding at the active site. The substrate specificity of the $\Delta 9$ desaturase has been further investigated by Pollard (40) in studies which utilized a vast array of fatty acid substrates varying in the degree and type of unsaturation. The results fitted the model as proposed by Brett et al. (38).
Unlike the Δ9 desaturase which will readily introduce the double bond into saturated fatty acids, the Δ6 desaturase from rat liver would appear to be totally inactive with such substrates (40). However, Cook and Spence (100) have shown that double bonds were introduced into the 6- position of both 16:0 and 18:0 by microsomes isolated from fetal and neonatal rat brains and would suggest that they are different enzymes. The substrates of primary interest to the Δ6 desaturase are those whose first double bond is in the 9- position and preferably unsaturated in a divinyl rhythm thereafter (40). Thus 9-18:1 is poorly desaturated, 9, 12-18:2 better, and 9,12,15-18:3 the fastest. The increase in the rate of desaturation with the increase in double bonds has also been reported by other investigators (23). A chain length profile of 9- monoenoic acids shows a low, but nonetheless maximum at 9-16:1, whereas Bernert and Sprecher have reported a higher rate for 9-18:1 as compared to 9-16:1 (23). A chain length profile of 9,12- fatty acids was also performed and the two regions of maximum activity would imply that two Δ6 desaturases exist in rat liver (40). The double bond requirement of the enzyme would suggest that for binding to occur, specific kinks in the fatty acid molecule must be present and that they must be situated at specific locations along the fatty acid chain. The presence of double bonds within the molecule may also allow for the interaction of π binding sites on the enzyme with the π electrons of the substrate (40). Brenner (41) has confirmed the Δ6 desaturase's requirements for its substrate and hypothesized that binding occurs from the double bond in the 9- position to the terminal methyl group, that binding of the 9- double bond is essential, and that the additional double bonds distal to the 9-
double bond strengthens the binding. Using Vandenheuvel's projection of fatty acids (75) and the known substrate specificity of the Δ6 desaturase, a tentative model has been proposed of the binding and desaturating site of the enzyme (41).

Although the Δ5 desaturase will not readily produce a double bond in the 5- position of saturated fatty acids and prefers unsaturated substrates whose first double bond is in the 8- position (40), desaturation in the 5- position can also occur with unsaturated substrates whose first double bond is instead at position 11 (101,102). With substrates containing the 8,11,14- series of double bonds, the enzyme showed a definite chain length specificity in that both 8,11,14-18:3 and 8,11,14-22:3 were not desaturated and those with intermediate chain lengths were (103,104). The chain length specificity has essentially been substantiated with an 8,11- system (23). In studies similar to those as reported by Brett et al. (38), a number of methyl substituted 8,11,14-20:3 isomers were synthesized and used in determining rates of Δ5 desaturation by Do and Sprecher (42). A rapid decline in the enzymatic rate was observed as the methyl group was moved closer to the site of desaturation. These findings along with the other substrate specificity studies would suggest, as with the Δ9 and Δ6 desaturases, that the geometry of the binding site is very limited and probably best defined by the most preferred substrate.

From the preceding discussion it is apparent that a paucity of information still exists for malonyl-CoA dependent microsomal chain elongation. Two areas thought worthy of investigation were 1, the substrate specificity of the overall chain elongation process and those of
the individual reactions, i.e. condensation, \( \beta \)-hydroxy dehydration, and 2\text{--trans} reduction, and the relationship of those enzymes to one another, and 2, the fatty acyl binding site associated with the chain elongating enzyme as elucidated by substrate specificities.

Concerning the first point, Nugteren (19) was first to recognize that chain elongation involved the condensation of long chain fatty acyl-CoA's with malonyl-CoA and the subsequent intermediary steps, but the substrate specificities of these enzymes was not shown. Also, relatively little was known with respect to the rates of conversion of the individual reactions and that of chain elongation, and whether the individual enzymes might actually be of a multifunctional nature. Thus, it was decided that a systematic investigation using an extensive series of isomers of a known chain elongated fatty acid would be helpful in establishing the substrate specificity of the chain elongating enzyme. In addition, parallel substrate specificity studies with the \( \beta \)-hydroxy and 2\text{--trans} analogues of the parent fatty acids would assist in establishing the specificity of the \( \beta \)-hydroxy fatty acyl-CoA dehydrase and \( \alpha,\beta \)-enoyl-CoA reductase. Seubert (52) had predicted from the substrate specificity of the \( \alpha,\beta \)-enoyl-CoA reductase with short chain 2\text{--trans} fatty acids that a fatty acid with a double bond in the 6-position, e.g. 6,9-18:2 or 6,9,12-18:3, would be chain elongated the fastest and such a study would either confirm or refute this prediction. Comparison of the rates of condensation and overall chain elongation for the isomeric series would allow for the confirmation of the rate limiting nature of the initial reaction and allow for the formulation of a mechanism which might be common to the chain elongation reactions.
occurring during polyunsaturated fatty acid metabolism. The ability of a particular fatty acid or acids in the isomeric series to be chain elongated could also provide pertinent information as to the reason why a fatty acid is either desaturated or chain elongated as it is metabolized in vivo.

Previous studies had suggested that the level of the condensing enzyme can be manipulated by dietary alterations with the level of the β-hydroxy fatty acyl-CoA dehydrase and α,β-enoyn-CoA reductase remaining unaffected (50). Also, Podack, et al. (52) had shown that the ratio of chain elongation activity and enoyl-CoA reductase activity remained essentially constant during the partial purification from beef adrenal cortex. This would suggest that after the initial condensation, the intermediates are converted by an enzyme complex composed of the noninvestigated β-keto fatty acyl-CoA reductase, and the readily measurable β-hydroxy fatty acyl-CoA dehydrase and α,β-enoyn-CoA reductase. This enzyme complex might possibly be multifunctional in nature. In that traditional methods of enzyme isolation and possible copurification can not be used to determine if in fact a multifunctional enzyme might be present, another tissue having the ability to chain elongate fatty acids could be investigated. The rates of the initial condensation and chain elongation reactions as well as those of dehydration and reduction could be measured and compared to the same rates as determined with liver microsomes. This approach might be successful in demonstrating whether the dehydrase and reductase are of a multifunctional nature or are individual and descrete.
Concerning the second point, extensive substrate specificity studies have obviously been successful in elucidating the fatty acyl binding site for the desaturases (38,40,41). Thus, it was with the anticipation that manipulations in chain length, position of unsaturation, and the degree of unsaturation of the acyl portion of the fatty acid molecule would successfully elucidate the preferred structure for microsomal chain elongation and provide initial evidence as to the geometry and type of binding. In addition, a systematic approach would also reconcile a number of disputed rates and either confirm or contradict the predictions of other investigators (52).
CHAPTER II
MATERIALS AND METHODS

Reagents and animals

Coenzyme A, ATP, NADPH, and bovine serum albumin (essentially fatty acid free) were purchased from the Sigma Chemical Company, Saint Louis, Missouri. Malonyl-CoA was obtained from P and L Biochemicals, Milwaukee, Wisconsin. N-hydroxysuccinimide and N,N'-dicyclohexyl carbodiimide were obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin. Ethyl chloroformate was from Eastman Chemical Company, Rochester, New York, and triethylamine was from Matheson, Coleman, and Bell of Cincinnati, Ohio. All other reagents and solvents were reagent grade and further purified as indicated.

The Na\textsuperscript{14}CN used in the synthetic procedures was purchased from NEN, Boston, Massachusetts; Amersham, Arlington Heights, Illinois, was the supplier of [1-\textsuperscript{14}C] 9,12,15-18:3. The following fatty acids were kindly supplied by Dr. Sprecher and were prepared by total organic synthesis as will be outlined: [1-\textsuperscript{14}C] 6,9-18:2, [1-\textsuperscript{14}C] 5,8,11-18:3, [1-\textsuperscript{14}C] 5,8,11-20:3, [1-\textsuperscript{14}C] 7,10,13-20:3, [3-\textsuperscript{14}C] \beta-hydroxy-9,12-20:2, and [3-\textsuperscript{14}C] 2-trans-9,12-20:3.

All animals used in this study were male Sprague Dawley rats maintained on a normal chow diet and sacrificed by cervical dislocation at total body weights between 200 and 300 grams.
Analytical procedures

A Beckman Acta II spectrophotometer was used for both ultraviolet and visible spectrophotometric analysis. Thin layer plates of varying thicknesses were prepared from a 1:2 (w/v) slurry of silica gel G (Merck, Darmstadt) and water. For argentation chromatography a 12.5% solution of AgNO₃ was substituted for the distilled water. The plates were air dried, activated for one hour at 95-100°C, and stored desiccated until used. Most separations were afforded by 70/31/1, PE/EE/AA (v/v), for normal thin layer chromatography and 80/20, PE/EE (v/v), for argentation chromatography. Fatty acyl-CoA derivatives were analyzed by ascending paper chromatography on Whatman No. 1 using a solvent system consisting of 5/2/3, n-butanol/AA/H₂O (v/v). A F and M Model 810 gas chromatograph, Avondale, Pennsylvania, equipped with a thermal conductivity detector and a Packard effluent gas cartridge fraction collector was used for the separation of fatty acid methyl esters. The separations were afforded by 10 foot long, 0.25 inch diameter, stainless steel columns packed with Hi-EFF-2BP (15% ethyleneglycol succinate on Gas-Chrom P, 80-100 mesh, Applied Science Labs, State College, Pennsylvania). A helium flow rate of 60 ml/min was used and the oven was maintained at various temperatures (185-205°C) depending on the type of separation desired. All [¹⁴C] determinations were made with a Packard TriCarb liquid scintillation spectrometer, Model 3380, using either a toluene based cocktail (5 grams PPO plus 0.5 grams dimethyl POPOP/liter toluene) for lipid soluble compounds or a dioxane based cocktail (14 grams PPO plus 0.6 grams dimethyl POPOP plus 200 grams naphthalene/1.6 liters dioxane plus 0.4 liter H₂O) for water soluble compounds (105).
Preparation of the isomeric series of 4,7- through 11,14- octadecadienoic acids

Synthesis of fatty alcohols (VII). All fatty acids used in this study were kindly supplied to the principle investigator by Dr. Sprecher as fatty alcohols containing one deficient methylene group at the derivatized end. Hence, the numbering system and total number of carbons was one less that the desired fatty acid (e.g., 6,9-17:2-1-ol would result in the fatty acid, 7,10-18:2). The alcohols were then converted to either the cold or [1-14C] fatty acids, as will be described in detail.

The general procedure for polyunsaturated fatty acid synthesis as described by Osbond et al. (106) was used, and the following scheme for 6,9-17:2-1-ol illustrates the series of reactions necessary for its synthesis. Modification of either the tail halide or front acetylenic alcohol permitted the synthesis of the different 4,7- through 11,14- isomers.

\[
\begin{align*}
\text{CH}_3-(\text{CH}_2)_{6}\text{-Br} + \text{HC}=\text{CH}_2\text{-OH} & \rightarrow \text{CH}_3-(\text{CH}_2)_{6}\text{-C}=\text{C}-\text{CH}_2\text{-OH} \\
\text{CH}_3-(\text{CH}_2)_{6}\text{-C}=\text{C}-\text{CH}_2\text{-Br} & \rightarrow \text{CH}_3-(\text{CH}_2)_{6}\text{-C}=\text{C}-\text{CH}_2\text{-Br} \\
\end{align*}
\]

\[
\begin{align*}
1. \text{Na, NH}_3 & \rightarrow \text{CH}_3-(\text{CH}_2)_{6}\text{-C}=\text{C}-\text{CH}_2\text{-OH} \\
2. \text{H}^+ & \rightarrow \text{CH}_3-(\text{CH}_2)_{6}\text{-C}=\text{C}-\text{CH}_2\text{-OH} \\
1. \text{EtMgBr} & \rightarrow \text{CH}_3-(\text{CH}_2)_{6}\text{-C}=\text{C}-\text{CH}_2\text{-Br} \\
2. \text{HC}=\text{C}-(\text{CH}_2)_{5}\text{-OH} & \rightarrow \text{CH}_3-(\text{CH}_2)_{6}\text{-C}=\text{C}-\text{CH}_2\text{-Br} \\
\text{Lindlar} & \rightarrow \text{CH}_3-(\text{CH}_2)_{6}\text{-C}=\text{C}-(\text{CH}_2)_{3}\text{-CH}_2\text{-OH} \\
\text{reduction} & \rightarrow \text{CH}_3-(\text{CH}_2)_{6}\text{-C}=\text{C}-(\text{CH}_2)_{3}\text{-CH}_2\text{-OH} \\
\end{align*}
\]

2-propargyloxytetrahydropyran (II) was allowed to react with Na metal in liquid NH₃ after which it was coupled with 1-bromoheptane (I) to give the pyrano derivative of 2-decyln-1-ol. The pyrano group was removed by acid hydrolysis to give 2-decyln-1-ol (III), and was then converted to 1-bromo-2-decyne (IV) with PBr₃. IV was converted to the
Grinard derivative by the addition of EtMgBr and coupled to 1-hydroxy-6-heptane (V) to give 1-hydroxy-6,9-heptadecadiyne (VI). Stereospecific hydrogenation of VI with Lindlar's catalyst afforded 1-hydroxy-6,9-heptadecadiene (VII).

Synthesis of labeled octadecadienoic acid (XI). The purification, derivatization, repurification, and saponification of [1-\(^{14}\)C] 7,10-18:2 is illustrative of the other octadecadienoic acids synthesized by the principle investigator. The following scheme outlines the series of reactions used in the synthesis of the labeled 7,10-18:2.

\[
\begin{array}{c}
\text{CH}_3\text{SO}_2\text{Cl} \\
\text{R-CH}_2\text{-OH} \xrightarrow{\text{pyridine}} \text{R-CH}_2\text{-OSO}_2\text{CH}_3 \\
\text{VII} \xrightarrow{\text{Na}^{14}\text{CN}} \text{R-CH}_2\text{-}^{14}\text{CN} \\
\text{X} \xrightarrow{\text{MeOH/HCl}} \text{X} \\
\text{X} \xrightarrow{1. \text{KOH/EtOH}} \text{CH}_3\text{-COOCH}_3 \\
\text{R-CH}_2\text{-}^{14}\text{COOCH}_3 \xrightarrow{2. \text{HCl}} \text{R-CH}_2\text{-}^{14}\text{COOH} \\
\text{XI} \\
\end{array}
\]

\( R = \text{CH}_3\text{-}(\text{CH}_2)_6\text{-}(\text{CH=CH-CH}_2)_2\text{-}(\text{CH}_2)_3\text{-} \)

However, since the synthesis of the labeled and nonlabeled fatty acids are essentially the same, only the synthesis of the labeled fatty acid will be described.

The mesylate derivative of heptadeca-6,9-dien-1-ol (VIII), Before derivatization, sufficient 6,9-heptadecadien-1-ol for the final acid was purified on a 2.5x45 cm Unisil column by eluting with increasing amounts of ethyl ether in petroleum ether. The purified alcohol, as determined by TLC monitoring in 70/30/1, PE/EE/AA, appeared in the 15 to 25% EE in PE fractions. The following procedure is essentially that of Bauman and Mangold (107): In a 50 ml, three necked flask equipped with a magnetic stirring bar, inlet tube for purified N\(_2\), and a dropping funnel, 1.02 g (4 mmoles) of 6,9-heptadecadien-1-ol was dissolved
in 14 ml pyridine. The flask was chilled and 0.687 g, 0.465 ml (6 mmoles) of methanesulfonyl chloride was added dropwise. The ice bath was removed and stirring was continued for another 3-5 hours at room temperature. The reaction was extracted with 26 ml H₂O and 35 ml ethyl ether in a 125 ml separatory funnel. After separation, the H₂O phase was reserved for further extraction. The ether phase was extracted consecutively with 9 ml H₂O, 2 N H₂SO₄ until acidic, 9 ml H₂O, 1% K₂CO₃ until neutral or basic, and 9 ml H₂O, and dried over sodium sulfate. The reserved H₂O phase and the other basic H₂O extracts were combined and treated with 35 ml ether. After washing the ether extract with 9 ml H₂O, it was used to extract the original acidic phases (2 N H₂SO₄ added if necessary). The ether phase was then treated with 9 ml H₂O, 1% K₂CO₃ until basic, 9 ml H₂O, and combined with the initial ether extract. The solvent was removed with a rotary evaporator giving 1.12 g crude VIII. In that the majority of the product was the mesylate and further purification occurred later on in the synthesis, the methanesulphonate derivative was used without further purification.

6,9-heptadecadieny1-1-¹⁴C-cyanide (IX). The following procedure is essentially that of Bauman and Mangold (108) except with the substitution of Na¹⁴CN for NaCN: Dimethyl sulfoxide (6.0 ml, dried over molecular sieves) was added to the vial containing 1 mCi Na¹⁴CN (40-60 mCi/m mole) and the contents permitted to stir until the Na¹⁴CN dissolved. Cold NaCN (35.8 mg, 0.73 mmoles) was weighed into a 3 neck 50 ml flask fitted with an N₂ inlet, dropping funnel, and condensor fitted with a drying tube, and 3.0 ml of the Na¹⁴CN/DMSO solution was added to it. The mesylate derivative of heptadeca-6,9-dien-1-ol (0,325
g, 0.987 mmole assuming pure) in a total of 20 ml DMSO was added to
the reaction via the dropping funnel and the reaction was allowed to
stir for 1.5 hours at 80°C. The reaction was cooled and 19 ml ether
and 7.6 ml H₂O added. The reaction mixture was transferred to a 125 ml
separatory funnel with an additional 19 ml ether, and the phases allowed
to separate after shaking. The aqueous layer was extracted with 4x7.6
ml ether, the combined ether phases washed with two 7.6 ml portions of
H₂O, and dried over anhydrous sodium sulfate. The ether was removed on
a rotary evaporator and converted directly to the methyl ester,

The desired specific activity for this acid was 1.5x10⁶ dpm/µmole
(0.675 µCi/µmole). Nonradioactive fatty acids were synthesized in a
similar manner except a 50% molar excess of NaCN was used.

Methyl [1-¹⁴C] 7,10-octadecadienoate (X). The methyl ester of
[1-¹⁴C] 7,10-octadecadienoate (X) was prepared from the 6,9-hepta-
decadienyl-¹⁴C-cyanide compound (IX) by the addition of 25 ml ice cold
25% HCl in MeOH. The reaction was allowed to stir overnight under a
N₂ atmosphere and extracted with 3x25 ml PE after the addition of 50 ml
H₂O. The combined PE extracts were extracted with H₂O until neutral,
dried over anhydrous sodium sulfate, and the PE removed on a rotary
evaporator giving 283 mg crude methyl [1-¹⁴C] 7,10-octadecadienoate (X).
The purity was checked by both TLC and GLC.

The methyl ester was purified by Argentation thin layer chroma-
tography by applying ca. 50 mg crude preparation per 20x20 cm plate and
developing in 80/20, PE/EE. The plates were lightly sprayed with 2',
7'-DCF and the methyl ester band scrapped into a small beaker and mixed
with a small quantity of ether. The ether suspension was added to a
1.7 cm OD 250 ml Kontes column with reservoir containing a glass wool plug and 1-2 cm silica gel G filtering bed. The methyl ester was eluted with approximately four bed volumes of EE into a clean, tarred receptacle and the ether removed under a gentle stream of N₂ yielding 161 mg (0.547 mmoles) pure methyl [1-¹⁴C] 7,10-octadecadienoate (X). The purity in reference to both mass and radioactivity was checked by GLC and the specific activity determined (Table 2).

Table 2 lists the octadecadienoic acids synthesized, specific activities, percent purity, and R₁₈:₀ as determined by GLC. All acids had a radioactive purity of greater than 95%.

In addition, a variety of other long chain, unsaturated fatty acids were synthesized according to the generalized scheme as outlined and are listed in Table 3 along with their percent radiochemical purity and specific activities.

[1-¹⁴C] 7,10-octadecadienoic acid (XI). Of the 161 mg pure methyl [1-¹⁴C] 7,10-octadecadienoate synthesized, 40.0 mg was saponified and the remainder sealed under N₂ for further use. The methyl ester was hydrolyzed by the addition of 5 ml 4% KOH in 9/1, EtOH/H₂O. The reaction was allowed to stir overnight under N₂ after which the medium was acidified with 1.0 N HCl and extracted three times with CHCl₃. The combined CHCl₃ extracts were washed with H₂O until neutral, dried over anhydrous sodium sulfate, and reduced to dryness yielding 36.1 mg (0.13 mmoles) [1-¹⁴C] 7,10-octadecadienoic acid (XI). The completeness of saponification was checked by TLC.

The free acid of [1-¹⁴C] 7,10-octadecadienoic acid (36.1 mg, 0.129 mmoles, 280.45 gmw) was converted to the potassium salt by the addition
Table 2

Percent purity\(^{a}\), specific activity\(^{b}\), and \(R_{18:0}\)\(^{c}\) of the chemically synthesized [1-\(^{14}\)C] octadecadienoic acids

<table>
<thead>
<tr>
<th>18:2 isomer</th>
<th>4,7-</th>
<th>5,8-</th>
<th>6,9-</th>
<th>7,10-</th>
<th>8,11-</th>
<th>9,12-</th>
<th>10,13-</th>
<th>11,14-</th>
</tr>
</thead>
<tbody>
<tr>
<td>percent purity(^{a})</td>
<td>97.6</td>
<td>96.8</td>
<td>97.4</td>
<td>97.7</td>
<td>95.8</td>
<td>95.3</td>
<td>96.8</td>
<td>97.1</td>
</tr>
<tr>
<td>specific activity(^{b})</td>
<td>0.52</td>
<td>0.61</td>
<td>0.25(^{d})</td>
<td>0.60</td>
<td>0.44(^{d})</td>
<td>0.65</td>
<td>0.58</td>
<td>0.62</td>
</tr>
<tr>
<td>(R_{18:0})(^{c})</td>
<td>1.40</td>
<td>1.39</td>
<td>1.44</td>
<td>1.47</td>
<td>1.45</td>
<td>1.54</td>
<td>1.58</td>
<td>1.66</td>
</tr>
</tbody>
</table>

\(^{a}\)As determined by radioactive GLC fractionation of representative methyl esters.  
\(^{b}\)As determined from the 20mM K\(^{+}\)/albumin complex of the fatty acid and expressed as µCi/µmole.  
\(^{c}\)As determined by GLC (F and M Model 810, 10 foot column packed with 15% ethyleneglycol succinate on Gas-Chrom P, 80-100 mesh, oven temp 190°C).  
\(^{d}\)Synthesized for a different study.
Table 3

Percent purity$^a$ and specific activity$^b$ of miscellaneous chemically synthesized $[1-^{14}\text{C}]$ fatty acids

<table>
<thead>
<tr>
<th>fatty acid</th>
<th>percent purity$^a$</th>
<th>specific activity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-18:1</td>
<td>98.6</td>
<td>0.36</td>
</tr>
<tr>
<td>5,8-16:2</td>
<td>98.0</td>
<td>0.30</td>
</tr>
<tr>
<td>7,10-14:2</td>
<td>98.0</td>
<td>0.42</td>
</tr>
<tr>
<td>7,10-16:2</td>
<td>97.6</td>
<td>0.47</td>
</tr>
<tr>
<td>7,10-20:2</td>
<td>97.5</td>
<td>0.49</td>
</tr>
<tr>
<td>4,7,10-18:3</td>
<td>97.2</td>
<td>0.82</td>
</tr>
<tr>
<td>6,9,12-18:3</td>
<td>97.7</td>
<td>0.70</td>
</tr>
<tr>
<td>7,10,13-18:3</td>
<td>99.5</td>
<td>0.64</td>
</tr>
<tr>
<td>8,11,14-18:3</td>
<td>98.3</td>
<td>0.40</td>
</tr>
</tbody>
</table>

$^a$As determined by radioactive GLC fractionation of representative methyl esters.

$^b$As determined from the 20mM K$^+/albumin$ complex of the fatty acid and expressed as $\mu$Ci/$\mu$ mole.
of 0.258 mmole of 0.1 N KOH and diluted to 20 mM by the addition of 3.86 ml H₂O. All other octadecadienoic acids were handled in a similar manner.

**Synthesis of the coenzyme A derivatives of [1-¹⁴C] octadecadienoic acids**

The coenzyme A derivatization, purification, and characterization of [1-¹⁴C] 7,10-18:2 is illustrative of the other octadecadienoic acids derivatized with CoASH. It represents a slight modification in the use of N-hydroxysuccinimide esters of fatty acids (109) for the synthesis of coenzyme A derivatives of long chain fatty acids as described by Al-Arif and Blecher (110).

\[
\begin{align*}
\text{R-CH}_2-^{14}\text{COOH} + \text{HO-NN'DCHCDI} & \rightarrow \text{R-CH}_2-^{14}\text{COO-N}^\cdot \\
\text{III} & \rightarrow \text{R-CH}_2-^{14}\text{COSCoA} \\
\text{IV} & \\
\text{R=CH}_3-(\text{CH}_2)_6-(\text{CH=CH-CH}_2)_2-(\text{CH}_2)_3- \\
\end{align*}
\]

N-hydroxy succinimide derivative of [1-¹⁴C] 7,10-octadecadienoic acid (III). [1-¹⁴C] 7,10-18:2 (I) (56.9 mg, 203 µmole, 280 gmw) was dissolved in ca. 1 ml of freshly distilled ethyl acetate, followed by 41.8 mg (203 µmole, 206.3 gmw) of urea free N, N' dicyclohexylcarbodi-imide (NN'DCHCDI) in 0.2 ml freshly distilled ethyl acetate. The reaction was allowed to proceed at room temperature for 3 hours under N₂ during which time urea precipitation occurred. The supernatant was carefully sucked off and filtered through glass wool, blown down (additional urea precipitation occurred) to a workable volume, streaked on a 0.5 mm 20x20 cm silica gel G plate and chromatographed in 80/20/1, PE/EE/AA. The plate was dried in a N₂ atmosphere until all traces of acetic acid
were gone, sprayed with 2',7' dichlorofluorescein, and the N-hydroxy-
succinimide ester of [1-\(^{14}\)C] 7,10-18:2 (III) band scrapped off. The
imide was eluted with ethyl ether from the silica gel using a small
column stoppered with glass wool and containing a filtering bed of
silica gel G. The yield was 67.9 mg (III) (180 \(\mu\)moles, 377 gmo). The
purity of the imide was checked by TLC and counting 1 cm sections of
the developed plate. A purity of 92.9% was found with the primary con-
taminant being unreacted free fatty acid (6.9%).

\[ [1-^{14}\text{C}] 7,10\text{-octadecadienoyl-CoA (IV).} \]

The N-fatty acyl succini-
mide ester of \([1-^{14}\text{C}] 7,10\text{-18:2 (III)} \] (16.6 mg, at 92.7% purity, 40.8
\(\mu\)moles) was dissolved in 2 ml freshly distilled THF. The lithium salt
of coenzyme A (41.8 mg, 42.8 \(\mu\)moles pure CoA assuming 90.2% total CoA
and 93% of that as reduced CoASH) was weighed into a small vial,
NaHCO\(_3\) (35.2 mg, 40.8 \(\mu\)moles) was added to the CoASH and the contents
dissolved in 1 ml \(O_2\) free H\(_2\)O. The aqueous solution was added to the
THF solution and either THF or H\(_2\)O added to induce clarity. The final
volume was recorded and the reaction allowed to stir at room tempera-
ture under a \(N_2\) atmosphere. Immediately after the start of the re-
action and during the reaction itself, 5\(\lambda\) aliquots were removed and
analyzed for free -SH with DTNB (5,5'-dithiobis(2-nitrobenzoic acid))
as described by Ellman, \textit{et al.} (III). After completion of the re-
action, as determined by -SH group analysis, the solution was trans-
ferred to a clean 15 ml tappered centrifuge tube with H\(_2\)O rinses and
the THF removed with a stream of \(N_2\). The CoA derivative of \([1-^{14}\text{C}] 7,
10\text{-18:2 (IV)} \) was precipitated by the addition of 4 ml 5% HClO\(_4\) and the
flocculant material centrifuged until a clear supernatant appeared.
A Pasteur pipette, whose tip was plugged with glass wool, was used to remove this supernatant and all subsequent supernatants. The precipitate was washed with 8.0 ml 0.8% HClO₄ and recentrifuged. The resulting precipitate was washed with 4x2.5 ml distilled acetone and 3x3 ml distilled ethyl ether, and dried under a gentle stream of N₂. The precipitate was extracted with 1x1 ml plus 3x0.5 ml H₂O with centrifugations between extractions. A small amount (ca, 10 mg) NaHCO₃ was added to neutralize any HClO₄ during extraction. The CoA derivative was reprecipitated with 2 ml 5% HClO₄ and the centrifuged pellet washed with 4 ml 0.8% HClO₄ and 3x1.5 ml acetone yielding 26.1 mg (25.0 μmoles, 61.3% yield by amide) of a white powder after drying under N₂. The CoA derivative of [1⁻¹⁴C] 7,10-18:2 (IV) was dissolved in 2.0 ml pH 3.0 H₂O and analyzed spectrophotometrically. Using a molar extinction coefficient of 14.0x10⁴ M⁻¹cm⁻¹ at 260 nm, a yield of 21.1 μmoles (51.7% yield from imide) of the CoA derivative was obtained. A few λ of the aqueous solution of the CoA derivative was spotted on a sheet of Whatman No. 1 and chromatographed by the ascending method in the solvent system as previously described. The chromatogram was cut into 1 cm wide sections and counted, indicating a purity of 93.3%. The specific activity of the CoA derivative was determined at 1.43x10⁶ dpm/μmole. The specific activities and percent purities of the other CoA derivatives that were synthesized appear in Table 4.

**Synthesis of [3⁻¹⁴C] 2-trans eicosatrienoic acids**

[1⁻¹⁴C] 5,8-18:2, [1⁻¹⁴C] 7,10-18:2, and [1⁻¹⁴C] 8,11-18:2 were used as the starting materials for the synthesis of the desired [3⁻¹⁴C] 2-trans 20:3 isomers using the general procedure of Stoffel and Pruss
(112). [3-¹⁴C] 2-trans-10,13-20:3 was prepared according to the following scheme and is illustrative of the three 2-trans 20:3 acids synthesized:

\[
\begin{align*}
&\text{R-CH}_2-^{14}\text{COOCH}_3 \xrightarrow{\text{LiAlH}_4} \text{R-CH}_2-^{14}\text{CH}_2-\text{OH} \xrightarrow{\text{MSCl}} \text{R-CH}_2-^{14}\text{CH}_2-\text{OSO}_2\text{CH}_3 \\
&\text{DMSO} \xrightarrow{\text{NaHCO}_3} \text{CH}_2(\text{COOH})_2 \xrightarrow{\text{H}} \text{R-CH}_2-^{14}\text{C}=\text{C-COOH} \\
&\text{R=CH}_3-(\text{CH}_2)_5-(\text{CH}=\text{CH-CH}_2)_2-(\text{CH}_2)_4-
\end{align*}
\]

[1-¹⁴C] 8,11-octadecadien-1-ol (II). Methyl [1-¹⁴C] 8,11-octadecadienoate (I) (0.87 g, 2.96 mmol, 294 g.mw) was dissolved in ca. 20 ml dry EE and added dropwise to a suspension of 0.8 g LiAlH₄ (Alfa) in ca. 40 ml dry EE with continuous stirring. A three neck flask fitted with a N₂ inlet tube, condenser, and dropping funnel was used. The reaction was allowed to stir for ca. 3 hours at room temperature after which it was refluxed for 1.5 hours. The flask was cooled and the excess LiAlH₄ hydrated by the dropwise addition of H₂O saturated ether. The lithium salts were dissolved in 20 ml 20% H₂SO₄ and the reaction immediately extracted with EE giving 0.743 g of [1-¹⁴C] 8,11-octadecadien-1-ol (II).

The mesylate derivative of [1-¹⁴C] 8,11-octadecadien-1-ol (III). In a 50 ml three neck flask equipped with a magnetic stirring bar, inlet tube for N₂, drying tube, and dropping funnel, 0.324 ml (0.479 g, 4.2 mmol) of methanesulfonyl chloride (MSCl) was added dropwise to
0.743 g (2.79 mmoles) of [1-\(^{14}\)C] 8,11-octadecadien-1-ol in 14 ml dry pyridine cooled in an ice bath. The reaction was allowed to stir for 5 hours and extracted as previously described for the mesylate derivative of [1-\(^{14}\)C] 6,9-heptadecadien-1-ol yielding 0.952 g (2.77 mmoles, 344 gmw).

[1-\(^{14}\)C] 5,8-octadecadien-1-al (IV). In a 50 ml, two neck flask fitted with a condensor and N\(_2\) inlet tube, the 0.952 g (2.77 mmoles, 344 gmw) of mesylate (III) was heated with 5.0 ml DMSO and 0.5 g NaHCO\(_3\) at 160°C for 10 minutes with stirring. The reaction vessel was cooled rapidly and cold distilled H\(_2\)O added. The reaction was extracted with ether and the ether dried over anhydrous sodium sulfate yielding 0.502 g. TLC against the appropriate standards indicated 76.2% aldehyde (0.383 g, 264 gmw, 1.45 mmoles), 13.7% mesylate (0.069 g, 0.20 mmoles, 344 gmw) and 9.2% alkene (0.046 g, 0.19 mmoles, 248 gmw) or 2.64 mmoles total.

[3-\(^{14}\)C] 2-trans-10,13-eicosatrienoic acid (V). Malonic acid (0.45 g, 4.11 mmoles) was added to 1.0 ml dry pyridine in a 15 ml two necked flask fitted with a condensor and N\(_2\) inlet tube. The [1-\(^{14}\)C] 8,11-octadecadien-1-al (IV) in 0.65 ml pyridine was added after the addition of 0.30 ml piperidine. The flask was heated for 1 hour at 55°C and then 5 hours at 80 to 90°C. The reaction was allowed to cool, 7 ml conc. HCl in 25 ml H\(_2\)O was added, and the reaction extracted with EE. The dried ether extract yielded 61.0% [3-\(^{14}\)C] 2-trans-10,13-eicosatrienoic acid (V) (0.297 g, 0.97 mmoles, 306 gmw), 16.6% alcohol (0.081 g, 0.30 mmoles, 266 gmw), and 13.2% alkene (0.064 g, 0.26 mmoles, 248 gmw) as determined by TLC. The EE extract was purified in a preparative manner by TLC and the free acid band eluted giving 0.301 g.
Argentation chromatography of methyl [3-14C] 2-trans-10,13-eicosatrienoate. The free acid was methylated with ca. 20 ml 25% HCl in MeOH at room temperature, overnight, under N₂. The reaction medium was diluted with H₂O, extracted with PE, and yielded 0.305 g crude methyl [3-14C] 2-trans-10,13-eicosatrienoate. The 0.305 g methyl ester was streaked on six 20x20 cm 0.5 mm plates and developed by argentation chromatography in 60/40, PE/dibutyl ether. After complete evaporation of the dibutyl ether in a N₂ atmosphere, the plates were sprayed with 2', 7'-DCF and the position of the methyl [3-14C] 2-trans-10,13-eicosatrienoate was recognized as described by Gunstone et al. (113). The bands were removed and the component eluted with ether giving 165.5 mg methyl ester. GLC indicated a purity of 95.2%.

[3-14C] 2-trans-10,13-eicosatrienoic acid. The methyl [3-14C] 2-trans-10,13-eicosatrienoate was saponified in 15 ml 0.5 N NaOH in 60/40, H₂O/t-butanol overnight with stirring under N₂ according to Davidoff and Korn (114). Extraction of the reaction medium with ether after acidification gave 0.154 g (0.502 mmol) of chromatographically pure free acid.

Synthesis of Coenzyme A derivatives of [3-14C] 2-trans eicosatrienoic acids

The coenzyme A derivatives of [3-14C] 2-trans-7,10-20:3, [3-14C] 2-trans-9,12-20:3, and [3-14C] 2-trans-10,13-20:3 were synthesized from their mixed anhydrides as described by Vagelos and Alberts (115). The reaction sequence with [3-14C] 2-trans-10,13-20:3 is representative of the synthesis.
Mixed anhydride of ethyl hydrogen carbonate and [3-\(^{14}\)C\] 2-trans-10,13-20:3 (II). Triethyl amine (14.8 mg, 19.4 \(\mu\)mol, 139.1 \(\mu\)moles) and freshly distilled ethyl chloroformate (15.1 mg, 11.1 \(\mu\)mol, 139.1 \(\mu\)moles) were added to 35.3 mg [3-\(^{14}\)C\] 2-trans-10,13-20:3 (116.0 \(\mu\)moles) in 2.0 ml dry benzene and the reaction allowed to proceed for ca. 1 hr, room temperature with stirring and under a \(\text{N}_2\) atmosphere. The solvent was removed with a gentle stream of \(\text{N}_2\), and the resulting precipitate extracted with three 1 ml volumes of freshly distilled THF. The THF was removed and the precipitate dissolved in 2.0 ml THF. The THF solution was analyzed for mixed anhydride as described by Lapidot et. al. (108) and indicated a yield of 70 \(\mu\)moles of the mixed anhydride of ethyl hydrogen carbonate and [3-\(^{14}\)C\] 2-trans-10,13-20:3 (II).

[3-\(^{14}\)C\] 2-trans-10,13-eicosatrienoyl-CoA (III). The THF solution of the mixed anhydride (70 \(\mu\)moles) was used directly in the synthesis of the CoA derivative of [3-\(^{14}\)C\] 2-trans-10,13-20:3 (III) as described for the CoA derivative of [1-\(^{14}\)C\] 7,10-18:2. Using a molar extinction coefficient of 15.1x10^3 M\(^{-1}\)cm\(^{-1}\) at 260 nm, a yield of 32.95 \(\mu\)moles was obtained. The specific activities and percent purities of the three 2-trans 20:3-CoA's synthesized appear in Table 4.

**Synthesis of [3-\(^{14}\)C\] \(\beta\)-hydroxy-eicosadienoic acids**

The [3-\(^{14}\)C\] \(\beta\)-hydroxy-eicosadienoic acid analogues of [1-\(^{14}\)C\] 5,8-18:2, [1-\(^{14}\)C\] 7,10-18:2, and [1-\(^{14}\)C\] 8,11-18:2 were synthesized with minor modification as described by Stoffel and Pruss (112). [3-\(^{14}\)C\]...
\( \beta \)-hyrdoxy-10,13-20:2 was prepared according to the following scheme and is illustrative of the three [3-\(^{14}\)C] \( \beta \)-hydroxy eicosadienoic acids synthesized.

\[
\text{R-}^{14}\text{COOH} \xrightarrow{\text{(COCI)}_2} \text{R-}^{14}\text{COCI} \xrightarrow{\text{Na}^+ [\text{CH}_3-\text{CO-CH}_2-\text{COOCH}_3]} \text{R-}^{14}\text{CO-CH}_2-\text{COOCH}_3
\]

1. \( \text{NaBH}_4 \) \( \xrightarrow{\text{OH}} \) \( \text{R-}^{14}\text{CH-CH}_2-\text{COOH} \)
2. \( \text{OH}^- \) \( \xrightarrow{\text{IV}} \)

\[ \text{R=CH}_5-(\text{CH}_2)_5-(\text{CH=CH-CH}_2)_2-(\text{CH}_2)_5- \]

[1-\(^{14}\)C] 8,11-octadecadienoyl chloride (II). One ml of benzene and 1.0 ml of oxalyl chloride (freshly distilled, 11.8 mmoles) were added to 0.420 g of [1-\(^{14}\)C] 8,11-18:2 (I) (1.50 mmoles, 280 gmw) and the reaction permitted to stir under \( \text{N}_2 \) overnight. The excess oxalyl chloride was removed under a stream of \( \text{N}_2 \) and repeated additions of benzene, and used immediately for the following synthesis.

Methyl [3-\(^{14}\)C] \( \beta \)-keto-10,13-eicosadienoate (III). The sodium salt of methyl acetoacetate was prepared by the addition of 36.8 mg sodium (1.6 mmoles, 23 gmw) to 0.173 ml methyl acetoacetate (1.6 mmoles, 116.1 gmw) in 2.5 ml benzene. The reaction was allowed to reflux for 6 hrs and then stand overnight. One ml of benzene was added to the acid chloride (II) and the acid chloride transferred with benzene washes to the warmed sodium methyl acetoacetate. The reaction was permitted to stir 1.5 hrs at room temperature and reflux 0.5 hrs with both steps under a \( \text{N}_2 \) atmosphere. The benzene solution was washed with 1/1, \( \text{EtOH/H}_2\text{O} \), and then 1/9, \( \text{EtOH/H}_2\text{O} \). The combined aqueous phases were
extracted with ether and the ether extract added to the initial benzene phase. After drying over anhydrous sodium sulfate, 0.552 g of crude methyl [3-14C] β-keto-10,13-20:2 (III) was obtained. The crude methyl ester was purified in a preparative manner by TLC as previously described giving 0.463 g of 96.5% pure methyl [3-14C] β-keto-10,13-20:2 (III) (1.38 mmol, 336 gmw).

[3-14C] β-hydroxy-10,13-eicosadienoic acid (IV). Twenty-five ml of dry methanol and 100 mg NaBH₄ (26.45 mmol) were added to III and the reaction permitted to stir under N₂ overnight. Water was added to the reaction mixture and the aqueous methanol extracted with ethyl ether. The dried ether extract (0.414 g) was purified by preparative TLC using 70/30/1, PE/EE/AA, and yielded 0.108 g of 98.1% pure methyl [3-14C] β-hydroxy-10,13-20:2. It is suggested that the reaction be followed by TLC to prevent overreduction. The methyl [3-14C] β-hydroxy-10,13-20:2 was saponified in 5 ml 4% KOH, 9/1 EtOH/H₂O, overnight with stirring and under N₂. The reaction was acidified, diluted with H₂O, and extracted with CHCl₃. The CHCl₃ extract of crude free β-hydroxy fatty acid was purified by preparative TLC giving 29.4 mg of pure [3-14C] β-hydroxy-10,13-20:2 (IV) (90.7 µmol, 324 gmw).

Synthesis of [3-14C] β-hydroxy-eicosadienoyl-CoA derivatives

The coenzyme A derivatives of [3-14C] β-hydroxy-7,10-20:2, [3-14C] β-hydroxy-9,12-20:2, and [3-14C] β-hydroxy-10,13-20:2 were synthesized from their N-hydroxysuccinimide derivatives as described for [1-14C] 7,10-octadecadienoyl-CoA. The specific activities and percent purities of the individual CoA's appears in Table 4.
Table 4
Percent purity\(^a\),\(^b\) and specific activity\(^c\) of the chemically synthesized
\([1-^{14}\text{C}]\) octadecadienoyl, \([3-^{14}\text{C}]\) \(\beta\)-hydroxy eicosadienoyl,
and \([3-^{14}\text{C}]\) 2-trans eicosatrienoyl-CoA's

<table>
<thead>
<tr>
<th>parent 18:2 acid</th>
<th>5,8-</th>
<th>7,10-</th>
<th>8,11-</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta)-hydroxy 20:2</td>
<td>--</td>
<td>7,10-</td>
<td>--</td>
</tr>
<tr>
<td>2-trans 20:3</td>
<td>--</td>
<td>--</td>
<td>9,12-</td>
</tr>
<tr>
<td>percent purity methyl ester(^a)</td>
<td>97.5</td>
<td>98.6</td>
<td>96.9</td>
</tr>
<tr>
<td>percent purity CoA derivative(^b)</td>
<td>97.7</td>
<td>--</td>
<td>95.6</td>
</tr>
<tr>
<td>specific activity(^c)</td>
<td>0.32</td>
<td>0.28</td>
<td>0.65</td>
</tr>
</tbody>
</table>

\(^a\) As determined by radioactive GLC fractionation of representative methyl esters.

\(^b\) As determined by ascending paper chromatography of representative CoA derivatives.

\(^c\) As determined from 20mM CoA derivative, pH 3.0, and expressed as \(\mu\text{Ci}/\mu\text{mole},\)
Preparation of albumin and K⁺ salt of fatty acids or albumin and CoA derivative complexes for use as substrates

Either 0.25 ml of the 20 mM K⁺ salt of the [¹⁴C] fatty acid or 0.25 ml of the 20 mM CoA derivative of the [¹⁴C] fatty acids (includes β-hydroxy and 2-trans intermediates) were combined with 1.0 ml 2.5 mM BSA (fraction V, essentially fatty acid free, 66,000 gmw) and 0.417 ml H₂O to give 3.0 mM fatty acid solutions with a fatty acid to albumin ratio of 2:1. All substrates were stored under N₂ and frozen at -20°C until used.

Preparation of rat liver and rat testicular microsomes

In the preparation of liver microsomes, the liver was quickly removed after a midline incision was made, minced in ca. 5 volumes ice cold buffer containing 0.25 M sucrose, 5 mM MgCl₂ and 0.01 M potassium phosphate buffer, pH 7.4, filtered through cheese cloth, and homogenized with a Potter-Elvehjem homogenizer in 3 volumes of the same buffer. Initial centrifugation at 17,000xg for 15 min in a refrigerated RC-2B Sorvall centrifuge removed cellular debris, nuclei, and mitochondria. Centrifugation of the resulting supernatant at 100,000xg for 60 min in a Beckman Model L2-65B ultracentrifuge afforded a microsomal pellet and supernatant. The supernatant was carefully decanted, the adhering fatty film wiped away, and each pellet suspended in 2.5 ml buffer using a Potter-Elvehjem homogenizer. The protein concentration was measured by the method of Lowry (116) (usually gave ca. 25 mg/ml at the above dilutions) and diluted to the desired concentration. A second protein determination after the dilution was performed to confirm the concentration.
Rat testicular microsomes were prepared as described by Murono and Payne (117), and both fresh and lyophilized preparations were used. Normally, approximately 3 to 4 grams of tissue was obtained after decapsulation of both testes. The testes were homogenized in 4 volumes of microsomal suspension buffer using a Potter-Elvehjem homogenizer, centrifuged at 500xg for 15 min, followed by 10,000xg for 30 min. The final centrifugation at 100,000xg for 90 min yielded a small pellet of white microsomes. The pellet was resuspended in 2.0 ml buffer using a Potter-Elvehjem homogenizer and the protein was determined by the method of Lowry (114). For the lyophilized preparations, the same procedure was used except the number of sacrificed rats was greater. In a typical preparation, 15 rats weighing between 150 and 250 g yielded 53 g wet weight tissue and 306 mg microsomal protein. The lyophilized microsomal suspension gave 1.5 g dry weight which was stored at -20°C until needed.

All operations after the initial sacrifice were performed either in an ice bucket or in a cold room maintained at 4°C.

**Incubations and measurements of in vitro conversions**

Activation to fatty acyl-CoA. Incubations were performed in 15 ml test tubes in a Dubnoff metabolic shaker maintained at 37°C. The following components were present unless otherwise noted: 10 μmoles MgCl₂, 10 μmoles ATP, 0.3 μmoles CoASH, 65 mM potassium phosphate, pH 7.4, and 150 nmoles [1-14C] octadecadienoic acid as the K⁺/albumin complex in a total volume of 1.1 ml. The incubations were started by the addition of 0.1 mg microsomal protein in 0.4 ml microsomal suspension buffer and permitted to shake for 3 min unless specified otherwise. The reaction was terminated by the addition of 7.5 ml Dole's reagent (113), mixed,
and the upper heptane layer pipetted into a scintillation vial. The reaction was then extracted twice with 4.5 ml heptane after the addition of 3.0 ml H2O. The combined heptane extracts were allowed to evaporate to dryness and counted with 10 ml toluene based scintillation cocktail. The aqueous phase was transferred to a scintillation vial, the volatile components blown off, and counted with 10 ml Snyder's cocktail (105). The number of counts found in the aqueous layer divided by the total counts from both the heptane and aqueous extracts gave the percent conversion.

Chain elongation. Incubations were performed in 25 ml rubber stoppered vials in a Dubnoff metabolic shaker maintained at 37°C. The following components were present unless noted otherwise: 10 μmoles MgCl2, 10 μmoles ATP, 2 μmoles NADPH, 0.3 μmoles CoASH, 0.3 μmoles malonyl-CoA, 0.085 mmoles potassium phosphate, pH 7.4, and 150 nmoles [1-14C] octadecadienoic or other fatty acid as the K+/albumin complex in a total volume of 1.1 ml. When the CoA derivative of the fatty acid was used, both ATP and CoASH were eliminated from the incubation. Prior to the initiation of the reaction, all vials were flushed with N2. The incubations were started after a 1 min preincubation period at 37°C by the addition of 5.0 mg microsomal protein in 0.4 ml microsomal suspension buffer and permitted to shake for 3 min unless specified otherwise. The reactions were terminated by the addition of 0.25 ml 4 N NaOH and 5 ml methanol, and then allowed to saponify for 1 hour at 37°C with shaking. After saponification, the reaction medium was acidified and extracted according to Folch (119). The vials were extensively shaken, centrifuged, and the bottom CHCl3 layer taken to dryness after drying over anhydrous
sodium sulfate. The lipids were converted to their methyl esters by refluxing with 5% anhydrous HCl in methanol for 1.5 hrs, extracted, and analyzed by radioactive GLC fractionation using the appropriate standards where necessary. The amount of chain elongation was calculated from the radioactivity present in the chain elongated fatty acid product versus the radioactivity present in both the substrate and product.

Condensation. Incubations to measure the rate of condensation were performed exactly as for chain elongation except NADPH was eliminated from the incubation. The reactions were analyzed as described by Bernert and Sprecher (50) using thin layer chromatography to separate substrate from product. The lipids from each reaction were applied to 5 cm wide origins on 20x20 cm 0.5 mm silica gel G plates and developed in 70/30/1, PE/EE/AA using the appropriate standards. These standards were palmitic acid, 8-keto stearic acid, 2-heptadecanone, and methyl stearate. The location of the standards was ascertained by spraying with 2',7' DCF, and the bands corresponding to these standards counted with 10 ml toluene based scintillation cocktail. Although a difference in the degree of unsaturation and chain length existed between substrate and products and the standards used, careful fractionation showed that the Rf's were identical (Figure 4). The components representing substrate were free octadecadienoic acid and some iatrogenic methyl octadecadienoate, and the components representing product were 3-keto eicosadienoic acid and 2-keto nonadecadiene. Conversions were measured as the sum of products divided by the total radioactivity times nmoles substrate,
Figure 4. Assay of the condensation reaction with the illustration of the workup products and their separation by thin layer chromatography (silica gel G, 70/30/1, PE/EE/AA). The workup products for [1-14C] 7,10-octadecadienoyl-CoA condensation would be 1, [1-14C] 7,10-octadecadienoic acid, 2, methyl [1-14C] 7,10-octadecadienoate, 3, [3-14C] β-keto 9,12-eicosadienoic acid, and 4, [2-14C] 9,11-nonadecadien-2-one.
Dehydration. Incubations to measure the rate of dehydration were performed exactly as for chain elongation, except ATP, NADPH, CoASH, and malonyl-CoA were eliminated from the reaction medium. The substrate in this reaction was 150 nmoles [3-14C] 3-hydroxy eicosadienoyl-CoA bound to albumin. The incubations were initiated by the addition of 0.25 mg microsomal protein in 0.4 ml suspension buffer and permitted to shake for 20 min unless specified otherwise. Analysis of the methyl esters was performed by TLC rather than GLC as described by Bernert and Sprecher (50). The methyl esters from each reaction were streaked on 5.0 cm wide origins of 20x20 cm 0.5 mm silica gel G plates and developed in 70/30/1, PE/EE/AA, against the appropriate standards. These standards were methyl 3-hydroxy stearate and methyl stearate. The location of the standards was ascertained by spraying with 2',7' DCF, and the bands corresponding to these standards counted with 10 ml toluene based scintillation cocktail. Although a difference in the degree of unsaturation and chain length existed between the substrate and product and the standards used, careful fractionation showed that the Rf's were identical (Figure 5). The component representing substrate was 3-hydroxy eicosadienoic acid and the component representing product was 2-trans eicosatrienoic acid. Conversions were calculated by dividing the amount of radioactivity in the product by the radioactivity in the substrate plus product times the nmoles of substrate. Incubations in which NADPH was included in the reaction medium were also performed in which case the faster moving methyl esters were eluted from the silica gel and the methyl 2-trans eicosatrienoate and methyl eicosadienoate separated by GLC fractionation.
Figure 5. Assay of the dehydration reaction with the illustration of the workup products and their separation by thin layer chromatography (silica gel G, 70/30/1, PE/EE/AA). The workup products for [3-14C] β-hydroxy 9,12-eicosadienoyl-CoA dehydration would be 1, methyl [3-14C] β-hydroxy 9,12-eicosadienoate, and 2, methyl [3-14C] 2-trans 9,12-eicosatrienoate,
Reduction. Incubations to measure the rate of α,β-enooy fatty acyl-CoA reduction were performed exactly as for chain elongation, except that ATP, CoASH, and malonyl-CoA were eliminated from the reaction medium. The substrate in this reaction was 150 nmoles [3-14C] 2-trans eicosatrienoyl-CoA bound to albumin. The incubations were initiated by the addition of 0.25 mg microsomal protein in 0.4 ml suspension buffer and permitted to shake for 2 min, unless otherwise specified.

Analysis of the methyl esters was performed by a combined TLC and GLC technique, essentially as described by Bernert and Sprecher (50). Equal portions of the methyl esters from each reaction were streaked on 2.5 cm wide origins of 20x20 cm 0.5 mm silica gel G plates and developed in 70/30/1, PE/EE/AA, against the appropriate standards. These standards were methyl β-hydroxy stearate and methyl stearate. The location of the standards was ascertained by spraying with 2',7' DCF, and the bands corresponding to these standards from only one of the 2.5 cm columns counted with 10 ml toluene based scintillation cocktail. This manipulation allowed for the determination of the reverse, hydration reaction. From the other 2.5 cm wide column only the band containing the 2-trans fatty acid substrate and the reduced fatty acid product was removed and eluted with ethyl ether. Radioactive GLC fractionation against the appropriate standards allowed for the separation of the faster moving eicosadienoic acid product from the slower moving 2-trans eicosatrienoic acid substrate. Figure 6 depicts the reactions and analysis for these incubations. The reverse, hydration reaction was also measured in which case NADPH was totally eliminated from the incubation medium and the reaction products analyzed as described for reduction.
Figure 6. Assay of the 2-trans reduction reaction with the illustration of the workup products and their separation by thin layer chromatography (silica gel G, 70/30/1, PE/EE/AA) and gas chromatography (F and M Model 810, 15% ethyleneglycol succinate on Gas-Chrom P). The workup products for [3-14C] 2-trans 9,12-eicosatrienoyl-CoA reduction would be 1, methyl [3-14C] 2-trans 9,12-eicosatrienoate, 2, methyl [3-14C] Δ3,9,12-eicosatrienoate, 3, methyl [3-14C] 9,12-eicosadienoate, and 4, methyl [3-14C] β-hydroxy 9,12-eicosadienoate. Separation of 4 from 1, 2, and 3 was afforded by TLC and separation of 1, 2, and 3 was afforded by GLC (see page 64).
Figure 6. (continued)
CHAPTER III
MALONYL-CoA DEPENDENT CHAIN ELONGATION IN MICROSOMES

The substrate specificity and rates of conversion for an isomeric series of octadecadienoic acids and selected 3-hydroxy and 2-trans analogues

RESULTS

Activation to the Coenzyme A derivative

In that the CoA derivative of each octadecadienoic acid isomer used in these chain elongation studies were not chemically synthesized, it was necessary to determine whether each isomer would be activated in vitro to its CoA form at a rate sufficient for the subsequent chain elongation reaction. Initial investigations with [1-\(^{14}\)C] palmitic acid and [1-\(^{14}\)C] palmitoyl-CoA indicated that 99.0% of the radioactivity for [1-\(^{14}\)C] palmitic acid and 0.7% of the radioactivity for [1-\(^{14}\)C] palmitoyl-CoA was found in the heptane fraction for zero time incubations. Thus it was assumed that the substrate octadecadienoic acid and product octadecadienoyl-CoA would be properly distributed in the heptane and aqueous fractions during the activation studies.

Figure 7 shows the profile of activation for the 4,7- through 11,14-octadecadienoic acid isomeric series used in this study. Time and protein curves for 6,9-octadecadienoic acid activation indicated that linear conditions were used for the determination of this profile.
Figure 7. Rates of activation to the CoA derivative for an isomeric series of [1-\(^{14}\)C] octadecadienoic acids as determined with the standard incubation conditions.
Rates varied between 76.9 nmoles/min.mg microsomal protein for 10,13-18:2 activation to 136.7 nmoles/min.mg microsomal protein for 4,7-18:2 activation. The rate of 7,10-18:2 activation was also measured under typical incubation conditions for chain elongation, i.e. 5 mg protein at 3 min. Although the reaction did not remain linear with respect to time at these higher protein concentrations, essentially all 150 nmoles of the substrate were activated. At 0.25 min 71.4 nmoles were activated suggesting that sufficient fatty acyl-CoA would be synthesized for the subsequent chain elongation reactions, whose rates seldom exceed 5.0 nmoles/min.mg microsomal protein.

Microsomal chain elongation

Figure 8 clearly indicates that a sharp substrate specificity exists for the chain elongating enzyme system in rat liver microsomes. Of the eight octadecadienoic acids used to measure chain elongation, only the naturally occurring 6,9-18:2 and unnatural 7,10-18:2 were chain elongated at any appreciable rate. These rates were 2.89 nmoles/min.mg and 5.20 nmoles/min.mg respectively. All other acids, excluding 10,13-18:2, were chain elongated at negligible rates as indicated in Table 5.

To ascertain if the conditions used to determine the octadecadienoic acid profile (3 min, 5 mg microsomal protein, 0.2 mM malonyl-CoA, 1.33 mM NADPH, and 0.1 mM fatty acid K+/albumin) were indeed linear with respect to time and protein concentration, and saturating with respect to malonyl-CoA, NADPH, and fatty acid, these variables were analyzed with 7,10-18:2 as the substrate. Figures 9 and 10 indicate that the
Figure 8. Rates of chain elongation (◦) and condensation (x) for an isomeric series of [1-14C] octadecadienoic acids as determined with the standard incubation conditions.
Figure 9. Time dependent rates of chain elongation for [1-\(^{14}\)C] 7,10-octadecadienoic acid as performed with the standard incubation conditions with 5.0 mg microsomal protein; K+/albumin complex (•) and CoA derivative/albumin complex (x).
Figure 10. Protein dependent rate of chain elongation for [1-$^{14}$C] 7,10-octadecadienoic acid as performed with the standard incubation conditions for 3.0 min using the K$^+$/albumin complex.
Figure 11. Kinetics of [1-^{14}C] 7,10-octadecadienoic acid chain elongation as a function of the malonyl-CoA (•) and NADPH concentrations (o) as performed with the standard incubation conditions.
Figure 12. Kinetics of 7,10-octadecadienoic acid chain elongation as a function of the [1-14C] 7,10-18:2-CoA concentration and performed with the standard incubation conditions at 1 min with 5 mg microsomal protein.
chain elongation of 7,10-18:2 is linear with respect to time and protein concentration, and Figure 11 indicates that the concentrations of malonyl-CoA and NADPH used were indeed saturating for this reaction.

Although the initial activation studies (Figure 7) indicated that sufficient octadecadienoyl-CoA would be synthesized in vitro to eliminate the possibility that this step could be rate limiting for the subsequent chain elongation reaction, the coenzyme A derivative of [1-\(^{14}\)C] 7,10-18:2 was nonetheless chemically synthesized to determine if indeed this was true. Chain elongation of 7,10-octadecadienoyl-CoA with respect to time appears in Figure 9 and gives a rate identical to that of the free acid.

The v/s curves for the chain elongation of 7,10-octadecadienoyl-CoA is given in Figure 12. The substrate is clearly saturating at the conditions used for the assay of chain elongation and in fact suggests substrate inhibition occurs at the higher concentrations. A double reciprocal plot indeed confirms the substrate inhibition (Figure 12), but due to apparent deviations in Michaelis-Menten behavior, a \(K_m\) and \(V_{\text{max}}\) could only be estimated at 143.0 \(\mu\)M and 32.3 nmoles/min·mg microsomal protein, respectively.

It should be noted that despite involved analytical procedures, the linearity of both the time and protein curves indicates that the methods used in measuring chain elongation are indeed representative of the actual in vitro rates.

The condensation reaction

In that the 7,10-18:2 was the most rapidly chain elongated fatty acid of the octadecadienoic acid series and preliminary investigations
Figure 13. Time dependent rates of condensation for [1-\(^{14}\text{C}\)] 7,10-octadecadienoic acid performed with the standard incubation conditions and 5.0 mg microsomal protein using the K\(^+\)/albumin complex.
Figure 14. Protein dependent rates of condensation for [1-\(^{14}\)C] 7,10-octadecadienoic acid performed with the standard incubation conditions for 2.0 min using the K\(^+\)/albumin complex.
Figure 15. Kinetics of 7,10-octadecadienoic acid condensation as a function of the [1-14C] 7,10-18:2-CoA concentration and performed with the standard incubation conditions at 1 min with 2.5 mg microsomal protein.
in our laboratory with 16:2 and 6,9-18:2 condensation suggested that the conditions used for chain elongation would not be kinetically justifiable for the measurement of the 8-keto fatty acyl-CoA reaction, the proper conditions for the measurement of this initial step were established using the 7,10- isomer.

The time and protein curves for the condensation of malonyl-CoA with 7,10-18:2 appear in Figures 13 and 14, and show that the conditions previously used for chain elongation, i.e. 5 mg protein and 3 min, would not have produced linearity for this enzymatic step. All subsequent condensation reactions were assayed at 1 min with 2.5 mg protein. Figure 15 represents the substrate saturation curve for 7,10-18:2 condensation, but due to an apparent deviation in normal Michaelis-Menton behavior, a Km and Vmax could only be estimated at 200 μM and 40 nmoles/min·mg, respectively. A similarity to the values obtained for 7,10-18:2 chain elongation is noted.

With the optimal conditions for condensation determined with 7,10-18:2, the rates of condensation for the isomeric series of octa-decadienoic acids were measured. From Figure 8 it can clearly be seen that both the substrate specificity and absolute rates for condensation are identical to those of chain elongation for the isomeric series.

**The dehydration reaction**

Optimal conditions for the 8-hydroxy fatty acyl-CoA dehydrase reaction were determined using [3-14C] 8-hydroxy-9,12-eicosadienoyl-CoA. Initial in vitro activation studies with 8-hydroxy-9,12-eicosadienoic acid suggested that the free acid might not be activated to the CoA
Figure 16. Time dependent rates of dehydration (■) and dehydration plus reduction (x) for [3-14C] 8-hydroxy-9,12-eicosa-dienoyl-CoA as performed with the standard incubation conditions and 0.5 mg microsomal protein.
Figure 17. Protein dependent rates of dehydration (o) and dehydration plus reduction (△) for [3-\textsuperscript{14}C] ß-hydroxy-9,12-eicosadienoyl-CoA as performed with the standard incubation conditions for 3 min.
Figure 18. Kinetics of $[3^{-14}C]$ 8-hydroxy-9,12-eicosadienoyl-CoA dehydration and reduction as a function of the NADPH concentration and performed with the standard incubation conditions; 2-trans 9,12-eicosatrienoic acid (□), 9,12-eicosadienoic acid (●), and total converted product (○).
Figure 19. Kinetics of \([3-^{14}C] 3\)-hydroxy-9,12-eicosadienoyl-CoA dehydration and reduction as a function of the substrate concentration and performed with the standard incubation conditions at 1 min with 0.23 mg microsomal protein.
derivative by fatty acyl-CoA synthetase at a rate sufficient for the subsequent dehydration step and thus necessitated the synthesis of the individual CoA derivatives of [3-14C] β-hydroxy-7,10-20:2, [3-14C] β-hydroxy-9,12-20:2, and [3-14C] β-hydroxy-10,13-20:2 as previously described.

The time and protein curves for the conversion of [3-14C] β-hydroxy-9,12-20:2-CoA in the presence and absence of NADPH appears in Figures 16 and 17. Without NADPH the β-hydroxy fatty acid is converted exclusively to the 2-trans-9,12-eicosatrienoic acid intermediate, while with increasing concentrations of NADPH the β-hydroxy fatty acid is converted predominately to the reduced 9,12-eicosadienoic acid, as indicated in Figure 18. The v/s curve for the dehydration of [3-14C] β-hydroxy-9,12-eicosadienoyl-CoA to [3-14C] 9,12-eicosadienoic acid is given in Figure 19 and gives the kinetic parameters of 95.7 nmoles/min·mg for the Vmax and 10.7 μM for the Km when plotted in a double reciprocal manner. With the optimal conditions for protein, time, and substrate concentration established, the individual rates for the three different β-hydroxy eicosadienoyl-CoA's were determined and appear in Table 5. They are 32.0 nmoles/min·mg for the β-hydroxy-7,10- isomer, 41.7 nmoles/min·mg for the β-hydroxy-9,12- isomer, and 64.3 nmoles/min·mg for the β-hydroxy-10,13- isomer of eicosadienoic acid.

The reduction reaction

Time, protein, and NADPH concentration curves appear in Figures 20, 21, and 22, respectively, for 2-trans-9,12-20:3-CoA reduction. In the absence of exogenous NADPH no reduction occurred, the product being
Figure 20. Time dependent rates of reduction for $[3^{14}C] 2$-trans-$9,12$-eicosatrienoyl-CoA as performed with the standard incubation conditions and $0.25\ mg$ microsomal protein.
Figure 21. Protein dependent rates of reduction for $[3^{-14}C]$ 2-trans-9,12-eicosatrienoyl-CoA as performed with the standard incubation conditions for 2.0 min.
Figure 22. Kinetics of $[3^{-14}C]$ 2-trans-9,12-eicosatrienoyl-CoA reduction as a function of the NADPH concentration as performed with the standard incubation conditions; 9,12-eicosadienoic acid (○) and 3-hydroxy-9,12-eicosadienoic acid (●).
Figure 23. Time dependent rates of hydration for [3-14C] 2-trans-9,12-eicosatrienoyl-CoA with (x) and without (○) NADPH as performed with the standard incubation conditions,
Figure 24. Time dependent rates of hydration for $[3-^{14}C]$ 2-trans-9,12-eicosatrienoyl-CoA with NADPH (x), with NADPH plus NADP (.), with NADP (o), and without NADPH and NADP (O) as performed with the standard incubation conditions.
Figure 25. Kinetics of [3-$^{14}$C] 2-trans-9,12-eicosatrienoyl-CoA reduction as a function of the substrate concentration and performed with the standard incubation conditions at 1 min with 0.23 mg microsomal protein.
Table 5
Rates$^a$ of chain elongation, condensation, dehydration, and reduction for an isomeric series of octadecadienoic acids and associated $\beta$-hydroxy and 2-trans intermediates

<table>
<thead>
<tr>
<th>Parent 18:2 Acid</th>
<th>4,7-</th>
<th>5,8-</th>
<th>6,9-</th>
<th>7,10-</th>
<th>8,11-</th>
<th>9,12-</th>
<th>10,13-</th>
<th>11,13-</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chain Elongation</strong></td>
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<tr>
<td>0.28±0.02</td>
<td>0.48±0.02</td>
<td>2.89±0.27</td>
<td>5.20±0.11</td>
<td>0.57±0.10</td>
<td>0.50±0.03</td>
<td>0.84±0.03</td>
<td>0.24±0.02</td>
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<tr>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(12)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
<td><strong>Condensation</strong></td>
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<tr>
<td>0.74±0.03</td>
<td>0.57±0.02</td>
<td>2.79±0.10</td>
<td>4.30±0.17</td>
<td>0.42±0.02</td>
<td>0.47±0.02</td>
<td>0.68±0.02</td>
<td>0.30±0.01</td>
<td></td>
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<tr>
<td>(2)</td>
<td>(2)</td>
<td>(2)</td>
<td>(8)</td>
<td>(2)</td>
<td>(2)</td>
<td>(2)</td>
<td>(2)</td>
<td></td>
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<tr>
<td><strong>Dehydration</strong></td>
<td></td>
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<tr>
<td>32.0±2.6</td>
<td>41.7±3.7</td>
<td>64.3±3.4</td>
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<tr>
<td>(7)</td>
<td>(9)</td>
<td>(7)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Reduction</strong></td>
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<td></td>
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<tr>
<td>48.7±3.9</td>
<td>87.4±3.6</td>
<td>52.5±2.9</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>(8)</td>
<td>(5)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

$^a$All rates expressed as nmoles product formed min$^{-1}$ (mg microsomal protein)$^{-1}$ ± SE. The number of determinations for each rate appears in the parentheses.
exclusively the hydration adduct, β-hydroxy-9,12-20:2. The reverse reaction still occurs with the addition of NADPH, and the appearance of the hydration product occurs in an interesting time dependent manner as illustrated in Figure 23. Figure 24 indicates that in the absence of NADPH, only the hydrated product is synthesized with essentially no 9,12-20:2 (1.56 nmoles) being formed at 10 min. With the addition of 2.0 μmoles NADPH, 110.2 nmoles of 9,12-20:2 was produced at 10 min. However, at concentrations of 1.3 mM for both NADPH and NADP, 9,12-20:2 synthesis is sharply curtailed to 68.5 nmoles in 10 min. Although minor amounts of β-keto-9,12-20:2 could be detected when NADPH was excluded and NADP included in the incubation, no 7,10-18:2 was found.

The v/s curve for the reductase reaction measured with 2-trans-9,12-20:3-CoA as substrate appears in Figure 25 and when treated appropriately gives a Km of 18 μM and a Vmax of 137 nmoles/min·mg microsomal protein. The rates of reduction for 2-trans-7,10-20:3-CoA and 2-trans-10,13-20:3-CoA, measured under the linear conditions as determined for 2-trans-9,12-20:3-CoA, are listed in Table 5, and their rates are 48.7 and 52.5 nmoles/min·mg microsomal protein, respectively, as compared to 87.4 nmoles/min·mg microsomal protein for the 2-trans-9,12-20:3-CoA.

**Enzyme levels in rat testicular microsomes**

Table 6 illustrates the absolute rates of chain elongation and condensation as measured with [1-14C] 7,10-18:2-CoA for both rat liver and rat testicular microsomes. The rates for the testicular microsomes are 1.14 and 1.25 nmoles/min·mg microsomal protein, respectively. Although the rates are considerably less for the testicular conversions,
Table 6

Comparison of the rates\(^{a}\) of chain elongation, condensation, dehydration, and reduction for 7,10-octadecadienoic acid and associated \(\beta\)-hydroxy and 2-trans intermediates for rat liver (RLM) and rat testicular (RTM) microsomes

<table>
<thead>
<tr>
<th></th>
<th>chain elongation</th>
<th>condensation</th>
<th>dehydration</th>
<th>reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RLM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate(^{a})</td>
<td>5.21±0.11 (12)</td>
<td>4.31±0.17 (8)</td>
<td>41.7±3.7 (9)</td>
<td>87.4±3.6 (8)</td>
</tr>
<tr>
<td>ratio of activity to chain elongation</td>
<td>1.00</td>
<td>0.83</td>
<td>8.02</td>
<td>16.80</td>
</tr>
<tr>
<td><strong>RTM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate(^{a})</td>
<td>1.14±0.07 (7)</td>
<td>1.25±0.08 (4)</td>
<td>23.7±0.35 (8)</td>
<td>9.02±0.16 (11)</td>
</tr>
<tr>
<td>ratio of activity to chain elongation</td>
<td>1.00</td>
<td>1.10</td>
<td>20.8</td>
<td>7.90</td>
</tr>
<tr>
<td>ratio liver to testicular activity</td>
<td>--</td>
<td>3.45</td>
<td>1.86</td>
<td>9.36</td>
</tr>
</tbody>
</table>

\(^{a}\)All rates are expressed as nmoles product formed min\(^{-1}\) (mg microsomal protein)\(^{-1}\) ± SE. The number of determinations for each rate appears in the parentheses.
an approximate equality between the two values is again observed. The low level of fatty acyl-CoA synthetase necessitated the use of the CoA derivatives. In addition, the rate of dehydration for [3-\(^{14}\)C] \(\beta\)-hydroxy-9,12-20:2-CoA and the rate of reduction for [3-\(^{14}\)C] 2-trans-9,12-20:3-CoA were also measured using rat testicular microsomes, and are 23.7 and 9.02 nmoles/min·mg microsomal protein, respectively. These rates, as well as the ratios of activity to overall chain elongation, appear in Table 6 along with the comparable conversions as measured with rat liver microsomes. The rate of dehydration for the testicular microsomes is approximately one half that of the liver microsomes, and for reduction, one tenth. However, when the enzymatic rates are compared to chain elongation, the resulting ratios indicate that the dehydrase is considerably more active and that the reductase considerably less active in rat testicular microsomes as compared to rat liver microsomes. The activities of the individual enzymatic conversions for testicular microsomes were also directly compared to the same conversions for liver microsomes (Table 6). The ratios indicate that the activity of the condensing enzyme is 3.45 times greater in liver than in testicles, only 1.86 times greater for the dehydration step, and 9.36 times greater for the reduction step.

**DISCUSSION**

These studies were initiated to elucidate the nature of the enzyme systems responsible for the chain elongation of polyunsaturated fatty acids in reference to substrate specificity of the initial condensation step, the rate limiting nature of any of the enzymatic conversions, and
the substrate specificity of the intermediary conversions. Experiments were also performed to determine if the enzymes involved in chain elongation might be of a multifunctional nature.

Previously published results suggested that the microsomal enzyme responsible for fatty acid activation to fatty acyl-CoA's, i.e., fatty acyl-CoA synthetase, shows a low degree of substrate specificity and a high rate of synthesis relative to known microsomal chain elongation rates (88-92). However, because extensive conclusions were to be drawn from enzymatic rates, the rates of activation of the isomeric octadecadienoic acid series were determined using linear conditions. Although all 18:2 acids were not activated at exactly the same rate, all isomers were converted at rates which greatly exceeded known or anticipated rates of chain elongation. Using protein concentrations identical to that of chain elongation, 71.4 nmoles of 7,10-18:2 was activated in 0.25 min suggesting adequate in vitro synthesis of the fatty acyl-CoA's. Thus it was assumed that the synthesis of the individual CoA derivatives of each 13:2 isomer would not be necessary and that activation would have little or no effect on the subsequent chain elongation rates to be measured. This was shown to be the situation with 7,10-18:2 chain elongation where both the free acid and the CoA derivative had identical rates. As this acid was the most rapidly chain elongated, the unavailability of the immediate substrate for chain elongation, i.e. fatty acyl-CoA, would be most critical for this isomer as compared to the others which were chain elongated at lesser rates.

In contrast to the high ratio of activation to chain elongation, in vitro synthesis of β-hydroxy and 2-trans fatty acyl-CoA's from their
free acids was initially shown to be insufficient for the subsequent dehydration and reduction steps. This necessitated the synthesis of the CoA derivatives of all intermediates tested in order to obtain linear rates of conversion.

The chain elongation profile for the eight octadecadienoic acids used in this study clearly shows a sharp specificity for only two isomers, the 6,9-18:2 and 7,10-18:2. Although chain elongation rates for 6,9-18:2 were known for animals maintained on a fat free diet (51), it helped to serve as an internal standard to insure that the incubation and workup conditions were functioning. The interesting features of the profile are 1, the fact that only two isomers are chain elongated, the other six apparently being very poorly converted by the chain elongating enzyme; 2, despite the hypothesis of Seubert and Podack (52) predicting preference in chain elongation for fatty acids with their first double bond in the 6- position, that 7,10-18:2 is chain elongated the most rapidly (checked with two independently made acids) in the isomeric series; and 3, that unlike the left hand side of the curve where 6,9-18:2 is chain elongated at a rate approximately one half that of 7,10-18:2, there is no equivalent intermediary rate on the right hand side for 8,11-18:2. It seems to be an "all or none" phenomenon. These aspects of substrate specificity will be discussed in more detail in the second part of this chapter.

The validity of the conditions used to measure chain elongation for the isomeric series was confirmed with the rapidly converted 7,10-18:2. Time, protein, malonyl-CoA, NADPH, and substrate saturation curves
indicated that the measured rates were performed under linear conditions and are thus kinetically comparable. Other studies of not only chain elongation but also desaturation have relied on single point assays which do not always reflect the true rate of conversion.

The development of an assay for the initial reaction of chain elongation, i.e. condensation or β-keto fatty acyl-CoA synthetase, by Nugteren (19), and its subsequent refinement in our laboratory (50), permitted the determination of malonyl-CoA condensation rates with the eight octadecadienoic acids used in this study. Although it was anticipated that condensation rates for both 6,9- and 7,10-18:2 would be similar to their rates of chain elongation, it was important to confirm not only the equivalency of these two rates, but also show that just as the other isomers were not chain elongated, they would also not be enzymatically condensed with malonyl-CoA. If the other isomers were in fact converted to their respective β-keto 20:2 intermediates, then the specificity observed for chain elongation would not reside with the initial condensation step.

As with chain elongation, the conditions necessary to produce linear kinetics were determined, again using 7,10-18:2. The condensation reaction appears to decrease in rate more rapidly with respect to time and protein concentration than chain elongation, suggesting that the synthesized β-keto-9,12-20:2-CoA might be involved in some type of feedback inhibition. Thus when condensation was measured with the same conditions as for chain elongation (i.e., 5 mg protein and 3 min), considerably less β-keto-9,12-20:2 was synthesized as compared to 9,12-20:2. It is only at shorter time periods and with less protein that the rate
approximates that of chain elongation. Again, there is essentially no difference in the rate of condensation when 7,10-18:2-CoA is substituted as the substrate instead of the K⁺ salt.

With the proper conditions for condensation determined with 7,10-18:2, the seven other isomers were tested. A similar profile in respect to rate and substrate specificity was observed, demonstrating that the β-keto fatty acyl-CoA synthetase is responsible for the specificity of chain elongation.

The three β-hydroxy 20:2-CoA's and three 2-trans 20:3-CoA's used to determine dehydrase and reductase activity were chosen from the octadecadienoic acid profile of chain elongation/condensation because they represented the intermediates of chain elongation of the most rapidly converted acid, 7,10-18:2, and two poorly converted acids, 5,8- and 8,11-18:2. Although the β-hydroxy and 2-trans intermediates of palmitic acid and 6,9-18:2 chain elongation, two acids which are chain elongated relatively rapidly, had been tested as substrates for dehydrase and reductase steps (50), no intermediates of non-chain elongated fatty acids had ever been investigated. Thus, dehydration of the three β-hydroxy 20:2-CoA's at approximately equivalent rates and reduction of the three 2-trans 20:3-CoA's at approximately equivalent rates would imply that unlike the initial condensation enzyme, both the dehydrase and reductase are very nonspecific. This situation would imply that all β-hydroxy and 2-trans intermediates, irrespective of their degree of unsaturation or chain length, would be dehydrated or reduced by a common β-hydroxy fatty acyl-CoA dehydrase and a common α,β-enoyl-CoA reductase. Or, the obviously restrictive binding site of the condensing enzyme is
not present in the dehydrase or reductase. These findings thus suggest that the different chain elongation steps occurring in the polyunsaturated fatty acid pathways would be handled by individual condensing enzymes and common dehydrases and reductases. Once condensation had occurred, the intermediates would be funneled to a common site for dehydration and reduction. It would be assumed that the β-keto fatty acyl-CoA reductase would also be of a common nature. It would also suggest that after condensation, the enzyme might be of a multifunctional nature, rather than individual and discrete. In addition, rates of dehydration and reduction greater than the initial condensation reaction confirms the rate limiting nature of the initial reaction. It is also interesting to note that with the exception of the final reduction step in 8,11-18:2 chain elongation, all the individual reactions measured in the chain elongation of 5,8-, 7,10-, and 8,11-18:2 have rates greater than the preceding steps, thus insuring completion of the reaction sequences.

Dehydrase activity was measured with [3-14C] β-hydroxy-9,12-20:2-CoA both with and without NADPH. In the presence of NADPH, the 2-trans-9,12-20:3 produced by the β-hydroxy fatty acyl-CoA dehydrase is reduced to 9,12-20:2. Obvious increases in the rate of dehydration were observed when 1.3 mM NADPH was included. The rate changes suggest that without NADPH present, the synthesized 2-trans-9,12-20:3 is involved in feedback inhibition, or that the dehydrase/reductase enzymes are controlled by the NADPH concentration. Higher levels in the concentration of NADPH increases the appearance of 9,12-20:2 from 2-trans-9,12-20:3. Without NADPH, essentially no 2-trans-9,12-20:3 is reduced and a plateau
in reduction occurs at 1.3 mM NADPH. The presence of 2-trans-9,12-20:3 at the higher concentrations is probably a reflection of the lag in the enzymatic conversions of the two step sequence.

An interesting observation was made when the time dependent hydration of [3-14C] 2-trans-9,12-20:3-CoA was measured. When NADPH was excluded from the incubation, a typical increase in β-hydroxy-9,12-20:2 was found. However, addition of NADPH to allow for the reduction of 2-trans-9,12-20:3 did not prevent the initial rapid increase in hydration, but did prevent its sustained synthesis. This situation could occur either from the dehydration of the synthesized β-hydroxy-9,12-20:2 back to the 2-trans-9,12-20:3 and eventual reduction to 9,12-20:2, or from the oxidation of the synthesized β-hydroxy-9,12-20:2 to β-keto-9,12-20:2 with perhaps eventual thiolytic cleavage to give 7,10-18:2. The oxidation of the β-hydroxy-9,12-20:2 could possibly occur by the cycling of oxidized NADPH from the enoyl-CoA reductase reaction. To determine if in fact oxidation of the β-hydroxy-9,12-20:2 was indeed occurring, incubations were also performed in which NADP was included. However, the presence of NADP did not promote the disappearance of the β-hydroxy-9,12-20:2, and although minor amounts of β-keto-9,12-20:2 could be detected, no 7,12-18:2 was found. If both NADPH and NADP were included, their combined presence had a mediating effect on the sustained hydration and suggests that the reactions between the β-hydroxy stage and final chain elongated fatty acid might be controlled by subtle changes in the levels of NADPH and NADP,
The following evidence suggested that the enzymes responsible for the conversions after the initial condensation reaction might be of a multifunctional nature, such as for liver fatty acid synthetase (120): 1, a limited co-purification of chain elongation activity and α,β-enoyl-CoA reductase activity from adrenal cortex microsomes (52); 2, previous feeding studies had shown that the level of the condensing enzyme could be manipulated by diet although the level of the dehydrase and reductase remained constant to each other (50); and 3, the lack of specificity in respect to the position of double bonds for both the dehydrase and reductase, as established by these studies. Thus, if the enzymes co-purified together, the dehydrase and reductase ratio remained constant during dietary manipulations, and unlike the condensing enzyme, the dehydrase and reductase lacked any degree of specificity, then perhaps the β-keto fatty acyl-CoA reductase, β-hydroxy fatty acyl-CoA dehydrase, and α,β-enoyl-CoA reductase are structurally incorporated into a single polypeptide chain which has multicatalytic sites for the different enzymatic conversions. In that the standard methods of demonstrating a single polypeptide chain with multicatalytic sites, i.e., single point mutations or co-purification to constant enzymatic ratios (121), could not be used with the enzymes responsible for chain elongation, a different approach was used. This involved using a different tissue having chain elongation activity, e.g., rat testicular microsomes. The ratio of dehydration to chain elongation is considerably greater in rat testicular microsomes as compared to rat liver microsomes, and the ratio of reduction to chain elongation is considerably lower, implying a nonmultifunctional nature
of the two enzymes. The possibility also existed that a single poly- peptide chain might contain only two active sites, rather than all of the active sites. Such would be the case if the condensing enzyme and perhaps the dehydrase or reductase were part of the same molecule. However, examination of the ratio of rat liver activity to rat testicular activity indicates that this is not the situation, since none of the values are identical. Admittedly this rather limited study lacks the comprehensiveness necessary to prove the individuality of the different enzymes. This would be possible with the use of other microsomal sources showing chain elongation activity and the use of the β-keto intermediate, which unfortunately is difficult to synthesize. It does, nonetheless, provide initial evidence suggesting that the enzymes are individual and discrete,
The substrate specificity and proposed binding site
of microsomal chain elongation

RESULTS

In an attempt to further elucidate what structural features of the fatty acid molecule are necessary for recognition by the chain elongating enzyme, various incubations were performed with rat liver microsomes and fatty acids whose acyl moieties were altered in respect to chain length, the number of double bonds, and the position of those double bonds. This was done in both a systematic approach to initially define what elementary structural features were necessary for chain elongation and in a limited approach on selected fatty acids containing structural features of presumed interest.

It should be emphasized that all conversions were measured under linear conditions with respect to time and protein, and are thus directly comparable to each other. This approach was taken in preference to single point assays at an extended incubation period. The later suffers from the possibility that a preferred substrate may be converted very quickly but whose total conversion is similar to a substrate which is converted very slowly but continuously until the incubation is terminated.

Figure 8 shows the chain elongation profile of the eight octadecadienoic acids having the naturally occurring allyl rhythm of double bonds, and clearly shows that a rather restricted substrate specificity exists for the enzyme. Only the two isomers, 6,9-18:2 (2.89 nmoles/min·mg) and 7,10-18:2 (5.20 nmoles/min·mg) are converted to any appreciable degree. All other acids, excluding 10,13-18:2, are chain
elongated at rates less than 0.5 nmoles/min·mg, while the 10,13- isomer is chain elongated at 0.84 nmoles/min·mg.

In Figure 26 a similar chain elongation profile for an isomeric series of octadecatrienoic acids is observed. Again the preferred substrate contains the 7,10- sequencing of double bonds, with the 7,10,13-18:3 being converted at 4.70 nmoles/min·mg. The single major difference between the dienoic and trienoic fatty acid profile is that 5,8,11-18:3 (2.83 nmoles/min·mg) is apparently rapidly converted, while its homologue (5,8-18:2) with one less double bond, is converted at a negligible rate. Although the 4,7,10-18:3 shows a slight increase in the ability to be chain elongated, as compared to 4,7-18:2, both 8,11,14- and 9,12,15-18:3 are converted very slowly just as their dienoic acid homologues are.

The effect of altering the chain length while maintaining the preferred 7,10- sequencing was also investigated. Figure 27 indicates that there is an increase in the ability of the fatty acid to be chain elongated with an increase in the number of methylene groups until C18 is reached. After 18 carbons a 94.6% decrease in rate is observed, with the 7,10-20:2 being chain elongated at only 0.28 nmoles/min·mg. Coincidentally with the octadecadienoic acid profile, it is 7,10-18:2 which has the fastest rate. Table 7 also illustrates a 76.2% decrease in rate when 5,8,11-18:3 is extended distally by two carbons, and a 85.1% decrease when 7,10,13-18:3 is also extended by two carbons.

In that recognition of the fatty acid substrate by the chain elongating enzyme might occur from the terminal methyl end, a number of acids were paired to test this hypothesis. Table 8 lists the grouped
Figure 26. Rates of chain elongation for an isomeric series of [1-\(^{14}\)C] octadecatrienoic acids as determined with the standard incubation conditions.
Figure 27. Rates of chain elongation for $[1^{-14}C]$ 7,10- fatty acids of increasing chain length as determined with the standard incubation conditions.
Table 7
Comparative rates of chain elongation for fatty acids with identical proximal and dissimilar distal moieties

<table>
<thead>
<tr>
<th>fatty acid</th>
<th>rate(a)</th>
<th>% parent acid(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7,10-18:2</td>
<td>5.20±0.11 (12)</td>
<td>100.0</td>
</tr>
<tr>
<td>7,10-20:2</td>
<td>0.28±0.03 (2)</td>
<td>5.4</td>
</tr>
<tr>
<td>5,8,11-18:3</td>
<td>2.84±0.19 (4)</td>
<td>100.0</td>
</tr>
<tr>
<td>5,8,11-20:3</td>
<td>0.92±0.04 (4)</td>
<td>32.8</td>
</tr>
<tr>
<td>7,10,13-18:3</td>
<td>4.70±0.30 (6)</td>
<td>100.0</td>
</tr>
<tr>
<td>7,10,13-20:3</td>
<td>0.70±0.05 (5)</td>
<td>14.9</td>
</tr>
</tbody>
</table>

\(a\) All rates are expressed as nmoles product formed min\(^{-1}\) (mg microsomal protein\(^{-1}\)) ± SE. The number of determinations for each value appears in the parentheses.

\(b\) Percent rate of parent acid.
Table 8
Comparative rates of chain elongation for fatty acids with identical distal and dissimilar proximal moieties

<table>
<thead>
<tr>
<th>fatty acid</th>
<th>rate(^{a})</th>
<th>% parent acid(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-18:1 (ω9)</td>
<td>0.16 ± 0.01 (5)</td>
<td>5.2</td>
</tr>
<tr>
<td>6,9-18:2 (ω9)</td>
<td>2.89 ± 0.27 (4)</td>
<td>100.0</td>
</tr>
<tr>
<td>5,8,11-20:3 (ω9)</td>
<td>0.92 ± 0.04 (4)</td>
<td>32.2</td>
</tr>
<tr>
<td>6,9-18:2 (ω9)</td>
<td>2.89 ± 0.27 (4)</td>
<td>100.0</td>
</tr>
<tr>
<td>5,8-16:2 (ω8)</td>
<td>1.27 ± 0.04 (3)</td>
<td>24.2</td>
</tr>
<tr>
<td>7,10-18:2 (ω8)</td>
<td>5.20 ± 0.11 (12)</td>
<td>100.0</td>
</tr>
<tr>
<td>7,10,13-20:3 (ω7)</td>
<td>0.70 ± 0.05 (5)</td>
<td>24.6</td>
</tr>
<tr>
<td>5,8,11-18:3 (ω7)</td>
<td>2.84 ± 0.19 (4)</td>
<td>100.0</td>
</tr>
<tr>
<td>9,12-18:2 (ω6)</td>
<td>0.50 ± 0.03 (4)</td>
<td>13.2</td>
</tr>
<tr>
<td>6,9,12-18:3 (ω6)</td>
<td>3.80 ± 0.25 (3)</td>
<td>100.0</td>
</tr>
<tr>
<td>10,13-18:2 (ω5)</td>
<td>0.84 ± 0.08 (4)</td>
<td>17.9</td>
</tr>
<tr>
<td>7,10,13-19:3 (ω5)</td>
<td>4.70 ± 0.30 (6)</td>
<td>100.0</td>
</tr>
</tbody>
</table>

\(^{a}\)All rates are expressed as nmoles product formed min\(^{-1}\) (mg microsomal protein\(^{-1}\)) ± SE. The number of determinations for each value appears in the parentheses.

\(^{b}\)Percent rate of parent acid.
Table 9

Comparative rates of chain elongation for fatty acids as a function of double bonds and position

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Rate $^{a}$</th>
<th>% Parent Acid $^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Section I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-18:1</td>
<td>0.16 ± 0.01 (4)</td>
<td>5.2</td>
</tr>
<tr>
<td>6,9-18:2</td>
<td>2.89 ± 0.27 (4)</td>
<td>100.0</td>
</tr>
<tr>
<td>7-18:1</td>
<td>0.12 ± 0.01 (4)</td>
<td>2.1</td>
</tr>
<tr>
<td>10,13-18:2</td>
<td>0.84 ± 0.08 (4)</td>
<td>16.2</td>
</tr>
<tr>
<td>7,10-18:2</td>
<td>5.20 ± 0.11 (12)</td>
<td>100.0</td>
</tr>
<tr>
<td><strong>Section II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10,13-18:2</td>
<td>0.84 ± 0.08 (4)</td>
<td>17.0</td>
</tr>
<tr>
<td>7,10-18:2</td>
<td>5.20 ± 0.11 (12)</td>
<td>116.4</td>
</tr>
<tr>
<td>7,10,13-18:3</td>
<td>4.70 ± 0.30 (6)</td>
<td>100.0</td>
</tr>
<tr>
<td><strong>Section III</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9,12-18:2</td>
<td>0.50 ± 0.03 (4)</td>
<td>13.2</td>
</tr>
<tr>
<td>6,9-18:2</td>
<td>2.89 ± 0.27 (4)</td>
<td>76.1</td>
</tr>
<tr>
<td>6,9,12-18:3</td>
<td>3.80 ± 0.25 (3)</td>
<td>100.0</td>
</tr>
<tr>
<td><strong>Section IV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8,11-18:2</td>
<td>0.57 ± 0.10 (4)</td>
<td>20.1</td>
</tr>
<tr>
<td>5,8-18:2</td>
<td>0.48 ± 0.02 (4)</td>
<td>17.2</td>
</tr>
<tr>
<td>5,8,11-18:3</td>
<td>2.84 ± 0.19 (4)</td>
<td>100.0</td>
</tr>
</tbody>
</table>

$^{a}$All rates are expressed as nmoles product formed min$^{-1}$ (mg microsomal protein)$^{-1}$ ± SE. The number of determinations for each value appears in the parentheses.

$^{b}$Percent rate of parent acid.
acids according to the omega (ω) designation, with the parent fatty acid being expressed as 100%. In all cases the paired fatty acid has a rate considerably less than the parent fatty acid. The pairing of 5,8-16:2 (ω8) with 7,10-18:2 (ω8), and 7,10,13-20:3 (ω7) with 5,8,11-20:3 (ω7), is perhaps most illustrative, since both pairs have the same number of double bonds.

The relationship between the number of double bonds, their positions, and the ability of the fatty acid substrate to be chain elongated is illustrated in Table 9. In Section I, 9-18:1 is chain elongated at only 5.2% of the rate of its parent acid, 6,9-18:2. Also, 7-18:1 (2.1%) and 10,13-18:2 (16.2%) are also converted at rates considerably less than their parent fatty acid, 7,10-18:2. Section II indicates the same relationship with the two trienoic acids, 7,10,13-18:3 and 6,9,12-18:3, as parent fatty acids. Neither 10,13-18:2 (17.0%) or 9,12-18:2 (13.2%) are converted to any appreciable degree, although 7,10-18:2 (116.4%) and 6,9-18:2 (76.1%) are. The exception appears with 5,8,11-18:3 (100.0%), where 8,11-18:2 (20.1%) and 5,8-18:2 (17.2%) are chain elongated at a rate much slower than the parent octadecatrienoic acid.

DISCUSSION

Prior to these studies no systematic approach to determine the substrate specificity of the microsomal chain elongating enzyme of rat liver had been undertaken. Although Nugteren (19) did investigate the changes in rate which accompany the lengthening of the saturated fatty acid substrate from hexanoic to octadecanoic acid, and the rate of conversion of a few selected unsaturated fatty acids, the particular
analytical method that was used does not necessarily reflect the absolute rate. An example would be the chain elongation of 9,12-18:2 and 6,9,12-18:3. The former is depicted as being converted at 0.2 nmoles/min·mg and the later at 2.5 times that rate. However, from these studies with a continual adherence to linear kinetics, 9,12-18:2 was converted at 0.50 nmoles/min·mg and 6,9,12-18:3 at 3.80 nmoles/min·mg, or 7.6 times faster. It is also difficult to determine from Nugteren's studies (19) whether the fatty acids that were chain elongated are converted by an enzyme showing broad specificity, i.e., both saturated and unsaturated fatty acids, or by two or more enzymes.

Sprecher (50) and Bernert and Sprecher (51) were able to further define the relationship between the number of chain elongating enzymes using the two substrates palmitic acid and 6,9-18:2. From feeding and N-ethylmaleimide inhibition studies, they were able to speculate that rat liver microsomes contained a chain elongating system for saturated fatty acids, specifically palmitic acid since no other saturated fatty acids were investigated, and for unsaturated fatty acids, In the later system only 6,9-18:2 was investigated, although from other studies (23), a few selected polyunsaturated fatty acids had also been used to determine their ability to be chain elongated. However, these studies only explored the relationship between the amount of a particular acid found in vivo, and its ability to be either chain elongated and or desaturated in vitro. Other than these two studies, a paucity of substrates have been used to establish the substrate specificity for the chain elongating enzyme,
Figure 8 clearly illustrates the rather limited ability of rat liver microsomes to chain elongate an isomeric series of octadecadienoic acids. Only 6,9- and 7,10-18:2 are converted at any appreciable rate. It is interesting to note that the profile is nonsymmetrical, since 8,11-18:2 does not have an intermediary rate such as 6,9-18:2 does. A slight increase in rate over the apparent base line rate of approximately 0.3 to 0.5 mmols/min·mg is observed for 10,13-18:2. Both of these anomalies will be speculated upon in a later section. The importance in this profile lies in the fact that rat liver microsomes have now been shown to chain elongate only a limited number of dienoic acids and that the others are apparently nonacceptable to the enzyme. The low, but nonetheless real ability of the poorly converted acids to be chain elongated will also be discussed in a later section. Although the 2,5-, 3,6-, 12,15-, 13,16-, and 14,17- isomers were not synthesized and used to complete the full series of octadecadienoic acids, it would be assumed from their isomeric analogues that they too would not be chain elongated. Unlike the two methyl isomer studies of Brett et al., (38) and Do and Sprecher (42) with the Δ9 and Δ5 desaturases where movement of the methyl group away from the site of desaturation on the fatty acyl chain permitted normal or near normal rates of desaturase activity, the opposite effect has occurred with chain elongation. Only acids with specific double bonds near the center of the fatty acyl chain have allowed conversion to occur. Thus these additional isomers would obviously not have been necessary.

A similar profile for the chain elongation of trienoic acids, as for the dienoic acid series, is observed in that maximum chain
I elongation occurred when the first double bond was in the 7- position. The differences between the dienoic acid profile and the trienoic acid profile lie in the fact that the rate for 6,9,12-18:3 is slightly above that for 6,9-18:2 and that the rate for 7,10,13-18:3 is slightly below that for 7,10-18:2. Also, a dramatic increase in rate is observed for 5,8,11-18:3 as compared to 5,8-18:2. Even 4,7,10-18:3 is chain elongated more rapidly than 4,7-18:2. The ability of fatty acids to be chain elongated is abruptly terminated when the first double bond occurs in the 8- position for both the dienes and trienes and is an interesting feature of both profiles.

The influence of altering the chain length of the fatty acid while maintaining the preferred 7,10- double bonds shows a coincidental maximum rate for 7,10-18:2. The shape of the profile is reminiscent of the octadecadienoic/octadecatrienoic isomer profile in that the curve shows a gradual increase in the rate of chain elongation to a maximum followed by a dramatic decrease. Similar decreases in rate in going from an octadecatrienoic acid system to an eicosatrienoic acid system is observed for fatty acids whose double bonds are in the 5,8,11- and 7,10, 13- positions.

Both the octadecadienoic and octadecatrienoic acid profiles plus the chain length profile immediately indicate that the ability of the chain elongating enzyme to bind a particular fatty acid substrate in preparation for eventual condensation with malonyl-CoA is very carefully regulated by well defined regions within the enzyme. It is obviously not an arbitrary event, but rather a highly selective process.
With the elucidation of various structural features necessary for the chain elongation of the fatty acid substrate, i.e., double bonds in either the 6,9- or 7,10- positions with 18 carbons, the possibility existed that recognition by the enzyme for the fatty acid substrate did not necessarily occur from the carboxyl end to the first double bond, but rather from the terminal methyl group to the second double bond. Thus 6,9-18:2 (ω9) and 7,10-18:2 (ω8) would be chain elongated because they had either nine or eight methylene groups after the second double bond, and the ability of a fatty acid to be chain elongated would thus be governed by their omega (ω) classification. This appears not to be the mechanism of recognition in that the fatty acids, paired by their omega number, all show enormous differences in rates. A limiting feature of this comparison is the fact that many of the paired fatty acids do not have the same total number of double bonds. However, since both 5,8-16:2 (ω8) (24.2%) and 7,10,13-20:3 (ω7) (24.6%) have the same number of double bonds as their respective paired parent acids, that is 7,10-18:2 (ω8) (100.0%) and 5,8,11-18:3 (ω7) (100.0%), recognition obviously does not occur because a fatty acid has the proper distance between the terminal methyl group and the second or last double bond. Recognition thus occurs in a fatty acid which has the proper distance between the carboxyl group and the first double bond, i.e., at positions 6- and 7-.

The possibility also existed that 6,9-18:2 and 7,10-18:2 were both chain elongated either because they had a double bond in either the 6- or 7- position and no other double bonds were necessary, or because they
had a double bond in either the 9- or 10- position and no other double bonds were necessary. It was shown that neither the double bond being in the 7- position, e.g., 7-18:1 (2.1%) or in the 9- and 10- positions, e.g., 9-18:1 (5.2%) and 10,13-18:2 (16.2%) is essential. An analogous situation is illustrated with trienoic acids. Only dienoic acids with double bonds in the 6,9- and 7,10- positions will be chain elongated. An exception appears with 5,8,11-18:3 which is chain elongated (2.84 \text{ nmoles/min\cdot mg}), although 5,8-18:2 (0.48 \text{ nmoles/min\cdot mg}) is not. It is suggested that this trienoic acid substrate represents a second binding site specific for fatty acids with three double bonds.

With the further definition of the particular structural features necessary for maximum chain elongation and the realization of the lack of broad specificity, i.e., it seems to be a "go, no go" situation, the low, but nonetheless real rates of the other fatty acids must be recognized. In that what shall now be termed the dienoic acid chain elongating system is so specific, perhaps these other acids are converted by the now recognized saturated fatty acid chain elongating system for palmitic acid (50). Although all of these "no go" substrates (ca. 0.5 \text{ nmoles/min\cdot mg}) would be very dissimilar to the dienoic acid system, perhaps they are all similar enough to each other with their multi double bonds to be chain elongated by this system. So, superimposed on the octadecadienoic acid profile of chain elongation with only two suitable substrates, 6,9- and 7,10-18:2, for the dienoic acid system, is the low baseline rate of chain elongation for 4,7-, 5,8-, 8,11-, 9,12-, and 11,14-18:2's by perhaps the saturated acid system. The only exception to this possibility is 10,13-18:2 which shows a slight increase in rate
over baseline conversions. At this moment it is difficult to explain why this occurred. It is also suggested that the same situation may exist with the trienoic acids tested for their ability to be chain elongated. The 4,7,10-18:3 (0.74 nmoles/min·mg), with a structural similarity to 5,8,11-18:3 and a slightly higher than baseline rate, is perhaps elongated by the proposed trienoic acid system, while 8,11,14- and 9,12,15-18:3's, both of which are unable to fit the requirements of double bonds being in the 6,9- or 7,10- positions, are converted nonetheless by perhaps the saturated fatty acid chain elongating system.

So, superimposed on the octadecatrienoic acid profile of chain elongation are the three systems. The first, the dienoic acid system which chain elongates the 6,9,12- and 7,10,13-18:3's because of their parent 6,9- and 7,10- double bond positioning, the second, the trienoic acid system which chain elongates the 4,7,10- and 5,8,11-18:3's, and the third, the saturated system which chain elongates the 8,11,14- and 9,12,15-18:3's.

These results clearly indicate that the substrate during the chain elongation process must interact specifically with the enzyme and allows one to speculate on the type of binding and configurational nature of the binding site. In this discussion, it is actually the β-keto fatty acyl-CoA synthetase, or more commonly called condensing enzyme, which actually exhibits the observed specificity, since from earlier work it was shown that both the β-hydroxy fatty acyl-CoA dehydrase and α,β-enoyl-CoA reductase, lack any extreme degree of substrate specificity. This is also assumed to be true of the β-keto fatty acyl-CoA reductase. So,
in reference to the substrate specificity studies and binding site configuration, chain elongating enzyme and condensing enzyme will be used interchangeably.

Preceding chain elongation two events must occur. First, the fatty acyl-CoA molecule must be recognized by the enzyme as a CoA derivative and be bound at the active site where condensation with malonyl-CoA occurs. It has previously been shown that it is the CoA derivative of the fatty acid which serves as the substrate for chain elongation (17). This binding could either be covalent through a thiol group present at the active site of the enzyme, or noncovalent by the interaction of the CoA molecule with various groups contained near the active site.

Nugteren (19) proposed that the fatty acid remains as a CoA derivative during condensation, although Podack et al. (54) suggested that the acyl moiety remains enzyme-bound during chain elongation. However, their results were based only on experiments performed with the terminal enzyme of chain elongation, i.e. \( \alpha,\beta \)-enoyl-CoA reductase, and are not necessarily applicable to the initial condensation step. However, either a covalent or noncovalent bond at the active site would help position the carbonyl group of the fatty acid molecule relative to any additional binding which might occur. The additional binding could be referred to as the second event which must occur during the recognition process. From these studies it is obvious that the enzyme is very specific in chain elongating only those fatty acids which contain certain double bonds and have a selected chain length as previously discussed. In reference to whether the acyl molecule is covalently linked to the enzyme through a thiol group or to whether it is noncovalently linked by binding
of the CoA moiety to a specific CoA binding site, this can only be un-
equivocally answered when the enzyme is obtained in pure form.

Two possibilities exist for the binding of the acyl portion of the fatty acid with the enzyme itself. The first would involve the interaction of the hydrocarbon chain with nonpolar, hydrophobic regions of the enzyme situated in a planar or unrestricted region on the surface of the enzyme. Since it is generally accepted that the enzymes of chain elongation and desaturation are an integral component of the micro-
somal membranes, partly because of their difficulty in being separated from it, it can be assumed that regions of hydrophobicity exist on the enzyme molecule which would enable it to not only interact with the hydrophobic regions of the lipid bilayer, as proposed by Singer and Nicolson (122), but also bind nonpolar substrates or nonpolar moieties of polar substrates. The former situation is illustrated by the fact that both cytochrome b5 (43) and stearoyl CoA desaturase (45) show a high degree of hydrophobicity, and the latter by the binding which occurs between free fatty acids and serum albumin (123). In reference to the binding site of the chain elongating enzyme, the nonpolar regions of the molecule would be arranged in such a pattern as to allow for the maximum binding with specific substrates, i.e., 7,10-18:2, as illustrated in Figure 28, A1. This situation would also allow for the chain elongation

1 The structures used in the conception of the binding sites are the ac-
cepted planar projections by Vandenheuvel (75) of fatty acids with cis-
double bonds being coplanar and producing a pronounced bend or kink in an otherwise linear molecule. Although the hydrocarbon portion of a polyunsaturated fatty acid in no way is exclusively hydrophobic due to the presence of polarizable π electrons on the double bonds, at this point in the discussion the fatty acid tail will be considered as a single, nonpolar entity.
Figure 28. Possible model of the substrate-binding site involving the interaction of the fatty acyl moiety (\_\_\_\_) with a planar or unrestricted region (\_\_\_\_) on the surface of the chain elongation enzyme. A, coplanar projection of 7,10-18:2. B, coplanar projection of 6,9-18:2 and distal portion of 7,10-16:2. C, coplanar projection of 7,10-20:2 and distal portion of 8,11-18:2. All orientations assume fixed carbonyl positioning and fatty acids are projected according to Vandenheuvel (75).
of other fatty acids such as 6,9-18:2 and 7,10-16:2, both of which show slightly slower rates, and which would be conceivably bound slightly less by the apolar region (Figure 28, B). However, this situation would not explain the dramatic loss in the ability of some fatty acids to be chain elongated as exemplified by 8,11-18:2 and 7,10-20:2 (Figure 28, C). Both of these acids have the same degree of deviation from 7,10-18:2 as 6,9-18:2 and 7,10-16:2 have, except in the opposite mode, yet neither are chain elongated to any appreciable degree. If 6,9-18:2 and 7,10-16:2 can be chain elongated by being positioned less than ideally over the apolar region, then 8,11-18:2 and 7,10-20:2 should be too. Since they are not, it can be assumed that additional features must define the geometry of the binding site.

The second possibility would involve a cleft on the surface of the enzyme into which the fatty acid must pass and be bound in order for condensation to occur as illustrated in Figure 29, Brett et al. (38) and Pollard (40) have proposed a similar type of binding site for the Δ9 desaturase, although of a configuration which favors the binding of those fatty acids to be desaturated rather than chain elongated. This restriction on the binding site would allow for 6,9-18:2 and 7,10-16:2 to be chain elongated and also offer an explanation why 7,10-20:2, 8,11-18:2, or the monoenoic acids which were tested are not chain elongated. They simply do not fit into the cleft. Thus it would account for the apparent "all or none" situation as observed for the tested substrates. The exact geometry of the cleft would presumably be defined by 7,10-18:2 which shows the maximum rate of chain elongation of all fatty acids investigated. First, the site must possess sufficient non-polar
Figure 29. Proposed model of the substrate-binding site involving the interaction of the fatty acyl moiety (---) in a cleft ( \ \ \ \ \ ) of the chain elongation enzyme. A, coplanar projection of 7,10-18:2. B, coplanar projection of 6,9-18:2 and distal portion of 7,10-16:2. C, coplanar projection of 7,10-20:2 and distal portion of 8,11-18:2. All orientations assume fixed carbonyl positioning and fatty acids are projected according to Vandenheuvel (75).
regions to allow binding to the hydrocarbon chain. Second, the cleft must contain sufficient curvature and it must be positioned at a definite length from the carbonyl/CoA end to allow binding of the bent hydrocarbon chain. And third, the binding site must have some restrictive barrier distal to the carbonyl/CoA end to prevent the binding of fatty acids containing an excess of methylene groups. A cleft binding site with these geometrical limitations would adequately explain the substrate specificities as observed from these studies.

The fatty acid triad of 5,8-16:2, 5,8-18:2, and 5,8,11-18:3 are perhaps perfect examples to illustrate changes in rate with changes in the fatty acid molecule. From the initial octadecadienoic acid profile of chain elongation 5,8-18:2 showed a rate that was only 9.2% of the 7,10-18:2 rate. Using the proposed model the extremely slow rate could be explained either by the inability of the binding site to properly hold (Figure 30, A, circle 1) the acyl portion during condensation or by the fact that the distal barrier (Figure 30, A, circle 2) prevents the 5,8-18:2 from entering the site just as it did the 7,10-20:2 fatty acid. With the addition of a double bond in the 11-position (Figure 30, B), the rate substantially increases to 54.6% of the 7,10-18:2 rate, possibly due to the movement of the fatty acid tail away from the restrictive barrier. An increase in rate to 24.6% of the rate for 7,10-18:2 is also observed with 5,8-16:2 when two terminal methylene groups are removed and suggests that the acyl portion is now able to enter the binding site (Figure 30, C). However, it is strange that the rate of increase that was observed with 5,8-16:2 is not as great as that with 5,8,11-18:3, especially since the dienoic acid would allow for a greater amount of
Figure 30. Selected features of the fatty acyl moiety (—) and the cleft (\_\_\_\_\_\_) involved with binding in the proposed model. A, coplanar projection of 5,8-18:2 indicating improper distance for binding to occur (circle 1) or blockage by distal barrier (circle 2). B, coplanar projection of 5,8,11-18:3 indicating movement of the acyl tail away from the distal barrier due to the additional 11-cis double bond. C, coplanar projection of 5,8-16:2 indicating possible entrance into cleft due to shorter length.
interaction of the fatty acid tail with the non-polar binding regions. This discrepancy suggests the possibility of a second condensing enzyme for polyunsaturated fatty acids that could conceivably be responsible for the chain elongation of trienoic acids. Instead of 5,8,11-18:3 fitting into the dienoic cleft (Figure 30, B), it would have its own binding site more closely tailored to three double bonds. It would also be imagined that as a 5,8- dienoic acid is shortened, its rate should slowly increase until its total length approximates the position of the last double bond of the trienoic acid. This, however, awaits further investigation with additional substrates, a different enzyme source other than liver, or enzyme purification. Only then could the existence of a dienoic and trienoic chain elongating system be unequivocally established.

To this point in the discussion the type of binding between the fatty acid tail of the substrate and the chain elongating enzyme(s) has only been alluded to as being hydrophobic, although it was rightfully suggested that both London-Van der Waals dispersion forces and polarization forces are undoubtedly involved. This was done for the sake of simplicity in that the amphipathic fatty acyl-CoA molecule can be more easily divided between the strongly electrostatic forces associated with the coenzyme A end and the more subtle forces associated with the acyl end. Concerning the three types of interactions that this molecule can participate in, Salem (124) has estimated the binding energies for different groups at a distance of 5 angstroms from each other. It was estimated that energies of approximately 4 kilocalories per mole are involved in electrostatic interactions, only 0.002 kilocalories per mole
are involved in polarization interactions, and 0.4 kilocalories per mole are involved in London-Van der Waals interactions. For 7,10-octadecadienoyl-CoA at physiological pH's this would involve a total energy of ca. 17 kilocalories if the molecule were situated 5 angstroms from a hypothetical binding site capable of maximum interactions with all groups involved, as illustrated in Figure 31. Although the coenzyme A moiety with three possible net charges as well as numerous other ionizable groups, shows a theoretical binding energy of 12 kilocalories, it was not the intention of this study to investigate possible coenzyme A interactions with the enzyme. The type of involvement of the two double bonds of the 7,10-18:2 molecule is discussed in the following paragraph. The interaction by London-Van der Waals dispersion forces of the numerous hydrophobic methylene groups and possible nonpolar regions of the binding site is illustrated in Figure 27 by the fact that an increase in rate is observed for fatty acids containing double bonds in the 7,10-positions until C20 is reached. The exceedingly slow rate for 7,10-20:2 is suggested to be caused by the inability of the acid to enter the cleft where binding occurs. Since the only alteration which occurs in this sequence of fatty acids is the chain length, then the methylene groups which are added obviously participate in the binding. A semi-quantitative estimate of the number of methylene groups involved in London-Van der Waals interactions can be obtained by comparing the increase in rate of chain elongation with the increase in binding energy when methylene groups are added. Using this technique, there is a direct relationship between the number of methylene groups, excluding the isolated one at C9, in 7,10-14:2, 7,10-16:2, and 7,10-18:2, and
Figure 31. Possible binding energies from electrostatic forces, polarization forces, and London-Van der Waals dispersion forces of 7,10-octadecadienoyl-CoA at a distance of 5 Å from the fatty acyl-CoA binding site and computed according to Salem (124).
their rates of chain elongation. This suggests that both the methylene groups between C1 and C7, and those after C11 participate in binding.

Two additional features of the proposed binding cleft can only be speculated upon at this time. The first would involve the width of the binding site, and the second the involvement of the double bonds. In reference to the width feature, Brett et al. (38), Pollard (40), and Do and Sprecher (42), using methyl substituted fatty acids, suggested a very close enfolding of the substrates by the enzyme for the Δ9 and Δ5 desaturases. Methyl groups located on or near the methylene groups to be desaturated were able to greatly reduce the rate of desaturation. This implied a very tight fit at the active center which would be anticipated if hydrogen abstraction is to occur there. Since the enzymatic conversion which occurs during chain elongation takes place at the carbonyl end and not on the hydrocarbon chain itself, perhaps a minimal amount of lateral movement in the cleft can be tolerated. However, since rather restrictive features have been defined for the other two dimensions, perhaps it would also exist for this third, as yet un-investigated, dimension. The type of involvement associated with the double bonds could be either geometrical due to their kinking effect on the otherwise straight methylene chain, binding due to π-binding sites on the enzyme interacting with the polarizable π-electrons of the double bonds on the substrate, or both. Although Brenner (41) has proposed a model for Δ6 desaturation involving Δ9-cis, Δ12-cis, and Δ15-cis π-bond interactions with the enzyme itself, such proclamations as to the reasons why at least two double bonds are necessary before chain elongation can occur would be unwarranted at this time. It has already been
shown that of the monoenoic acids tested, all were converted at a negligible rate. It is interesting to note that intramolecular rotation about a carbon-carbon single bond of an otherwise linear fatty acid molecule to the synclinal or gauche position produces a conformation similar to a cis-double bond. Although the energy state for this conformation is only slightly higher than the preferred antiplanar or trans-conformation, it would require the molecule to rotate through the rather energy demanding anticlinal state. The possibility of intramolecular rotations would suggest that octadecanoic acid could assume a conformation similar to all cis-7,10-18:2 providing rotation to the synclinal or gauche position occurs about C7-8 and C10-11. It would also suggest that rotation about C9-10 of 6-cis-18:1 or rotation about C10-11 of 7-cis-18:1 would produce conformations similar to 6,9-18:2 and 7,10-18:2. In that the measured rates of chain elongation for both of these monoenoic acids were extremely slow, it could be assumed that these types of conformational changes probably do not occur and that the presence of cis-double bonds is necessary to alter the fatty acid molecule into such a conformation which is then mimicked by the binding site. However, the possible importance of cis-double bonds in polarization type binding can not be ascertained from the substrates which are presently available.

Examination of the linolenate, linoleate, oleate, and palmitoleate pathways (Figure 2) of polyunsaturated fatty acid metabolism as established by Klenk (17) and by Mead (18) reveals the obvious feature that after the introduction of the cis-double bond in the 9- position, fatty acids are metabolized by alternating desaturations and chain elongations. It is also generally recognized that the desaturations which occur at
C9-10 (Δ9 desaturase), C6-7 (Δ6 desaturase), and C5-6 (Δ5 desaturase) are performed by different desaturating enzymes, and with the recent description of two chain elongating enzymes (50), one for palmitic acid and the other for 6,9-18:2, it suggests that the desaturations and chain elongations must occur in an alternating sequence in order to convert the fatty acid into a product which is then recognized by the next enzyme as its substrate. The substrate specificities of the chain elongating enzyme(s) as established by these studies do in fact confirm this observation. From the octadecadienoic and octadecatrienoic acid profiles of chain elongation, three interesting features are brought forth. First, of those fatty acids which are eligible for desaturation, e.g., 8,11-18:2, 9,12-18:2, 8,11,14-18:3, and 9,12,15-18:3, none are chain elongated to any appreciable degree. Second, of those fatty acids which are chain elongated, none can conceivably be desaturated by a Δ9, Δ6, or Δ5 desaturase. Pollard (40) has shown that allene and conjugated double bonds are not formed during any microsomal desaturations. And third, only those fatty acids whose products of chain elongation are eligible for desaturation are in fact chain elongated. The chain elongation product of 7,10-18:2 is 9,12-18:2, which has been shown to have a rapid rate of in vitro desaturation (40). Thus it seems that the organism, in this case rat liver microsomes, would make a concerted effort not to chain elongate and desaturate abnormal fatty acids were they introduced into the organism. It also suggests that because of differences in substrate specificities, the organisms do not permit competition by the

2Desaturation at C4-5 (Δ4 desaturase) has not yet been conclusively established.
desaturases and chain elongating enzymes for a particular substrate.

It is either eligible for chain elongation or desaturation.

We are thus presented with the dilemma of why fatty acids with
double bonds in the 7- and 10- positions, e.g., 7,10-14:2 (ω4), 7,10-16:2
(ω6), 7,10-18:2 (ω8), and 7,10,13-18:3 (ω5), are indeed chain elongated.
None of these acids appear in any of the four families of polyunsaturat­
ted fatty acids, although it has been suggested that acids such as
7,10,13,16-22:4 serve as intermediates in the formation of 4,7,10,13,16-
22:5 (125). However, this conversion would be a desaturation and not a
chain elongation. Nonetheless, these 7,10- acids are chain elongated
either with or without reason. Since biological systems usually do not
perform unnecessary functions, this dilemma has provided this investi­
gator the opportunity of suggesting two possible reasons why 7,10- acids
are converted.

The first involves the continuing speculation on the method of
introducing a cis-double bond at C4.5. Although the existence of the
Δ9, Δ6, and Δ5 desaturases is firmly established, aerobic desaturation
of Δ4 precursors has not been shown. Ayala et al. (125) were unable
to demonstrate in vitro desaturation of 7,10,13,16-22:4 with either rat
liver or rat testicular preparations, and Bernert and Sprecher (23)
found that both 7,10,13-20:3 and 7,10,13,16-22:4 proceeded at negligible
rates with rat liver microsomes in the formation of 4,7,10,13-20:4 and
4,7,10,13,16-22:5, respectively. However, Bridges and Coniglio (126)
have shown that the injection into rat testes of 9,12-18:2 and
5,8,11,14-20:4 rapidly labeled 4,7,10,13,16-22:5. It is now proposed
that 7,10,13-20:3 and 7,10,13,16-22:4 are indeed precursors of 4,7,10,13-
20:4 and 4,7,10,13,16-22:5, respectively, but by a mechanism that would
not involve direct desaturation at the C\textsubscript{4}-5. Instead it would involve Δ6 desaturation of a partially chain elongated acid with the 7,10,13-configuration of double bonds by the following scheme:

\[ \text{CoAS-C-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH=CH-R} \]

**Step 1**

\[ \text{malonyl-CoA} \]

\[ \text{CoS-C-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH=CH-R} \]

**Step 2**

\[ \text{NADPH} + \text{O}_2 \]

**Step 3**

\[ \text{acetyl-CoA} \]

\[ \text{R = e.g. -CH}_2\text{-CH=CH-CH}_2\text{-CH=CH-CH}_2\text{-CH=CH-CH}_2\text{-CH=CH-CH}_3 \]

Step 1 would involve the condensation of a fatty acid containing the 7,10- etc. double bond configuration with malonyl-CoA to form the well characterized β-keto intermediate (19). Thus the 7,10- etc. double
bonds now become 9,12- etc. double bonds. At this point either the β-keto intermediate could be reduced, dehydrated, and reduced again by the normal sequence of conversions in chain elongation (C.E.), or it could serve as a substrate for the Δ6 desaturase (step 2). Perhaps the total number of double bonds and chain length would actually determine whether the β-keto intermediate would be eligible for desaturation or reduction. If desaturation were to occur (step 2), a double bond would be introduced at C₆₋₇ by the well characterized Δ6 desaturase (40). It is noteworthy to include the fact that Pollard has indicated an increase in the rate of Δ6 desaturation with an increase in the number of double bonds and chain length (40). Unfortunately no Δ6 desaturation studies have been performed on fatty acids containing any type of bulky group on C₃. Step 3 would involve the loss of acetyl-CoA by β-keto fatty acyl-CoA thiolase, an enzyme found in both the cytosol and mitochondrial fractions (87). The 6,9,12- etc., sequencing of double bonds would thus produce the desired 4,7,10- etc., fatty acid. The cofactors for desaturation would be NADPH (NADH) and O₂ as well as malonyl-CoA. Ayala (125) and Bernert and Sprecher (23) did not include malonyl-CoA in their incubations with 7,10,13,16-22:4 or 7,10,13-20:3.

The second possible reason why 7,10- acids are chain elongated would involve the continuing need in animals of certain essential fatty acids commonly considered to be 9,12-18:2 and 9,12,15-18:3. Thus rat liver microsomes might deliberately chain elongate 7,10-18:2 because it is similar enough to 7,10-16:2, which would produce 9,12-18:2 if chain elongated. By the same reasoning 7,10,13-18:3 is chain elongated in that conversion of 7,10,13-16:3 would give 9,12,15-18:3. These studies
have shown that 7,10-16:2 is indeed rapidly chain elongated and both 7,10-16:2 and 7,10,13-16:3 have been shown in vivo to be essential fatty acid precursors (127). Although 9,12-18:2 and 9,12,15-18:3 are considered to be the common essential fatty acids in one's daily diet and they are readily available in numerous foods, there are a number of sources of either 7,10-16:2 or 7,10,13-16:3 such as soybean oil (128), cashew nuts (129), ferns (130), and mosses (131). Perhaps this particular chain elongation is a remnant from when diets were less refined and more varied than today, and represents the organisms continuing effort of being able to convert every available resource into an essential component of its diet.
An isomeric series of $[1^{-14}C]$ octadecadienoic acids was synthesized and used in determining the rates of malonyl-CoA dependent chain elongation with rat liver microsomes. The 7,10- isomer was converted at approximately twice the rate of the frequently measured and naturally occurring 6,9- isomer. All other isomers were chain elongated at negligible rates. Elimination of NADPH from the incubation medium allowed for the determination of the respective rates of the initial condensation reaction for the same isomeric series. The substrate specificity and rates of β-keto fatty acid formation were essentially equivalent to those of chain elongation.

To determine if the substrate specificity, as observed for the chain elongation and condensation reactions, would be duplicated by the β-hydroxy fatty acyl-CoA dehydrase and α,β-enoyl-CoA reductase reactions associated with chain elongation, the $[3^{-14}C]$ β-hydroxy eicosadienoyl-CoA and $[3^{-14}C]$ 2-trans eicosatrienoyl-CoA intermediates of the most rapidly converted and two poorly converted parent octadecadienoic acids were synthesized. The enzymatic activities of the dehydrase and reductase, as measured with these two sets of intermediates, were approximately equivalent indicating a broad substrate specificity. In addition, the high rates of conversion confirm the rate limiting nature of the initial condensation reaction in microsomal chain elongation. Although an analogous series of β-keto fatty acyl-CoA's was not synthesized, due to
the difficulty of these compounds to be chemically prepared, these results suggest that fatty acids are condensed with malonyl-CoA by specific β-keto fatty acyl-CoA synthetases and that a common β-keto fatty acyl-CoA reductase, β-hydroxy fatty acyl-CoA dehydrase, and 2-trans enoyl-CoA reductase are responsible for the later conversions.

Previous evidence has indicated that separate condensing enzymes are present in the microsomes and that the dehydrase and enoyl-CoA reductase remain in a fixed ratio to each other. From the foregoing results it could be concluded that once condensation occurs, the remaining reactions are handled by a multifunctional enzyme with a broad specificity. To determine if the dehydrase and enoyl-CoA reductase are of a multifunctional nature, the activities of these enzymes were measured with [3,14C] β-hydroxy-9,12-eicosadienoyl-CoA and [3,14C] 2-trans-9,12-eicosatrienoyl-CoA with microsomes from rat liver and rat testicles. Comparison of the rates to the initial rate of condensation and overall rate of chain elongation, as measured with [1,14C] 7,10-octadecadienoyl-CoA, for the two enzymatic sources indicates that neither enzyme remains in a fixed ratio and suggests that these enzymes are not of a multifunctional nature.

The extremely limited substrate specificity of microsomal chain elongation, as observed with the isomeric series of octadecadienoic acids, indicated that the acyl binding site of the enzyme was geometrically restrictive and that it could be probed with the use of fatty acids containing specific structural features. A cleft binding site has been proposed and the available evidence suggests that it is curved and restrained distally to the carbonyl carbon, such that it is unable to
bind monoenoic acids and certain dienoic acids, and will only interact with particular dienoic and trienoic acids of specific chain lengths. Since maximum chain elongation activity was observed with 7,10-octadecadienoic acid, it is assumed that the cleft is well defined geometrically by this fatty acid.
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