

Aspects of fatty acid metabolism in  
Galleria mellonella, (L.) (Lepidoptera:  
Pyralidae): Isolation of the elongation  
system, with calculated fractional turnover  
rates of octadecanoic and 9-octadecenoic acids.

by

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B.Sc., Simon Fraser University, 1968

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APPROVAL

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Title of Thesis: Aspects of fatty acid metabolism in Galleria melonella (L.) (Lepidoptera: Pyralidae): Isolation of the elongation system, with calculated fractional turnover rates of octadecanoic and 9-octadecenoic acids.

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## ABSTRACT

The major fatty acids of Galleria mellonella (L.) (Lepidoptera:Pyralidae) were found to be C 14:0 (myristic or tetradecanoic acid), 0.3%; C 16:0 (palmitic or hexadecanoic acid), 38.0%; C 16:1, 4.7%; C 18:0 (stearic or octadecanoic acid), 0.8%; C 18:1, 49.8%; C 18:2, 6.4%. After injection of  $^{14}\text{C}$ -1-acetate, for short exposure periods, activity appeared solely in octadecanoic and 9-octadecenoic acids. The formation of octadecanoic acid was due to elongation of hexadecanoic acid, and the fractional turnover rate for this reaction was calculated to be  $7.76 \times 10^{-6}$ /minute. Formation of 9-octadecenoic acid was by desaturation of octadecanoic acid, and the fractional turnover rate for this reaction was calculated to be  $1.9 \times 10^{-8}$ /minute. The halflives were C 18:0,  $8.93 \times 10^4$  minutes; C 18:1 (9),  $3.65 \times 10^7$  minutes; and the turnover times, C 18:0,  $1.29 \times 10^5$  minutes; C 18:1 (9),  $5.26 \times 10^7$  minutes. The turnover fluxes were calculated to be C 18:0,  $8.50 \times 10^{-7}$  mg/minute; C 18:1 (9),  $1.28 \times 10^{-7}$  mg/minute. No evidence of de novo synthesis was found until after 4 hours exposure.

## PREFACE

In 1967, Bracken and Barlow reported in the Canadian Journal of Zoology (45: 57-61) that the parasitic Ichneumonid wasp, Exeristes comstockii (Cress.) duplicates the fatty acid pattern of its host. It was later shown that another Ichneumonid parasite, Itopectis conquisitor (Say), exhibits a similar relationship as E. comstockii (Thompson and Barlow 1970 unpublished results). This was contrary to previous studies, which demonstrated that most insects retain definite characteristics of their fatty acid patterns, regardless of the fatty acid composition of their diets (Barlow 1964, Yendol 1970).

After the initial studies on the Ichneumonid parasites were completed, experiments were conducted to study this phenomenon further, and the mechanisms responsible for the parasite's duplicated pattern. Host transfer experiments (i.e. transfer of 2nd instar E. comstockii to a new host of different species, for complete development), involving  $^{14}\text{C}$ -l-acetate injections indicated that the two hosts, Lucilia sericata (Meigen) and Galleria mellonella (L.), synthesized fatty acids at different rates, and that the parasite synthesizes fatty acids at the same rate as the host upon which it is reared (Barlow and Bracken 1969 unpublished results). However, since

very little was known about the fatty acid metabolism of the hosts themselves, a study of these hosts appeared to be the next step.

Since I was interested in the synthetic pathways for fatty acids, and the possibility of studying incorporation curves and reaction rates in vivo, I undertook to study fatty acid metabolism in G. mellonella.

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To all the other members of the Department of Biological Sciences who assisted me during this period, I am indeed grateful.

## INTRODUCTION

Biochemical studies with insects have indicated that these have similar synthetic pathways for saturated and monounsaturated fatty acids as higher animals. Zebe and McShan (1959) first demonstrated the presence of the de novo synthetic pathway. After injection of  $^{14}\text{C}$ -acetate into Prodenia eridania activity appeared in palmitic, oleic, myristic and lauric acids. The syntheses required malonate, ATP, coenzyme A and glutathione. This pathway was later isolated and further characterized by Tietz (1961) in Locusta migratoria. In both cases, a significant amount of acetate was incorporated into fatty acids when malonate was omitted from the preparation, indicating the simultaneous occurrence of elongation.

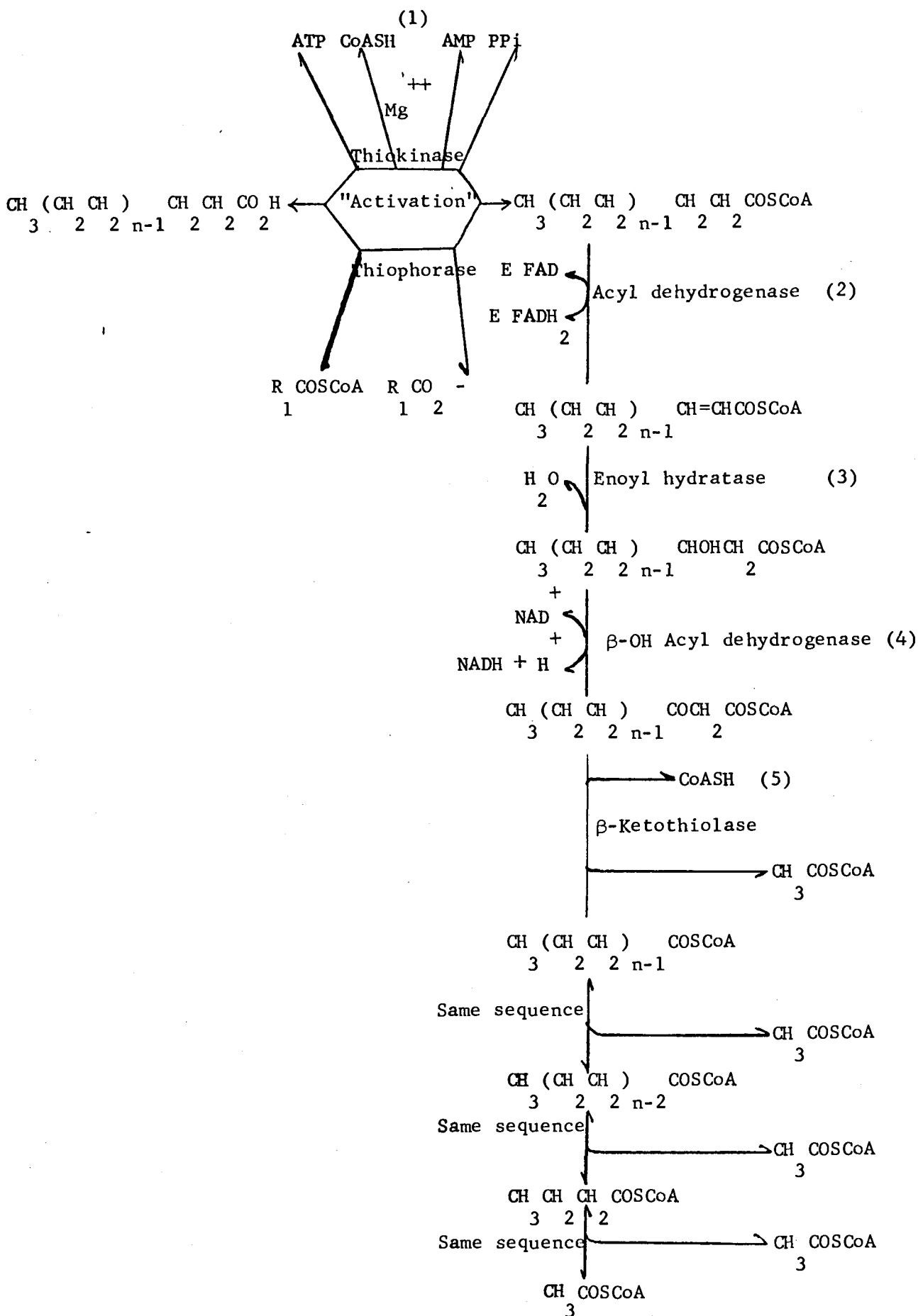
Bade (1964) first demonstrated the formation of a monounsaturated fatty acid by direct desaturation of the corresponding saturate. Labelled stearic acid was converted to correspondingly labelled oleic acid, in Eurycotis floridana. Similar results were obtained with Bombyx mori (Sridhara and Bhat 1965). Labelling patterns, consistent with those in the studies just described, were also obtained with Anthonomus grandis (Lambremont, Stein and Bennett 1965), and Hyalophora cecropia (Stephen and Gilbert 1969). In all these studies either no activity or trace amounts were detected in polyunsaturates.

It is evident from previous studies that little quantitative information on fatty acid synthesis and turnover in insects is available. Few researchers have attempted to analyze specific activities, fractional turnover rates or compartmentation. The purpose of these experiments was to study some aspects of fatty acid synthesis in vivo in G. mellonella: 1) to elucidate the mechanisms of fatty acid synthesis in G. mellonella, 2) to determine fractional turnover rates and 3) to study some effects of physiological compartmentation on incorporation of  $^{14}\text{C}$ -l-acetate.

LITERATURE REVIEW

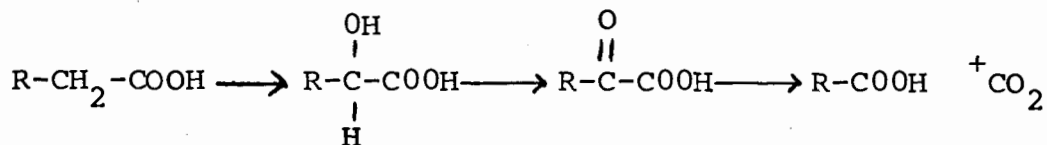
Since the majority of studies on fatty acid synthesis have been carried out on microorganisms and higher animals, it is essential to describe this work, in order to understand the principles of fatty acid synthesis and to see how this work is applied to insects.

In 1904, Knoop first outlined the  $\beta$  oxidation scheme for fatty acid oxidation. Dakin (1923) confirmed the presence of Knoop's scheme, and attempted to account for the ketone compound "by-products". MacKay, Wick and Barnum (1940) solved this problem by proposing the  $\beta$  oxidation-condensation scheme. Leloir and Munoz (1939) first observed fatty acid oxidation in cell free preparations of guinea pig liver, allowing identification and characterization of the individual enzymatic reactions. In 1951, Lynen and Reichert identified the structure of Knoop's activated fatty acids as A-acyl derivatives of coenzyme A, accounting for the fact that none of the proposed intermediates of  $\beta$  oxidation could be detected. Such were the key steps in the elucidation of the  $\beta$  oxidation scheme. The pathway can be summarized as follows: fatty acids are converted to their acyl-Co A derivatives, followed by oxidation to the  $\alpha$   $\beta$  unsaturated forms; these undergo hydration and oxidation to the corresponding  $\beta$  keto derivatives, after which, thiolytic cleavage yields one acetyl Co A unit, and the acyl Co A derivative minus two carbon units. The scheme can be illustrated as follows:



Although all the enzymes of the  $\beta$  oxidation scheme have not been found in any one insect, workers have isolated various enzymes and preparations that support the supposition that  $\beta$  oxidation occurs in insect tissues. Hoskins, Cheldelin and Newburgh (1956) detected the acetate activating enzyme in Apis melifera, and Nelson (1958) described this enzyme in Hyalophora cecropia. Zebe (1960) isolated the condensing enzyme responsible for the entry of activated acetate into the tricarboxylic acid cycle, and  $\beta$  ketoacyl thiolase, the enzyme responsible for step 5 in the oxidation process.

More recent work has shown the presence of other mechanisms of biological oxidation, including  $\alpha$  and  $\omega$  oxidation. Mead and Levis (1963) have described  $\alpha$  oxidation in rat brain tissue as the oxidation of long chain fatty acids to  $\alpha$  hydroxy acids and  $\alpha$  keto acids, followed by oxidative decarboxylation yielding  $\text{CO}_2$  and the corresponding acid less one carbon unit. The scheme can be illustrated as follows:



More recently, Hitchcock and James (1966) have isolated such a system from Ricinis gibsonii (castor oil plant) leaves.

Wakabayashi and Shimazono (1963) have described  $\omega$  oxidation as a two step pathway involving the formation of  $\omega$  hydroxy acids from long chain fatty acids, followed by

oxidation to the corresponding dicarboxylic acids.  $\beta$  oxidation then proceeds from this newly formed carboxyl end. This process was demonstrated in cell free systems of guinea pig liver. Sorbic acid amide, octatrienoic acid amide and capric acid amide were all converted to the corresponding dicarboxylic acid monoamides. After incubation of  $^{14}\text{C}$ -2-sorbic acid amide with microsomes, the formation of  $\omega$  hydroxy  $^{14}\text{C}$ -2-sorbic acid amide was detected, and was followed by oxidation to muconic acid.

Since the steps of the  $\beta$  oxidation scheme are reversible, it was thought for many years that the biosynthesis of fatty acids was occurring via the reversal of this pathway. This original concept was first proposed by Lynen (1953), "Fatty acid synthesis is accomplished through repetition of a cycle of four consecutive reactions. a) Condensation of two molecules of acetyl Co A to form acetoacetyl Co A and Co A; b) reduction of acetoacetyl Co A to  $\beta$  hydroxybutyryl Co A; c) dehydration of  $\beta$  hydroxybutyryl Co A to crotonyl Co A; and d) reduction of crotonyl Co A to butyryl Co A with another molecule of acetyl Co A, to form  $\beta$  keto caproyl Co A and Co A, and so forth. The cycle is repeated eight times until stearyl Co A is formed." This scheme can be followed by studying the  $\beta$  oxidation scheme, previously shown, in reverse. This viewpoint was further supported by other workers. Dituri and Gurin (1953) demonstrated

that particle free extracts of rat liver, containing an aqueous extract of mitochondria could carry out fatty acid synthesis. However, in these experiments it was found impossible to demonstrate conversion of acetyl Co A to butyryl Co A by recombining the purified oxidation enzymes from the mitochondria. Also, Langdon (1957) suggested, in reference to the enzymatic steps requiring  $\text{NADH} + \text{H}^+$ , such as the  $\beta$  hydroxyacyl dehydrogenase step, that the  $\text{NADH} + \text{H}^+ : \text{NAD}^+$  ratio, in the mitochondria, is too low to favor the reductive process.

Van Baalen and Gurin (1953) while studying differences in cofactor requirements for the oxidative and reductive processes, suggested that there may be two separate systems for the synthesis and oxidation of fatty acids.

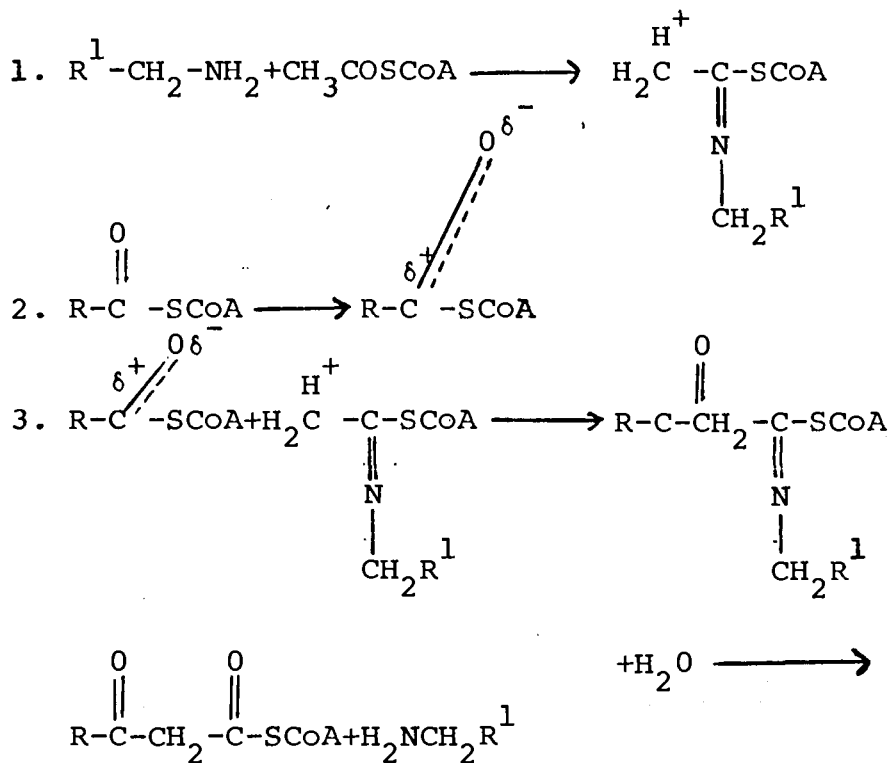
Later experiments showed more conclusively that fatty acid synthesis occurs via a modified reversal of the  $\beta$  oxidation scheme. Langdon (1957) showed that crotonyl Co A was reduced by  $\text{NADPH} + \text{H}^+$  in the presence of an enzyme,  $\text{NADPH} + \text{H}^+$  crotonyl Co A reductase, from soluble extracts of rat liver. This reaction is represented by the reverse of step 2 of the first cycle in the previous illustration of  $\beta$  oxidation. Seubert, Greull and Lynen (1957) isolated this enzyme from pig liver mitochondria, and demonstrated the synthesis of octanoyl Co A and capryl Co A from hexanoyl Co A and acetyl Co A in the presence of  $\text{NADH} + \text{H}^+$ ,  $\text{NADPH} + \text{H}^+$ , thiolase, enoyl hydratase,



$\beta$  hydroxyacyl dehydrogenase, and NADPH-specific enoyl Co A reductase, the newly isolated enzyme.

Wakil, McLain and Warshaw (1960) demonstrated that intact mitochondria from rat liver could synthesize long chain fatty acids from acetyl Co A. The mitochondria were incubated anaerobically with acetyl Co A,  $\text{NADH} + \text{H}^+$ ,  $\text{NADPH} + \text{H}^+$ , and ATP. The fatty acids synthesized were stearic acid, 40%; palmitic acid, 20%; and lauric acid, 20%. Upon decarboxylation of stearic acid, the carboxyl carbon had over twice the specific activity as that of the whole molecule, indicating that acetate was being enzymatically added to the carboxyl end of shorter chain acids. The synthesis was primarily dependent on ATP, but  $\text{NADH} + \text{H}^+$  and  $\text{NADPH} + \text{H}^+$  were required for optimal activity. An unidentified cofactor was also required. After the identification of this scheme, Wakil termed it the "elongation" or mitochondrial scheme for fatty acid synthesis. The requirement for ATP and a heat stable cofactor would not be necessary for simple reversal of the  $\beta$  oxidation system, but a number of common intermediates and enzymes are involved. In these experiments, NADPH-specific crotonyl Co A reductase, described previously, was not detected. The unidentified cofactor necessary for the operation of this preparation was later identified by Wakil (1961) as the coenzyme, pyridoxamine phosphate. After dialysis of the enzyme preparation, the synthesis only proceeded

after the addition of this coenzyme. When pyridoxamine phosphate concentrations reached  $10^{-3}$  M there was 2 to 3 times the incorporation of  $^{14}\text{C}$ -l-acetyl Co A into long chain fatty acids. The role of this coenzyme was found to be in the condensation step of acetyl Co A with the fatty acyl Co A acceptor, and therefore eliminates the requirement for thiolase in this reaction. Wakil reports that thiolase has an extremely low equilibrium constant as well as a great tendency to catalyze condensation of two acetyl Co A units to acetoacetyl Co A, indicating that this enzyme may not be involved in the synthetic process. The involvement of pyridoxamine phosphate provides specificity to the condensation of acetyl Co A with an intermediate chain acyl Co A, and the formation of an acetyl Co A pyridoxal complex results in the formation of a Schiff base described in the pyridoxal coenzyme models proposed by other researchers, including Cornforth (1959). Wakil states that this base may activate the methyl group of the acetyl Co A molecule by imposing an electronegative charge, thus favoring condensation with a relatively positively charged carbonyl group of acyl Co A allowing the elongation to proceed. The role of pyridoxamine phosphate can be shown as follows:



R<sup>1</sup> represents the rest of the pyridoxamine phosphate molecule.

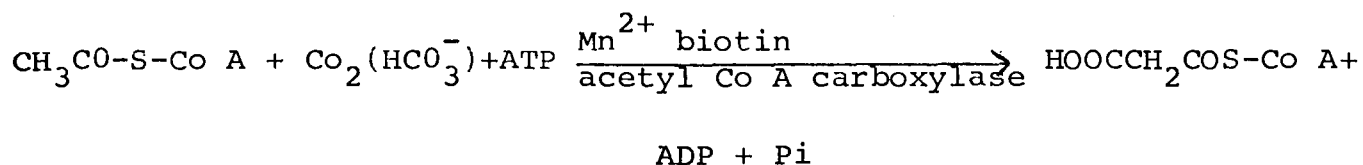
R represents the acyl group of the Co A derivative

Before the elongation system was fully characterized, evidence was accumulating in support of a second mechanism of fatty acid synthesis located in the cytoplasmic portion of the cell, and not associated with the mitochondria. This system was first detected in avian liver, and the system was found to be free of all the β oxidation enzymes. Brady and Gurin (1950) demonstrated the conversion of octanoic acid to stearic acid in avian liver extracts. Bicarbonate was found to be essential to the reaction, in contrast to the elongation system, which is bicarbonate independent. Gibson, Titchener and Wakil (1958)

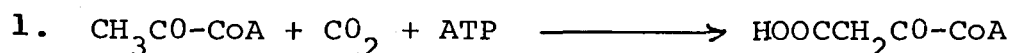
further characterized the system, showing its dependence upon bicarbonate, ATP,  $Mg^{2+}$  or  $Mn^{2+}$ , as well as acetyl Co A. Barron, Squires and Stumpf (1961) demonstrated that enzyme extracts from avocado mesocarp required  $CO_2$  for fatty acid synthesis. Squires and Stumpf (1959) obtained similar results for intact yeast cells. Since Gibson et al. found that  $H^{14}CO_3^-$  was not incorporated into long chain fatty acids in those organisms which require  $CO_2$ , he proposed that bicarbonate served a catalytic role. He suggested that acetyl Co A was first carboxylated to malonyl Co A before synthesis of the fatty acids occurred. Brady (1958) first demonstrated the conversion of malonyl Co A to fatty acids with crude avian liver preparations. These preparations catalyzed the reduction of acetyl Co A by  $NADPH + H^+$  to acetaldehyde. Brady then proposed that synthesis of the carbon chain was occurring by the reduction of acyl Co A derivatives to the corresponding aldehydes by condensation between the carbonyl carbon of the acyl derivatives with the methylene carbon of malonyl Co A. Cornforth (1959) discusses the physical and chemical factors involved in such a mechanism.

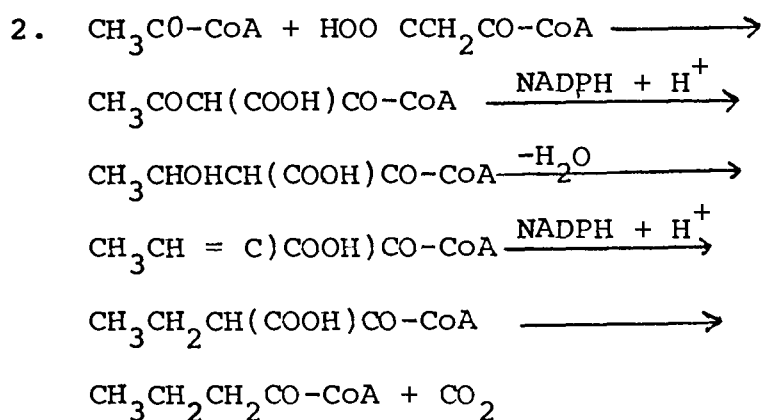
Wakil (1958) isolated and characterized the first intermediate in the synthesis of fatty acids from acetyl Co A. Upon hydrolysis the derivative was indistinguishable from malonic acid, and the intermediate could be produced by reacting acetyl Co A and  $CO_2$ . Wakil concluded that the first step in the synthesis of fatty acids by this pathway was the

enzymatic carboxylation of acetyl Co A to a malonyl derivative in the presence of biotin, ATP and  $Mn^{2+}$ . The subsequent successive condensation and reductive steps leading to the fatty acids are catalyzed in the presence of  $NADPH + H^+$ . The reaction can be summarized as follows:

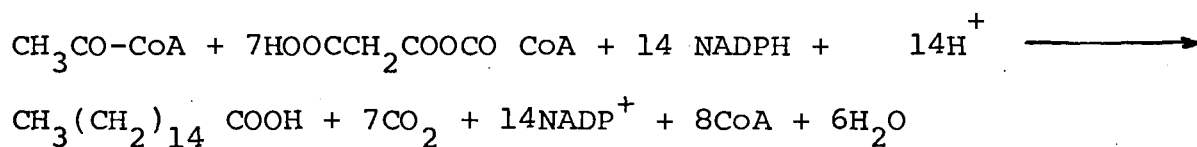


The characterization of acetyl Co A carboxylase was the first major step toward the elucidation of this second pathway of fatty acid synthesis, which Wakil termed the de novo pathway. Wakil and Ganguly (1959) reported that malonyl Co A could be readily converted to palmitate in the presence of a second enzyme preparation with  $NADPH + H^+$ . Addition of acetyl Co A significantly increased the rate of synthesis. Butyryl Co A and octanoyl Co A could also be converted into palmitate in the presence of malonyl Co A. This preparation was shown not to contain any of the  $\beta$  oxidation enzymes. The oxidation of  $NADPH + H^+$  in the system required the combined presence of malonyl Co A, and some unsubstituted fatty acyl Co A. No  $\beta$  oxidation intermediates could replace these Co A esters. At that time the following scheme was proposed for the de novo synthesis of fatty acids:





The fatty acids synthesized de novo by these enzyme preparations are primarily palmitic acid with small amounts of myristic and lauric acids, in contrast to the elongation system, in which the primary acids synthesized are stearic, myristic and lauric acids. The synthesis of one mole of palmitic acid requires 7 moles of malonyl Co A, and one mole of acetyl Co A. The overall reaction can be shown as follows:

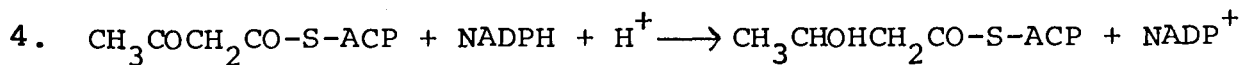
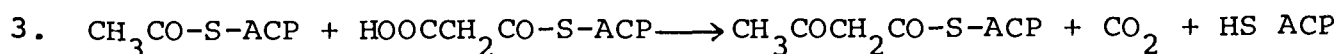
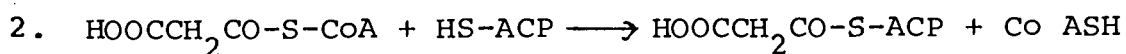
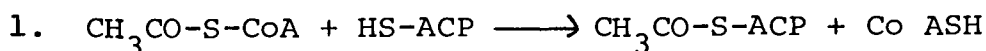


Bressler and Wakil (1961) have described in some detail the conversion of malonyl Co A into long chain fatty acids. Using avian liver extracts, they have demonstrated that palmitic acid and not its Co A derivative is the main product, and that acetyl Co A contributes carbons 15 and 16 of the palmitate, while malonyl Co A contributes carbons 1-14. However, short chain acyl Co A derivatives such as butyryl Co A, hexanoyl Co A and octanoyl Co A were only slightly incorporated into palmitic acid, and these did not accumulate during the

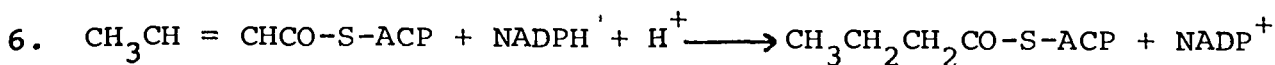
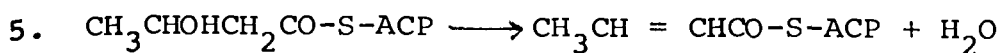
synthesis of acetyl Co A. They then concluded that free acyl Co A derivatives were not intermediates in this synthetic mechanism. Wakil and Bressler (1962) demonstrated that propionyl Co A will substitute for acetyl Co A in the initial condensation with malonyl Co A to yield odd chain fatty acids.

Bressler and Wakil demonstrated a requirement for a protein linked sulfhydryl group in fatty acid synthesis in avian liver. Lynen and Tada (1961) were able to isolate an acetoacetyl-S-enzyme when yeast preparations were incubated with  $^{14}\text{C}$ -acetyl Co A and malonyl Co A in the absence of  $\text{NADPH} + \text{H}^+$ . They concluded that acyl-S-enzymes were intermediates in fatty acid synthesis. Goldman, Alberts and Vagelos (1963) showed that palmitic and vaccenic acids were synthesized from malonyl Co A and acetyl Co A when incubated with soluble extracts from Esherichia coli. These extracts were found to contain two fractions, a heat labile protein and a heat stable protein fraction, both of which were necessary for synthesis. Wakil, Pugh and Sauer (1964) reported that their preparations from avian liver contained 5 protein fractions, but all attempts to fractionate these were unsuccessful. Working with E. coli, they confirmed the results of Goldman, and showed that the heat stable protein serves as a coenzyme, rather than an enzyme. The sulfhydryl group of this coenzyme acts as an acyl acceptor and donor and all the synthetic reactions occur while the acyl chain is linked to this protein. The heat stable protein was

designated the "acyl carrier protein", abbreviated ACP. Their experiments with avian liver extracts suggested that this system behaves similarly to that of E. coli, and the requirement for a polypeptide coenzyme is probably a general characteristic of all de novo fatty acid synthesizing systems. These workers summarize their results as follows: 1) A heat stable protein, now referred to as acyl carrier protein, ACP, participates in fatty acid synthesis; 2) This protein accepts acetyl and malonyl groups from their Co A derivatives, forming covalently linked acetyl and malonyl derivatives; 3) Acetyl ACP and malonyl ACP react to form acetoacetyl ACP; 4) In the presence of NADPH + H<sup>+</sup>, malonyl ACP and acetoacetyl ACP react to form long chain fatty acids; 5) Acetyl Co A, malonyl Co A, NADPH + H<sup>+</sup>, and ACP yield a mixture of β hydroxyacyl ACP of chain lengths C<sub>8</sub>, C<sub>10</sub>, C<sub>12</sub>, C<sub>14</sub>. These β hydroxyacyl derivatives are converted to their saturated homologues or palmitic acid on incubation with NADPH + H<sup>+</sup> and malonyl Co A. 6) The final product of fatty acid synthesis is free palmitic acid, but stoichiometric amounts of ACP and palmityl ACP may be isolated. The scheme can be shown as follows:

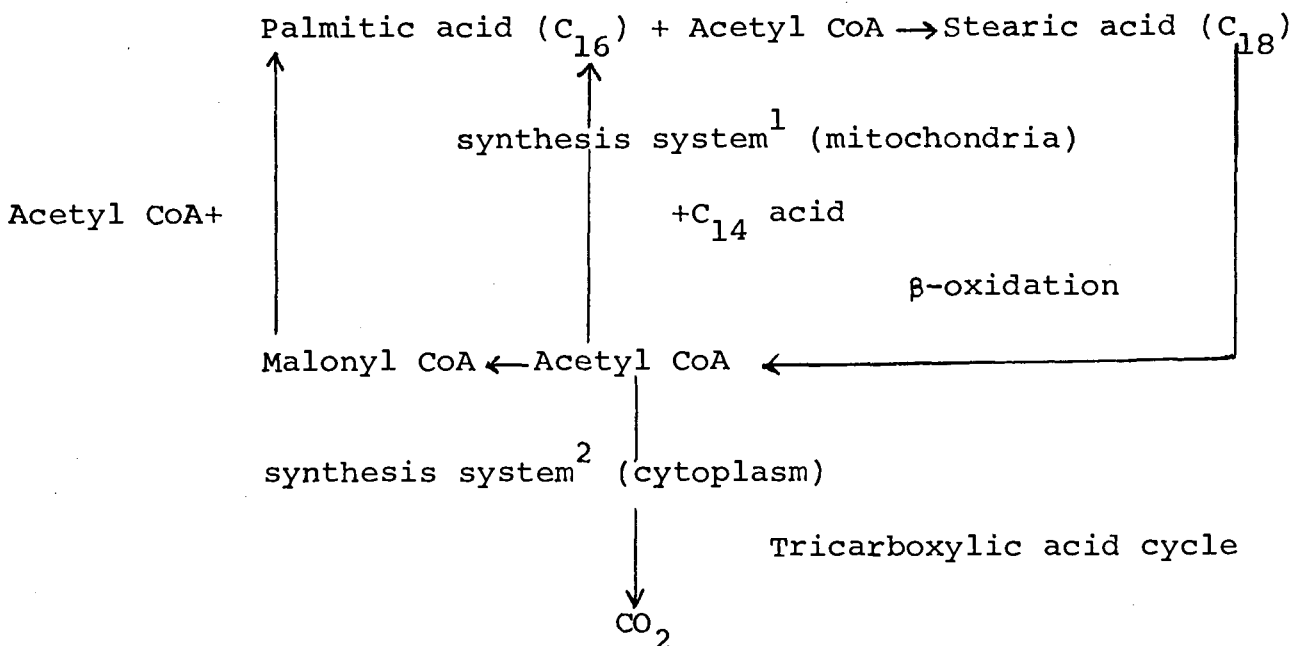






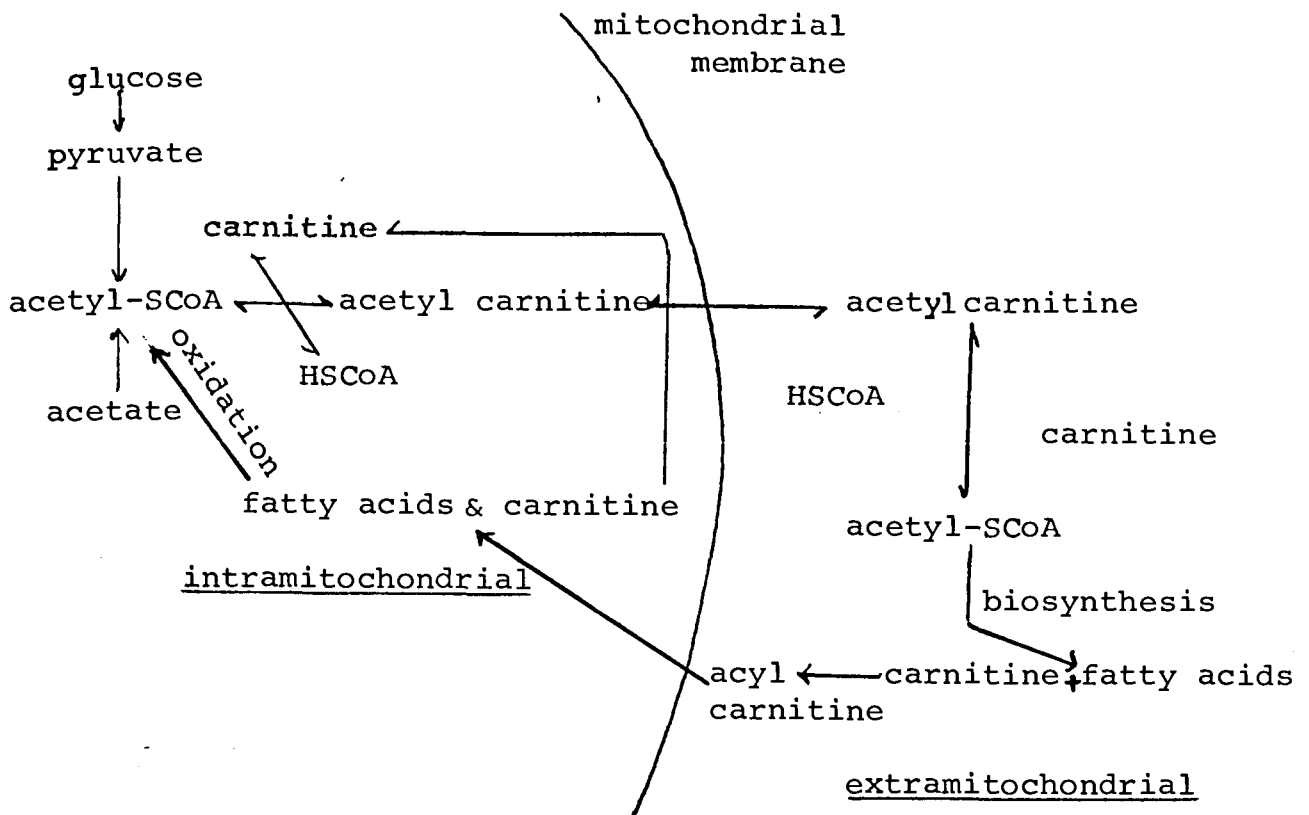
Repetition of steps 3 to 5, 6 more times, yields palmityl-S-ACP, which is then hydrolyzed to palmitic acid and the acyl carrier protein. More recently, Powell, Elovson and Vagelos (1969) have demonstrated that pantothenate is a prosthetic group of the two coenzymes Co A and ACP. The pantothenate is present in both as 4<sup>1</sup>-phosphopantethiene, to which the acyl groups are covalently bound as thioesters. It was also shown that Co A is the immediate precursor of ACP. Prescott, Elovson and Vagelos (1969) have described in more detail, the specificity of acyl carrier protein synthetase.

Wakil (1961) has schematically illustrated the inter-relationship of the elongation scheme and the de novo scheme for fatty acids synthesis as follows:



From the previous discussion, it is apparent that acetyl Co A is the basic building block for fatty acid synthesis. After the elucidation of various pathways for the synthesis of fatty acids, certain physiological problems became apparent. Determination of the origin of acetate was one of the first problems encountered. Cytoplasmic origins of acetyl Co A are limited to the ATP dependent cleavage of citrate and the acetate thiokinase reaction. Since the main source of acetyl Co A is intramitochondrial; by the oxidation of pyruvate, and the mitochondrial membrane is relatively impermeable to acetyl Co A, the question arose as to the source of acetyl Co A. Srere (1962) has demonstrated that citrate stimulates the synthesis of fatty acids, and suggested that the diffusion of citrate from the mitochondria into the cytoplasm may serve as a primary source of acetyl Co A. The citrate arising within the mitochondria from the condensation of oxaloacetate with acetyl Co A.

Mahler and Cordes (1966) have briefly described carnitine mediated transport of acetyl Co A across the mitochondrial membrane. The role of carnitine in the transport of acetate can be shown schematically as follows:



Friedman and Fraenkel (1955) were the first to describe the group transfer of acetate from coenzyme A to carnitine. Beenackers (1963) further characterized the role of carnitine trans-acetylase, the enzyme involved in the transfer of acetate, and demonstrated its role as a mediator of acetyl Co A between the mitochondria and the cytoplasm in rat heart muscle.

A similar problem to that just described involved the possible source of reduced  $\text{NADP}^+$  and  $\text{NAD}^+$  necessary for the reductive steps of fatty acid synthesis in the cytoplasm. Klingenberg and Bucher (1960) described the  $\alpha$  glycerophosphate cycle to explain the source of these coenzymes. By this pathway, hydrogen generated in the mitochondria could be made available to the cytoplasm and vice versa. Hydrogen is transferred

between the two pools as  $\alpha$  glycerophosphate, which passes readily across the mitochondrial membrane.  $\alpha$  glycerophosphate dehydrogenase within the mitochondria accepts hydrogen directly from the electron transport chain, and reacts with dihydroxyacetone phosphate to form  $\alpha$  glycerophosphate. After diffusion across the mitochondrial membrane, the  $\alpha$  glycerophosphate reacts with NAD-dependent extramitochondrial  $\alpha$  glycerophosphate dehydrogenase releasing dihydroxyacetone phosphate and  $\text{NADH} + \text{H}^+$ . Also, Krebs, Gascoyne and Notton (1967) suggested that a malate-oxalacetate system is used in mammals to transfer reducing equivalents across the mitochondrial membrane.

Since the elucidation of various aspects of saturated fatty acid synthesis, much work has been done on the various facets of these mechanisms in many animals, including several insects. However, less is known about the synthetic mechanism for unsaturated fatty acids, the mono- and polyenoic acids.

The formation of long chain monounsaturated fatty acids from their corresponding saturated acids was first described by Schoenheimer and Rittenberg (1936). Jacob, (1956) described in some detail an enzyme system from rat liver which could desaturate preformed fatty acids. Bloomfield and Bloch (1960), have demonstrated, in yeast cells, the synthesis of palmitoleic and oleic acids, both  $\Delta 9$  monounsaturated fatty acids, from their corresponding saturates, palmitic and stearic acids respectively, by direct desaturation. The proposed

scheme for synthesis can be shown as follows:

1. Palmitic acid  $\xrightarrow[\text{Mg}^{2+}]{\text{Co A ATP}}$  Palmityl-Co A
2. Palmityl-Co A  $\xrightarrow[\text{NADPH+H}^+]{\text{O}_2}$  (oxy-palmityl-Co A)
3. (oxy-palmityl-Co A)  $\longrightarrow$  Palmitoleyl Co A
4. Palmitoleyl Co A  $\xrightarrow{\text{Thiolase}}$  Palmitoleic acid + Co A

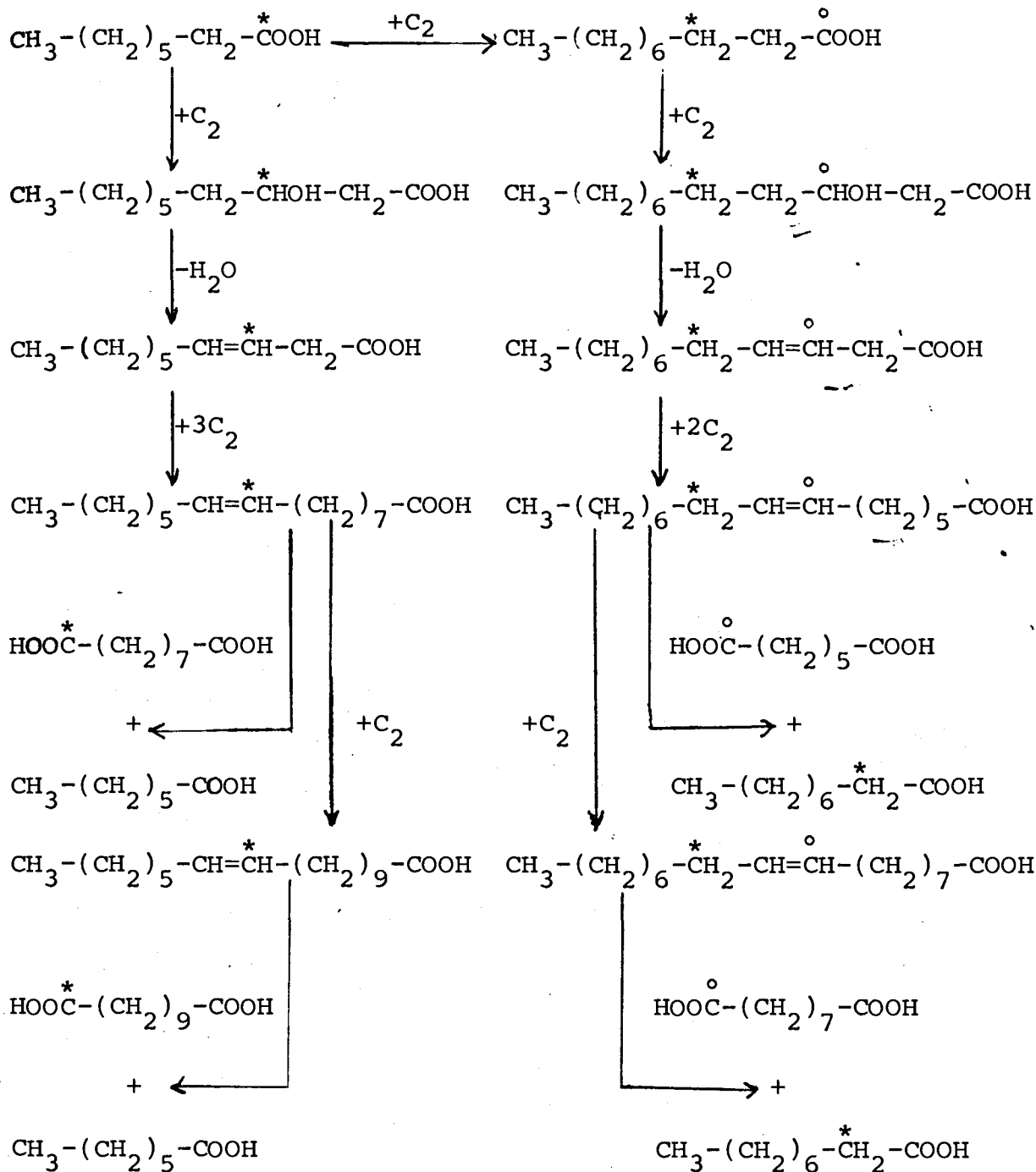
The cofactors necessary for the process were Co A, ATP, NADPH + H<sup>+</sup>, Mg<sup>2+</sup> and O<sub>2</sub>. Light, Lennarz and Bloch (1962) obtained evidence to suggest that hydroxy stearate and palmitate intermediates, irreversibly bound to the enzyme, are involved in this scheme. When labelled 9 or 10-hydroxy stearic acid or 9 or 10 hydroxy stearyl Co A were incubated with yeast, 9 and 10 acetoxy stearic acids, and their ethyl esters were formed, rather than the monoenoic acids. It appeared likely that some intermediate is formed in the oxidation of the saturated acids, and that this can be converted to one of the monoenoic acids or, in small yield, to hydroxy acid. This direct desaturation system, described by Mead (1965) as the aerobic mechanism of monounsaturate synthesis, has also been found in Mycobacterium phlei, Anabena variabilis (a blue green alga), and Penicillium chrysogenum by Lennart, Scheuerbrandt and Bloch (1962). In the bacterium, M. phlei, the cofactors Fe<sup>2+</sup>, and a flavin are required, in addition to those cofactors previously mentioned.

More recently, Schroepfer and Bloch (1965) have described the stereo-specific conversion of stearic acid to oleic acid in the bacterium, Corynebacterium diphtheriae. Since oleic acid (cis-9-octadecenoic acid) is the sole product of the desaturation of stearic acid, the reaction is characterized by positional and geometrical stereospecificity. Four stereospecifically labelled monotritiostearic acids were prepared from the enantiomorphic pairs of 9-hydroxy octadecanoate and 10-hydroxy octadecanoate. The four labelled stearic acids were incubated with growing cultures of a strain of Corynebacterium diphtheriae, and the oleate produced by the organism was isolated in each case. Loss of tritium occurred with the 9D and 10D tritio stearic acids, but not with the two L-tritio compounds. The conversion of stearate to oleate is, therefore, stereospecific with respect to the removal of hydrogen at carbons 9 and 10. Isotope effects observed in the formation of oleate suggested that hydrogen removal at carbon 9 precedes hydrogen removal at carbon 10 of stearate.

Goldfine and Bloch (1961) demonstrated in the anaerobic bacteria, Clostridium butyricum and Clostridium kluyveri, a different mechanism of synthesis for monounsaturated acids. In this case, the long chain saturates were not the sources of their corresponding monounsaturated analogues. No conversion of labelled stearic or palmitic acids to oleic or palmitoleic acids

was observed. Laurate and myristate underwent chain elongation, but did not give rise to olefinic products, the C 18 unsaturated acids. However,  $^{14}\text{C}$ -1-octanoate and  $^{14}\text{C}$ -1-decanoate were incorporated equally into both saturates and unsaturates. These workers proposed that a chain elongation process, combined with the introduction of a double bond during elongation, as the predominant mode of synthesis.

Scheuerbrandt et al. (1961) have studied this mechanism in more detail. The monounsaturated acids of C. butyricum consist of two pairs of isomers, 7- and 9-hexadecenoic and 9- and 11-octadecenoic acids. The mechanism of synthesis proposed involves the two carbon addition to octanoate or decanoate, followed by a  $\beta$ ,  $\gamma$  elimination of water from the presumed  $\beta$  hydroxy acid intermediates, followed by addition of further two carbon units without reduction of the double bond. As the two carbon units are added, the distance between the double bonds, and the carboxyl end of the molecule increases. The mechanism can be illustrated as follows:



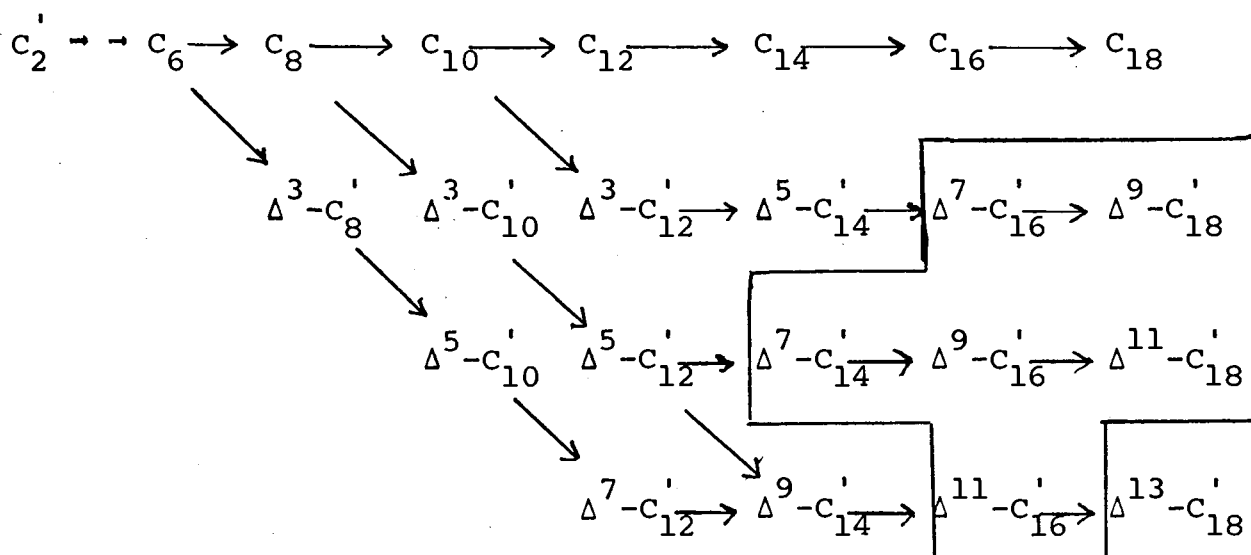
Octanoate is the precursor of the unsaturated acids which contain the double bond between carbons 7 and 8, and the decanoate is the precursor of the two corresponding isomeric acids with the double bond between carbons 9 and 10, counting from the methyl end in each case.



After incubation of the bacterium separately in  $^{14}\text{C}$ -1-octanoate and  $^{14}\text{C}$ -1-decanoate the individual fatty acid fractions were isolated, and the hexadecanoate and octadecanoate fractions, both consisting of the isomers previously described, were cleaved, and the resulting dicarboxylic acids were analyzed. The relative proportions of these esters indicated that the monounsaturated fatty acid isomers were present in the following ratios: 7-hexadecenoic acid to 9-hexadecenoic acid, 60:40, and 9-octadecenoic acid to 11-octadecenoic acid, 37:63. When  $^{14}\text{C}$ -1-octanoate was the precursor, only the C9 dicarboxylic acid from the hexadecenoic acids, and the C11 dicarboxylic acid from the octadecenoic acids was radioactive; whereas in the experiment with  $^{14}\text{C}$ -1-decanoate,  $^{14}\text{C}$  was found predominantly in the C7 dicarboxylic acid from the hexadecenoic acids, and the C9 dicarboxylic acids from the octadecenoic acids. This labelling pattern can be seen in the previously illustrated scheme. The possibility of direct interconversion of double bond isomers was therefore ruled out. The eventual location of the double bond is determined by the length of the medium sized acid at the point where the pathways to saturated and unsaturated acids diverge. Since the chain length of this intermediate is variable, more than one double bond isomer can be produced, as illustrated. This is in contrast to the previously described aerobic mechanism in yeast, in which the

corresponding long chain saturated fatty acids are the precursors, affording only a single monounsaturated acid.

Scheuerbrandt and Bloch (1962) have demonstrated that the non-oxidative pathway, or anaerobic pathway, operates not only in obligate anaerobes, but in various aerobic members of the Eubacteriales as well, including E. coli, Lactobacillus arabinosus, Pseudomonas fluorescens and Rhodopseudomonas spheroides. These workers have further summarized their work by proposing the following scheme (an elaboration of the scheme just illustrated) to show the relationship between this type of synthetic mechanism, and the monoenoic acids actually found in these species (shown in the closed area of the illustration):

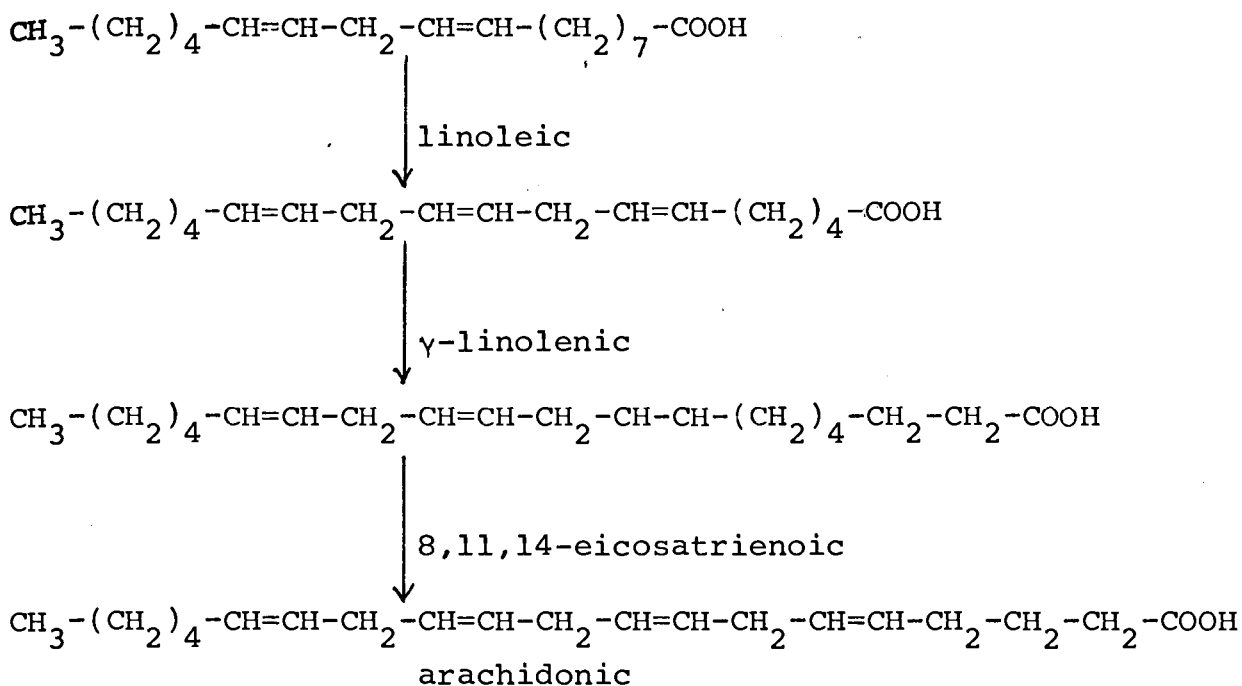


In addition, these workers have demonstrated that this pathway, still referred to as the "anaerobic" pathway, results in primarily 11-octadecenoic acid, as the major monoenoic acid, with smaller amounts of 9-hexadecenoic (palmitoleic) acid and 7-tetradecenoic acid; while the "aerobic" mechanism of synthesis,

discussed previously, results in the  $\Delta$  9 monoenoic acids, palmitoleic and oleic acids, with small amounts of 11-octadecenoic acid formed by elongation of palmitoleic acid. In the organisms employing the "anaerobic" mechanism, no polyunsaturates were detected.

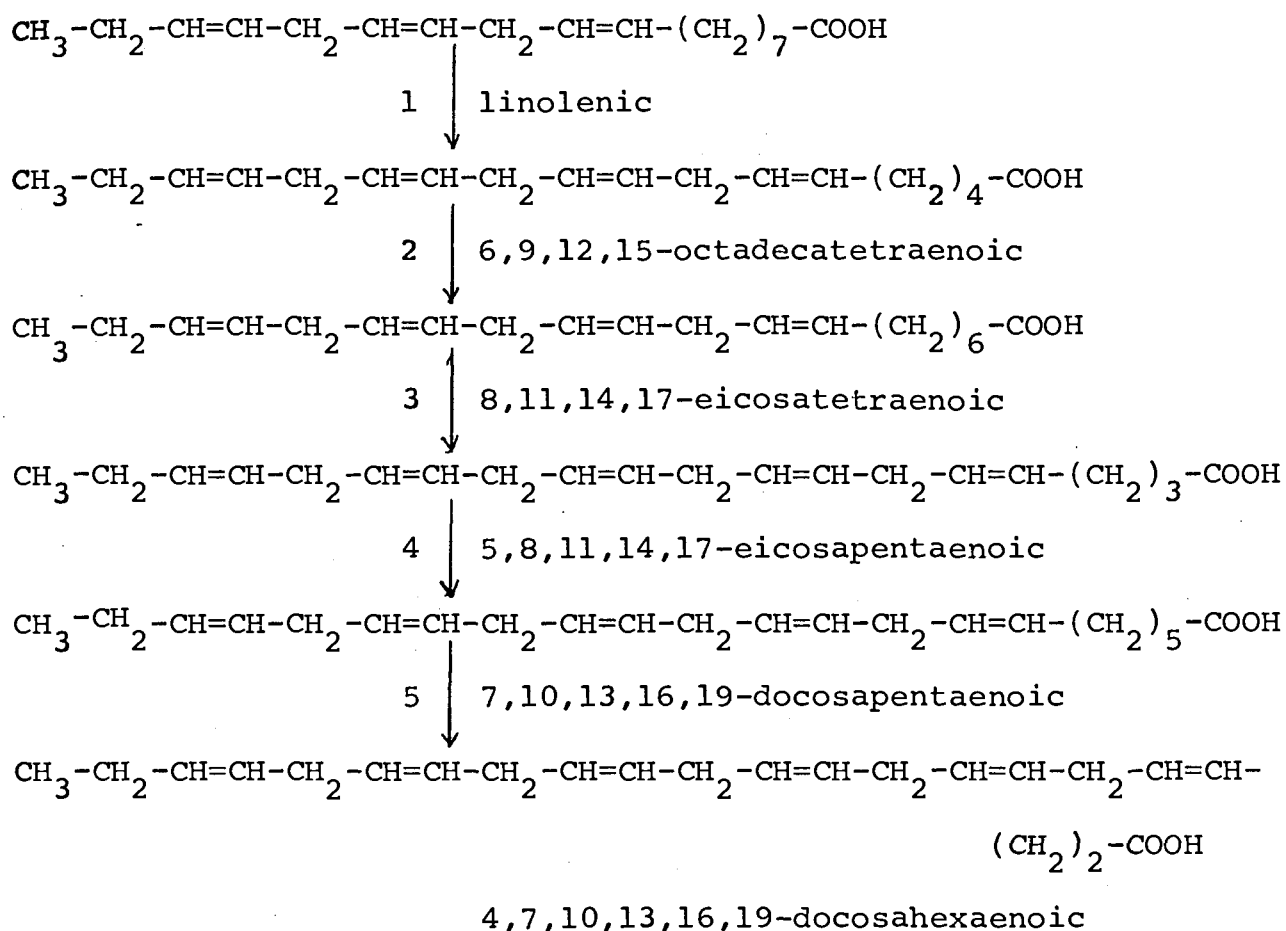
Bernhard and Schoenheimer (1940) demonstrated with tracer studies, that animal tissues could not synthesize polyunsaturates. This view was upheld for many years until the work of Mead and others demonstrated that animals could, in fact, synthesize such acids. Mead, Steinberg and Howton (1953) demonstrated the formation of arachidonic acid from a C 18 precursor by elongation with an acetate derivative. Steinberg et al. (1957) found that arachidonic acid was being formed from linoleic acid, and proposed that the conversion consisted of three steps; one involving chain elongation, and two involving dehydrogenation. The sequence of the steps, however, was not determined. Indirect evidence was obtained to indicate that linolenic acid is converted to eicosapentaenoic acid, a polyunsaturate.

Mead (1961) described the following scheme for the synthesis of arachidonic acid:



The synthesis involves the formation of polyunsaturated intermediates. The pathway was traced by a series of experiments involving the administration of  $^{14}\text{C}$  labelled acids to rats, followed by location of the  $^{14}\text{C}$  in arachidonic acid.  $^{14}\text{C}$ -1-acetate activity appeared only in the carboxyl group of the C 20 acid.  $^{14}\text{C}$ -1-linoleic acid activity appeared in carbon 1, as a result of its breakdown to acetate, and in carbon 3. Activity from  $^{14}\text{C}$ -1-linolenic acid appeared almost exclusively in carbon 3 of the C 20 acid, demonstrating rapid conversion of the former into the later. Finally, the activity from 8, 11, and 14 eicosatrienoic 2, 3  $^{14}\text{C}$  acid appeared only in the corresponding carbons of arachidonic acid. Starting from the existing double bonds of the precursor acid (in this case, linoleic acid) additional double bonds are introduced in the

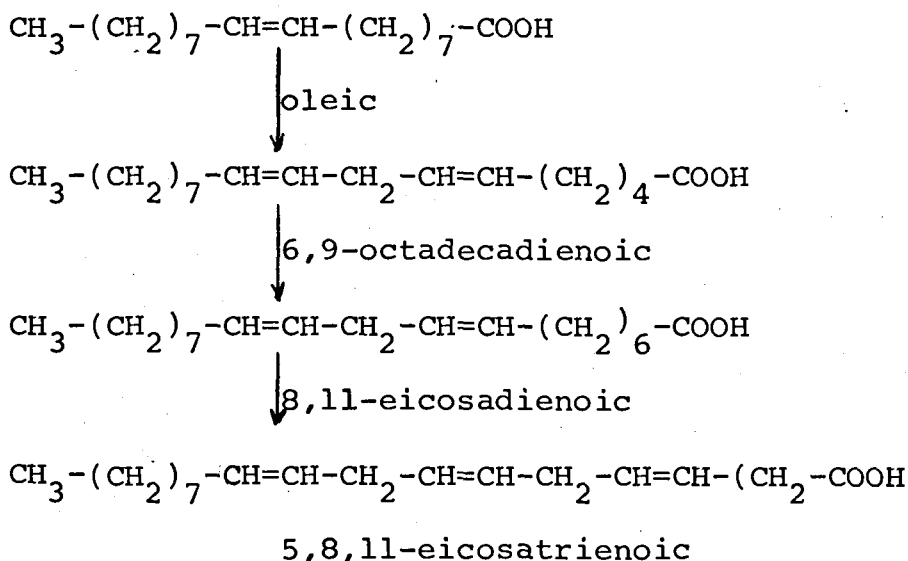
1:4 relationship toward the carboxyl group, until the next additional double bond would be in the  $\alpha \beta$  or  $\beta \gamma$  positions. Chain lengthening then permits the addition of one or more double bonds. Similar experiments with linolenic acid as the precursor acid led to the following pathway resulting in the synthesis of eicosapentaenoic, docosapentaenoic and docosahexaenoic acids:



Mead has therefore demonstrated that the polyunsaturated fatty acids of the animal body could be built up from dietary unsaturated acids by these previously described pathways.

Prior to these studies by Mead, it had been shown

that polyunsaturated acids could be derived from monounsaturated acids, which are themselves synthesized from acetate. Klein and Johnson (1954) while studying the nature of the trienoic acid which accumulates in the tissues of fat deficient animals, found that the acid was primarily 5, 8, 11-eicosatrienoic acid. Comparison of the activity incorporated into this acid from  $^{14}\text{C}$ -1-acetate with that of  $^{14}\text{C}$ -1-oleic acid isolated from the same rats, revealed that the former acid was derived from the later by a pathway such as this:



In addition to the eicosatrienoic acid, the 7, 10, 13-isomer, formed from palmitoleic acid, was also found. The ratio of the former to the later was of the same magnitude as the ratio of palmitoleic to oleic acid.

Mead (1961) has stated that at the present time it seems logical that three enzyme systems are operative in the formation and interconversions of polyunsaturated fatty acids.

There include, a polydehydrogenase, which dehydrogenates mono- or polyenoic acids in positions in the 1:4 relationship from the existing double bonds towards the carboxyl group: an acyl transferase, which adds two carbons when chain lengthening is necessary; and a system which disposes of unsaturated acids with double bonds  $\alpha \beta$  or  $\beta \gamma$  to the carboxyl group.

Yuan and Bloch (1961) have demonstrated in Torulopsis utilis the conversion of oleic acid to linoleic acid. Since the reaction required oxygen, it was suggested that the reaction was similar to that of the "aerobic" pathway, described previously, for the synthesis of oleate from stearate. This idea was supported by the previously described work of Scheuerbrandt and Bloch, 1962, in which no polyunsaturates were found in those organisms which display the "anaerobic" mechanism of monoenoic acid synthesis.

Harlan and Wakil (1962) demonstrated that subcellular particles from rat liver were capable of incorporation of acetyl Co A into saturated and unsaturated fatty acids. Saturated fatty acids were synthesized via a de novo pathway, or by elongation of shorter fatty acyl Co A derivatives. These workers report that their mitochondrial system from rat liver converted <sup>14</sup>C-1-myristate to palmitate, 46%, stearate, 17%, arachidate, 11%, and behenate, 4%. <sup>14</sup>C-1-oleate was converted to C 18 dienoic, C 20 trienoic and dienoic and C 22 trienoic and dienoic

acids. Harlan and Wakil (1963) demonstrated that the process of desaturation leading to the formation of mono- and polyenoic acids could be coupled with both the elongation and the de novo synthetic mechanisms.

Olson (1966) has summarized the pathways of fatty acid metabolism as follows:

PATHWAYS OF FATTY ACID METABOLISM\*

System	Substrates	Cofactors	Product	Cell fraction
<i>De novo</i> formation	Acetyl-CoA + Malonyl-CoA	NADPH, ACP	C <sub>16:0</sub>	Soluble complex
Elongation	1) Long chain acyl-CoA + acetyl-CoA (sat. or unsat.)	NADPH, NADH	C <sub>n+1</sub> :y acyl-CoA (y = 0-6)	Mitochondria
	2) Long chain acyl-CoA + malonyl-CoA (C <sub>10:0</sub> -C <sub>16:0</sub> )	NADPH	C <sub>n+1</sub> :0 acyl-CoA	Microsomes
	3) Unsat. acyl-CoA + malonyl-CoA (C <sub>18:3</sub> > C <sub>18:2</sub> > C <sub>18:1</sub> )	NADPH	Unsat. C <sub>n+1</sub> acyl-CoA	Microsomes
	4) Unsat.-HO-C <sub>18</sub> + acetyl-CoA	NADPH, NADH	Unsat., HO-C <sub>20</sub>	Mitochondria (plant)
Condensation	C <sub>n</sub> acyl-CoA (n = 8, 10, 16, 24)	NADH, Mn, ATP	C <sub>2n</sub> acyl-CoA	Soluble
Cyclopropane acid formation	Unsat. phosphatidyl ethanolamine	S-Adenosyl-methionine	Cyclopropane-phospholipid	Soluble
β-Oxidation	C <sub>n</sub> acyl-CoA (sat. or unsat.)	NAD, FAD	Acetyl-CoA (n = even) Propionyl-CoA (n = odd)	Mitochondria
ω-Oxidation	C <sub>n</sub> acid (n = 8-14 > 16, 18)	NADH, Fe <sup>2+</sup> , O <sub>2</sub>	ω-HO-C <sub>n</sub>	Microsomes (liver) Soluble (bacteria)
α-Oxidation	RCH <sub>2</sub> COOH	NAD, Fe <sup>2+</sup> , ATP DMPH <sub>4</sub> or Ascorbate	RCOOH	Microsomes (brain)
Desaturation	R(CH <sub>2</sub> ) <sub>x</sub> CH=CH(CH <sub>2</sub> ) <sub>y</sub> CO-CoA	O <sub>2</sub> , NADPH (Fe <sup>2+</sup> , FAD)	R(CH <sub>2</sub> ) <sub>x</sub> CH=CH(CH <sub>2</sub> ) <sub>y</sub> CO-CoA	Microsomes
Hydration	R(CH <sub>2</sub> ) <sub>x</sub> CH=CH(CH <sub>2</sub> ) <sub>y</sub> CO-CoA	H <sub>2</sub> O	D-R(CH <sub>2</sub> ) <sub>x</sub> $\overset{\text{OH}}{\text{CH}}-\text{CH}(\text{CH}_2)_y\text{CO-CoA}$	Soluble

\* Abbreviations not previously specified include: HO-, hydroxy; DMPH<sub>4</sub>, dimethyltetrahydropteridine.

It is evident from the previous discussion that the major pathways of fatty acid synthesis described occur in a great many higher and lower forms, throughout the animal and plant Kingdoms. It is likely, therefore, that insects have similar pathways. The purpose of these experiments is to study fatty acid synthesis in vivo in G. mellonella.



MATERIALS AND METHODS

G. mellonella larvae were obtained from a colony reared on a Pablum based diet (Dutky, Thompson and Cantwell 1962). Seventh instar larvae were starved for 24 hours, and then anaesthetized with a mixture of CO<sub>2</sub> and air (2:1 v/v). A solution of <sup>14</sup>C-l-acetate, sodium salt (52.9 mCi/mM) in distilled water (2 μCi/μl), was injected into the hemocoel with a 25 μl microsyringe, dorsally and posteriorly, through the 5th or 6th body segment. The reduced gut volume following starvation minimized bleeding after puncture and injection. Injections were made directly into the hemocoel to minimize any contribution by gut microflora (Bucher and Williams 1967), even though insect microflora probably have little or no influence on fatty acid synthesis (Lambremont 1965; Lambremont, Stein and Bennett 1965).

Preliminary studies with various dosages of <sup>14</sup>C-l-acetate showed that approximately 1 μCi/10 mg body weight was needed to obtain significant activities in the fatty acids, after short exposure periods. Subsequently 1 μCi/9 mg insect weight was injected in all the experiments, to allow direct comparison and analysis of specific activities of all insects. After determination of the initial incorporation phases of acetate into lipids, exposure times of 10, 20 and 30 minutes were found convenient to study fatty acid synthesis. The maximum

radiation dosage, without correction for  $^{14}\text{CO}_2$  expiration was calculated to be 1.83 Rads/gram for insects exposed for 30 minutes. Three replicates of two insects each were exposed for each of the three time periods.

The exposure times chosen allowed measurements of incorporation and turnover following initial compartmentation effects, and before decrease of specific activity from the fat pool, or component pools, characteristic of a pulse-like feeding. This will be discussed in more detail later. In a control test to determine if a pulse-like feeding was occurring, the fatty acid samples were all dissolved in hexane, followed by distillation under diminished pressure and radioassay of the collected hexane, with which any acetic acid present would have co-distilled. No activity was detected, indicating that all free radioacetate had been used or bound some time prior to 10 minutes after injection. Indeed, the turnover of acetate is exceedingly rapid.

After exposure, the insects were homogenized in a tissue grinder, the total lipid extracted (Bligh and Dyer 1959), saponified (Lepper 1950) and esterified with diazomethane (Shlenk and Gellerman 1960). The fatty acids were fractionated, according to their degree of saturation on silicic acid-silver nitrate columns (De Vries 1963). Fatty acid analysis was carried out on a Carlo-Erba gas liquid chromatograph with hot

wire detector and fraction collector. Two meter glass columns (4 mm I.D.) were packed with 15% diethylene glycol succinate on Chromosorb W (AW), mesh 60/80. The carrier gas was helium. Methylated fatty acid standards (myristic acid (C 14:0), palmitic acid (C 16:0), palmitoleic acid (C 16:1), stearic acid (C 18:0), oleic acid (C 18:1), linoleic acid (C 18:2), linolenic acid (C 18:3)) were run before and after each sample, allowing for identification and quantitative calculations. The unsaturated fatty acids used in the standard mixture, palmitoleic, oleic, linoleic and linolenic acids, are specific isomers, each of which belongs to a group of isomers represented as C 16:1, C 18:1, C 18:2 and C 18:3 respectively. However, since the GLC technique used only separates the mixture according to chain length and degree of unsaturation, any one of the isomers in a group may be used as a standard for that group.

The individual fatty acid fractions were collected from the column effluent in side arm collection tubes immersed in a dry ice-acetone bath. Their activity was determined in a Beckman LS-250 liquid scintillation spectrometer. The position of double bonds and the distribution of radioactivity within individual radioactive unsaturated fatty acids were then determined by oxidative cleavage of the unsaturated fractions at the double bond (Von Rudloff 1956; Davidoff and Korn 1963), followed by GLC analysis and radioassay of the monocarboxylic

(derived from the methyl end of the longer chain unsaturated acid) and dicarboxylic (derived from the carboxyl end of the acid) acid products. Standard mixtures of monocarboxylic and dicarboxylic acids were run before and after each sample for identification purposes. The cleavage products were, in turn, collected from the column effluent in glass cartridges containing anthracene coated with silicone oil 550 (50% w/w) (Karmen, Guiffrida and Bowman 1962). The cartridges were then radioassayed directly.

Experiments consisting of exposure for 4 hours, followed by fatty acid analysis and radioassay were also carried out.

Radioactivity loss due to oxidation and  $^{14}\text{CO}_2$  expiration was measured by trapping the expired  $^{14}\text{CO}_2$  in a mixture of 2 aminoethanol and ethylene glycol monomethyl ether (2:1 v/v). The loss was calculated as the percent dosage lost.

## RESULTS AND DISCUSSION

The percentage of individual major fatty acids of G. mellonella calculated from gas chromatograms (Fig. 1) are as follows: C 14:0, 0.3%; C 16:0, 38.0%; C 16:1, 4.7%, C 18:0, 0.8%; C 18:1, 49.8%; C 18:2, 6.4%. No polyunsaturates except C 18:2 were detected. The fatty acid composition is, in general, consistent with that found by other workers (Niemierko and Cepelewicz 1950; Barlow 1964; Young 1964; Yendol 1970). The composition is influenced both by synthetic processes (Table I, II) and by the diet (Fig. 2) on which the insects are reared (Yendol 1970).

Analysis of lipids from insects exposed to  $^{14}\text{C}$ -1-acetate for 10, 20 and 30 minutes (Table I) revealed radioactivity only in the C 18:0 and C 18:1 fatty acid fractions (Table II). Oxidative cleavage of the C 18:1 fraction yielded the dicarboxylic acids, nonanedioic (89%) and undecanedioic (nonanedicarboxylic) acids (11%) (Fig. 3). The C 18:1 monounsaturated fraction is therefore composed of 89% 9-octadecenoic acid and 11% 11-octadecenoic acid. Analysis was not carried out to determine which specific geometric isomers of these two acids were present. All collected activity was found in nonanedioic acid, that is, 11-octadecenoic acid did not incorporate detectable quantities of  $^{14}\text{C}$ -1-acetate during the exposure period.

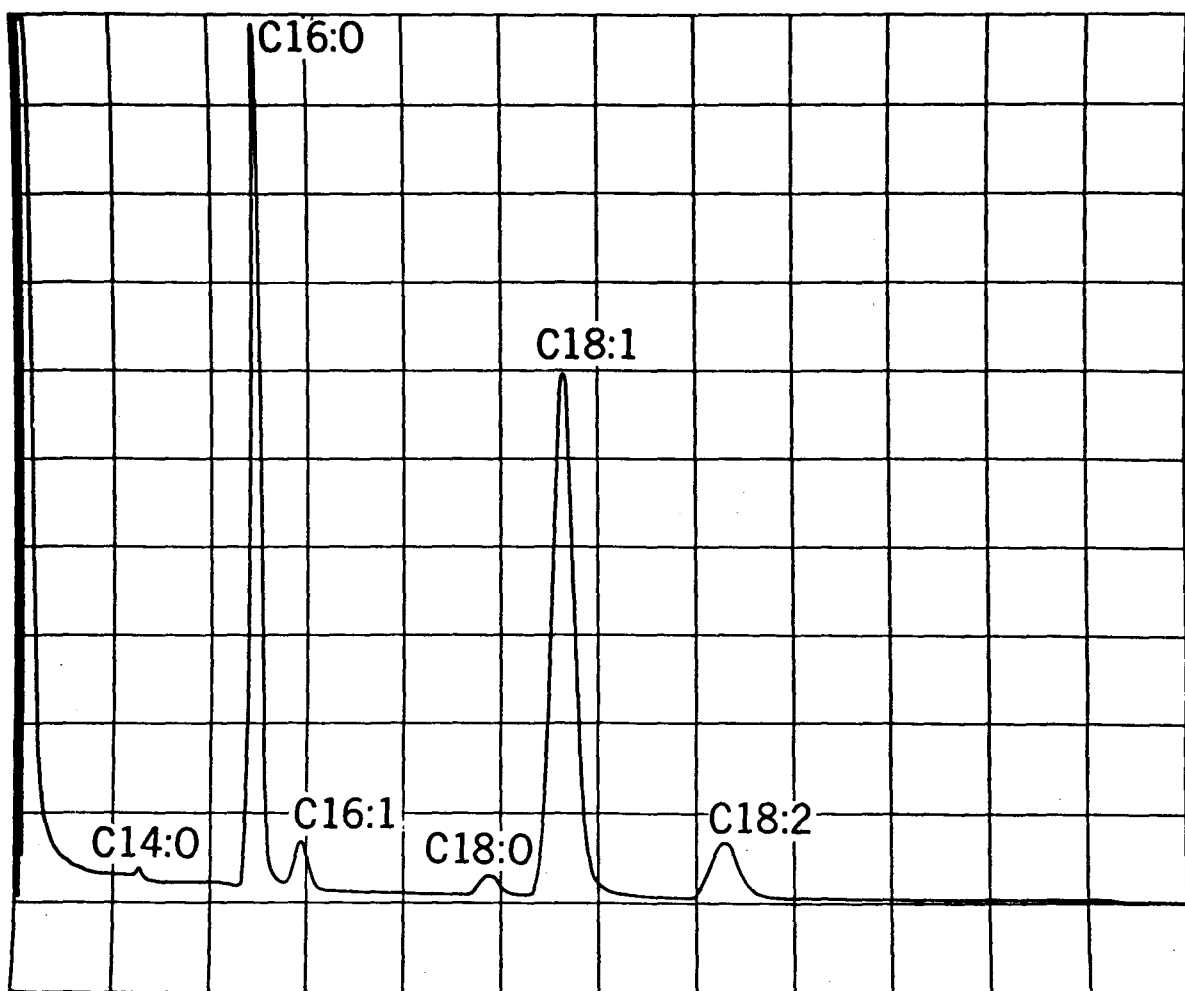


Figure 1 A chromatogram of the fatty acids of Galleria mellonella (L.) (Lepidoptera:Pyralidae).

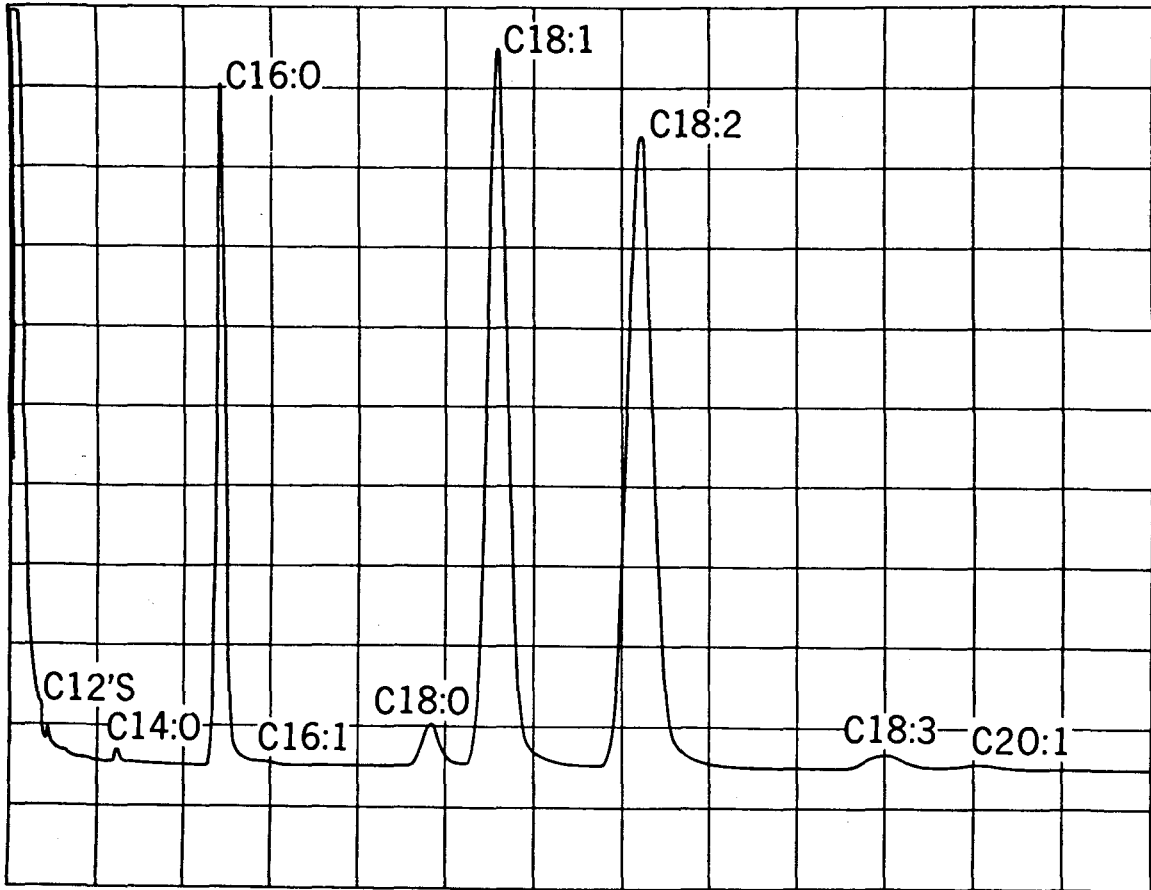


Figure 2 A chromatogram of the fatty acids in the diet on which Galleria mellonella (L.) (Lepidoptera: Pyralidae).

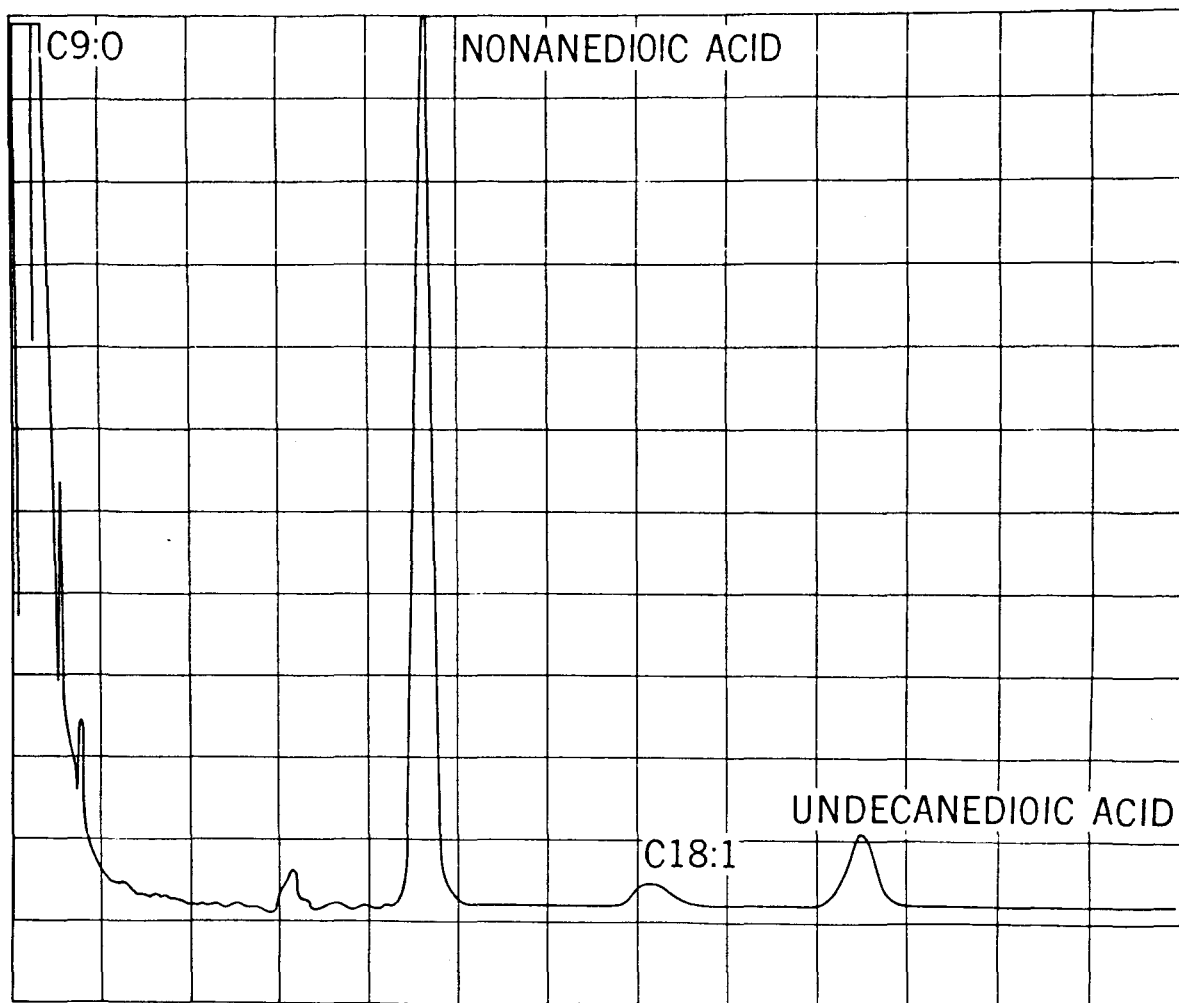


Figure 3 A chromatogram of the oxidative cleavage products of the C 18:1 fatty acid fraction of Galleria mellonella (L.) (Lepidoptera:Pyralidae)



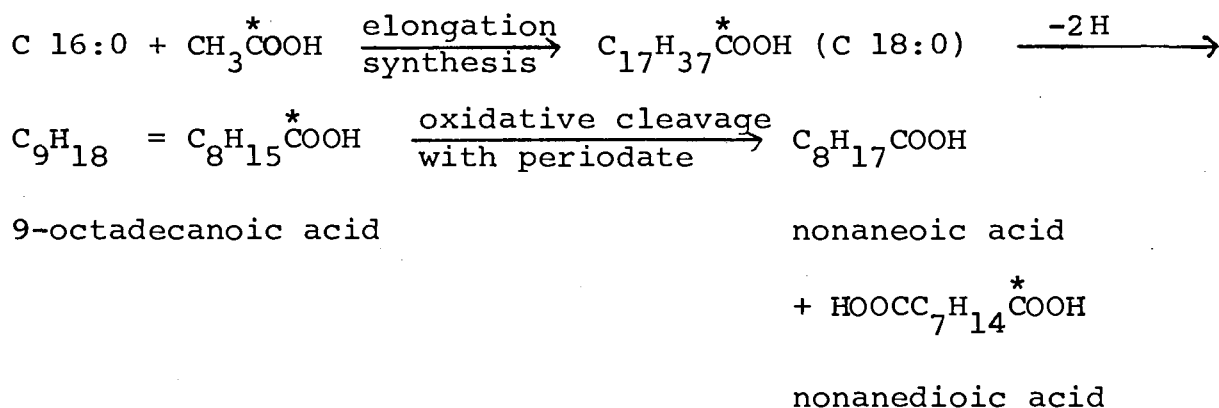
Table I - Injection and extraction data for Galleria mellonella (L.) (Lepidoptera:Pyralidae) exposed to <sup>14</sup>C-1-acetate for 10, 20 and 30 minutes.

Exposure Time (min)	Replicate	Insect Weight (mg)	Injection (μl)	Total Lipids (mg)	Lipids in % of Insect Weight	Fatty Acids (mg)	Fatty Acids % of Lipid Weight
10	1	142.08 159.20	7.9 8.8	13.89	9.22	11.34	81.64
	2	118.40 140.67	6.6 7.1	20.64	13.70	12.42	60.17
	3	137.75 115.30	7.7 6.4	17.36	13.72	14.52	83.64
20	1	123.41 133.75	6.9 7.4	19.31	15.01	14.41	74.62
	2	128.45 117.40	7.9 6.5	17.67	14.37	15.05	85.17
	3	122.25 119.10	6.8 6.5	13.29	11.01	10.54	79.31
30	1	126.50 129.95	7.0 7.2	18.99	14.81	16.51	86.94
	2	132.59 119.25	7.4 6.6	17.45	13.85	-	-
	3	117.70 125.60	6.2 7.0	15.58	12.8	13.26	85.11

Table II - Activity of fatty acids in Galleria mellonella (L.) (Lepidoptera:Pyralidae) after exposure to  $^{14}\text{C}$ -l-acetate for 10, 20 and 30 minutes.  $\bar{x}$  is the mean of the replicates.

Exposure Time (min)	Replicate	Specific Activity of Fatty Acids (dpm/mg)	Specific Activity of C 18:0 (dpm/mg)	Specific Activity of C 18:1 (dpm/mg)
10	1	5,030	19,769	354
	2	8,358	32,658	774
	3	9,107	36,111	317
		$\bar{x} = 7,498$	$\bar{x} = 29,513$	$\bar{x} = 482$
20	1	7,445	28,373	1,409
	2	13,749	53,900	1,096
	3	23,487	93,475	474
		$\bar{x} = 14,893$	$\bar{x} = 58,583$	$\bar{x} = 993$
30	1	2,453	9,508	317
	2	31,313	122,971	2,282
	3	45,066	179,196	1,066
		$\bar{x} = 38,190$ (for 2+3 only)	$\bar{x} = 151,084$ (for 2+3 only)	$\bar{x} = 16,741$ (for 2+3 only)

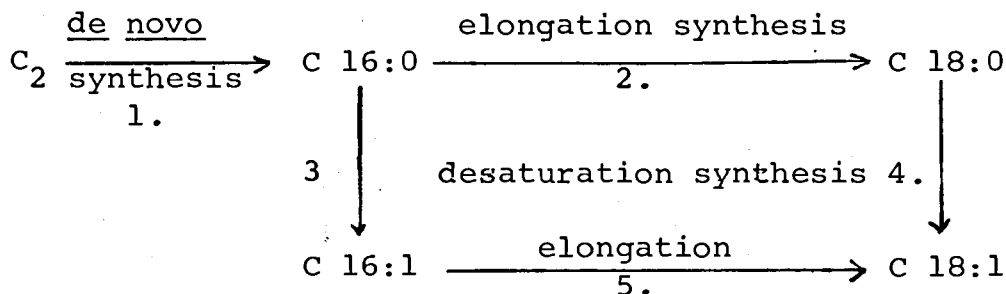
Since insects synthesize monounsaturates by direct desaturation of the corresponding saturated fatty acids (Bade 1964; Sridhara and Bhat 1965), the distribution of labelled atoms will be the same in the monounsaturated fatty acids and their corresponding saturated precursors. Since all the activity detected from the cleavage products of the C 18:1 fraction was found in nonanedioic acid (derived from the carboxyl end of 9-octadecenoic acid), the corresponding saturated acid, octadecanoic acid, is probably being formed by elongation of a shorter chain fatty acid, usually hexadecanoic acid, with  $^{14}\text{C}$ -1-acetate. The reactions occurring can be summarized as follows:



There was no detectable de novo synthesis involving the incorporation of  $^{14}\text{C}$ -1-acetate, during this exposure period. If de novo synthesis had occurred, an equal labelling would occur in both the monocarboxylic and dicarboxylic acid cleavage products. In addition, no elongation of 9-hexadecenoic acid (C 16:1) with  $^{14}\text{C}$ -1-acetate was detected. The elongation of this acid can be illustrated as follows:



If this reaction were occurring activity would be detected in the C 11 dicarboxylic acid product after cleavage of the C 18:1 fraction, since after elongation of C 16:1 (9), the position of the double bond increases two carbon units, from Δ 9 to Δ 11. However, no activity was detected in undecanedioic acid, eliminating the possibility that this reaction was occurring to any extent. The relationship between the two synthetic pathways with respect to C 16 and C 18 acids can be illustrated as follows:



Reactions 2 and 4 have been isolated and no evidence of reactions 1, 3 and 5 was found.

Evidence of de novo synthesis, involving the incorporation of <sup>14</sup>C-l-acetate was not detected until after 4 hours exposure, even though the insects were anaethetized with CO<sub>2</sub>. Increased de novo synthesis occurs with increased bicarbonate ion, used in the conversion of acetyl Co A to malonyl Co A (Gibson, Titchener and Wakil 1958; Wakil 1958). After 4 hours exposure, activity was detected in C 16:0. In an individual collection, the specific activities of individual fatty acid

fractions were as follows: C 16:0, 4 disintegrations/chromatogram unit; C 18:0, 59 dpcu; C 18:1, 1 dpcu. Detection of radioactivity in the C 16:0 fraction is consistent with previous studies demonstrating that the primary product of de novo synthesis is hexadecanoic (palmitic) acid (Wakil 1961; Wakil, Pugh and Sauer 1964). However, no activity was detected in tetradecanoic (myristic) or dodecanoic (lauric) acids, both secondary products of de novo synthesis, which Wakil (1961) reported to appear simultaneously with hexadecanoic acid. Although the specific activities of the fatty acids collected is still very low after 4 hours exposure, the total activities collected were significant and activity in tetradecanoic and dodecanoic acids should have been detected. Lambremont, Stein and Bennett (1965), Bade (1964) and Sridhara and Bhat (1965) have also not detected the synthesis of these acids by de novo synthesis in insects. Therefore, in G. mellonella, and perhaps in other insects, the only product of de novo synthesis appears to be hexadecanoic acid. The shorter chain precursors probably remain bound to the acyl carrier protein, and are, therefore, difficult to detect.

No evidence was obtained to indicate that the saturates and unsaturates are formed by two completely independent mechanisms. Sedee (1961) concluded that, since the unsaturates became more highly labelled than the saturates, and since the two did not obtain the same isotope content during the exposure

period, the unsaturates could not be directly formed from the corresponding saturates by dehydrogenation. However, if the saturates have a higher fractional turnover rate than the unsaturates, they could very likely have a lower specific activity after a considerable exposure time. Strict precursor-product relationships cannot always be expected or found in a complex living organism.

Since only octadecanoic and 9-octadecenoic acids were labelled in G. mellonella, exchange reactions between the terminal carbons of various fatty acids, and between fatty acids and the acetate pool (Barron 1966) were probably minimal.

Regression analyses were carried out on the data in Table II. The specific activities of replicate 1, exposed for 30 minutes, are exceedingly low, and do not reflect the characteristic increasing specific activities observed between the other replicates and the insects exposed for shorter periods. It was assumed, therefore, that something detrimental had happened to one or both of the insects in this group, and these values were not used in the subsequent regression analyses. The equation of the regression curve representing the incorporation of  $^{14}\text{C}$ -1-acetate into the total fatty acid fraction (Table II) was  $y = 1473.4x - 9755.5$  (Fig. 4) ( $y$  = specific activity of fatty acids, dpm/mg;  $x$  = time, minutes), with a correlation coefficient of 0.94. Similarly, the equation for the curve representing

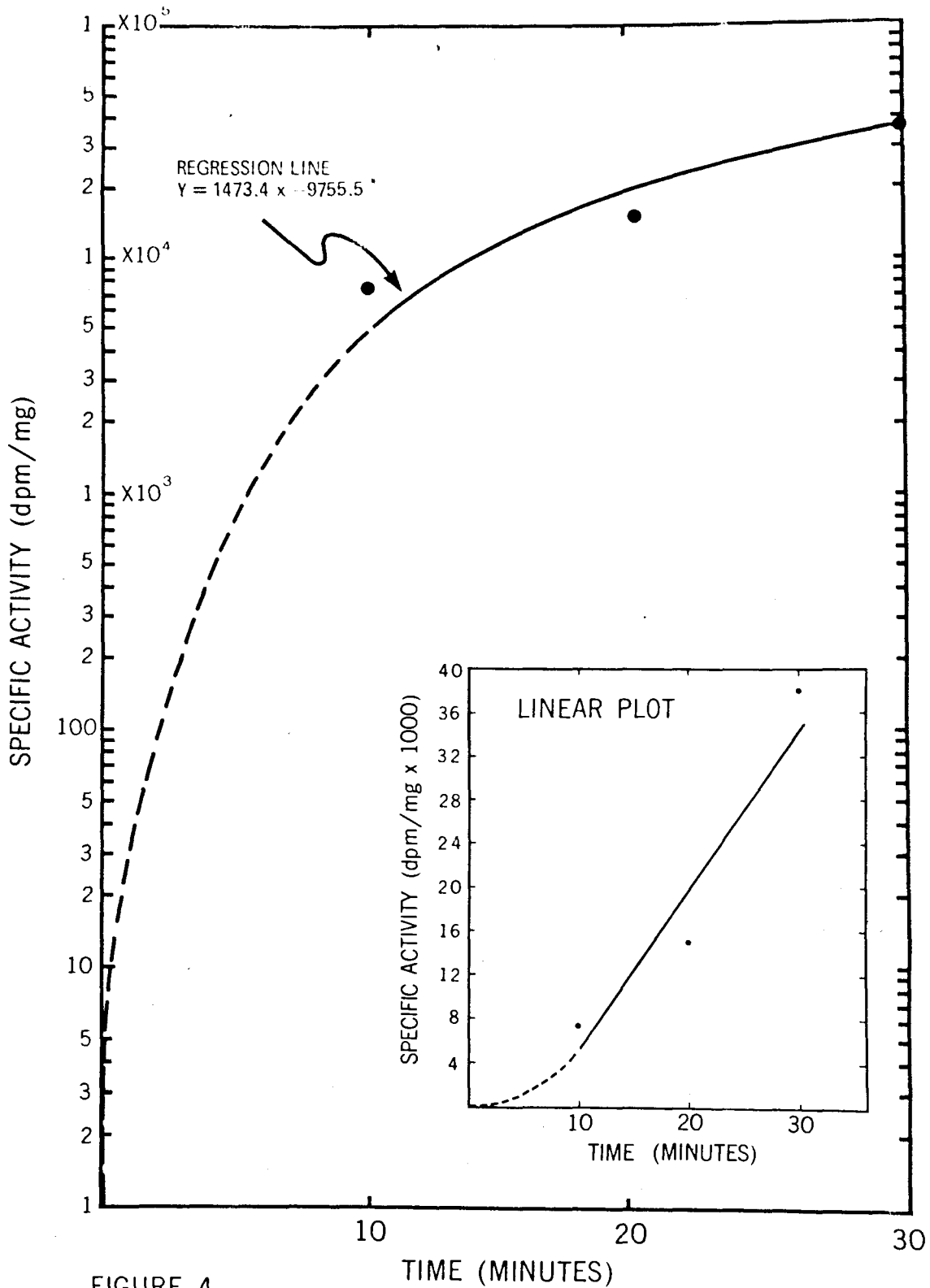


FIGURE 4—

THE INCORPORATION OF  $^{14}\text{C}$ -1-ACETATE INTO THE TOTAL FATTY ACID FRACTION OF *GALLERIA MELLONELLA* (L.) (LEPIDOPTERA:PYRALIDAE) ● REPRESENTS THE MEAN AT EACH TIME PERIOD

\*Significant at 95%

incorporation into C 18:0 was  $y = 5834.6x - 38591.7$  (Fig. 5), with a correlation coefficient of 0.86; and into C 18:1,  $y = 59.1x - 137$  (Fig. 5), with a correlation coefficient of 0.75. The regression curves representing incorporation of  $^{14}\text{C}$ -l-acetate into C 18:0 and C 18:1, from 10 to 30 minutes (Fig. 5) illustrate precursor-product characteristics (Zilver-smit, Entenman and Rishler 1943).

The fractional turnover rates of C 18:0 and C 18:1 (9) were calculated from the incorporation data; and various forms of compartmentation, which are characteristic of in vivo incorporation studies, were discussed.

Table III and Figure 6 summarize and illustrate a typical incorporation of  $^{14}\text{C}$ -l-acetate into lipid components of individual insects, G. mellonella, after various exposure periods. The experiment was carried out on several individual sets of insects, and resulted in the same phases of incorporation illustrated in Figure 6. The exposure times of 10, 20 and 30 minutes were chosen to study fatty acid synthesis, after consideration of the following criteria: 1) They could be reasonably replicated (Table II), such that mathematical determinations of the equations of the incorporation curves could be made, and fractional turnover rates could be calculated.

2)  $^{14}\text{C}$ -l-acetate incorporation was as close as possible (with consideration of 1) to the exponential phase (after 1 hour,



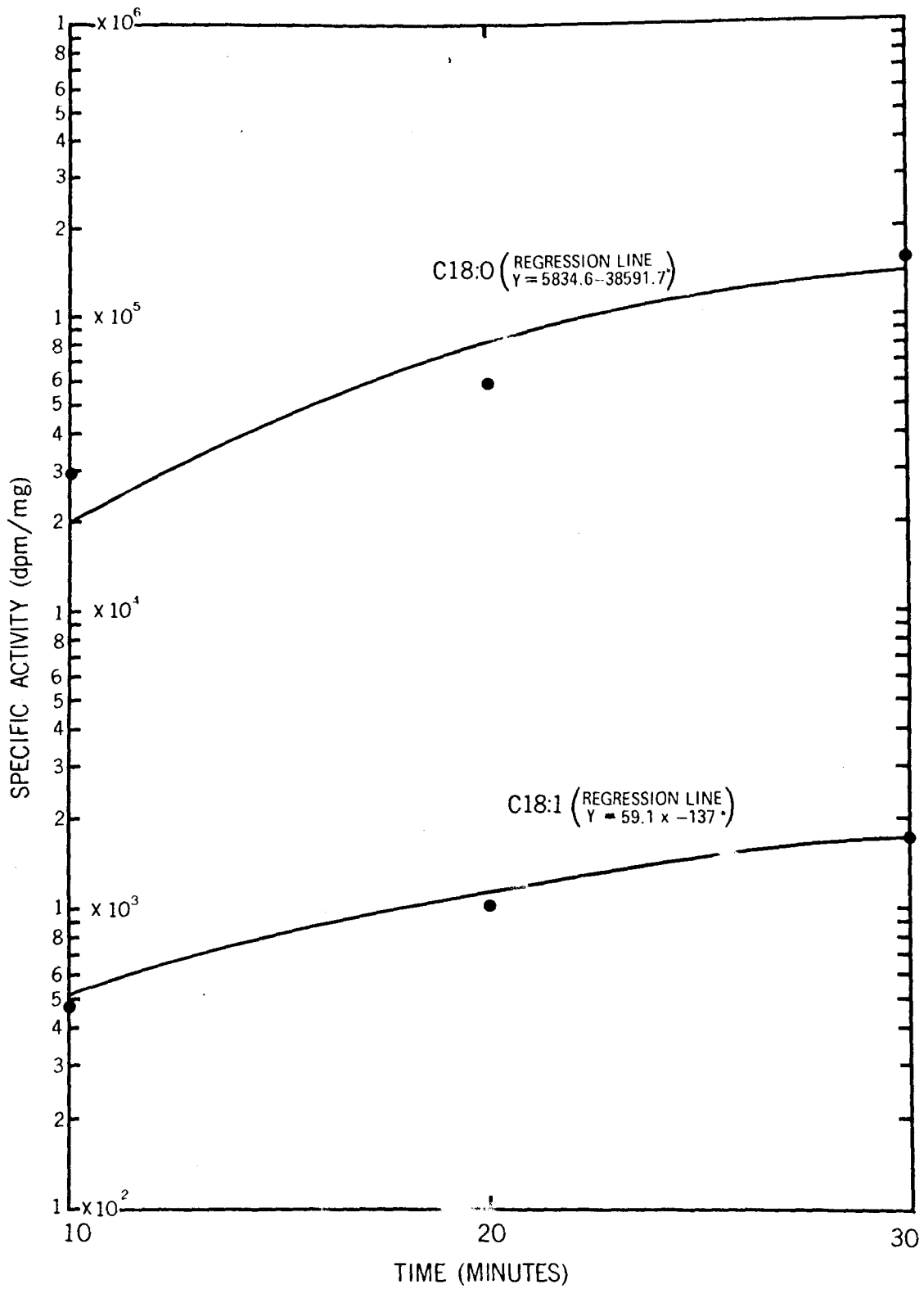


FIGURE 5— THE INCORPORATION OF  $^{14}\text{C}$ -1-ACETATE INTO OCTADECANOIC AND 9-OCTADECENOIC ACIDS IN *GALLERIA MELLONELLA* (L.) (LEPIDOPTERA: PYRALIDAE) ● REPRESENTS THE MEAN AT EACH TIME PERIOD

\*Significant at 95%

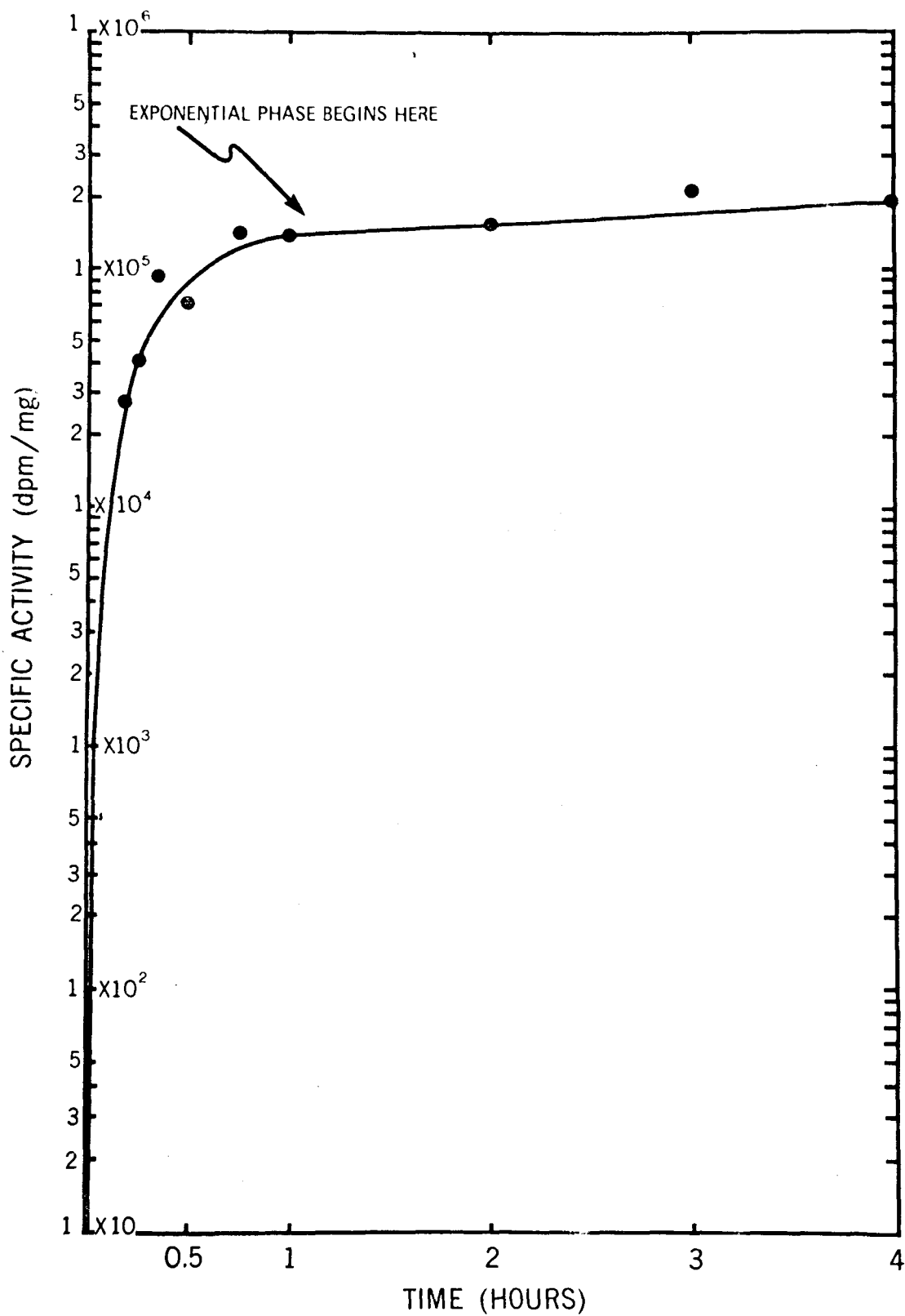


FIGURE 6— INCORPORATION OF <sup>14</sup>C-1-ACETATE INTO LIPIDS OF INDIVIDUAL INSECTS *GALLERIA MELLONELLA* (L.) (LEPIDOPTERA:PYRALIDAE) AFTER VARIOUS EXPOSURE TIMES.

Table III - Incorporation of  $^{14}\text{C}$ -l-acetate into lipids of individual insects, Galleria mellonella (L.) (Lepidoptera:Pyralidae) after various exposure times.

<u>Exposure Time</u> <u>(minutes)</u>	<u>Weight of Insect</u> <u>(mg)</u>	<u>Injection</u> <u>(<math>\mu\text{l}</math>)</u>	<u>Specific Acticity</u> <u>of Lipids (dpm/mg)</u>
1	153.11	8.5	669
5	219.28	12.2	6,035
10	136.45	7.6	28,459
15	150.78	8.4	43,280
25	117.30	6.5	98,472
30	208.52	11.6	74,697
45	167.28	9.3	146,809
60	190.90	10.6	148,990
120	273.43	15.2	157,140
180	196.35	10.9	214,099
240	218.30	12.1	202,362

Figure 6), when the system was equilibrated, and the incorporation rate is a true measure of the turnover rate. 3) They allowed the synthetic processes to be studied before the specific activity of any of the component fat pools decreased significantly due to a pulse type of exposure. Since these times are not quite in the exponential phase of incorporation, linear equations were determined.

If the regression curve representing the incorporation of  $^{14}\text{C}$ -1-acetate into total fatty acids is extrapolated to the origin (Fig. 4), it is evident that a multiphasic increase in specific activity is occurring. The rate of incorporation is slow at first and gradually increases. This lag period of incorporation, which lasts for approximately 10 minutes after injection, is more evident when the curve is plotted linearly (Fig. 4 - insert). The multiphasic increase in specific activity suggests that acetate is contained in a multicompartment system such as that described by Zilversmit and Shore (1952), Beaven (1964) and Montanari, Beaven and Brodie (1963). According to the model proposed in Figure 7, the acetate is first injected into pool 1, the hemolymph or "blood", and slowly diffuses into the second pool, the fat body tissues. At equilibrium, the increase in specific activity will be exponential (Fig. 6), that is, the rate will be constant, and is then a measure of the rate of fatty acid turnover. Since

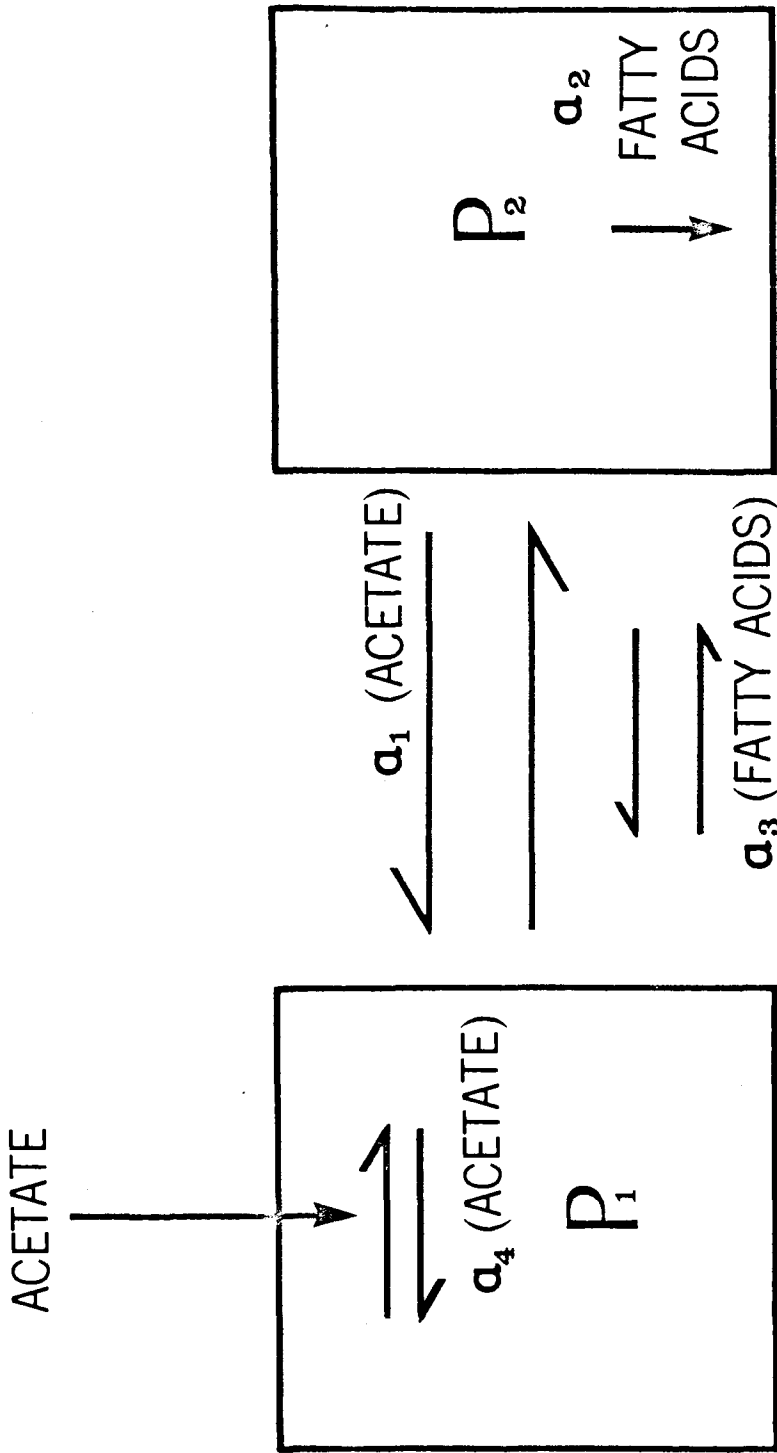


FIGURE 7—  
MODEL SYSTEM OF FATTY ACID SYNTHESIS AND COMPARTMENTATION IN *GALLERIA MELLONELLA* (L.) (LEPIDOPTERA:PYRALIDAE) FROM  $^{14}\text{C}$ -1-ACETATE.  $P_1$  AND  $P_2$  REPRESENT THE TWO COMPARTMENTS, THE HEMOLYMPH AND FAT BODY RESPECTIVELY.  $\alpha_1$  TO  $\alpha_4$  REPRESENT FRACTIONAL RATES OF TRANSPORT AND TURNOVER.

the hemolymph cannot synthesize fatty acids (Chino and Gilbert 1964), fat is synthesized only in pool 2. Therefore, the increase in fatty acid activity will be the least during the early period after injection, when the specific activity is the highest in pool 1 and lowest in pool 2. Following this early lag period, as the two pools approach equilibrium, the specific activity of acetate in pool 2 increases along with the rate of incorporation into fatty acids, which finally, at equilibrium, becomes exponential (Fig. 6, after 1 hour). Although the model is hypothetical, it appears to describe the data and incorporation curves obtained.

This model system can be further characterized by analysis of the experimental incorporation curve of fatty acids (Fig. 4). We can describe this experimental curve as being composed of two component curves with differing slopes, each representing primarily one phase of the multiphasic system previously described. The first component curve is composed of the lag phase portion of the experimental curve from 0 to 10 minutes, and is primarily representative of the equilibrium rate between the two pools. The average slope of this curve is 500 dpm/mg/minute, and the fractional transport rate represented by this slope is  $1.30 \times 10^{-6}$ /minute (calculated according to footnote 2). The second component curve, obtained by arithmetically subtracting the extrapolated first component curve from the

experimental curve after 10 minutes exposure, is a more correct measure of fatty acid synthesis in pool 2. The resolution of the experimental curve (Fig. 4) into these two component curves is shown in Figure 8. The fractional turnover rate of fatty acids (curve 2) is obtained by subtracting the fractional transport of the first component curve, determined previously, from the fractional turnover rate of the experimental curve from 10 to 30 minutes, and is, therefore,  $1.66 \times 10^{-6}$ /minute.

Similarly, the fractional turnover rates of octadecanoic and 9-octadecenoic acids were calculated to be  $7.76 \times 10^{-6}$ /minute, and  $1.9 \times 10^{-8}$ /minute respectively. The halflives<sup>3</sup> are: C 18:0,  $8.93 \times 10^4$  minutes; C 18:1 (9),  $3.65 \times 10^7$  minutes; and the turnover times<sup>4</sup> are: C 18:0,  $1.29 \times 10^5$  minutes; C 18:1 (9),  $5.26 \times 10^7$  minutes. The turnover fluxes<sup>5</sup> were calculated to be C 18:0,  $8.50 \times 10^{-7}$  mg/minute; C 18:1 (9),  $1.28 \times 10^{-7}$  mg/minute. These rates are consistent with those found for various other enzyme systems in vitro (Weast 1968).

It should be noted at this time, that although the characteristics of the incorporation curves have been described as indicative of hemolymph-fat body compartmentation, other alternatives are possible. The lag phase could be due to the distribution of label within the hemolymph itself (Fig. 7). Also, enzyme induction could account for the lag period, although it is unlikely that the enzymes for fatty acid synthesis are

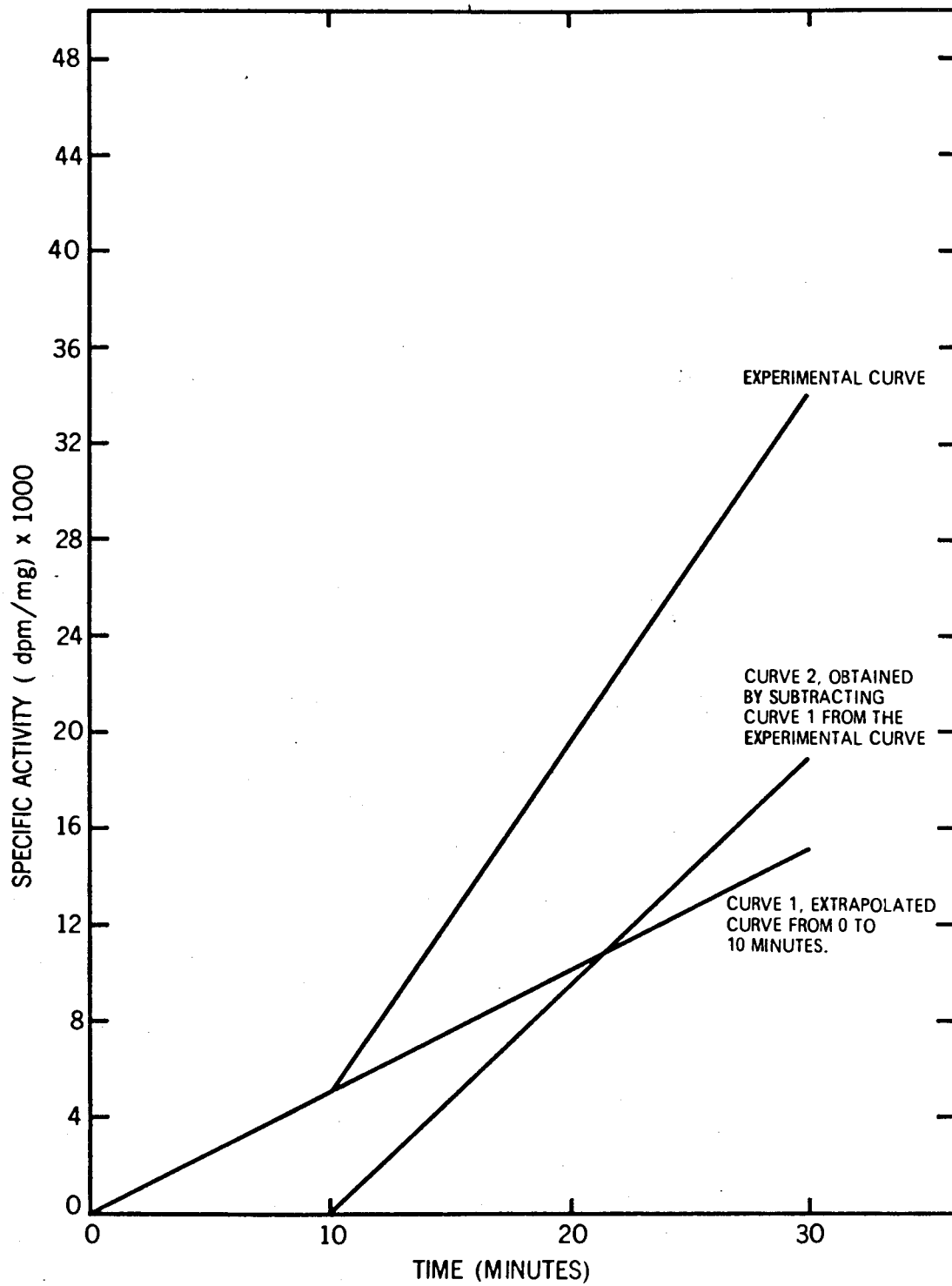


FIGURE 8—  
RESOLUTION OF THE EXPERIMENTAL CURVE (FIG. 4) INTO ITS TWO COMPONENT CURVES,  
ONE REPRESENTING COMPARTMENTALIZATION, THE OTHER, SYNTHESIS.



repressed, particularly at this stage of the life cycle, when the insect is synthesizing "energy stores" for pupation. Other possibilities include other types of compartmentation, both physiological and biochemical.

Regression analysis of the percent dosage lost as  $^{14}\text{CO}_2$  expiration (Table IV) determined the equation of the curve to be  $y = 5.9x + 306.7$  ( $y = \% \text{ dosage} \times 10^{-5}$ ;  $x = \text{time, minutes}$ ) with a correlation coefficient of 0.97. It can be calculated that  $4.83 \times 10^{-3}\%$  of the dosage is lost as  $^{14}\text{CO}_2$  (indirectly representative of oxidation) in 30 minutes, and  $2.08 \times 10^{-2}\%$  within 5 hours. This is not necessarily indicative of a slow oxidation rate, and slow turnover of acetate, since the amount of acetate injected was exceedingly small, and probably negligible in comparison to the total acetate pool.

Table IV - Loss of radioactivity in Galleria mellonella (L.) (Lepidoptera:Pyralidae), due to oxidation of  $^{14}\text{C}$ -l-acetate and  $^{14}\text{C}$   $\text{CO}_2$  expiration.

Trial	Percent activity lost ( $\times 10^{-5}$ ) after various exposure times (minutes)							
	10	20	30	60	120	180	240	300
1	75.3	129.5	239.6	-- $\alpha$	1506.2	1862.4	1464.4	1833.9
2	177	286.7	637.9	6405	1350.4	-- $\alpha$	2099.9	-- $\alpha$
3	251.1	-- $\alpha$	338.6	-- $\alpha$	931.9	1070.7	1583.9	1872.9
4	116.1	171.6	443.6	3588	970.9	-- $\alpha$	1999.1	-- $\alpha$

$\alpha$  Missing data were lost due to a technical error involving the sectioning of the absorber in the fractionation column and sampling before complete remixing of the absorber in the reservoir.

SUMMARY AND CONCLUSIONS

1. The turnover rate of acetate appears to be very rapid.
2. After short exposure periods to  $^{14}\text{C}$ -1-acetate, G. mellonella incorporates activity into octadecanoic (stearic) acid, consistent with the elongation synthetic mechanism. No de novo synthesis, involving the incorporation of  $^{14}\text{C}$ -1-acetate was detected until a few hours after initial exposure. It appears, therefore, that the two synthetic mechanisms of elongation and de novo synthesis can be studied independently, by varying the exposure periods to  $^{14}\text{C}$ -1-acetate.
3. The desaturation reaction, yielding 9-octadecenoic acid, corresponds with the incorporation of  $^{14}\text{C}$ -1-acetate into octadecanoic acid, in a typical precursor-product fashion, but the rate of incorporation is much slower.
4. No elongation of unsaturated fatty acids with  $^{14}\text{C}$ -1-acetate was detected in G. mellonella, in the first 30 minutes of exposure.
5. The fractional turnover rates calculated for octadecanoic acid, formed by elongation of hexadecanoic acid, and 9-octadecenoic acid, formed by desaturation of octadecanoic acid, are within the range of other in vitro enzyme systems.
6. The fractional turnover rate of 9-octodecenoic acid is much lower than that of octadecanoic acid, indicating that the unsaturates may be storage products.

7. Accurate calculations of fractional turnover rates in vivo are impractical at this time. It is evident that multiple physiological and biochemical reactions are occurring at the same time, and resolution of incorporation curves under such conditions is difficult. It should be noted, however, that such graphical resolutions have been carried out, and in some cases, have been proven correct and accurate by later studies. Although the importance of in vivo studies cannot be underestimated, in vitro studies would appear to be more conclusive, at this time.

FOOTNOTES

1. The first number refers to the carbon number and the second, the number of double bonds.
2. The weight of compound corresponding to a given activity can be calculated by using the following formula:

$$W = \frac{0.77 \times 10^{-8} \times \text{mCi} \times A \times t_{\frac{1}{2}}}{\text{number of labelled atoms per molecule}}$$

where W = weight of the compound in mg.

mCi = number of millicuries of activity

A = molecular weight

$t_{\frac{1}{2}}$  = half-life of the isotope involved in days.

The derivation, as described by Francis, et al. (1959), is as follows: The number of atoms in 1 curie of isotope

$$= N \times \frac{W}{A} \quad \text{where } N = \text{Avogadro's number}$$

W = weight of the isotope in grams  
in one curie

A = mass weight of the isotope

The disintegration rate,  $3.7 \times 10^{10}$  dps for 1Ci, is equal to the number of atoms present, multiplied by the disintegration or decay constant,  $K_D$  ( $K_D = \frac{0.693}{\text{half-life}}$ ). Since the disintegration rate is expressed in seconds, half-life must also be expressed in seconds. Thus, if the half-life of an isotope is  $t_{\frac{1}{2}}$  days, the equation is:

$$3.7 \times 10^{10} = \frac{0.693}{24 \times 60 \times 60 \times t_{\frac{1}{2}}} \times \frac{W}{A} \times (6.02 \times 10^{23})$$

$$W = \frac{3.7 \times 8.64 \times 10^{14} \times A \times t_{\frac{1}{2}}}{6.02 \times 0.693 \times 10^{23}}$$

$$= 0.77 \times 10^{-8} \times A \times t_{\frac{1}{2}}$$

and the wt. of an isotope in mg represented by a certain activity in mCi =  $0.77 \times 10^{-8} \times A \times t_{\frac{1}{2}} \times \text{mCi}$

In order to calculate the weight of a compound with a certain activity, the equation is multiplied by:

$$\frac{\text{molecular wt. of X}}{\text{mass wt. of the isotope}} \div \frac{\text{the number of labelled atoms}}{\text{number of atoms}}$$

For example, the shape of the curve in figure 4 represented from 10 to 30 minutes is equal to  $\frac{1473.4 \text{ dpm/mg}}{\text{minute}}$ . Therefore, the weight of compound which is represented by 1473.4 dpm can be calculated as follows:

$$W(\text{mg}) = \frac{(0.77 \times 10^{-8}) (\text{mCi}) \left( \frac{\text{molecular wt.}}{\text{mass wt. of the isotope}} \right) \times A \times t_{\frac{1}{2}}}{\text{number of labelled atoms}}$$

Since we have shown that the labelled molecules are labelled only once, in the C position:

$$W(\text{mg}) = (0.77 \times 10^{-8}) (\text{mCi}) (A) (t_{\frac{1}{2}}) \left( \frac{\text{molecular wt.}}{A} \right)$$

$$W(\text{mg}) = (0.77 \times 10^{-8}) (6.7 \times 10^{-7}) (14) (2.03 \times 10^6) \left( \frac{283}{14} \right)$$

$$= 2.96 \times 10^{-6} \text{ mg. Note that the two 14s cancel.}$$

The slope of the reaction is therefore:  $\frac{2.96 \times 10^{-6} \text{ mg/mg}}{\text{minute}}$

and the fractional turnover rate, K, is  $2.96 \times 10^{-6} / \text{minute}$ .

3. The half-life can be calculated from the following standard radioactive decay relationship:

$$\frac{1}{K} = \frac{-t_{1/2}}{\ln 2} = \frac{t_{1/2}}{0.693} \quad \text{where } K = \text{fraction turnover rate}$$

$$t_{1/2} = \text{half-life}$$

4. The turnover time,  $T_t = \frac{1}{K}$ , since  $KA =$  amount turned over in unit time or time 1, where  $A =$  the total amount of the substance turned over.

$\frac{1}{K}$  is then the time required for the turnover of an amount of  $A$  equal to the total amount originally present in the pool, and therefore is the average life of the molecules in the compartment.

5. The turnover flux,  $T_f$ , is a turnover rate expressed as mg/min, and is therefore dependent on the body pool size.

It is calculated as follows:

$$T_f = (K) (\text{body pool size})$$

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