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ABBREVIATIONS

ATP	Adenosine 5'-Triphosphate
ADP	Adenosine 5'-Diphosphate
ACP	Acyl Carrier Protein
BSA	Bovine Serum Albumin
BHT	Butylated Hydroxy Toluene
CoA	Coenzyme A
Cyt c	Cytochrome C
CL	Cardiolipin
CN	Cyanide
DAG	Diacylglycerol
DGDG	Digalactosyldiglyceride
DCS	N-Acylsphingosine-1-Sulfonic Acid
DDSA	Dodecynyl succinic Anhydride
DMP	Tri-(Dimethyl Amino Methyl)-Phenol
Destun	1,1,1,Trifluoro-4'(phenylsulfonyl)-methansulfo-o-toluidide
EDTA	Ethylene-diamine Tetraacetic Acid
ESG	Esterified Sterol Glycoside
ESR	External Standard Ratio
FAME	Fatty Acid Methyl Esters
FAS	Fatty Acid Synthetase
GLC	Gas-Liquid Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HSS	High Speed Supernatant

INT	2-(p-Iodophenyl)-3-(p-3-Nitrophenyl)-5-phenyl-tetrazolium hydrochloride
Lyso-PC	Lysophosphatidylcholine
MGDG	Monogalactosyldiglyceride
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Reduced Form
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Reduced Form
NL	Neutral Lipid
PVP	Polyvinyl Polipyrolidone
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PG	Phosphatidylglycerol
PS	Phosphatidylserine
PA	Phosphatidic Acid
POPOP	1,4-bis[2(5-phenyloxazolyl)]-benzene
PPO	2,5-Diphenyloxazole
R_f	TLC Mobility Relative To Solvent Front
SG	Steryl Glycoside
SQD	Sulfoquinovosyldiglyceride
SS	24-Methylenecholesterol Sulfate
SE	Sterol Ester
SAN 6706	4-chloro-5-(dimethylamino)-2-(α,α,α,-trifluoro-m-tolyl)-3(2H)-pyridazinone

SAN 9785	4-chloro-5-(dimentylamino)-2-phenyl-3(2H)-pyridazinone
TTGDG	Tetragalactosyl Diglyceride
TGDG	Trigalactosyl Diglyceride
TAG	Triacylglycerol
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl
UDP-GAL	Uridine Diphosphate Galactose
18:1-CoA	Oleoyl-Coenzyme A.
18:1-CoA	Linoleoyl-Coenzyme A

Fatty Acids are abbreviated as follows:

n:x, where n = number of carbon atoms and x = number of double bonds.

ABSTRACT

Under optimum conditions for desaturase activity, three and four products were observed with 18:1-CoA and 18:2-CoA as substrate, respectively, with either proplastids or microsomes. The two major products of 18:1-CoA metabolism were identified as $\Delta^{2,9}$-octadecadienoic acid and 3-hydroxyoleate, a product of hydroxylase activity, while the minor product was identified as $\Delta^{9,12}$-octadecadienoic acid, the product of Δ^{12}-desaturase activity. These products were characterized by the following methods: (a) comigration of products with authentic standards on 10% silver nitrate-silica gel and silica gel TLC plates and on radio-GLC; (b) quantitative hydrogenation; (c) permanganate-periodate oxidation; (d) by gas liquid spectrometric analysis.

The two major products of 18:2-CoA metabolism were identified as $\Delta^{2,9,12}$-octadecatrienoic acid and 3-hydroxylinoleate, a product of hydroxylase activity, while the two minor components were not identified further because of the low amount of material at our disposal. The identification of the two major products of 18:2-CoA metabolism was performed as for the 18:1-CoA metabolic products. No product of Δ^{15}-desaturase activity was detected.

The Δ^{12}-desaturase and hydroxylase activities were found associated mainly with the proplastids and to some extent with microsomes. These observations are in contrast to previous

findings which demonstrated the Δ^{12}-desaturase to be primarily of microsomal origin and the hydroxylase activity either of microsomal origin or soluble.

The Δ^{12}-desaturase of both proplastid or microsomes was also found to behave differently than the Δ^{12}-desaturase of other plant systems with respect to pyridine nucleotide requirements and response to cyanide: the enzyme was found to be insensitive to cyanide and did not have an absolute requirement for exogenous electron donors.

The hydroxylase system did not require exogenous electron donors absolutely, as with the Δ^{12}-desaturase, and was not significantly inhibited by azide and carbon monoxide, inhibitors of electron donors and cyt P-450 respectively. The absence of inhibition by azide and carbon monoxide is in contrast to the strong inhibition of ω- and midchain plant hydroxylating systems and the 3-hydroxylation system in slime mold, but is similar to the insenstivity of the hydroxylating system involved in the formation of ricinoleic acid (12-hydroxyoleate) which does not utilize cyt P-450.

Both hydroxylation and desaturation enzyme activities were found to act on acyl-CoA derivatives and not on phospholipids or glycolipids as substrates; this observation, as far as the Δ^{12}-desaturase is concerned, is also in contrast to the findings in other plant systems showing 18:1 → 18:2 desaturation occurring on phospholipids, mainly PC.

Résumé

Sous des conditions optimales pour l'activité de désaturase, trois ou quatre produits ont été observés avec 18:1-coenzyme A et 18:2-coenzyme A comme substrat, respectivement, avec les proplastides ou les microsomes des cultures de cellules de fèves soja. Les deux produits majeurs du métabolisme du 18:1-CoA ont étés identifiés comme l'acide $\Delta^{2,9}$-octadecadiénoique et l'acide 3-hydroxyoléique, pendant que le produit mineur a été identifié comme l'acide $\Delta^{9,12}$-octadecadiénoique, le produit de l'activité Δ^{12}-désaturase. Ces produits ont été identifiés par les méthodes suivantes: (a) comigration des produits avec des substances authentiques par chromatographie sur couche mince de silice-nitrate d'argent (10%), par chromatographie sur couche mince de gel de silice et par radio chromatographie en phase gazeuse; (b) hydrogénation quantitative; (c) oxidation avec le permanganate-periodate; (d) analyse par spectrométrie de masse.

Les deux produits majeurs du métabolisme du 18:2-CoA ont été identifiés comme l'acide $\Delta^{2,9,12}$-octadecatriénoique et l'acide 3-hydroxylinoléique, un produit de l'activité hydroxylasique, pendant que les deux constituants mineurs n'ont pas été identifiés plus précisement parce qu'il n'y avait que très peu de substance. La méthode employée pour identifier les deux produits majeurs du métabolisme 18:2-CoA était la même que pour les produits métaboliques 18:1-CoA. On n'a pas détecté de produits de l'activité Δ^{15}-désaturasique.

Les activités de désaturase et d'hydroxylase sont associées surtout avec les proplastides et jusqu'à un certain point avec les microsomes. Ces observations contrastent avec les résultats d'autres recherches qui ont démontré que la Δ^{12}-désaturase est surtout d'origine microsomale et que l'hydroxylase est d'origine microsomale oùt est soluble.

Les Δ^{12}-désaturases des proplastides et des microsomes agissent différemment de la Δ^{12}-désaturase d'autres systèmes végétaux si on compare les exigences des cofacteurs pyridines nucléotides et la réaction au cynaure, on trouve que l'enzyme est insensible au cyanure et n'a pas besoin de pyridine nucléotides réduits

Le système hydroxylasique n'a pas besoin absolue en donneurs exogènes d'électrons ainsi que la Δ^{12}-désaturase et son activité n'est pas réduite notamment par l'azide et le monoxide de carbone, inhibiteurs des donneurs d'électrons et du cytochrome P-450 respectivement. L'absence d'inhibition par l'azide et le monoxide de carbone contraste avec l'inhibition très forte des systèmes des hydroxylases végétales (ω- et demi-chaîne) et du système de la 3-hydroxylation dans les moisissures "slime", cette comportement est semblable à l'insensibilité du système hydroxylasique responsable de la formation de l'acide ricinoléique (12-hydroxyoléique) qui n'utilise pas le cytochrome P-450.

On a trouvé que les activités des deux systèmes, hydroxylasique et désaturasique, agissent sur les dérivés acyl-CoA mais pas sur les phospholipides ou les glycolipides comme substrats. En ce qui concerne la désaturation, cette observation, contraste avec les résultats obtenus avec les autres systèmes de plantes qui démontrent la désaturation 18:1 → 18:2 sur les phospholipides, surtout la phosphatidylcholine.

INTRODUCTION

A. Introduction

I. General

A living cell consists of a discrete mass of cytoplasm separated from its environment by a membrane, called "plasma membrane", which defines the boundaries of the cell. The cell can take on a number of different shapes according to its physiological role and the extent of interaction of the plasma membrane with the cytoskeleton. The plasma membrane in forming a continuous barrier around the cell also limits the diffusion of substances in and out of the cytoplasm as result of its lipid core, which being hydrophobic, restricts or controls the passage of water and other charged particles such as anions and cations. The organization of the complex membrane lipids also plays a role in the permeability of the membrane.

One of the most prominent features of the eukaryotic cell is the abundance of subcellular organelles partitioned off by intracellular membranes. These intracellular membranes are similar to the plasma in that the membrane is made up by two layers of polar lipid molecules, but differ radically with respect to their metabolic functions which depend on their constituent proteins. One of the most important functions of these intracellular membranes is to provide a matrix to which enzymes may bind and also form a suitable environment within which

certain enzymes work best. For example, plant membranes are characterized by their high content of polyunsaturated fatty acids such as linoleic and linolenic acids and the properties (e.g. fluidity) which they impart on the membrane are known to affect the activity of many membrane bound enzymes.

A second, and very important function of intracellular membranes is to define compartments within the cell creating a specific environment able to support the function of specialized organelles. Thus, the interesting problems associated with membranes are concerned with the role of lipids as support material for membrane bound enzymes as well as the control mechanism involved in the regulation of membrane lipid or fatty acid biosynthesis. Thus interesting problems associated with the study of membranes are the questions of control of membrane lipid composition and the control of temperature-induced fatty acid changes in membranes lipids.

The present study is concerned with the mechanism and control of fatty acid biosynthesis and membrane lipid composition in plants. We have chosen soybean cell suspension cultures as a useful model for the study of membrane lipids in plants. Specifically, the aim of the present research was to demonstrate in vitro desaturation of acyl-CoA chains (18:1 and 18:2-CoA) by subcellular fractions prepared from these soybean cell suspensions. This, in turn, would help us to answer the question concerning the site of biosynthesis of linoleic (18:2) and linolenic (18:3)

fatty acids, the major constituent fatty acids in plant cells, and the question whether acyl-CoA or phospholipids or both are the actual substrates for desaturation. The answer to these questions should lead to a better understanding of the biosynthetic pathway for 18:2 and 18:3 fatty acids and its regulation, and will thus provide a basis for exploration of some of the more challenging problems, such as the effect of growth temperature on fatty acid composition of membranes and the corresponding activity of membrane bound enzymes. During the course of these studies we found that hydroxyacids were major products of the metabolism of 18:1 and 18:2-CoA of proplastid and microsomal fractions of soybean cultures. We have therefore also investigated the characteristics of the membrane-bound hydroxylation system associated with these cell fractions.

Before proceeding with the experimental section, a brief account of the composition and metabolism of lipids and fatty acids in plants will presented in order to provide a background on this subject.

II. Plant Fatty Acids

a) Major Saturated and Unsaturated Fatty Acids

The major fatty acids of plants are saturated and unsaturated, normal monocarboxylic acids with an even number carbon chain. The saturated fatty acids are lauric (dodecanoic, 12:0), myristic (tetradecanoic, 14:0), palmitic (Hexadecanoic, 16:0), and stearic (octadecanoic, 18:0) while the unsaturated fatty acids are oleic (cis-9-octadecenoic, 18:1), linoleic (cis, cis-9,12-octadecadienoic, 18:2) and linolenic (cis,cis,cis-9,12, 15-octadecatrienoic, 18:3) as summarized in Table I (1). Together these fatty acids account for most of the acid content of higher plants; for instance these fatty acids account for 94% of the total fatty acids of commercial plant oils and 89-97% of the total fatty acids of leaf tissues.

b) Minor Saturated and Unsaturated Fatty Acids

The minor fatty acids of plants are short chain saturated acids such as hexanoic, octanoic, and decanoic acids, differing from the major fatty acids only in carbon chain length. Usually they represent only undetectable proportions of the total fatty acids except in certain oils such as coconut (Cocus nucifer) oil which contain up to 1, 8, and 7% of these acids respectively (2). Longer chain fatty acids such as arachidic (eicosanoic), behenic (docosanoic) and lignoceric (tetracosanoic) acids, have been observed in waxes of seeds (3) such as in sunflower (4), and in leaves (55). Branched chain fatty acids are rare in plants; the simplest case is the accumulation of

Table I

Structures of the Major Fatty Acids[a,b] in Plants

World istribution (%)	Common name	Abbreviation	Structure	Systematic name
4	Lauric	12:0	$CH_3(CH_2)_{10}COOH$	Dodecanoic acid
2	Myristic	14:0	$CH_3(CH_2)_{12}COOH$	Tetradecanoic acid
11	Palmitic	16:0	$CH_3(CH_2)_{14}COOH$	Hexadecanoic acid
4	Stearic	18:0	$CH_3(CH_2)_{16}COOH$	Octadecanoic acid
34	Oleic	18:1(9c)	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	Octadecenoic acid
34	Linoleic	18:2(9c 12c)	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	Octadecadienoic acid
5	Linolenic	18:3(9c 12c 15c)	$CH_3(CH_2CH{=}CH)_3(CH_2)_7COOH$	Octadecatrienoic acid

[a]From Hitchcock and Nichols (2).

[b]The major fatty acids are those responsible for a proportion of fatty acids present in most plant lipids: other acids are ubiquitous but usually present in small quantities (minor acids) or are found in a few sources only (unusual acids).

2-methyl-2-butenoic acid in roots of Angelica archagelica and Croton tiglium (2), while the breakdown of phytol present in chlorophyll may lead to the formation of phytanic acid (3,7,11,15-tetramethylpalmitate) and pristanic acids (2,6,10,14-tetramethylpentadecanoate). In addition, branched chain amino acids, leucine and isoleucine, may lead to the formation of branched chain acids in Antirrhinum majus (6).

Of the minor unsaturated fatty acids, the most important ones are palmitoleic acid (cis-9-hexadecenoic, 16:1), trans-3-hexadecenoic acid, (16:1) found in the C_2 position of phosphatidylglycerol of phostosynthetic tissues, and arachidonic acid. Arachidonic (cis,cis,cis,cis-5,8,11,14-eicosatraenoic, 20:4) acid is not usually found in higher plants, but in mosses and some algae it may represent 40% of the total fatty acids (4), while in some ferns it represents 26% of the total fatty acids (7). Some unsaturated fatty acids found in seed oils of a few plants species have unusual structures. They can be classified as follows: (1) unusual double bond positions, e.g. cis-11-octadecanoic acid (vaccenic acid), found also in bacteria (See section A.VI.b); (2) nonconjugated ethylenic acids with mixed cis-trans configuration, e.g. trans, cis, cis-2, 9, 12-octadecatrienoic acid found in mixed pollen (8); (3) conjugated ethylenic acids such as cis, trans, trans-9,11,13-octadecatrienoic acid, trans,trans,cis-8,10,12-octasecatrienoic acid, are widely distributed in plants (9) where they are found associated mainly

with seed oils; (4) Acetylenic fatty acids, e.g. stearolic acid (18:1 (9a)), have been reported from many plant species (2) and may be derived from normal fatty acids.

(c) Hydroxy Fatty Acids in Plants

The most common substituted saturated and unsaturated fatty acids in plants have hydroxyl groups as major substituents although aldehydes, ketones, epoxides as well as carboxyl groups may also be found.

The 2-hydroxyacids are widely distributed in nature, being found in small amounts in animals, microorganisms, and plants. In plants the 2-hydroxyacid derivatives of common fatty acids are found esterified to cerebrosides, ceramides and phytoglycolipid while in seed oils they are found in triglycerides. For example, Sastry and Kates (10) have isolated a glucocerebroside from runner-bean leaves (Phaseolus multiflorus) which contained only 2-hydroxyacids while in Phaseolus vulgaris Carter and Koob (11) have reported the presence of 2-hydroxyacids in phytoglycolipid, cerebroside and ceramide. In seed oils, the 2-hydroxyacids occur in triglycerides; for example, the seeds of Pachira insignis and Bombacopsis glabra contain 20% and 10% of 2-hydroxysterculic (2-hydroxy-9,10-methylene-oleic acid) acid respectively (12) while the seed oils of Thymus vulgaris have been found to contain 13% of 2-hydroxylinolenic acid (13).

The 3-hydroxy fatty acids are important intermediates in both fatty acid synthesis and β-oxidation of fatty acids and there is evidence, at least in some systems, that these hydroxy fatty acids exist only as intermediates bound to enzyme sulfhydryls as thiol ester (14), and therefore, never accumulate in the cell. Interestingly, Davidoff and Korn (15) have found that a soluble enzyme from slime mold Dictyostelium discoideun was able to transform saturated fatty acyl-CoA derivatives to a series of long chain intermediates, each of which accumulated in considerable quantity. These compounds included long chain D(-)-β-hydroxyacids which are important intermediates in fatty acid synthesis but not β-oxidation and long chain trans-α,β-unsaturated fatty acids which are the postulated intermediates in fatty acid oxidation and synthesis. In addition, long chain cis- and trans-β,α-unsaturated fatty acids, whose metabolic role is not clear, were also formed. These compounds were also produced during the incubation of palmitoyl-CoA with the mitochondrial fraction from guinea pig liver (15). The 3-hydroxyacids have also been reported from other natural sources; 3-D-hydroxymyristic acid has been isolated from E. coli (16), 3-D-hydroxylauric acid from a metabolite of Isaria cretacea (17), and 3-D-hydroxycapric acid from a metabolite of Pseudomonas aeruginosa (18). Several species of the red yeast Rhodotorula graminis (19) have been shown to produce extracellular glycolipids consisting of a mixture of mannitol and pentitol esters of 3-D-hydroxypalmitic

and 3-D-hydroxystearic acids.

Ricinoleic acid, 12-hydroxyoleic acid, is a well characterized component of commercial castor oil, accounting up to 90% of the fatty acids of the seed oil of Ricinus communis (20) where it occurs exclusively in triglycerides.

The triglycerides of seeds from a number of plants have been found to contain conjugated fatty acid dienols with the hydroxyl group present either before or after the conjugated diene grouping. The most important hydroxyacids of these seeds include dimorphecolic (9-hydroxy-trans,trans-10,12-octadecadienoic) acid which was shown to have an unusual trans, trans conjugated double bond and found to be a major constituent (66.5%) of Dimorphotheca sinuata seed oil (21,22) while others had the cis, trans-conjugated dienol system either of 9-hydroxy or 13-hydroxy isomers.

Monohydroxymonobasic and monohydroxydibasic acids occur as important components of cutin and suberin in plant cell walls. Of the monobasic acids, hydroxytetradecenoic and hydroxypentadecanoic acids are most frequently encountered in plant cutins and suberins (23,24), and the positional isomers of both these compounds occur with the hydroxyl groups located mainly in the 9- and 10-position. Of the monohydroxydibasic acids, positional isomers of monohydroxyhexadecane-1,16-dioic acids have been found to occur frequently as minor components of cutin (25). Similar positional isomers of hydroxypentadecane-1,15-dioic acid have also been widely reported by Hunneman and Eglinton (23) but their presence as natural products needs further corroboration.

Perhaps, the most important class of hydroxyacids found in cutins is the ω-hydroxymonobasic acids, which was first identified by Matic (26) and Brieskon and Böss (27), comprising 5-10% of the total monomers of cutin. The principal compounds are the C-16, C-18, C-18:1(cis-9), and C-18:2(cis,cis-9,12) acids. Although the diunsaturated acid (C-18:2) occurs much less frequently, it was found to account for as much as 15% of the total cutin monomers in cultivars of Molus pumila fruit (28); 16-hydroxy-hexadec-9-enoic acid has also been identified as a minor component of the fruit cutin (29). Other hydroxyacids such as the monohydroxyoxomonobasic, dihydroxymonobasic, epoxymono-hydroxymonobasic, epoxydibasic, dihydroxydibasic, and trihydroxymonobasic have also been reported (25) but a complete survey of the distribution of hydroxyacid derivatives is beyond the scope of this thesis.

III. Complex Plant Acyl Lipids

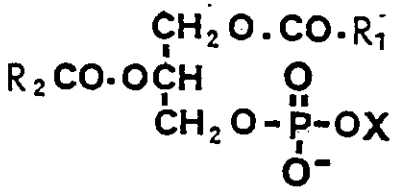
a) Glycerides

The most important member of this class of lipids is triacylglycerol (triglyceride), typical of plant seed oils, in which all three hydroxyl groups of the glycerol moiety are esterified with fatty acids. The less esterified 1,2-diacylglycerol and monoacylglycerol are important metabolic intermediates which do not ordinarily accumulate to any significant levels.

b) Glycerolipids or Phosphoglycerides

Glycerolipids are derived from phosphatidic acid, a key intermediate in glycerolipid synthesis and their structures are shown in Fig. 1. Their biosynthesis in plants is reviewed in detail elsewhere (30,31) and briefly in section A.VIII. Of the phosphoglycerides, phosphatidylcholine is the principal complex lipid in most plant tissues and membranes. Phosphatidylglycerol is also a major glycerolipid of plant tissues found predominantly in chloroplast membranes. It is also of interest because it contains high amounts of trans-3-hexadecenoic acid in the C-2 position. Although, phosphatidylglycerol (PG) has two asymmetric centres and can theoretically exist in any of the four possible sterioisomers, only one isomer is present in nature and shown to be (sn-3-phosphatidyl-sn-1-glycerol). Phosphatidylinositol (PI) is also a major phospholipid in many plant species, but unlike animal systems, plant systems do not synthesize the higher inositides, diphosphoinositide and triphosphoinositide. Phosphatidyl-ethanolamine (PE) is present in plants usually in lower content

FIGURE 1. The major phosphoglycerides.



Basic structure

Base moiety

Phospholipid

X= -H

Phosphatidic acid (PA)

X= $CH_2CH_2\overset{+}{N}(CH_3)_3$

Phosphatidylcholine (PC)

X= $CH_2CH_2NH_2$

Phosphatidylethanolamine (PE)

X= $CH_2CH_2NH.CO\,R$

N-Acyl Phosphatidylethanolamine

X= $CH_2CH(NH_2)COOH$

Phosphatidylserine (PS)

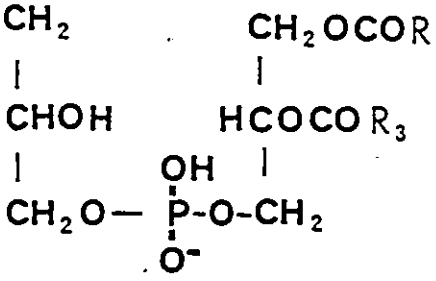
X= Glycerol

Phosphatidylglycerol (PG)

X= Inositol

Phosphatidylinositol (PI)

X=



Cardiolipin (CL)

than PC: recently N-acylderivatives of phosphatidylethanolamine have been found in some seeds (32). Phosphatidylserine (PS), although widely distributed in plants, comprises only a minor portion of the total phospholipids.

Disphosphatidylglycerol (cardiolipin, CL) is a major phospholipid of animal mitochondria and has been found also in plant mitochondria where it represents only a minor fraction. Other phospholipids which occur in plants in small amounts are lysophospholipids and phosphatidic acid; they are either intermediates in the biosynthetic pathway of phosphoglycerides, or cellular catabolites and/or artifacts generated during the lipid extraction procedure. Choline and ethanolamine plasmalogens are generally absent in higher plants but have been reported in some plants species (33). Bisphosphatidic acid, the fully acylated form of phosphatidylglycerol has been found in soybean suspension cultures (34) and has been implicated as a possible intermediate in the biosynthesis of polyunsaturated fatty acids (35).

c) Glycolipids

It is well established that plants tissues contain several polar lipids containing galactose which are called galatolipids. These galactolipids were first isolated and characterized from wheat flour in Carter's laboratory (36,37) and their presence confirmed in photosynthetic tissues (38,39) and runner bean leaves (40). The major galactolipids are the mono- and digalactosyldiglycerides whose structures are shown in Fig. 2.

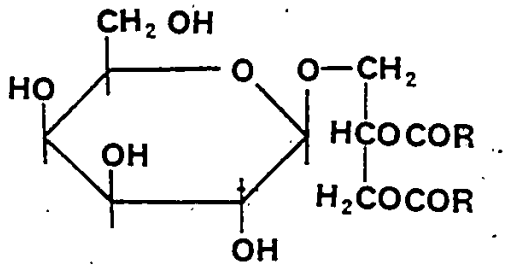
FIGURE 2. The Major Glycoglycerolipids

a. Monogalactosyldiglyceride, MGDG; 1,2-diacyl[β-D-galactopyranosyl (1'→3)]-sn-Glycerol.

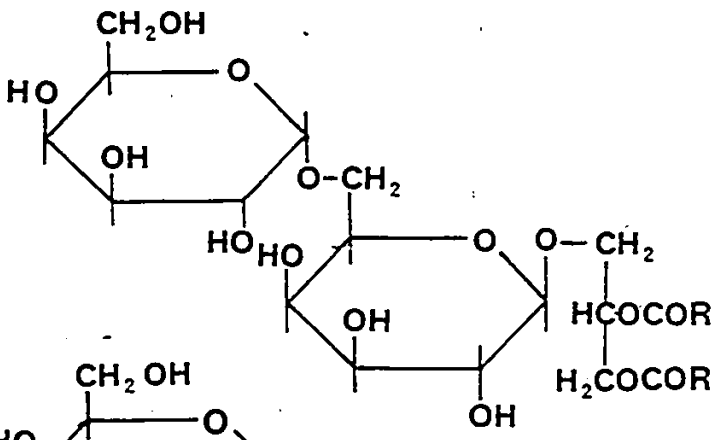
b. Digalactosyldiglyceride, DGDG; 1,2-diacyl[α-D-galactopyranosyl (1"→6')-β-D-galactopyranosyl (1'→3)]-sn-glycerol.

c. Trigalactosyldiglyceride, TGDG; 1,2-diacyl[α-D-galactopyranosyl (1"'→6")-α-D-galactopyranosyl (1"→6')-β-D-galactopyranosyl-(1'→3)]-sn-glycerol.

(a)


CH2 OH
HO
O
O—CH2
HCOCOR
OH
H2COCOR
OH

(b)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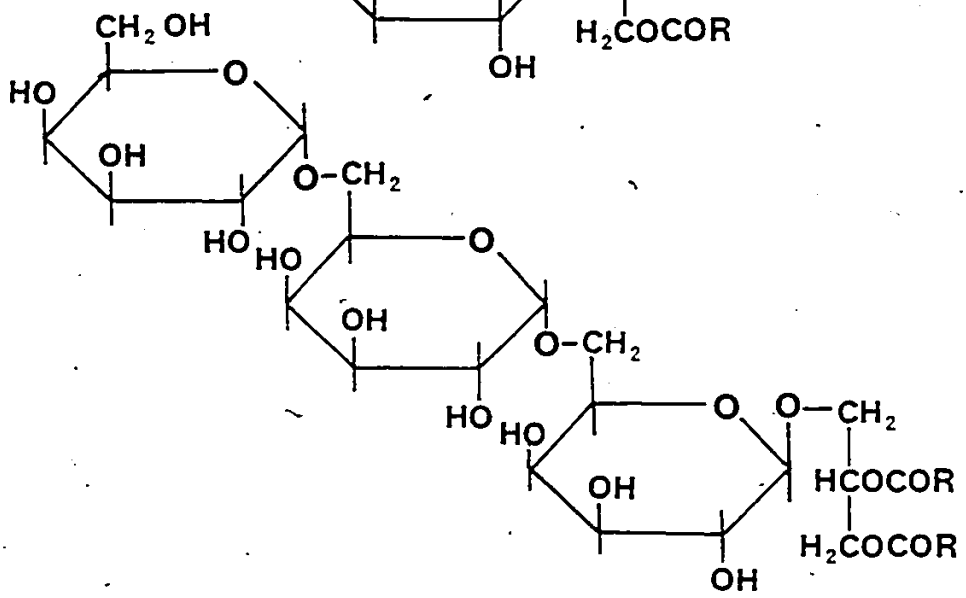
HO
O
OH
O-CH2
HO
HO
O
O—CH2
HCOCOR
OH
H2COCOR
OH

(c)


CH2 OH
HO
O
OH
O-CH2
HO
HO
O
OH
O-CH2
HO
HO
O
O—CH2
HCOCOR
OH
H2COCOR
OH

Galactolipids containing more than two galactose residues, namely trigalactosyl diglyceride (TGDG) and tetragalactosyl diglyceride (TTGDG) have been isolated from many plant species, but in marked contrast to monogalactosyldigyceride (MGDG) and digalactosyldiglyceride (DGDG) these galactolipids constitute only a minor percentage by weight of the total lipids in plants. In addition, several investigators (41-43) have isolated from spinach leaf homogenates a lipid which was more hydrophobic than MGDG and had a composition of glycerol, galactose and fatty acids in a mole ratio of 1:1:3. Further investigation revealed that the third fatty acid residue was esterified at the 6 position of the galactose (6-O-acyl-MGDG) (44). Investigation of spinach leaf homogenates revealed also the presence of other galactolipids which were characterized enzymatically, spectroscopically, and by nuclear magnetic resonance (nmr) as acyl-DGDG and 6-O-acyl-lyso-MGDG (45). These minor acylgalactolipids may be formed by acylation of MGDG, DGDG and lyso-MGDG exclusively in the 6-position of galactose perhaps by an acyl transferase located in the envelope membranes of the chloroplast (46). These major glycolipids, MGDG and DGDG, are concentrated within the chloroplast membranes (47, 30). In thylakoid membranes (stroma and grana lamellae) MGDG and DGDG are present in a ratio of 2:1 while the situation is reversed in the envelope fraction (48,49). It is interesting to note that the conclusions reached for mature chloroplasts are also valid for other types of plastids such as etioplasts, amyloplasts, and chromoplasts (50). While MGDG is synthesized in the envelope

from diacylglycerol and galactose (50-52). The fatty acids of either MGDG or DGDG can be either of microsomal origin in 18:3 plants or of chloroplast origin in 16:3 plants (53).

The MGDG molecule has a single galactose residue on its polar head group. Measurements of [^{13}C]-longitudinal relaxation times indicate that the head group motions of the thylakoid lipids DGDG, SQD, and PG are similar, but those of MGDG are considerably faster (54), indicating that the MGDG head group is smaller than those of the other lipids. Surface pressure versus area isotherms of galactolipids monolayers show that the MGDG film is less expanded than DGDG film (55) consistent with the fact that MGDG has a less bulky head group. The small polar head group and the relatively bulky hydrophobic region of MGDG results in the formation of a cone-shape molecule for this glycolipid (56). Some investigators (57) have concluded that MGDG does not form planar bilayers and that it may be responsible for the curvature in the lamellar membranes. In contrast, DGDG having a more polar and bulky group, is believed (50) to be strongly linked to proteins and probably plays an important role in stabilizing protein conformation in the thylakoid membranes.

d) Sulfolipids

Photosynthetic organisms have been shown to contain two main types of sulfur containing lipids: (1) a series of alkylsulfates and chloralkylsulfates (58,59), occurring mostly in Ochromonas sp. and others species of algae; (2) sulfoquinovosyl-diacylglycerol found in all green plants so far studies, as well

as in algae and photosynthetic bacteria (60-63). Furthermore while the latter sulfolipid is a major lipid in higher plants, in lower plant species it may represent a minor fraction of the total lipids. The structure of this sulfolipid has been established by Benson et al. (64) as shown in Fig. 3d.

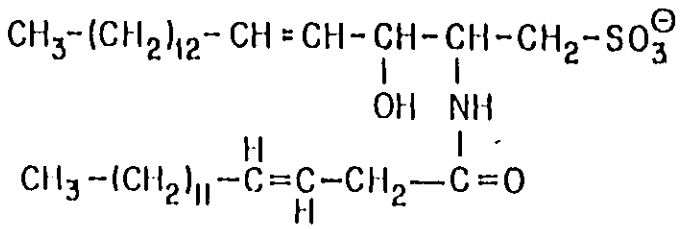
In photosynthetic tissues this sulfolipid is associated with the chloroplasts (65) where it is concentrated in the lamellar membranes (66). Available evidence indicates that this sulfolipid is evenly distributed between the stroma and grana membranes (67) and on the basis of labelling (68) and x-ray difraction studies (69) it was suggested that it may be associated with photosystem I. Other sulfur containing lipids were first detected in several photosynthetic diatoms (70,71) and later identified in the non-photosynthetic diatom Nitzschia alba as a ceramide sulfonic acid (N-acylsphingosine-1-sulfonic acid) (DCS) (72), 24-methylenecholesterol sulfate (SS) (73), phosphatidyl-sulfocholine (PSC) (70,71,74) as well as the ubiquitous sulfoquinovosyl diacylglycerol (SQD) (see Fig. 3a-c). Phosphatidyl sulfocholine completely replaced phosphatidylcholine in the nonphotosynthetic N. alba (75) but all of the photosynthetic diatoms contained both PC and PSC in varying proportions (71).

FIGURE 3. The major sulfolipids.

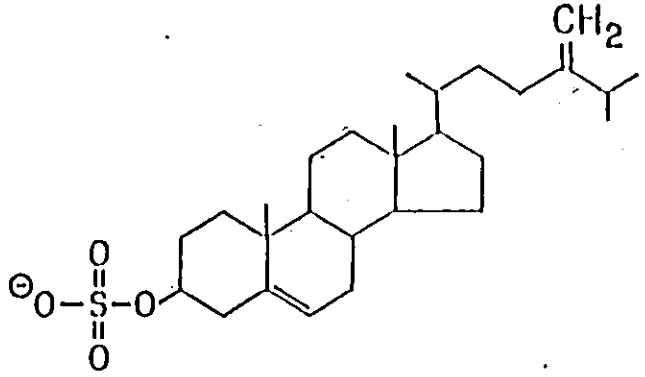
FIGURE 3

SULFOLIPIDS

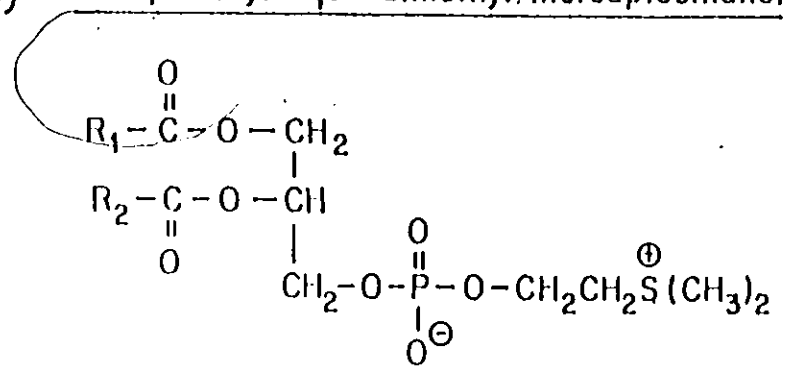
a) N-acyl-1-deoxysphingenine-1-sulfonate



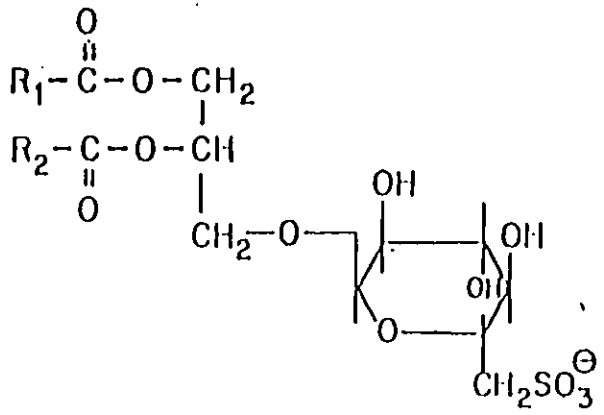
b) 24-Methylene cholesterol sulfate



c) Phosphatidyl S,S-dimethyl, mercaptoethanol



d) Sulfoquinovosyl diglyceride



IV. Distribution of Acyl Lipids and Fatty Acids in Plant Tissue

a) General

Cultured plant cells provide useful and manipulative systems for the study of plant biochemistry. The culturing of plant cells may be initiated from most plant parts (leaf, roots, seedling) of many plant species.

The initial step involves the sterilization of the plant organ followed by incubation of an explant on a suitable solidified growth medium under appropriate conditions (76,77). A callus culture, an undifferentiated, disorganized and proliferating mass of tissue is usually established within a month. Cell suspension cultures are obtained when a friable callus is placed in a liquid medium with suitable agitation to disperse callus clumps into single or small aggregates of cells.

The advantages of using plant cell culture in basic and applied research are numerous, and a few examples are cited below:

i. Cell cultures are available for study all year round.

ii. The life cycles of plant cell cultures are much shorter, whereas those of normal plants take months or years.

iii. The handling of plant cell cultures is much easier than that of normal plant cells, being handled like bacterial or yeast cells.

iv. A greater number of environmental and nutritional manipulations are possible with plant cell cultures than possible with normal plant cells. For instance, environmental

conditions prevailing during growth such as aeration, nutrients, illumination and temperature are known to affect the composition of lipid classes in plant cell cultures as well as the patterns of their constituent fatty acids.. Because of this, it is possible to direct the lipid metabolism toward the synthesis of a valuable commercial product or to study the phenomenon of chilling resistance in order to select chilling resistant cell lines.

v. Protoplasts can be generated more easily with plant cell cultures than normal plant cells.

vi. There is a greater freedom from contaminating organisms (e.g. bacteria) with plant cell cultures than normal plant cells.

These advantages make the plant tissue culture a valuable technique in agricultural programs aimed to breeding oil-bearing plants of high nutritional values, curing disease traits, and particularly important in the propagation of valuable varieties vegetatively.

b) Lipid Classes

Studies on photoautotrophic, photomixotrophic and heterotrophic cell cultures of a variety of plant species have revealed that the composition of lipid classes, as well as the pattern of the constituent fatty acids of these lipids is different. Generally, the lipids of photoautotrophic cell cultures contain higher proportions of monogalactosyldiacyl-glycerols and digalactosyldiacylglycerols, as well as fairly

TABLE 2

Galactolipids and phospholipids in heterotrophic, photomixotrophic and photoautotrophic cell suspension cultures of Peganum harmala*. Values are given in mg/g dry weight.

Lipid	Cell Suspension Culture		
	heterotrophic	photomixotrophic	photoautotrophic
Monogalactosyldiacylglycerols	0.7	2.75	4.1
Digalactosyldiacylglycerols	0.5	1.4	4.6
Sulfoquinovosyldiacylglycerols	0.25	0.7	1.1
Diacylglycerophosphocholines	3.85	3.9	8.65
Diacylglycerophosphoethanolamines	1.7	2.3	4.1
Diacylglycerophosphoglycerols	0.15	0.8	1.8
Diacylglycerophosphoinositols	0.8	1.0	1.1

*Taken from Barz et al (78).

TABLE 3

Constituent fatty acids of total lipids from heterotrophic, photomixotrophic and photoautotrophic cell suspension cultures of Peganum harmala*. Values are given in weight%.

Chain length: number of double bonds	Cell suspension culture		
	heterotrophic (%)	photomixotrophic (%)	photoautotrophic (%)
<16	0.7	0.7	2.2
16:0	24.0	24.0	27.3
16:1	0.1	0.1	0.7
16:3	-	-	tr
18:0	2.5	2.1	2.4
18:1	13.9	14.3	14.1
18:2	43.0	34.9	25.4
18:3	11.0	17.5	21.9
<18	4.2	5.6	5.3

*Taken from Barz et al. (78).

high amounts of sulfoquinovosyldiacylglycerols and phosphatidylglycerols (Table 2). Conversely, the photomixotrophic and heterotrophic cells cultures, from which the photoautotrophic cultures had been derived, contained much lower levels of these lipids (Table 2). The lipids of photoautotrophic cell cultures contained higher proportions of linolenic acid, but lower concentrations of linoleic acid than the corresponding photomixotrophic and heterotrophic cell cultures (Table 3). Thus one of these possible stages of culturing plant cells may be selected with the aim of studying the biosynthesis of specific classes of lipids as well as the biosynthesis of specific fatty acids.

Environmental conditions, which prevail during the growth of cell cultures, affect the relative abundance of different lipid classes and their respective fatty acid composition. For instance, an increase in the rate of aeration results in large proportions of sterols, steryl esters (SE), steryl glycosides (SG) and various sterylglycolipids (79); and while illumination does not affect the relative concentrations of lipid classes in heterotrophic cultures (79), a change in growth temperature results in slight changes in the concentration of lipid classes (80).

Concerning the fatty acids of cell cultures, palmitic acid (16:0) is the major saturated fatty acid while stearic acid (18:0) is present only in small proportions (Table 3); oleic (18:1), linoleic (18:2) and linolenic (18:3) are the major

unsaturated fatty acids (Table 3). In most cases, the levels of 18:2, predominantly associated with PC, are much higher than those of 18:3, predominant in glycolipids. The environmental factor which has the greatest effect on the relative proportions of fatty acids in cell suspension culture is temperature. Generally, the lipids in cell cultures grown at lower temperatures contain higher levels of 18:3 and 18:2 than the corresponding cultures grown at ambient temperature (80).

V. De novo Fatty Acid Synthesis

The enzymes that catalyse the synthesis of fatty acids are collectively referred to as the fatty acid synthesis (FAS) system, a multienzyme complex. The overall process catalysed by these enzymes is summarized by scheme I. Although a homologous series of fatty acids with chain length ranging from 12 to 20 carbons occur in most organisms, the main products of FAS are palmitic and stearic acids. As shown in scheme I, although coenzyme A derivatives of acetate and malonate serve as initial substrates or primers to the FAS, the intermediates of FAS are derivates of acyl carrier protein (ACP). ACP is composed of 4'-phosphopantetheine linked to a protein. In bacteria and eukaryotic plants, the intermediates of FAS occur as free ACP derivates however, in animals and fungi, the intermediates are linked to 4'-phosphopantetheine which is part of a multifunctional FAS protein and the intermediates are not released from the FAS until an appropriate acyl chain length is reached. Although the reactions of fatty acid biosynthesis are universal, the type of organization of the respective enzymes of FAS, as indicated above, vary with the organism and even in a single organism may vary with the growth condition.

Bloch and Vance (81) have distinguished two classes of enzymes involved in the de novo biosynthesis of fatty acids. Type-I FAS is a high molecular weight complex composed of two multi-functional polypeptide chains and is found in yeast, pigeon liver, mammary gland, some bacteria (e.g. Mycobacterium

SCHEME I

General Pathway for Fatty Acid Biosynthesis

I. $CH_3\text{-}CO\text{-}SCoA + ACP\text{-}SH \xrightleftharpoons[\text{acyltransacylase}]{\text{ACP}} CH_3\text{-}CO\text{-}S\text{-}ACP + CoASH$

II. $CH_3\text{-}CO\text{-}S\text{-}ACP + \text{synthase-SH} \xrightleftharpoons{\text{ß-ketoacyl-ACP synthase}} ACP\text{-}SH + CH_3\overset{O}{\overset{\|}{C}}\text{-S-synthase}$

III. $\begin{matrix}COOH\\|\\CH_2\end{matrix}\text{-CO-SCoA} + ACP\text{-SH} \xrightleftharpoons[\text{malonyltransacylase}]{\text{ACP}} \begin{matrix}COOH\\|\\CH_2\end{matrix}\text{-CO-S-ACP} + CoASH$

IV. $CH_3\text{-CO-S-synthase} + \begin{matrix}COOH\\|\\CH_2\end{matrix}\text{-CO-S-ACP} \xrightarrow[\text{ACP synthase (condensing enzyme)}]{\text{ß-ketoacyl-}} CH_3\text{-CO-}CH_2\text{CO-S-ACP} + \text{synthase-SH}$ (with CO_2)

V. $CH_3\text{-CO-}CH_2\text{-CO-S-ACP} + NADPH + H^+ \xrightleftharpoons[\text{ACP reductase}]{\text{ß-ketoacyl}} \begin{matrix}OH\\|\\CH_3\text{-CH-}CH_2\end{matrix}\text{-CO-S-ACP} + NADP^+$

VI. $\begin{matrix}OH\\|\\CH_3\text{-CH-}CH_2\end{matrix}\text{-CO-S-ACP} \xrightleftharpoons[\text{dehydratase}]{\text{ß-hydroxyacyl-ACP}} CH_3\text{-CH=CH-CO-S-ACP} + H_2O$

VII. $CH_3\text{-CH = CH-CO-S-ACP} + NADPH + H^+ \xrightleftharpoons[\text{reductase}]{\text{enoyl-ACP}} CH_3\text{-}CH_2\text{-}CH_2\text{-CO-S-ACP} + NADP^+$

Addition of C_2 units continues until C_{16} is reached; then the acyl group is transferred to an acceptor or cleaved to free fatty acid by palmitoyl-ACP thioesterase.

phlei) and Euglena gracilis (etiolated). Type-II FAS system is composed of a separated ACP and discrete enzyme components and has been found in most bacteria and plants.

The mechanism of fatty acid formation involves the transfer of acetyl-CoA and malonyl-CoA to ACP. These reactions are catalyzed by acetyl-CoA and malonyl-CoA: ACP transacylases, respectively which constitute part of the FAS (Scheme I, steps I and III). The acetyl moiety of acetyl-ACP is subsequently transferred to the sulfhydryl group ("parking" SH-group) of β-ketoacyl-ACP synthesis present at the active site, step II. The next reaction in the biosynthesis of fatty acids is the condensation of the acetyl and malonyl moieties with the loss of CO_2 to form acetoacetyl-ACP. This reaction is catalyzed by β-ketoacyl-ACP synthetase which is referred to as the condensing enzyme. It should be kept in mind that acetoacetyl-ACP intermediate may be either free or bound to the enzyme depending on the source of the FAS. The importance of malonyl-ACP as condensing agent rather than acetyl-ACP (step III) is explained on a thermodynamic basis in which the loss of CO_2 shifts the equilibrium of the reaction toward the condensation reaction. The condensation reaction (step IV) is followed by the reduction of acetoacetyl-ACP to D(-)-β-hydroxybutyryl-ACP (step V). This reaction is catalyzed by 3-ketoacyl-ACP: butyryl-ACP reductase and is specific for NADPH as a reductant and D(-)-stereoisomer as the substrate. The reduction is followed by the dehydration of the hydroxy intermediate to crotonyl-ACP (trans-2-butenyl-ACP)

catalyzed by D(-)-β-hydroxybutyryl-ACP dehydrase (step VI). This enzyme is highly specific for the 3-hydroxy-ACP derivative, and several dehydrases with different chain length specificities have been isolated from different sources (see section A.VI.b). The final chain modification involves the reduction of crotonyl-ACP to butyryl-ACP in a reaction catalyzed by enoyl-ACP reductase, (step VII). The above sequence of reactions are repeated with the malonyl-ACP moiety instead of acetyl-ACP until longer chain lengths, (C_{16} and C_{18}) more prevalent in nature, are formed. The terminal reaction of FAS regulated by β-ketoacyl-ACP synthetase appears to vary with the organism and may include: (1) transfer of acyl group to free ACP to form the ACP ester characteristic of bacteria and plants, (2) transfer of acyl group to CoA to form the CoA ester as is the case in fungi and yeast (3) hydrolysis to the free fatty acid as in mammals.

Recent genetic and biochemical studies on the Type I of the yeast (82-85) or animal (86-88) FAS, have reported that the FAS was composed of two multifunctional proteins, designated as α and β, for the yeast, and α and α for the animal system. The α and β subunits of the yeast contain 3 and 5 of the synthetase functions, respectively and is believed to exist as six copies of each subunit, α_6 β_6 (89) and the synthesis of the two subunits is coded by two unlinked genes (82), fas 2 and fas 1, respectively. The distribution of catalytic function and cofactors between the two subunits for the yeast FAS is summarized in Table 4 Fig. 4A.

TABLE 4

Distribution of Catalytic Functions and Cofactors between the α and β Subunits of the Yeast Fatty Acid Synthetase

Fas 1 (Subunit β)	Fas 2 (Subunit α)
Acetyl-CoA:ACP transacylase	4'-Phosphopantetheine (ACP function)
Malonyl-CoA (palmityl):ACP transacylase[a]	β-ketoacyl synthetase (condensing enzyme)
D(-)-β-hydroxybutyryl:ACP dehydrase	β-ketoacyl reductase
Enoyl reductase	
FMN	

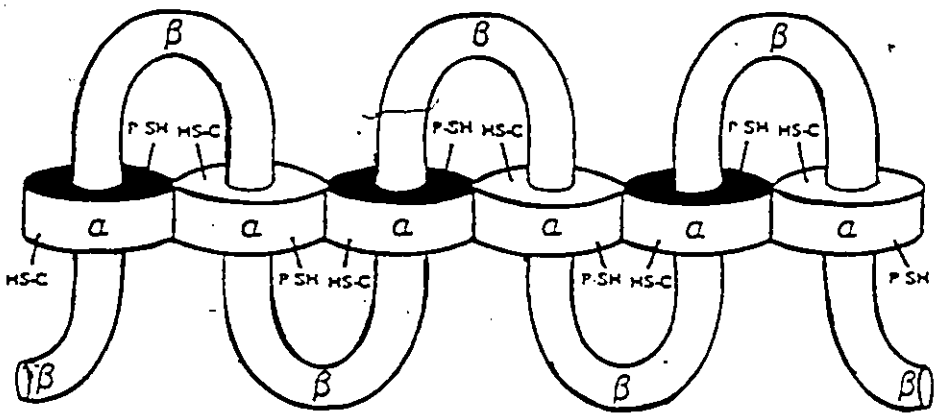
[a] In yeast, the malonyl and palmityl transacylase activities are associated with the same catalytic site (serine hydroxyl) whereas they are distinct sites in animal fatty acid synthetase complexes. Fatty acid synthetase complexes from various sources have been compared by Bloch and Vance (81).

FIGURE 4. Proposed models for yeast and animal FAS complex; Wakil et al (91).

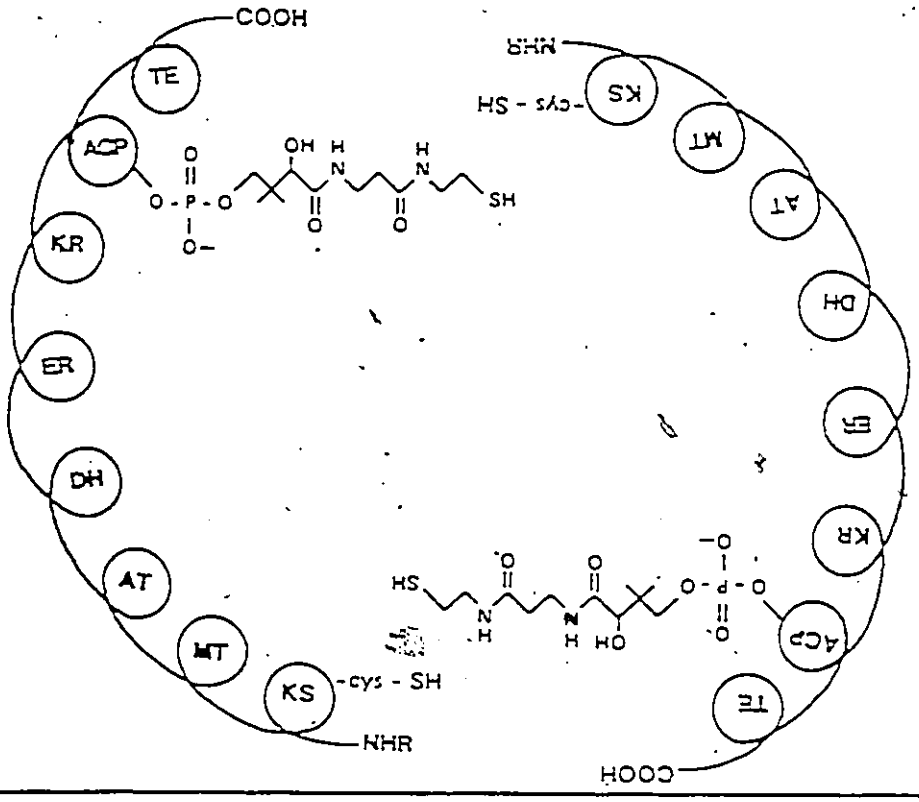
A. a linear model of the yeast FAS depicting the six sites of palmitate synthesis and the complimentary arrangement of the 4'-phospho-pantetheine-SH (P-SH) and the active cysteine-SH(HS-C) at the β-ketoacyl synthetase centres in each of these sites (the activity of each of the polypeptides is as stated in Table 4).

B. schematic representation of animal FAS complex showing the two multifunctional polypeptides and their head-to-tail association to form the enzymatically active homodimer (Abbreviations: AT, acetyl transacylase; MT, malonyl transacylase; KS, β-ketoacyl synthetase; KR, β-ketoacyl reductase; DH, dehydratase; ER, enoyl reductase; TE, thiolesterase; ACP, acyl carrier protein.

A


β
P SH
HS-C
α
HS-C
P SH
HS-C
β

B


COOH
TE
ACP
KR
ER
DH
AT
MT
KS
-cys - SH
NHR
OH
H
N
O
O-P-O
O-
SH

In contrast the animal fatty acid synthetase has two identical polypeptide subunits and each polypeptide contains the sites of the partial reactions required for palmitate synthesis (Fig. 4B).

Knowledge of the organization of FAS enzymes in higher plants has emerged more slowly than for other organisms. As plants are eukaryotes, a type I arrangement might be expected. However, the purification of plant acyl carrier protein similar to the E. coli protein, and the observation that the synthesis of fatty acids in plants appears to be compartmentalized in the plastid, rather than in the cytosoplasm as in animals, suggested that a prokaryotic type II organization might also exist in plants.

In plants, all of the nonassociated enzymes of FAS have ACP as their common acyl carrier. Thus, the localization of this protein in the leaf cell immediately identifies the site of the enzymes. By employing a radio-immunoassay system for the quantitation of ACP protein, Ohlrogge et al (90) were able to demonstrate that in spinach leaf, the ACP protein was localized in the stroma of the chloroplast. In addition, further work in spinach leaf has demonstrated that acetyl-CoA synthetase (91) and acetyl-CoA carboxylase (B.J. Nikolan, personal communication) were also localized in the chloroplast. Thus, the evidence is overwhelming that the chloroplast is the site for the synthesis of palmitoyl-ACP which is further

elongated to stearoyl-ACP followed by desaturation to oleoyl-ACP. Thus, the previously observed association between eukaryotes and type I FAS structure does not hold for plants, the most abundant class of eukaryotes.

Recently Shimakata and Stumpf (92) have isolated two different β-ketoacyl-ACP synthetase enzymes. The first synthetase produced primarily palmitate when incubated with the other FAS enzymes. However, upon addition of the second β-ketoacyl-ACP synthetase, the product shifted primarily to the production of stearate. Thus, it appears that the palmitate to stearate elongation requires a second condensing enzyme, whereas the other enzymes have broader substrate specificity which can act on all chain lengths from C_{12} to C_{16}. The acyl-ACP's synthesized are in turn hydrolysed by a oleoyl-ACP thioesterase, present in the chloroplast (93), acting on both palmitoyl-ACP and oleoyl-ACP to release the respective free acids. These fatty acids are subsequently converted to acyl-CoA by a acyl-CoA synthetase found associated with the envelope of the chloroplast (94-96) and either released into the cytoplasm of the cell or used for acylation of lipids.

Shimakata and Stumf (92), have speculated on the possible stoichiometry of the FAS complex in vivo, and suggested that there might be two types of noncovalent complexes. The major type of association, including stearyl-ACP desaturase and oleoyl-ACP hydrolase, would provide oleate for export from the

plastid, while the second type of complex lacking in palmitoyl-ACP elongase, might provide palmitoyl-ACP for direct acylation to e.g. glycerol-3-phosphate. Thus, the presence of two condensing enzymes would provide a method of channelling the different metabolic routes. In addition, the chloroplast, through its Photosystems I and II, is able to generate ATP, NADPH, and O_2 which are all required in the synthesis of these fatty acids.

This new information on the similarity between the organization of plant and prokaryotic FAS lends further support to the theory of an endosymbiotic origin of the chloroplast.

Recent studies on soybean (Glycine max) cell suspension cultures have also shown the FAS to be located in the proplastid fraction (97-98).

VI. Biosynthesis of Unsaturated Fatty Acids

a) General

The mechanism of biosynthesis of the unsaturated fatty acids follow two completely different pathways: The anaerobic pathways which occurs in prokaryotes (anaerobic and some aerobic bacteria) and the aerobic pathway which has been demonstrated in a variety of eukaryotic systems from mammals, bird, fishes, plant, algae and also in some bacteria, is summarized in Table 5, (99-102). While the anaerobic system is associated largely with primitive organisms (prokaryotes), the aerobic system arose when higher forms of life (eukaryotes) superceded the more primitive ones.

b) Anaerobic Pathway of Monoenoic Acid Biosynthesis

Anaerobic desaturation involves the retention of the cis-double bond formed during the course of biosynthesis of fatty acids by the FAS complex. The mechanism to explain how this might arise was put forward by Scheuerbrandt and Bloch (103) who were stuyding the biosynthesis of unsaturated fatty acids in the anaerobic bacterium Clostridium butyricum.

This organism, like most other bacteria, produces only monounsaturated fatty acids. Two pairs of monoene isomers are produced: Δ^7- and Δ^9-hexadecenoic and Δ^9- and Δ^{11}-octadecenoic acids. The formation of these isomers can be explained if there are branch points in the FAS complex at C_8 and C_{10} as

TABLE 5

Organisms Known to Synthesis Unsaturated Fatty Acids by A) Anaerobic and B) Aerobic Pathway

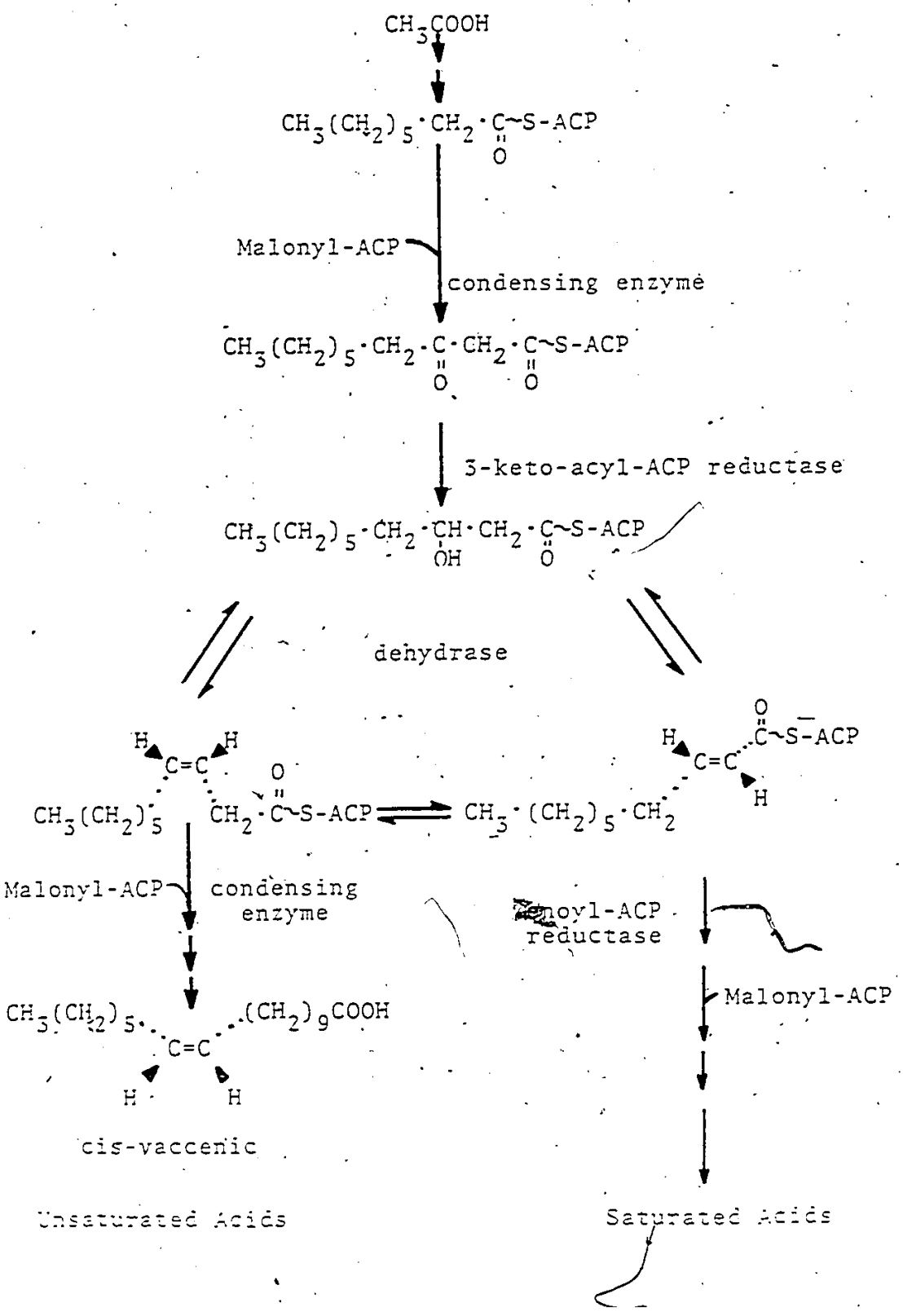
A)	Anaerobic Pathway[a]	
	Bacteria[b]	*Clostridium butyricum*
		C. kluyveri
		C. pasteurianum
		Escherichia coli[c]
		Lactobacillus arabinosus
		Pseudomonas fluorescens
	Photosynthetic bacteria[b]	*Rhodopseudomonas capsulata*
		R. gelatinosa
		R. palustris
		R. spheroides
		Rhodospirillum rubrum
B)	Aerobic Pathway[d]	
	Bacteria	*Bacillus megaterium*
		Corynebacterium diphteriae
		Micrococcus lysodeikticus
		Mycobacterium phleib
	Algae	*Anabaena variabilis*
		Ochromonas malhamensis
		Porphyridium cruentum
		Poteriochromonas stipitata
		Chlorella vulgaris
	Euglenids	*Astasia longa*
		Euglena gracillis[c,e]
	Yeasts	*Saccharomyces cervisiae*[c]
		Torulopsis utilis
	Metaphyta	Avocado (mesocarp)[e]
		Castor bean (leaf)[c,e]
		Castor bean (developing seeds)[c]
		Spinach (leaf)[b]
	Metazoa	Fish (liver)[c]
		Goat (liver)[c]
		Hen (liver)[c]
		Rat (liver)[c]
		Sheep (intestinal epithelium)[c]

a. Erwin and Bloch (99); Wood *et al* (100).
b. Whole cells.
c. Active subcellular systems have been prepared.
d. Erwin and Bloch (99); Bloch *et al* (101); James (102).
e. The "plant pathway" can be demonstrated in these systems.

shown in Fig. 5. At these branch points, the normal trans-2-enoyl-ACP is isomerized to cis-3-enoyl-ACP which is not a substrate for the enoyl-ACP reductase, but can undergo chain elongation. Therefore, the trans-isomer formed at the branch point will give rise to a long-chain saturated fatty acid while the cis-isomer will give rise to a long-chain cis-unsaturated fatty acid. The position of the double bond in the final products will depend on the position of the branch point. Subsequent studies on the FAS complex of E. coli (105) revealed the presence of an enzyme system which catalysed the dehydration of 3-hydroxy-decanoyl-ACP (β-hydroxy-decanoyl) to a mixture of trans-2 and cis-3-decenoates, and the inter-conversion of these two isomers. The enzyme was called β-hydroxy-decanoyl thioester dehydratase, and has been purified (106-107) from FAS by making use of the property that the dehydratase is stable on heating at 50°C for 10 min., while the synthetase is completely denaturated after only 2 min. under the same conditions. The purified enzyme has been shown to produce mainly trans-2-enoyl-ACP (85%) while the complete FAS system produced predominantly unsaturated fatty acids. The explanation given to reconcile these differences is that the rate of C_2 addition to the cis-isomer is very much faster than the corresponding reductive step of the trans-2-isomer, thereby resulting in the accumulation of unsaturated products.

FIGURE 5. The anaerobic pathway for the synthesis of monounsaturated fatty acids; Gurr and James (104).

FIGURE 5


CH3COOH
CH3(CH2)5·CH2·C~S-ACP
O
Malonyl-ACP
condensing enzyme
CH3(CH2)5·CH2·C·CH2·C~S-ACP
O O
3-keto-acyl-ACP reductase
CH3(CH2)5·CH2·CH·CH2·C~S-ACP
OH O
dehydrase
H C=C H
CH3(CH2)5
O
CH2·C~S-ACP
CH3·(CH2)5·CH2
H C=C H
O
C~S-ACP
Malonyl-ACP
condensing enzyme
Enoyl-ACP reductase
Malonyl-ACP
CH3(CH2)5 (CH2)9COOH
C=C
H H
cis-vaccenic
Unsaturated Acids
Saturated Acids

Using a specific inhibitor (3-decynoyl-N-acetyl-stearamine) Kass and Bloch (105) were able to specifically inhibit the synthesis of unsaturated fatty acids *in vivo* and *in vitro* while the production of saturated fatty acids was not affected. It seemed, therefore, that two dehydratases were present one for the synthesis of the trans-2-isomer and the other for the cis-isomer (see Fig. 5). This idea was confirmed by the finding of Silbert and Vagelos (108) that an *E. coli* mutant, lacking the specific dehydratase could synthesize saturated acids normally, but not unsaturated acids. A closer scrutiny has revealed that the β-hydroxy-decanoyl thioester dehydrase has the ability to catalyse both dehydration and isomerization reactions (109) by way of enzyme-bound trans-2-decenoyl thiolester. The enzyme has been shown to consist of two polypeptide chains of almost equal molecular weight each of which contains both dehydration and isomerization activities. The inhibitory effect of 3-decynoyl-N-acetyl-stearamine was due to its irreversible binding to functional groups of the enzyme that are involved in both activities.

Altogether three dehydratases have now been found to be involved in fatty acid synthesis in *E. coli* (110), one dehydratase specific for the dehydration of β-hydroxy-decanoyl-ACP, the second specific for the dehydration of shorter β-hydroxyacyl-ACP (C_4 to C_8) and the third specific for the dehydration of longer chain β-hydroxyacyl-ACP (111).

It seems, therefore, that although a specific dehydratase is essential for the synthesis of long chain unsaturated fatty acids, it is not responsible for the proportions of these acids: in vivo the relative rates of the two reactions, reduction and chain elongation, will determine the proportions of saturated and unsaturated acids. In particular it explains why octanoic acid is the highest homologue of the saturated fatty acid series, while the lower C_8 homologue intermediate leads to the formation of vaccenic acid, Δ^{11}-18:1, characteristic of bacteria as the only octadecenoic acid present in some strains of E. coli (112). While the 3-hydroxydecanoyl thiolester dehydrates has been found only in those organisms which synthesize their unsaturated fatty acids via the anaerobic pathway, this anaerobic pathway is not strickly confined to anaerobic bacteria but is also present in facultative aerobes (e.g. E. coli) and obligate aerobes (e.g. Pseudomonas) as shown in Table 5.

Other bacteria (e.g. Micrococcus lysodeikticus, Bacillus megaterium, Corynebacterium species and Mycobacterium phlei) synthesize their unsaturated fatty acids via the aerobic pathway of desaturation (Table 5) (99-113).

c) Aerobic Pathway of Fatty Acid Desaturation

1. Biosynthesis of Monoenoic Fatty Acids

The most important pathway of fatty acid unsaturation in eukaryotes is by an oxidative mechanism in which the double bond is introduced directly into the preformed long chain saturated fatty acid with oxygen and NADPH as cofactors. This pathway is almost universal and is used by yeasts, higher animals, protozoa, algae, higher plants and some bacteria (Table 5). Thus far, no organism has been found that contains both pathways of desaturation. The mechanism of desaturation has been particularly difficult to demonstrate because the desaturase enzymes are membrane bound and the substrates used are in the micellar form at concentrations usually employed in in vitro studies. Work by Oshino et al., (114) and Holloway and Katz (115), led to the identification of three microsomal bound proteins involved in desaturation of stearate to oleate in rat liver: a flavoprotein, NADH-cytochrome b_5 reductase; a heme-containing protein, cytochrome b_5; and the terminal desaturase itself which, because of its inhibition by low concentrations of CN is usually referred to as the cyanide sensitive factor. Strittmatter et al., (116) have purified the terminal desaturase and found it to be a single polypeptide chain of M.W. 53,000 containing one non-heme iron atom per mole of enzyme. The iron can be reduced in the absence of substrate, by NADH and the electron transport protein (NADH-cytochrome b_5 reductase and cyt b_5); when iron is lost the

activity of the enzyme is lost as well. A possible function of the reduced iron is to bind oxygen which, after an activation process not well understood, can accept the hydrogens abstracted from the substrate. The postulated roles of these components in the overall desaturation process is illustrated in Fig. 6. Briefly, the reducing equivalents from the electron donors (NADH or NADPH) are transferred sequentially to cytochrome b_5 reductase then to cytochrome b_5 and finally to the terminal desaturase; the reduced desaturase in turn reduces molecular oxygen to water with a concomitant re-oxidation of the enzyme. The desaturation of acyl chains can occur in the form of CoA or ACP thiolesters. The substrates for Δ^9-desaturation of saturated acids has been shown to be the acyl-CoA in yeasts (117); seed tissues (118), bacteria (119), and animal tissue (120,121). In contrast, Foot et al., (122) have suggested that Δ^9-desaturase in the psychrophilic bacterium Micrococcus cryophilus may take place at the level of phospholipids.

Although the above described aerobic desaturation pathway is very widespread, higher plants and some algae have a distinct desaturation pathway of their own. For example the pathway of oleate biosynthesis was shown to have myristate as a precursor and not palmitate and/or stearate even though the latter were effectively transferred without desaturation to acyl lipids (123-125). This anomaly was resolved by Nagai and Bloch (126) when they found that a soluble preparation

FIGURE 6

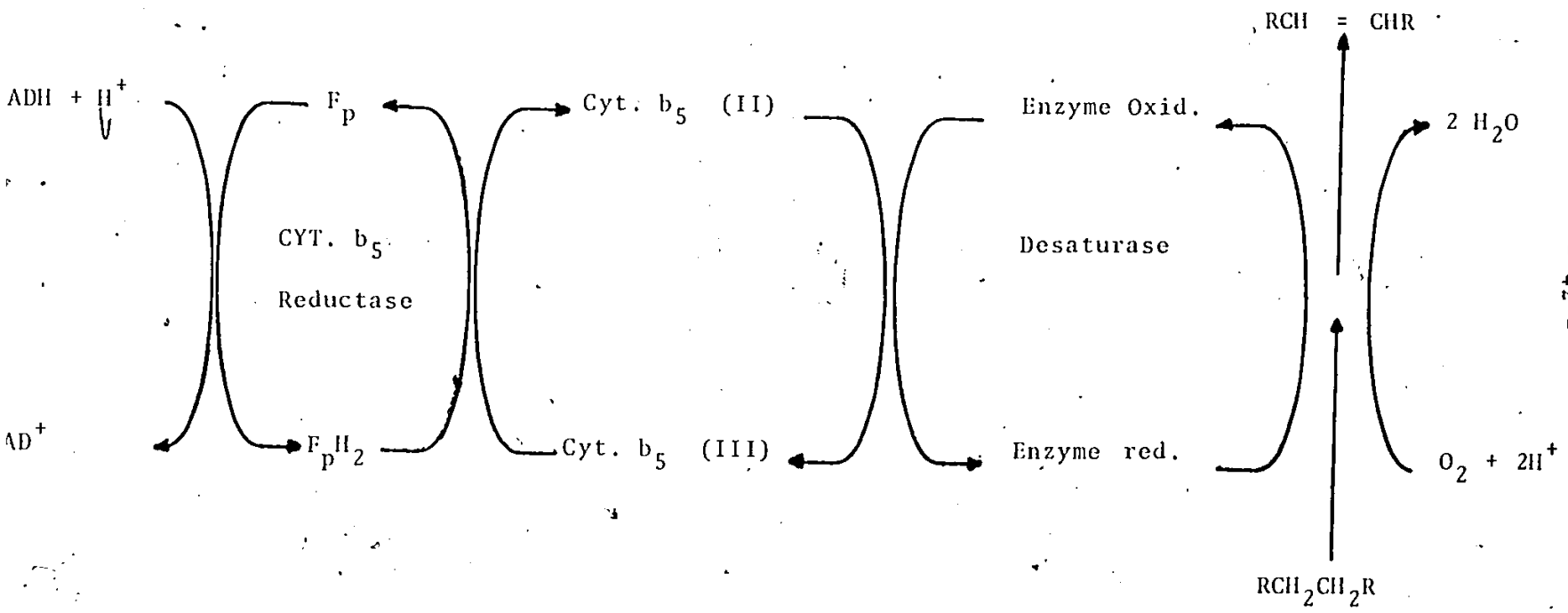
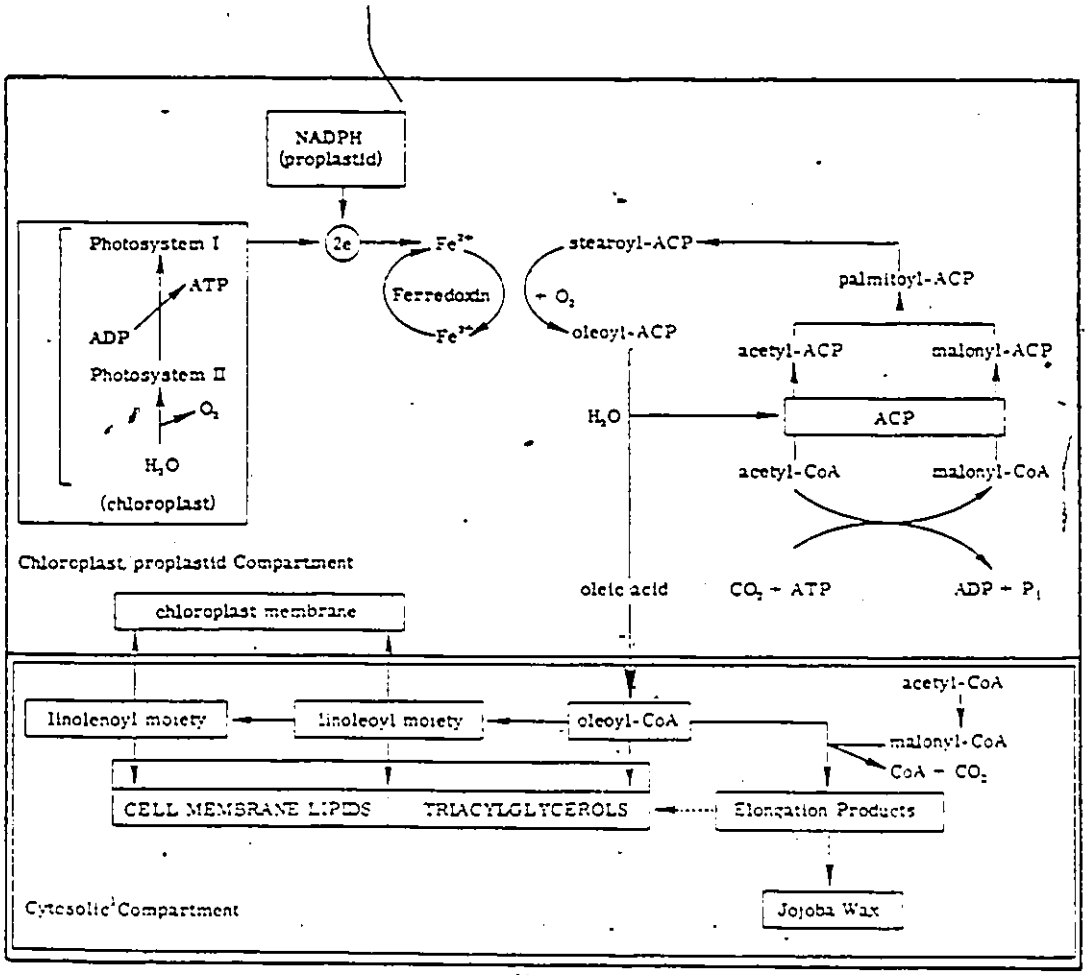


Fig. 6. Microsomal-bound lipid Δ^9-desaturase system. (Oshino et al., 114).

from the alga, Euglena gracilis which provides a link between animal and plant kingdoms, was able to convert stearic to oleic acid. When the organism was grown under photoautotrophic conditions, the required substrate was stearoyl-ACP; the CoA derivative was not a substrate. When the organism was grown in the dark on an organic medium, the CoA thiolester but not ACP derivative was the substrate. They also showed the existence of a stearoyl-ACP desaturase in spinach chloroplasts, and more recently these observations have been extended to include a variety of plant tissues (127). Thus the plant enzyme system uses stearoyl-ACP as substrate while oleoyl-ACP has been shown to be the product (128-129). In essence, the stearoyl-ACP desaturase in all plants examined is a soluble protein found either in the proplastids of the cell or in the stroma of the chloroplasts, unlike its counterpart in yeast and animal tissues in which the substrate is stearoyl-CoA and the enzyme is membrane bound. The plant stearoyl-ACP desaturase requires the input of two electrons either from NADPH or water (via photosystem I and II) and oxygen, and is inhibited by CN thus resembling the 18:0-CoA desaturase of liver microsomes. The desaturase system requires three components for activity: an NADPH: ferredoxin reductase, ferredoxin, and the terminal desaturase; the overall scheme is summarized in Fig. 7. The added stearate or palmitate cannot be converted to the active substrate and were therefore not available for desaturation. Since, however they were available for the synthesis of acyl lipids, esterification to

FIGURE 7. A generalized scheme showing the two-compartment system for the synthesis and desaturation of C_{18} fatty acids in the leaf cell or elongation etc. in the seed cell. For the sake of simplicity the origins of acetyl-CoA and NADPH are not defined. From P.K. Stumpf, (131).


NADPH
(proplastid)
Photosystem I
ATP
ADP
Photosystem II
O_2
H_2O
(chloroplast)
2e
Fe^{2+}
Ferredoxin
Fe^{3+}
stearoyl-ACP
palmitoyl-ACP
$+ O_2$
oleoyl-ACP
acetyl-ACP
malonyl-ACP
H_2O
ACP
acetyl-CoA
malonyl-CoA
Chloroplast proplastid Compartment
oleic acid
$CO_2 + ATP$
$ADP + P_i$
chloroplast membrane
acetyl-CoA
linolenoyl moiety
linoleoyl moiety
oleoyl-CoA
malonyl-CoA
$CoA - CO_2$
CELL MEMBRANE LIPIDS
TRIACYLGLYCEROLS
Elongation Products
Jojoba Wax
Cytosolic Compartment

their CoA thiolester must have occurred; a block therefore occurs during the process of transfer of the acyl chain from CoA to the enzyme. Nagai and Bloch (126) postulated that the enzymes which are lacking could be the CoA-ACP palmitoyl and stearoyl transferases. These enzymes are also absent or inactive in the organism such as Chlorella which exhibits the plant pathway when growing in a nutrient medium, but when the cells are suspended in an inorganic medium under illumination, the transferase enzymes are either activated or induced, and the direct desaturation of stearate can take place.

1.1 Specificity of Desaturation

Brett et al., (130) while studying the conversion of homologous ^{14}C-labelled saturated fatty acids to the corresponding monoenoic acids by four different systems (microsomes from goat, mammary gland, and hen liver, and whole cells of Torulopsis bombicola and Chlorella vulgaris) suggested the presence of a widely distributed desaturase system for long chain fatty acids capable of binding the substrate at its carboxyl group while correctly locating the C_{9-10} at the active centre of the enzyme. They suggested the presence of a second Δ^9-desaturase for shorter chain fatty acids. On the basis of their results they speculated on the likelyhood of conformational changes in both the enzyme and the substrate on binding and on the course of the desaturase reaction. A similar postulate was advanced by Pollard et al., (132) in which they proposed that the hydrocarbon chain of the substrate was held

at the active centre of the desaturase enzyme by a deep cleft of hydrophobic character with dimension of 26 Å in length and 4 Å in width. Such a model imposed a spatial constraint on the acyl chain favoring hydrogen removal at the C_9-C_{10} carbons. Evidence provided by Schroepfer and Bloch (133) and confirmed by Morris (134,135) and Morris et al., (136) showed that the stereospecificity of the hydrogen removal at one position of each double bond was absolute for the D-configuration. The Morris group (136) proceeded then to establish the overall stereochemistry by determining the relative configuration of the hydrogens removed, using racemic but geometrically specific vic-dideuterated substrates, either DL-cis-(or erythro) or DL-trans-(or threo). The conclusion was that the hydrogen atoms removed in all these substrates were of the cis or erythro configuration. The stereochemistry of hydrogen removal is identical whether the transformation is from a saturated to a monosaturated acid or from a monounsaturated to a diunsaturated acid. Consistent with their studies was the conclusion that desaturation involved a concerted removal of a pair of hydrogen atoms without the formation of oxygenated intermediates.

2. Biosynthesis of Polyenoic Fatty Acids

2.1 General

It is generally accepted that bacteria are unable to synthesize polyunsaturated fatty acids with the exception of a few bacteria. On the other hand, the biosynthesis of polyunsaturated fatty acids takes place in all plants and algae

by a mechanism involving sequential desaturation e.g. 18:1 → 18:2 → 18:3 → 18:4 and chain elongation, e.g. 18:4 → 20:4. The position of the second double bond is not made at random but it is usually spaced from a previous double bond to yield a product with the familiar methylene-interrupted fatty acid. Since the first double bond is usually introduced in the middle of the fatty acid chain, there is room at either end of the molecule for the introduction of the second double bond. In plants, fatty acids are usually desaturated between the existing double bond and the methyl end of the molecule; in contrast, in the animal kingdom, the second or third double bonds are introduced only between the pre-existing double bond and the carboxyl end of the molecule. Thus, the animal kingdom has lost the ability to desaturate the fatty acid between the existing double bond and the methyl end of the molecule.

2.2 Yeasts

Yuan and Bloch (137) were the first ones to study the biosynthesis of polyunsaturated fatty acid in the yeast Torulopsis utilis which contains a high proportion of linoleic acid; they observed that [1-^{14}C] oleic acid was rapidly converted to linoleic and α-linolenate by Torulopsis utilis cells, and that it occurred by an oxidative mechanism similar to that of Δ^9-desaturation. The first evidence for Δ^9-desaturation by subcellular fractions was provided by Mayer and Bloch (138). During the last decade there has been considerable discussion as to whether 18:1-CoA or 18:1 esterified to PC is the real

substrate for 18:1 desaturation. The argument now appears to be resolving in favor of 18:1-PC, a conclusion that is fully consistent with the evidence from in vivo studies. Evidence reported by Gurr et al., (139) with Chlorella, showed that Δ^{12}-desaturation of oleic acid might occur on the phospholipids, particularly with PC, as had been previously postulated by Nichols et al., (140). These findings were corroborated by Baker and Lynen (141) while studying Δ^{12}-desaturases of the fungus Neurospora crassa. More direct evidence was obtained by Talamo et al., (142), who found that synthetic 1,2-di-[^{14}C]-oleoyl phosphatidylcholine was converted very rapidly to dilinoleate-PC in a reaction requiring both the particulate fraction and the supernatant from Torulopsis utilis extracts.

Kates and Paradis (143), in attempting to explain the reciprocal changes of 18:1 → 18:2 in Candida lipolytica suggested that conversion of oleic to linoleic acid in C. lipolytica might occur by the operation of a desaturase system directly on the phospholipids.

To test the possibility of direct desaturation of phospholipids Pugh and Kates (144) incubated either [^{14}C, ^{32}P]lecithin or [^{14}C, ^{32}P]phosphatidylethanolamine with yeast microsomes in a reaction mixture containing oxygen and reduced pyridine nucleotides. These doubly-labelled phospholipids were obtained from cells of C. lipolytica grown in the presence of [^{14}C]acetate and [^{32}P]orthophosphate and were used as substrates in order to eliminate the possibility of hydrolytic breakdown

of the substrate and/or resynthesis by transacylation. When [^{14}C] and [^{32}P]-labelled lecithin of phosphatidylethanolamine were incubated with C. lipolytica microsomes in the presence of oxygen and reduced pyridine nucleotides, no breakdown of either substrate was observed. This was shown by the relatively constant $^{14}C/^{32}P$ ratios of the substrates and products isolated from the incubation mixture.

Both phospholipids isolated after the incubation, however, differed from the respective substrates in that they contained increased amounts of linoleic acid, the proportion of linoleic acid in both phospholipids increasing by 5-10%. The increased amounts of linoleic acid in the products must have originated from direct desaturation of the respective phospholipid substrates.

The alternative possibility was that the phospholipids used as substrates were first hydrolyzed by a phospholipase A_2 and the fatty acids activated by a thiokinase. The resulting CoA ester could in turn have been desaturated by an acyl-CoA desaturase and the desaturated product used to resynthesize the phospholipids. This possibility is, however, unlikely for two basic reasons; first no significant release of free fatty acids from the phospholipids were observed under the desaturating conditions, and second, CoA and ATP were not required for lipid desaturation of the phospholipid as would be expected if a thiokinase was involved at some

intermediary step. Therefore, it could be concluded from these studies that a direct desaturation of the membrane phospholipids had occurred. These membrane bound phospholipid and acyl-CoA desaturases both showed the same requirements for cytochrome b_5 reductase, cyt b_5 (lack of CO inhibition), and the terminal desaturase (cyanide sensitive) (145) (Fig. 6). Recent evidence (146) showed that microsomal membranes prepared from Candida lipolytica cells grown at 10°C had a higher lipid content and degree of unsaturation than membranes prepared from cells grown at 25°C, paralleling the respective microsomal desaturase activity. Regarding the identity of the actual substrate for the microsomal Δ^{12}-desaturase, recent evidence (147,148) suggests that the bulk of newly synthesized 18:2 occurs via phospholipid (steps A-C) as a substrate and to some extent from acyl-CoA as substrate (step A and D), as summarized in scheme II.

It was concluded from these studies that the phospholipid desaturase activity was inversely related to the 18:2/18:1 mole ratio of the membrane preparation or presumably to the fluidity of the membrane (149). A similar conclusion has been reached by Skriver and Thompson (150) for the palmitoyl-CoA desaturase of Tetrahymena and by Riordan (151) for the Mg^{2+}-ATPase of rat liver plasma membrane. Thus the activity of the membrane bound phospholipid desaturase system may be considered as self-regulating if one assumes maximum interaction and/or optimal conformation of the three proteins occurring in a membrane of low 18:2/18:1 ratio or low fluidity (Fig. 8B) and suboptimal interaction

SCHEME II

Proposed pathways for the synthesis of oleic and linoleic acids in the yeast Candida lipolytica microsomal membranes. Abbreviations used: PC, phosphatidylcholine; 18:1, oleate; 18:2, linoleate; taken from Ferrante et al., (147).

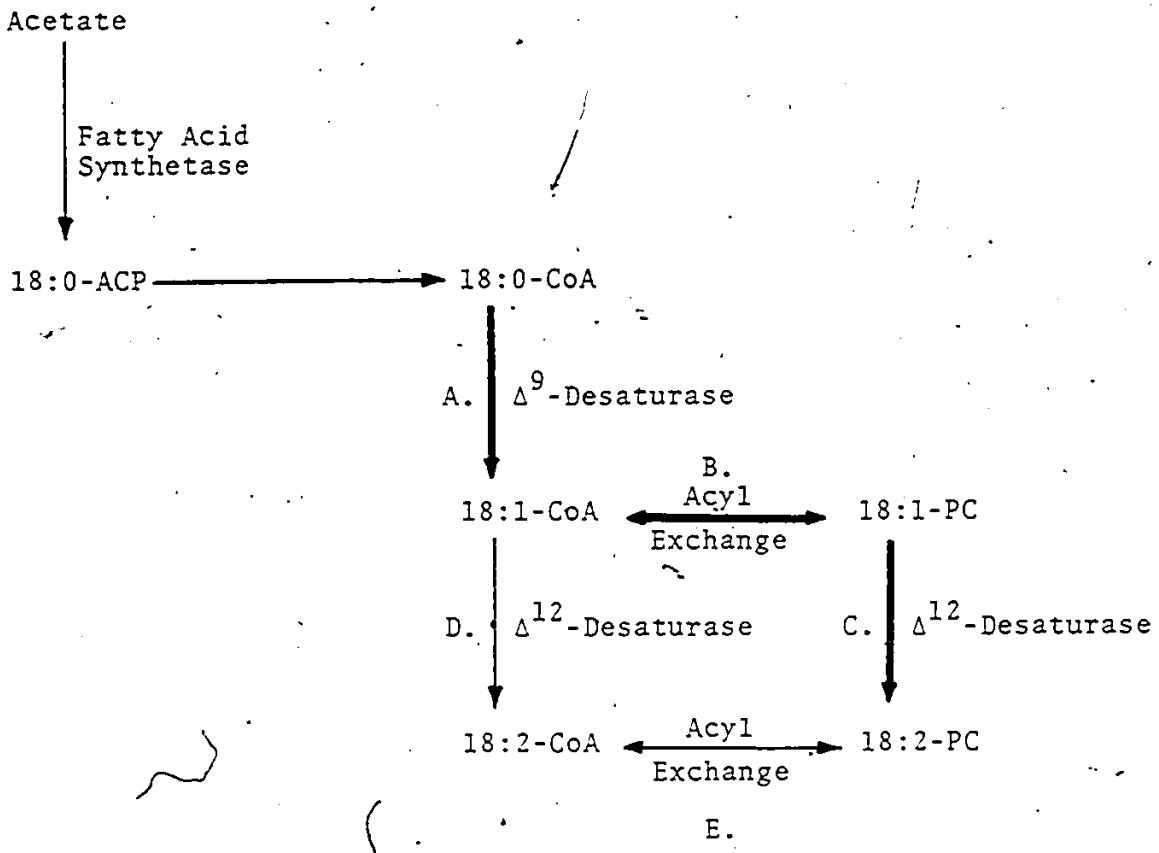
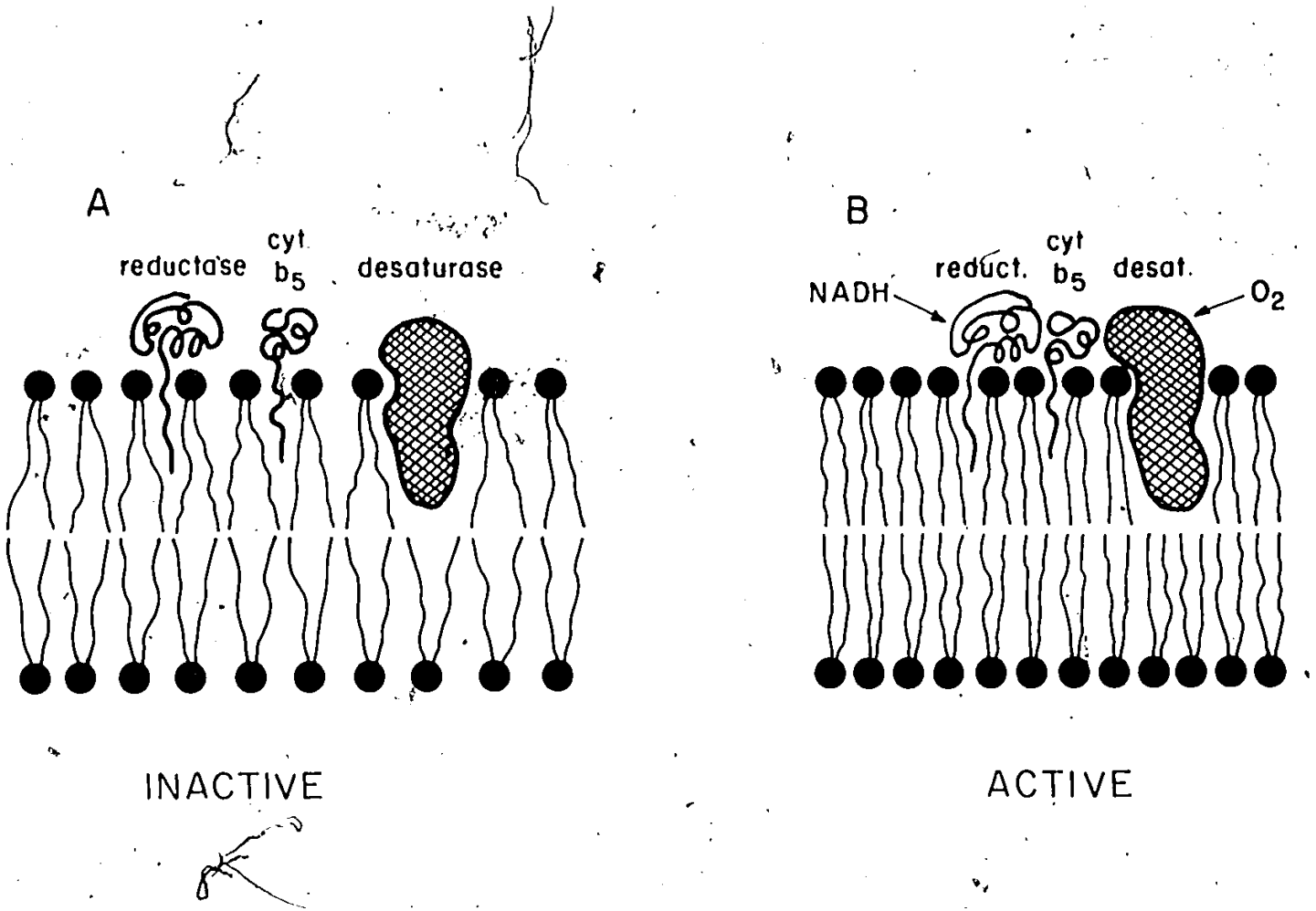

Acetate
Fatty Acid Synthetase
18:0-ACP
18:0-CoA
A. Δ9-Desaturase
B. Acyl Exchange
18:1-CoA
18:1-PC
D. Δ12-Desaturase
C. Δ12-Desaturase
18:2-CoA
Acyl Exchange
18:2-PC
E.

FIGURE 8. Hypothetical model for self-regulation of phospholipid desaturase activity by membrane fluidity (147).


A
reductase
cyt
b_5
desaturase
INACTIVE
B
NADH
reduct.
cyt
b_5
desat.
O_2
ACTIVE

conformation occurring in a membrane of high 18:2/18:1 ratio or high fluidity (Fig. 8A).

2.3 Plants

2.3.1 in vivo Studies

Kinetics of [1-^{14}C]acetate incorporation into pumpkin leaf lipids and the subsequent metabolism of fatty acids bound to glycerolipids suggested that the phospholipids, mainly phosphatidylcholine (PC), played an important role in the mechanism of long chain fatty acid desaturation in leaves. In addition it was suggested that the polyunsaturated fatty acids formed on PC were subsequently transferred to other glycerolipids and glycolipids within the cell. Of the radioactivity incorporated into lipid from [1-C^{14}]acetate, PC was found to contain the bulk of the label containing oleate and linoleate (152,153). The resulting unsaturated fatty acids of PC were subsequently transferred to MGDG (155,156). Recent studies have demonstrated high turnover rates of the acyl moieties of PC, PG and MGDG in leaves from a number of plant species labelled with [$^{14}CO_2$] (154-156) or [1-^{14}C]acetate (154, 155,157). In general, there is at the earliest labelling times a relatively specific association of labelled oleate and linoleate with PC, of labelled palmitate with PG, and of labelled linoleate and linolenate with MGDG. While the rapidly labelled PC was recovered in the microsomal fraction, the labelled PG and MGDG were recovered predominatly in the chloroplast fraction

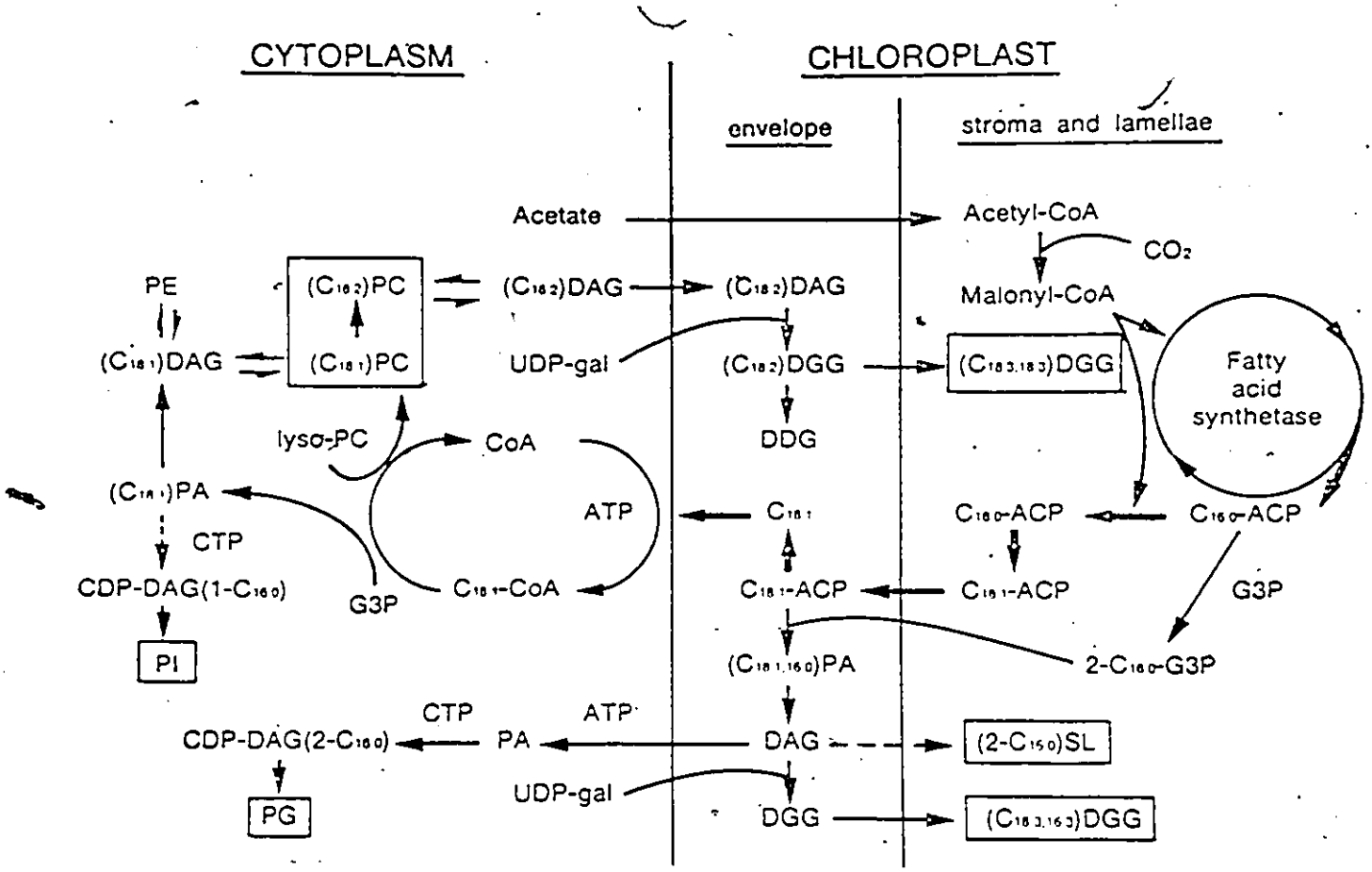
(154). In intact spinach leaves labelled with [1-^{14}C]acetate, a significant transfer of label from oleate of microsomal PC to linolenate of chloroplast MGDG occurred on prolonged incubation (158).

It was inferred from these and other studies (156,159) that PC acted as a carrier of the DAG backbone for the synthesis of MGDG. Similar conclusions have now been reached in developing oil seeds (159-161) which actively accumulate TAG. Roughan and Slack have proposed (158) that chloroplastic MGDG may be of dual origin (Fig. 9): (a) derived from di-18:2-DAG formed from microsomal di-18:2-PC; (b) derived from chloroplastic 18:1-16:0-DAG. This microsomal pathway, referred to as the "eukaryotic pathway", involves first the desaturation of di-18:1-PC to di-18:2-PC, then conversion to di-18:2-DAG and subsequently transferred to the chloroplast where it is converted to 18:2-MGDG and desaturated to 18:3-MGDG. This pathway accounts for the formation of MGDG molecular species containing C_{18}-fatty acids at both C-1 and C-2 positions of the glycerol (18/18-species). A similar sequence of reactions would account for the formation of minor proportions of 16/18-molecular species starting from 16/18.

In contrast, the "chloroplastic" or "prokaryotic pathway" involves a DAG made by the chloroplast which is characterized by the exclusion of C_{18} fatty acids from the C-2 position while containing 18/16 molecular species with minor proportions of 16/16 molecular species. The MGDG from

FIGURE 9. A model of plant lipid metabolism showing the interdependence of chloroplast (or plastids) and cytoplasm in the synthesis of typical membrane glycerolipids. (Taken from Roughan and Slack (158)). Abbreviations: DGG, diacylgalactosylglycerol; DDG, diacyldigalactosylglycerol; SL, sulphoquinovosyldiacylglycerol; for other abbreviation see page XV.

FIGURE 9


CYTOPLASM
CHLOROPLAST
envelope
stroma and lamellae
Acetate
Acetyl-CoA
CO2
Malonyl-CoA
PE
(C18:2)PC
(C18:2)DAG
(C18:2)DAG
(C18:1)DAG
(C18:1)PC
UDP-gal
(C18:2)DGG
(C18:3,18:3)DGG
Fatty acid synthetase
lyso-PC
CoA
DDG
(C18:1)PA
ATP
C18:1
C16:0-ACP
C16:0-ACP
CTP
C18:1-CoA
C18:1-ACP
C18:1-ACP
G3P
G3P
CDP-DAG(1-C16:0)
PI
(C18:1,16:0)PA
2-C16:0-G3P
CTP
ATP
CDP-DAG(2-C16:0)
PA
DAG
(2-C16:0)SL
UDP-gal
PG
DGG
(C18:3,16:3)DGG

the prokaryotic pathway resembles the MGDG from the primitive blue-green algae in its acyl positioning (162). In chloroplasts of 18:3- plants (Asteraceae and Fabaceae) (163) the 18/18 species represent more than 95% of the total MGDG, while in 16:3- plants (Apiaceae and Brassicacea) these molecular species account for only 20%. Chloroplasts isolated from 16:3- plants (163) synthesize 18:1/16:0-MGDG from water soluble precursors, made in a light-dependent reaction, which is in turn desaturated, in light independent reaction, to 18:3/16:3-MGDG. These results, demonstrate that isolated chloroplasts are autonomous in the biosynthesis of these 18:3/16:3 acids esterified to MGDG. In contrast chloroplasts isolated from 18:3- plants showed no significant change in the proportions of these polyenoic acids during the incubation.

However, when 16:3-plants were grown in the presence of [^{14}C]acetate, followed by the isolation of chloroplast and/or thylakoid MGDG, 10-17% of the acyl groups at the C-2 position were found to be C-18 fatty acids. These results demonstrate that the eukaryotic pathway provides the chloroplast with 18:2-DAG for synthesis of MGDG molecules which are subsequently, desaturated at both C_1 and C_2 positions to form 18:3/18:3-MGDG (Fig. 9). Recently, it has been reported (165) that the final step in desaturation of the fatty acid moiety (18:2 → 18:3) may occur directly on MGDG but not on DGDG.

The origin of 16:3 in different 16:3-plants may be explained by the desaturation of 16:0 to 16:3 at C-2 of MGDG and the varying proportions in which prokaryotic (18/16) MGDG is synthesized in chloroplasts of different plants, depends on the varying degrees of PA synthesis, PA phosphatase and galactosyltransferase enzyme activities which may be present in different 16:3-plants.

Further developments in the biosynthesis of 18:3 are discussed in the following section in "in vitro" studies.

2.3.2 In vitro Studies

In an early attempt to study the metabolism of [1-^{14}C] 18:1-CoA in safflower seed microsomes "in vitro", Vijay and Stumpf (164) concluded that the Δ^{12}-desaturase first desaturated 18:1-CoA to 18:2-CoA and then the 18:2 was esterified to PC. Unfortunately, the method used to differentiate between CoA and PC esterified 18:2 was shown to be unreliable (166). Later, Stymne and Appelqvist (167) indeed showed that the reverse was true: 18:1 was first transferred to phospholipid and then desaturated by the Δ^{12}-desaturase.

This mechanism of Δ^{12}-desaturation was shown to occur in other microsome systems such as fungi (168,142), yeast (145,147), leaves (169) and developing seed (170,171) using 18:1-CoA as substrate. More direct demonstration of phospholipid desaturation was achieved by the use of exogenous dioleoyl-PC added to the assay mixture (170) or by pre-labelling

membrane phospholipids under non-desaturating conditions (142,169-171).

One puzzle arising from these studies is the observation that only 30-50% of the 18:1-PC in the membrane is converted to 18:2-PC; if in fact oleoyl-PC was the actual substrate for the desaturase system, then a higher proportion of the oleoyl-PC might have been expected to be converted to linoleoyl-PC. One interpretation of this discrepancy comes from the suggestion that only oleoyl-PC "boundary" to the desaturase enzyme is actually available for desaturation and that the remaining oleoyl-PC in the bulk phase is too remote from the enzyme to serve as substrate. The 18:1-PC Δ^{12}-desaturase in plants is membrane bound and probably restricted to the endoplasmic reticulum (169,172). The desaturase system requires NADH and O_2 for activity and the enzyme is inhibited by CN- (171,173) as for the Δ^{9}-desaturase of liver microsomes (which uses 18:0-CoA as substrate) or Δ^{9}-desaturase of plant chloroplasts which uses 18:0-ACP.

At present there is limited information on the final desaturation step in plants namely, the conversion of linoleic acid to α-linolenic acid. In homogenates and in subcellular fractions from developing soybean bean cotyledons Stymne et al (167) showed marked differences in the properties of Δ^{12}- and Δ^{15}-desaturases indicating that the two systems

are distinct enzymes. These authors demonstrated that the Δ^{12}-desaturase resembled other desaturases in that NADH and O_2 were required and the enzyme was inhibited by cyanide. Other workers (165,174-176) have shown that while linoleoyl-PC accumulated under in vivo conditions, very little linolenoyl-PC could be detected as product and instead linolenoyl-MGDG appeared to be the main product. Further to the in vivo studies of Roughan et al., (158) and their proposal that di-18:2-PC was transferred to the chloroplast via di-18:2-DAG (Fig. 9) an alternative proposal was forwarded by Jones and Harwood (165) and Yamada and Ohnishi (177) who have demonstrated that cytoplasmic proteins (165) or phospholipid exchange proteins (177) were involved in the transfer of di-18:2-PC from microsomes to chloroplast. The di-18:2-PC, transferred to the chloroplast, is then converted to di-18:2-DAG followed by its conversion to di-18:2-MGDG. The di-18:2-MGDG is then desaturated to di-18:3-MGDG and subsequently converted to di-18:3-DGDG. This alternative pathway is reinforced by the finding by Jones and Harwood (181) that the actual substrate for the Δ^{15}-desaturase is MGDG and not DGDG (165). In conclusion, this alternative pathway is an extension of the work by Roughan et al., (158) in that it accounts for the labelling pattern of MGDG observed in vivo as well as the actual substrate for the Δ^{15}-desaturase (165).

2.4 Plant Cell Cultures

Stearns and Morton (178,179) have examined the capacity of soybean cell suspension cultures to incorporate [1-^{14}C] acetate into fatty acids. They observed that the ^{14}C derived from [1-C^{14}]acetate was rapidly incorporated into the saturated, monounsaturated diunsaturated and triunsaturated acyl moieties of phospholipids. Subsequently, Stumpf and Weber (180) not only confirmed these results but also studied the uptake and metabolism of [^{14}C]palmitate, [^{14}C]stearate, [^{14}C]oleate and [^{14}C]linoleate as a function of time. They observed that within minutes after addition to the medium, considerable portions of the exogeneous fatty acids entered the cells and were incorporated mainly into PC and TAG and to a lesser extent into PE. While the saturated fatty acids, [^{14}C]palmitate and [^{14}C]stearate were recovered unmetabolised after 5 hours of incubation, the unsaturated fatty acids, [^{14}C]oleate and to a lesser extent [^{14}C]linoleate were desaturated to [^{14}C]linoleate and [^{14}C]linolenate respectively.

When soybean cell cultures were incubated with [^{14}C] acetate for up to 22 hours (35) rather high proportions of ^{14}C were found in linoleate and linolenate of polar lipids. At the earliest time of incubation (16 min), PC accounted for the highest proportion of [^{14}C]oleate acid which decreased to a low value at 22 hours of incubation. In addition, while [^{14}C]linoleate was low at the earliest incubation time while

reaching a maximum at 2.6 hours and decreasing thereafter, [^{14}C]linolenate increased slowly up to 2.6 hours and then increased considerably between 2.6 hours and 22 hours of incubation. These results were consistent with the sequential desaturation of 18:1 → 18:2 → 18:3 and raised the interesting question concerning flow of ^{14}C through the major lipid components. It was suggested that the sequential desaturation of 18:1 might occur directly on PC and that there was a flow of ^{14}C from PC principally to PX (bis-PA) and MGDG plus DGDG, suggesting perhaps that PC might act as a donor of DAG backbone to the other polar lipids. The alternative pathway involved the direct sequential desaturation of acyl-CoA followed by acyl-exchange with PC, mainly, and to a lesser extent with the other lipids. However, further work was required to establish the actual pathway of biosynthesis of polyunsaturated fatty acids unambiguously in soybean cell suspension cultures.

2.5 Effect of Temperature

It is generally observed that growth of cells at low temperatures favours an accumulation of polyunsaturated fatty acids at the expense of the more saturated fatty acids (180-182). Two hypotheses have been put forward to explain this general observation: (1) as suggested several years ago (163), the availability of oxygen, which is an obligatory factor in the mechanism of desaturation, might influence directly the desaturase system; and (2) as proposed more

recently (149,183), the fluidity of the membrane might influence inversely the activity of the desaturase system (Fig. 8). The latter hypothetical model advances a very interesting self-regulatory mechanism for the control of membrane fluidity. The model proposes that the three proteins, two electron transporting proteins (cyt. b_5 reductase and cyt. b_5) and the terminal desaturase are optimally oriented only in a membrane of low fluidity (Fig. 8). In this physical state of the bilayer, a constraint is imposed on the conformational flexibility of the intergral proteins, as well as a reduced degree and rate of lateral diffusion that they can undergo within the plane of the bilayer. As more polyunsaturated fatty acids are formed, the lipid chains become more fluid due to the packing properties of the fatty acids, allowing increased lateral diffusion of the proteins and thus decreasing their interaction and hence the desaturase activity (Fig. 8). The system therefore constitutes a self-regulating mechanism, which shuts itself off when the membranes becomes too unsaturated or fluid and turns itself on again when the fluidity decreases due to insertion of less unsaturated fatty acids into the membrane lipids. In this way, an optimal physical state within the lipid bilayer of the cellular membranes would be maintained.

The synthesis of polyunsaturated fatty acids in plants may respond to variations in temperature in several ways. While the synthesis of 18:1 is generally favoured at high temperatures (184,185) more diverse responses are found for

the synthesis of polyunsaturated fatty acids. For instance, in the roots of alfafa exposed to frost hardening (186) or maize roots exposed to cold treatment (187) a stimulation in the biosynthesis of 18:2 has been observed. A similar observation has been reported in fungi (188) blue green algae (181), wheat seeldings (189) and in linseed and soya-bean cotyledons (190). In fungi (188) the biosynthesis of 18:2 is sometimes decreased at lower temperatures and in cotyledons of Pharbitis nil L., (191) the biosynthesis of 18:2 was more rapidly induced in plants grown at 27°C than those grown at 17°C.

However, it has been a general observation that seed oils produced by plants grown in a warm climate have lower proportions of polyunsaturated fatty acids than those grown in colder environments (192,193) and vice-versa.

These examples show that the three major unsaturated fatty acids in plants (oleic, linoleic and linolenic) respond to temperature in different ways. Thus, the biosynthesis of polyunsaturated fatty acids in plants may not be controlled by a simple physical factor such as the concentration of oxygen in the medium or membrane fluidity, as was mentioned above.

VII. Biosynthesis of Hydroxy Fatty Acids

The pathways for the biosynthesis of hydroxy fatty acids in plants is briefly summarized below: The 2-hydroxy-acids are probably formed in plants by α-oxidation. Two different processes of α-oxidation have been described in plants (194), one involving a hydrogen peroxide generating system (195) and a second system requiring molecular oxygen (2). The α-oxidation complex is membrane bound, probably in the endoplasmic reticulum, and is believed to act on free fatty acids. Although the physiologic role of the α-oxidation system is not fully defined, it is believed to be involved not only in the formation of 2-hydroxyacids but also in the formation of odd chain fatty acids from even numbered fatty acids (196).

A soluble preparation from maturing avocado mesocarp was found (15) to be highly active in forming 3-hydroxy fatty acids of medium chain lenghts (C_8 to C_{12}). While CoA, ATP and oxygen were required by this hydroxylating system, NADPH had no effect while NAD^+ or NADH were highly inhibitory. Inhibitor studies also revealed the involvement of thiol and heavy metals groups as well as Cyt. P-450. The biological significance of this very active hydroxylating system remains unknown and it has not been reported to act on unsaturated fatty acids.

The biosynthesis of ricinoleic acid, 12-hydroxyoleic acid, has been shown to occur with oleoyl-CoA as substrate (197) by a microsomal system requiring molecular oxygen as well as NADH. No other acyl-CoA or free fatty acid was hydroxylated by this system. As CO failed to inhibit this hydroxylation, cyt. P-450 is probably not involved. Studies with metal chelators suggested the probable involvement of iron in the system.

The ω-hydroxylation, which is the first step involved in the biosynthesis of hydroxyacids in plant cutin and suberin, has been shown to occur in microsomes of Vicia faba at the level of free fatty acids requiring NADPH and oxygen (198). This microsomal system was inhibited by the classic mixed function oxidase inhibitors such as metal ion chelators, azide and thiol directed reagents (198). The involvement of cyt P-450 was suggested on the basis of the extreme sensitivity of the ω-hydroxylase to carbon monoxide.

The midchain (C_8-C_{10}) hydroxylation, for the formation of dihydroxyacid components of cutin or suberin, also occurs by a microsomal mixed function oxidase (199) requiring NADPH and oxygen. This midchain hydroxylase is also inhibited by heavy metal chelators, azide and thiol directed reagents (199). Carbon monoxide inhibited this midchain hydroxylation and, unlike the ω-hydroxylase, the inhibition was reversed by light at 420-460 nm. Therefore, a typical cyt P-450 appears to be involved in this hydroxylation.

These few examples suggest that plants have different mechanisms for the biosynthesis of hydroxyacids and that the type of hydroxylating system observed (CO sensitive or insensitive), may depend on the tissue studied.

The mechanism of hydroxylation has also been demonstrated in many other systems such as slime mold (15) (see section A.II.c), bacteria (200), beeswax (201) yeast (202,203) and animal systems (204).

VIII. Biosynthesis of Phospholipids

The enzymatic steps of phospholipid biosynthesis in plants and the subcellular location of these steps is summarized in Fig. 10.

The biosynthesis of PA, a key intermediate in the biosynthesis of phospholipids may occur in the endoplasmic reticulum, mitochondria and plastids by the direct acylation of glycerol-3-phosphate (205) (Fig. 10). An alternative pathway which involves the direct phosphorylation of DG by a mitochondrial peanut kinase enzyme has been reported but Mazelis and Stumpf (206) but its contribution may be negligeable when compared to the acylation of glycerol-3-phosphate. Subsequently PA can be either hydrolysed to DG by PA-phosphatase (207) or can form CDP-diglyceride through the action of a cell-free CTP-phosphatidic acid cydidyl transferase enzyme (208). These latter intermediates, DG and CDP-diglycerides, are in turn utilized in the biosynthesis of the other phospholipids through the respective CDP-nitrogenous base or CDP-diglyceride pathway.

There are two major pathways for the biosynthesis of phosphatidylcholine (PC) in plants, the first involving DAG and CDP-choline catalysed by a microsomal CDP-choline: diglyceride phosphotransferase (209,210), referred to as the "nucleotide pathway", and the second mechanism involving the methylation of phosphatidylethanolamine (PE) with S-adenosyl-methionine (211,212). About 1-2% of PC synthesis by methylation (213) has also been reported associated with the inner mitochondrial

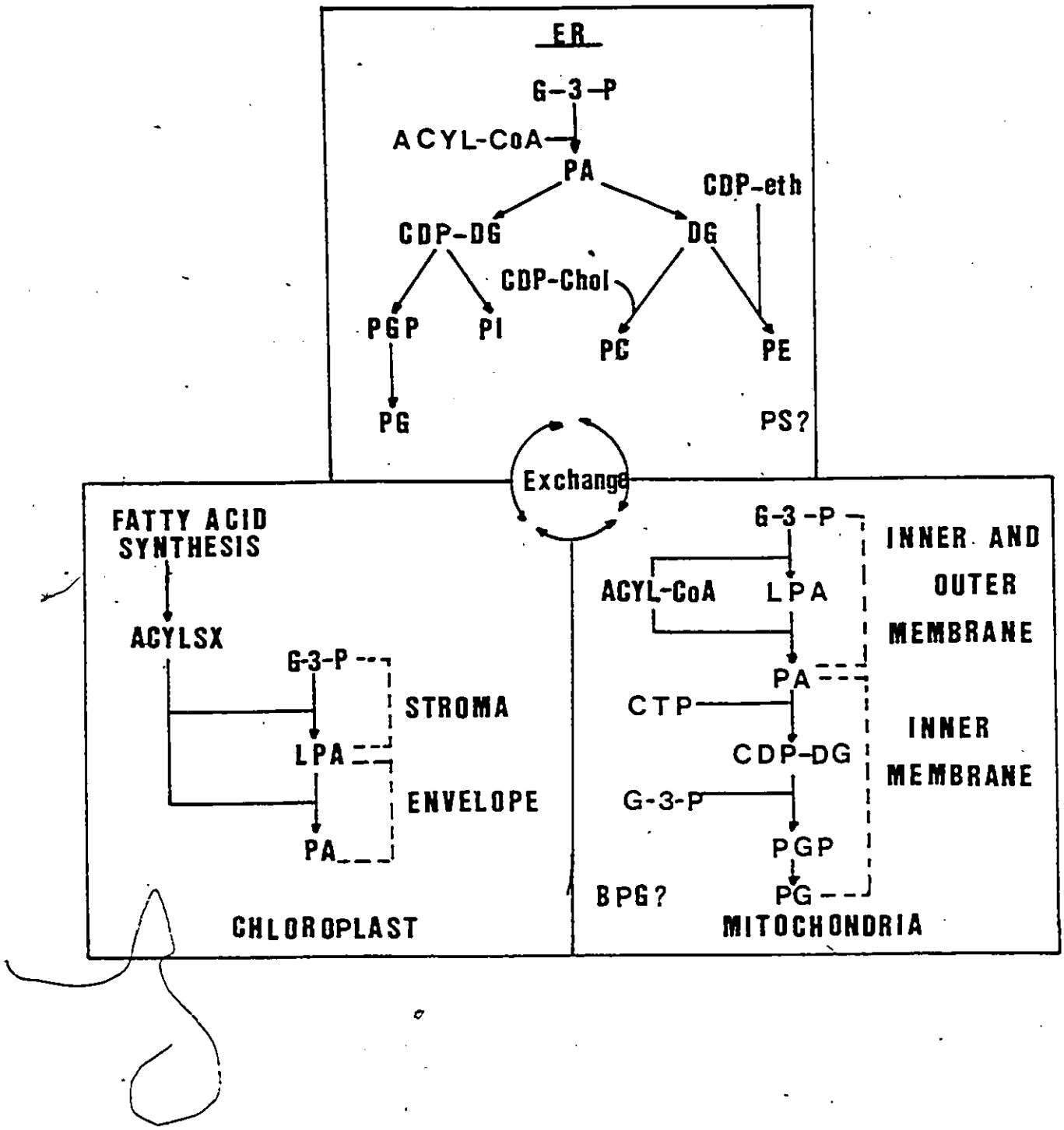

ER
G-3-P
ACYL-CoA
PA
CDP-eth
CDP-DG
DG
CDP-Chol
PGP
PI
PC
PE
PG
PS?
Exchange
FATTY ACID SYNTHESIS
ACYLSX
G-3-P
STROMA
LPA
ENVELOPE
PA
CHLOROPLAST
G-3-P
ACYL-CoA
LPA
INNER AND OUTER MEMBRANE
PA
CTP
CDP-DG
INNER MEMBRANE
G-3-P
PGP
BPG?
PG
MITOCHONDRIA

FIGURE 10. Pathways of phospholipid biosynthesis and their subcellular location in plants (214).

membranes.

Phosphatidylethanolamine (PE) can be synthesized in microsomes by either the nucleotide pathway (211,212) involving CDP-ethanolamine and DG catalysed by CDP-ethanolamine: digliceride phosphotransferase or by the decarboxylation of phosphatidylserine (211,212) catalysed by PS-decarboxylase enzyme.

The biosynthesis of phosphatidylserine (PS) and phoisphatidylinositol (PI) in plants occurs only by the CDP-DG pathway using serine for PI synthesis or inositol for the synthesis of PS catalysed by their respective phosphatidyl-transferase enzyme. The enzyme activities involved in the synthesis of PC, PE, PI and PS thus appear to be associated with the microsomal membranes (215).

The biosynthesis of PG in plants also occurs in microsomes by a reaction of CDP-DG with sn-glycerol-3-P with the intermediate formation of phosphatidyl glycerophosphate (PGP) which is dephosphorylated to PG (205). A major portion (50%) of the enzyme activity for the pathway of PA to PG has also been found associated with the inner mitochondrial membrane (216) (see Fig. 10).

The biosynthesis of cardiolipin (CL) has been demonstrated in E. coli to occur by a pathway involving two PG (217,218) while in animals the biosynthesis of CL occurs by a pathway involving PG plus CDP-DG (219,220). The latter pathway, expected for eukaryotes has not yet been satisfactorily demonstrated

in plants.

Since all the major enzymes involved in the biosynthesis of polar lipids are localized in the endoplasmic reticulum (221) the interesting question is how are the lipids transported to the organelles where they play major functional and/or structural roles. For instance, while PC is synthesized in the endoplasmic reticulum, it is also found in the outer envelope of the chloroplast membrane. Two mechanisms are envisaged for the movement or redistribution of lipids (222), one involving soluble phospholipid exchange proteins and the second is the hypothesis that proplastids and promitochondria derive directly from the endoplasmic reticulum.

IX. Aim of the Present Research

Previous *in vivo* studies have demonstrated that oleic and linoleic acid can be desaturated to the respective linoleic and linolenic acids and that this process of desaturation may occur at the level of phospholipids and glycolipids respectively (35). In the available literature, there are only two publications (97,98) which describe the *de novo* biosynthesis of fatty acids in proplastids isolated from heterotrophic soybean cell suspension-cultures.

The aim of the present study therefore, was first to demonstrate desaturation of 18:1 → 18:2 and 18:3 and then to study the mechanism of desaturation in subcellular fractions of soybean cell suspension cultures. Additionally, our aim was to establish whether the acyl-CoA or the phospholipid or both were actual substrates for the desaturase system. During the course of these studies it was found unexpectedly, that the major products of metabolism of 18:1 or 18:2-CoA were hydroxy fatty acids. Therefore the identity of these products as well as the properties of the enzyme system for their formation was investigated.

MATERIALS AND METHODS

B. Materials

I. Cultures

The soybean cell suspension culture was that of Glycine max (L) Merr., strain M.P. The suspension culture was initiated from callus cultures kindly donated by Agriculture Canada and maintained on solid B5 medium containing 2,4-dichlorophenoxyacetic acid as the only phytohormone (see Section C.I.a.).

II. Chemicals

All solvents were distilled before use. All other chemicals were of "reagent grade" quality unless otherwise specified. NADPH, NADH, CoA, ATP, Triton X-100, EDTA, cold oleoyl-CoA and linoleoyl-CoA, cytochrome c (Type III), 9- and 12-hydroxystearic methyl esters, p-iodonitrotetrazolium violet (INT), 1, 10-Phenanthroline, Phenazine methosulfate, Ferredoxin (Type III), Antimycin A, heptadecanoic acid methyl ester, oleic acid (cis-9-Octadecenoic acid), linoleic acid (all cis 9, 12-Octadecadienoic acid), linolenic acid (all cis 9,12,15-octadecatrienoic acid), nonanoic acid (pelargonic acid), azelaic acid (1,7-heptanedicarboxylic acid), pimelic acid, suberic acid, succinic acid, and succinic acid disodium salt were purchased from Sigma Chemical Co., St. Louis, Mo. [1-^{14}C]oleoyl-CoA was synthesized as described for [1-^{14}C]linoleoyl-CoA in Section C.XI.b. or purchased from Amersham Corporation (Oakville, Ontario, Canada) and the specific activity was adjusted to known values by addition

of unlabelled material.

Atebrine ·2HCl (Quinacrine); SAN 6706 [4-chloro-5-(dimethylamino-2-(α,α,α-trifluoro-m-tolyl)-3(2H)-pyridazinone] and SAN 9785 [4-chloro-5-(dimethylamino)-2-phenyl-3(2H)-pyridazinone]; Destun (1,1,1-trifluoro-4'(phenylsulfonyl)-methansulfo-o-toluidine); and hydroxyacids were gifts of Sterling-Wintrhrop Res. Inst., C. Willemot (Agriculture Canada, Ste-Foy, Canada), L.R.G: Valadon (University of London, Dept. of Botany, England) and A.P. Tulloch (National Research Council of Canada, Saskatoon, Sask.) respectively.

C. Methods

I. Growth of Soybean Cells

a) Growth Medium

The medium used for the growth of the soybean cells was the mineral salt medium of Murashige and Skoog (77) using the vitamins and hormone supplements described by Gamborg (76) as given in Table 6. Solid B5 medium, used for the propagation of callus cultures, contained 6-8 grams of agar per liter of ~~B5~~ liquid medium.

b) Culture of cells

For the growth of subcultures, sections of callus cultures, which had been grown for about 35 days in the dark on solid B5 medium, were transferred aseptically in a laminar flow cabinet to ~~125~~ ml Erlenmyer flasks each containing 40 ml of fresh B5 liquid medium and incubated on a G24 environmental incubator

TABLE 6

Composition of Mineral Salt Media for Plant Tissue and Cell Culture

Macronutrient	mg/l
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170

Micronutrient	mg/l
KI	0.83
H_3BO_3	6.20
$MnSO_4 \cdot 4H_2O$	22.30
$ZnSO_4 \cdot 7H_2O$	8.60
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
$Na_2 \cdot EDTA$	37.3
$FeSO_4 \cdot 7H_2O$	27.8
Sucrose	40000
pH	5.7

shaker (New Brunswick Scientific Co., Edison N.J., U.S.A.) rotating at 175 rpm under continuous illumination and at a temperature of 28-29°C. Good growth was usually established in 3-4 weeks; 5.0 ml of this subculture was used to initiate growth of soybean cell suspension cultures as described above. These cultures were subcultured every 7 days. Electron micrographs of typical soybean cells, grown as described above, are shown in Fig. 11: (A) a young soybean cell in the process of division showing a large vacuole with a small number of organelles; (B) an older soybean cell showing a much smaller vacuole with a large number of plastids and mitochondria. A clearer and higher magnification of these organelles is shown in Fig. 12.

For the study of the effect of light on desaturase, hydroxylase activities and fatty acid composition (see section D.II.1.d.), while one batch of cells was grown under continuous illumination, as described above, a second batch was grown in complete darkness. The basic difference between the two types of cells, dark grown and light grown, was in the lack of pigments formed with cells grown in the absence of light.

c) Harvesting of Cells

Unless specified, cells were harvested after 7 days of growth by suction filtration at room temperature in a Buchner funnel containing two layers of miracloth (Calbiochem-Boehring Corp., Lajolla, Clif.) weighed and quickly transferred in the cold room (4°C) for breakage in a precooled mortar and pestle. The yield of wet cells per 40 ml of culture was between 7-8 g.

FIGURE 11. Electron micrograph of typical soybean cells grown for seven days at 29°C as described in section C. A, young soybean cell (X 3,192); B, older soybean cell (X 3,500). N, nucleus; Nu, nucleolus; P, proplastids; M, mitochondria; CW, cell wall; CM, cell membrane; TM, tonoplast membrane; Ve, vesicles which may comprise a large part of band E on sucrose gradient (see Fig. 14); G, golgi apparatus; V, vacuole.

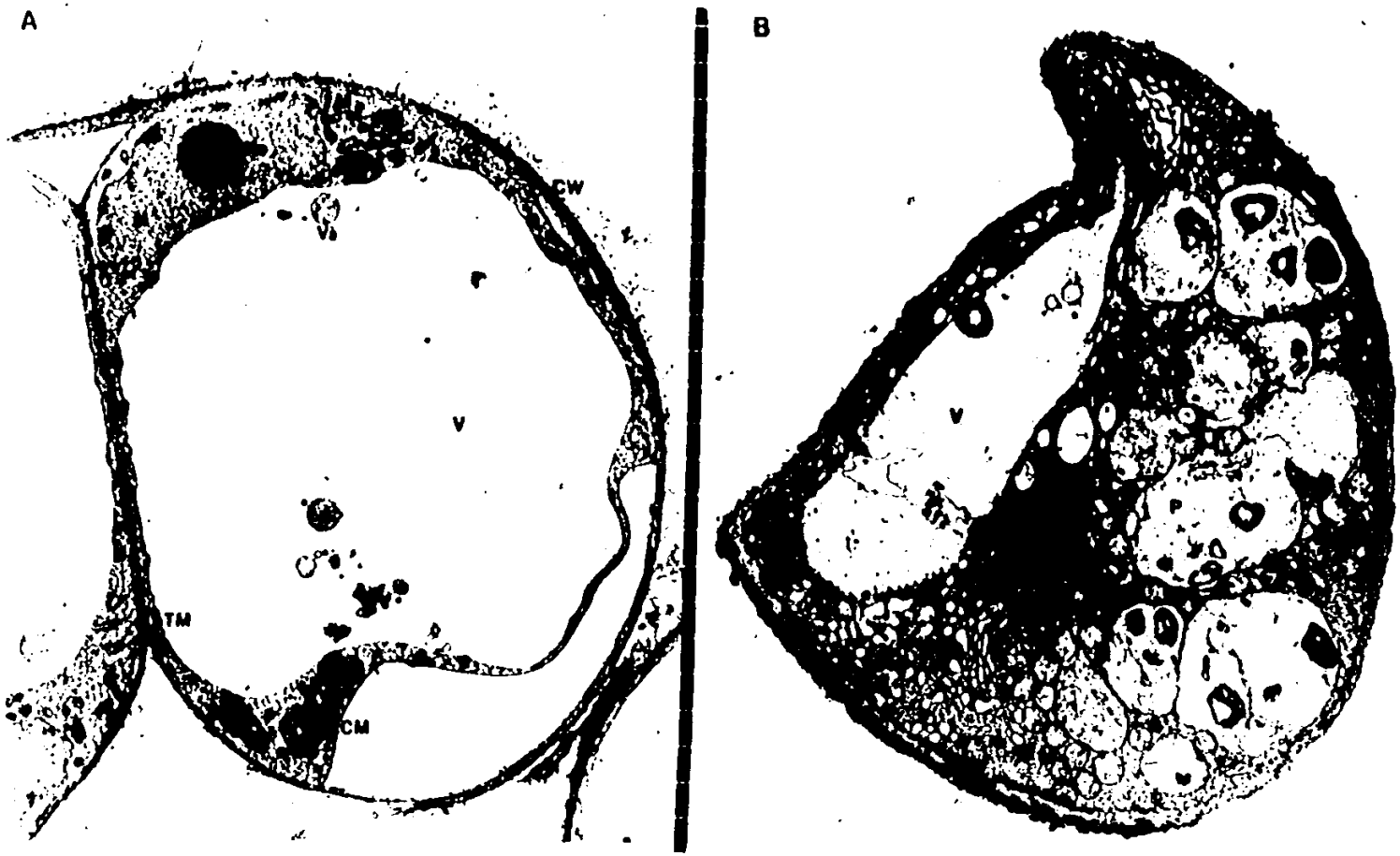

A
CW
V
V
TM
CM
B
V

FIGURE 12. Electron micrograph of a soybean cell showing the major organelles; P, proplastids; M, mitochondria; ER, endoplasmic reticulum; V, vacuole (X 7,000).


V
P
P
P
P

II. Preparation of Cell Free Extract and Subcellular Fractions

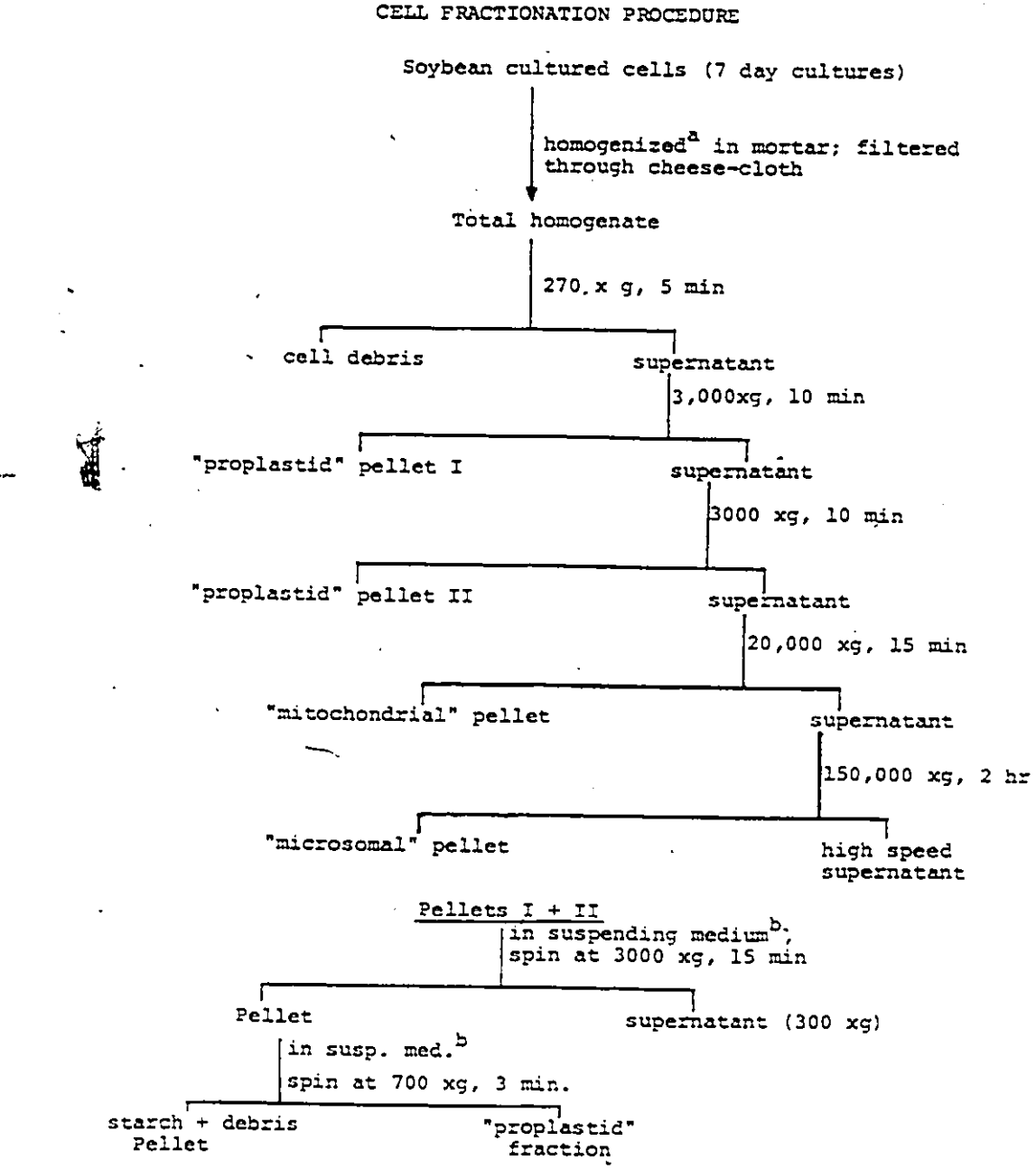
The cell free extracts were prepared as follows: The harvested cells were suspended in 2 volumes of homogenizing medium (Table 7) and carefully disrupted for 2 min. to ensure the isolation of intact organelles. The homogenate was filtered through 8 layers of gauze and the procedure was repeated 2 more times. Details for the isolation of subcellular organelles from the combined filtrates are given in the flow sheet (fig. 13).

III. Sucrose-Gradient Separation

Sucrose-gradient separations were necessary for the study of the intracellular localization of the oleoyl-CoA desaturase enzyme. The crude 3,000 x g pellets (pellet I and pellet II) were suspended in a small volume of suspending medium and layered on a discontinuous sucrose gradient* and the tubes centrifuged at 45,000 x g for 2 hours in the SW 28 head of Beckman model L2-65B ultracentrifuge. At the end of this time, the centrifuge was allowed to decelerate without the application of the brake. 1.0 ml fractions were collected with a Beckman Fraction Collector System followed by pooling those fractions corresponding to each clearly visible band (see Fig. 14). These sugar containing bands were diluted considerably

*Each Ultra-clear (25 x 89 mm) centrifuge tube (Beckman Instruments, Inc.) contained 5.0 ml each of 2.0 M (bottom) 1.75 M, 1.50 M, 1.0 M, 0.75 M (Top) sucrose solutions.

FIGURE 13.



[a] Homogenizing medium:

Phosphate buffer (pH 7.2) 10 mM, sorbitol 330 mM, $MgCl_2$ 1mM, KCl 10 mM, EDTA 1mM, BSA 0.1% and PVP 0.1%.

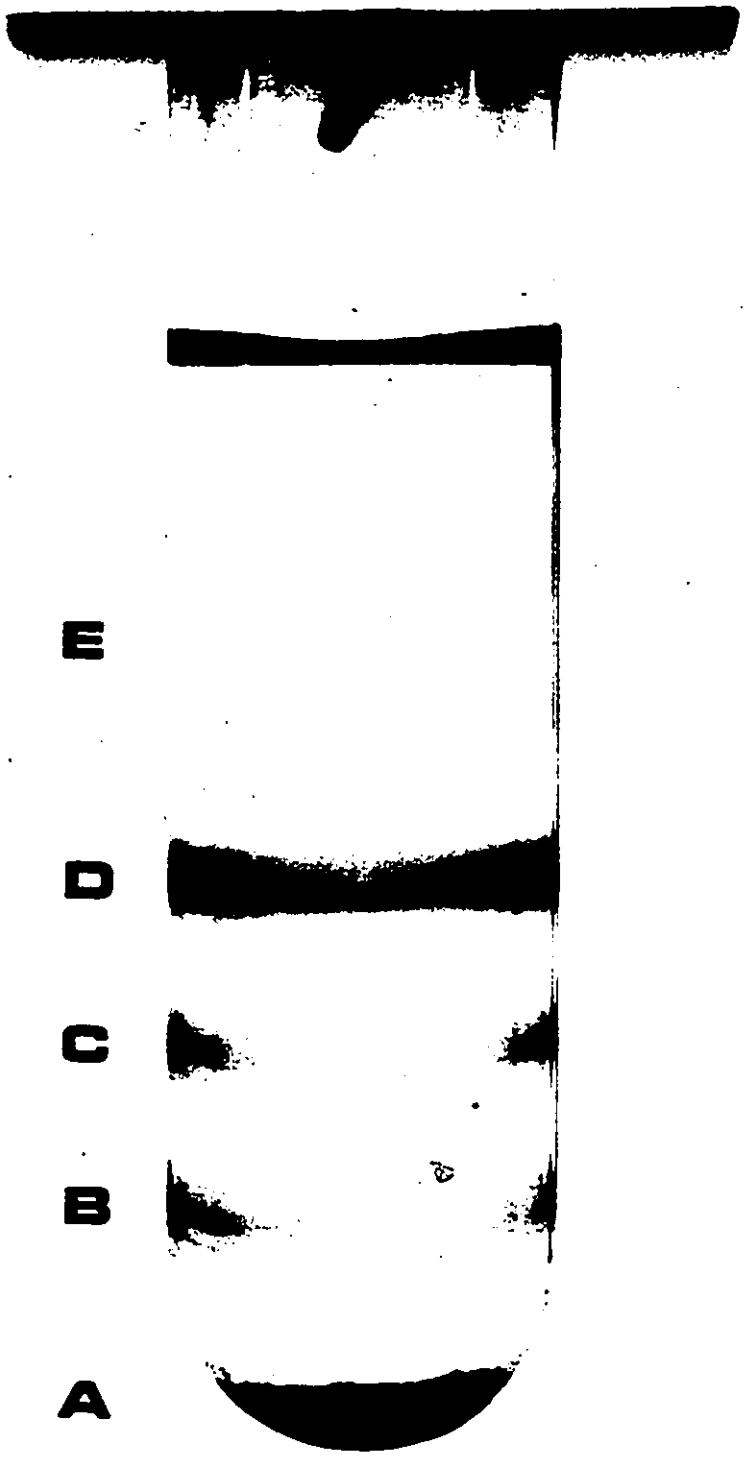
[b] tricine buffer (pH 8) 40 mM, sorbitol 330 mM, $MgCl_2$ 1mM.

TABLE 7

COMPOSITION OF MEDIA USED IN CELL FRACTIONATION

Compound	Homogenization Medium (mM)	Suspending Medium (mM)
Tricine pH 8.0	-	50
Phosphate Buffer pH 7.2	10	-
Sorbitol	330	330
$MgCl_2$	1	1
KCl	10	10
EDTA	1	-
PVP	0.1%	-
BSA	0.1%	-

FIGURE 14. Typical separation of crude proplastids (see Fig. 13) on sucrose gradient prepared as described in section C,III. Pellet A, containing mostly starch; Band B, peroxisomes; Band C, proplastids; Band D, mitochondria; Band E, vesicles derived from tonoplast and plasma membranes. For identification see electron micrographs and marker enzyme activities in section D,II.1.


E
D
C
B
A

with cold suspending medium and the organelle suspensions were then centrigued at 20,000 x g for 15 min. at 4°C in the SS34 head of Sorval RC-5B centrifuge. The individual pellets, A - E, obtained were finally suspended in a small volume of suspending medium and assayed for protein content (311), marker enzymes (see section C.V. and XIII) and oleoyl-CoA desaturase activities.

IV. Assay of Desaturase Activity and Hydroxy Fatty Acid Formation

The oleoyl-CoA and linoleoyl-CoA desaturase activities of proplastids or microsomes prepared from soybean cell suspension cultures were determined in a reaction mixture (1.0 ml) containing 100 mM Tricine buffer (pH 8.0), 200 µM NADPH: 40 mM $MgCl_2$; 40 mM ATP, 0.1 mM coenzyme A and either 100 µM oleoyl-CoA or 30 µM of linoleoyl-CoA with 0.2 mg enzyme protein. The incubation was carried out at 29°C for 30 min for oleoyl-CoA or one hour for linoleoyl-CoA, in 15 ml unstoppered tubes with shaking at 120 rpm.

Reactions were terminated by adding 0.2 ml of 20% methanolic KOH, followed by heating at 70° for an hour. The mixtures were subsequently acidified with 0.5 ml of conc. HCl; 0.3 ml water, 1.8 ml of methanol and 2.0 ml chloroform were added to form a biphasic system. The mixture was vortexed and centrifuged at 2,000 x g for 1-2 min. The lower chloroform phase, containing the free fatty acids was removed with a

Pasteur pipette into a clean 15 ml glass stoppered centrifuge tube. The upper methanol-water phase was washed with chloroform (2.0 ml) 3 to 4 times. The chloroform extracts were combined in 20 ml screw-capped test tubes and concentrated under a stream of N_2, with addition of benzene to remove any water; to the residue was added 2.0 ml of 2.5% of methanolic HCl and the mixture was heated under reflux for one hour at 70°C. After cooling, 0.2 ml of water was added and the fatty acid methyl esters (FAME) were extracted with 3 to 4 portions of 2.0 ml of petroleum ether. The combined petroleum ether extracts were brought to dryness in a 15 ml glass stoppered centrifuge tubes under a stream of N_2, using benzene to remove traces of water, and the residues were dissolved in 100 µl of chloroform. The fatty acid methyl esters obtained were analysed either according to their degree of unsaturation on 10% $AgNO_3$ impregnated silica gel G (0.5 mm thick) plates together with authentic standards, (18:0, 18:1, 18:2, 18:3 acids), in the solvent system chloroform-ethanol (200:5 v/v) or by radio-gas chromatography as outlined in Section C.VIV. The specific activity of the desaturase was expressed as nmoles (or µmoles) of product formed per min. or per hour (linoleoyl-CoA) per mg of either proplastic or microsomal protein.

V. Marker Enzyme Activities

a) Succinate Dehydrogenase Assay

Succinate dehydrogenase activity was measured as described by J.W. Porteous and B. Clark (223) in a system

(final vol, 1.0 ml) containing; potassium phosphate (pH 7.4) 50 mM: 2-(p-Iodophenyl)-3-(p-3-Nitrophenyl)-5-phenyl-tetrazolium hydrochloride (INT) 0.1%; sodium succinate, 50 mM; sucrose, 25 mM; and EDTA, 2 mM. The mixture was incubated at 0°C for 30 min. The reaction was initiated by addition of about 0.2 mg of protein followed by further incubation at 37°C for 15 min. Assays were done in duplicate in 15 ml centrifuge tubes. The reaction was stopped by addition of 1.0 ml of 10% trichloroacetic acid. Note that trichloroacetic acid was added prior to the protein for the zero-time blanks. The formazan or reduced INT was extracted with 4.0 ml of ethyl acetate and its absorbance measured at 490 nm (ε, 20.1 x 10^3 cm^2/mole). Specific activity was expressed as μmoles of INT reduced per min per milligram of protein.

b) Catalase Assay

The method of assay for catalase is that described by Hugo Aebi (224), involving the measurement of H_2O_2 decomposition at 240 mm (ε_{240}, 40 cm^2/μmole). The following were added to 3.0 ml (1 cm path) spectrophotometer cuvette; phosphate buffer (pH 7.0), 50 mM; and protein. The reaction was initiated by addition of 1.0 ml of 10.2 μM H_2O_2 into the sample cuvette and the decrease in absorbance was measured with a recorder for several minutes against a blank containing buffer alone. Specific activity was expressed as nanomoles of H_2O_2 removed per minute per milligram of protein.

c) UDP-Gal Transferase

The UDP-Gal transferase assay (51) was carried out in a reaction mixture (1.0 ml) containing the following: phosphate buffer (pH 7.5), 100 mM; UDP-Gal, 83.6 nmoles; $MgCl_2$, 10 mM; and diglyceride, 90 nmoles. The reaction was started by addition of 0.5 mg of protein followed by incubation at 30°C for 1 hour with gentle shaking. The reaction was stopped by addition of methanol-chloroform (2:1 v/v) followed by extraction of the lipids according to the method of Bligh and Dyer (225). The ^{14}C activity in the total lipids was counted in a liquid scintillation counter as described in section C.XI.c and the dpm converted to nmoles of [^{14}C]-galactose incorporated per milligram of protein per hour.

VI. Procedure for Electron Microscopy

The pellets of cell fractions prepared as described in section C.II, Fig. 14, were fixed overnight with 6% glutazaldehyde at 4°C. The gluteraldehyde was carefully removed with a Pasteur pipette and the pellets were washed 2 times with 3-5 ml of 0.1 M phosphate buffer (pH 7.2) for 1.5 hours total. The pellets were post-fixed in 2% OsO_4 for 4 hours at 4°C, then rinsed twice with phosphate buffer for 5 min. each time. The pellets were then subjected to a serial dehydration in 30%, 50%, 70% and 100% ethyl alcohol for 15 min. in each step. While the first three dehydrations were performed at 4°C the dehydration with 100% alcohol was done at room temperature. The alcohol was decanted and further

removed from the sample by addition and removal of propylene oxide followed by a mixture of Araldite-propylene oxide (1:1). The embedding material* was added to the sample and left overnight on a rotary shaker. The following day, the embedding material was gently decanted and replaced with fresh embedding material and left standing for an additional 1-2 hour. Fresh embedding material was added in a gelatin capsule followed by the addition of the pellet and kept first at 40°C for 6 hours then at 65°C for 72 hours. Samples were sectioned with a Reichert Ultramicrotome and the sections placed on a 300 mesh copper grid, stained first with uranyl acetate (saturated solution), then with lead citrate (Reinold stain). The sections were viewed with a Siemens 101 and/or Philipps 300 Electron Microscope. Fixing, staining, sectioning and viewing of the sample was carried out by Mr. Philip Chow-Chong, in the Department of Anatomy, University of Ottawa.

VII. Procedure for Hydrogenation

A chloroform solution of the lipid sample (1-10 mg) was transferred to a 15 ml centrifuge tube, and brought to dryness under a stream of N_2. The residue was dissolved in 1.0 ml of methanol and about 5 mg of platinum oxide catalyst was added.

*Embedding material (EPON-ARALDITE mixture) containing the following: DDSA, 30 gm; Araldite, 11.1 gm; and EPON 812, 15.5 gm; The ingredients were thoroughly mixed, followed by the addition of DMP-30 (1.4 ml) and mixing again.

Hydrogen gas was bubbled through the mixture for 15-30 min. at room temperature. On completion of the reaction, nitrogen was briefly bubbled through the mixture to remove excess hydrogen, and the catalyst was centrifuged down at 2,000 x g for 1 min. The methanol supernatant was transferred to a clean 15 ml centrifuge tube and the platinum residue washed 3 more times with methanol. The combined methanol solution was evaporated to dryness under a stream of N_2 and the residue dissolved in chloroform (100 µl).

VIII. Procedure for Methylation

A solution of the hydrogenated lipid (1-10 mg) was transferred to a 50 ml round bottom flask and the solvent removed in vacuo on a rotary evaporator. To the residue was added 4.0 ml of methyl iodide and 100 mg of silver oxide and the mixture was heated under reflux for 5 hours. On completion of the reaction, the mixture was transferred quantitatively into a 15 ml centrifuge tube using two or three 2.0 ml portions of ethyl ether each time to rinse the flask. The silver salts were centrifuge down at 2,000 x g for 1 min. and the supernatant transferred to a clean tube. The salts were rinsed twice more with 1.0 ml portions of ethyl ether. The combined ether solutions were concentrated under N_2, with addition of benzene, and the residue was dissolved in chloroform (100 µl).

IX. Analysis of Fatty Acids by Gas-Liquid Chromatography

Fatty acid methyl esters (FAME) were prepared as described in Section C.IV and analysed by GLC on a column,

2 m x 4 mm, of 10% SP-2330 on 100/120 chromosorb W AW or 5% OV-17 on chromosorb W, AW-DMCS 100/120. The inlet carrier gas (N_2) pressure was set at 0.7 Kg/cm^2, corresponding to a flow rate of about 30-40 ml/min. The gases fed to the detector were hydrogen (H_2) (1.0 Kg/cm^2) and compressed air (0.7 Kg/cm^2). The electrometer attenuation was set as required. Peak areas were measured by the retention time X peak height method of Carroll (226).

X. Periodate-permanganate Oxidation of Fatty Acids

This procedure was employed to determine the position of the ethylenic bonds in the fatty acids by oxidative cleavage with periodate-permanganate followed by the analysis of the cleavage products by GLC (227).

Each reaction mixture contained the following per mole of double bond: 1.0 ml of oxidation solution (20.86 g $NaIO_4$ + 395 mg $KMnO_4$ per liter), 1.0 ml of Na_2CO_3 (2.5 mg), 1.0 ml of t-butanol, and 1.0 mg of fatty acid. The reaction was carried out at room temperature in a 100 ml round bottom flask for one hour with stiring. On completion of the reaction, solid sodium metabisulfide was added until the permanganate colour disappeared, followed by the addition of 1.0 ml of IN NaOH. The t-butanol was removed in vacuo and the remaining mixture was transferred into a 15 ml centrifuge tube and acidified with 6N H_2SO_4. The products of oxidation were extracted with 4 portions of ethyl ether. The ethyl ether extracts were combined into a methanolysis tube and subjected to methanolysis

as described in Section C.IV. The methylated products were analysed by GLC at 90°C for monocarboxylic acids and 170°C for dicarboxylic acids along with authentic standards and oxidation products from standard fatty acids. The principal products included azelaic acid, nonanoic acid, and adipic acid, while C_3 (malonate) generated from the oxidation of 18:2 and 18:3, was not recovered due to its oxidative decomposition. It is also known that malonate is susceptible to decomposition during GLC analysis and has a low relative molar response in the flame ionization detector (228).

XI. Radioisotopic Procedures

a) Labelled Compounds

[1-^{14}C]oleic acid (50 mCi/mmole) and [1-^{14}C]linoleic acid (50 mCi/mmole) were purchased from Amersham Corporation. [1-^{14}C]oleoyl-CoA was either obtained from Amersham Co. or synthesized as [1-^{14}C]linoleoyl-CoA as described below.

b) Preparation of [1-C^{14}]linoleoyl-CoA or [1-C^{14}]oleoyl-CoA Substrates

The acyl-CoA esters of [1-C^{14}]oleic or [1-C^{14}]linoleic acids were synthesized by the method of Sanchez et al., (229) with modifications as follows: The anhydride of the [1-C^{14}] oleic or [1-C^{14}]linoleic acid (40 µmoles) was prepared in 15 ml centrifuge tubes by adding 0.5 ml of methylene chloride and triethylamine (40 µmoles), which had been dried over NaOH pellets. The reaction was left at room temperature for 30 min. After cooling to 0°C (on ice), 40 µmoles of ethylchloroformate was

added and the mixture was left for two hours at 0°C. The methylene chloride was removed under a gentle stream of N_2 and the residue was washed 3 times with 0.5 - 1.0 ml tetrahydrofuran. The combined tetrahydrofuran washings were transferred into a clean 15 ml tube and the tetrahydrofuran was removed under a stream of N_2; 2.0 ml of p-dioxane was added followed by 4.0 ml of 1M $KHCO_3$ containing 40 µmoles of CoASH. The tube was flushed with N_2, sealed and incubated at room temperature (25-30°C) in the dark, with gentle shaking for 90 min. The pH was then decreased to about 1 by dropwise addition of 2N HCl. The precipitate was collected by centrifugation at 2,000 x g for 2 min. and the pellet washed 5 times with 2.0 ml of diethyl ether and 3 times with 1.0 ml of 0.1 M HCl. The remaining pellet (acyl-CoA) was taken up in a known volume of 0.1 M acetate buffer pH 6.0. The concentration of acyl-CoA was determined by measuring the absorbance at 232 nm assuming ε_{232} of 15.4 mM^{-1} (230) and by quantitative GLC of the fatty acid ester prepared from acyl-CoA by transmethylation with 2.0 ml of sodium methoxide solution for 15 min. at 25°C. The FAME were extracted with petroleum ether, dried under N_2 and chromatographed as described in section C.IV and IX.

c) Measurement of Radioactivity

Solutions of ^{14}C lipid in chloroform were counted by liquid scintillation in 10 ml of a mixture of 0.3 g of POPOP, 55.0 g of PPO, 130 ml of CH_3OH, 100 ml of solubilizer (Bio-solv, Beckman) and 770 ml of toluene (J.T. Baker Chem. Co.,

Phillipsburg, N.J.); the instrument used was a LKB Rackbeta liquid scintillation counter (Wallac Oy., Turku Finland). Quenching was corrected by the external standard ratio (ERS) method using a series of Amersham-Searle standards containing equal amounts of ^{14}C toluene (2.03×10^5 dpm) with different amounts of the quenching agent, chloroform. The quench curve correction was programmed in the counter and the final counts given in dpm. Each sample was counted to 1% or less statistical error. ^{14}C spots on 10% $AgNO_3$ impregnated TLC plates were visualized by autoradiography as described below and were scraped directly into scintillation fluid prepared without the solubilizer and counted as described previously (147).

d) Autoradiography

^{14}C-labelled lipid spots on TLC plates were detected by exposing the chromatograms to a sheet of Kodak Blue Brand X-ray film for a suitable period of time depending on the ^{14}C activity of the labelled spot; 4 days for 1,000 dpm per spot on the TLC plates.

The film was developed and fixed by standard photographic procedure.

e) Radio-Gas Liquid Chromatography

Fatty acid methyl esters (FAME) were prepared as described in Section C.IV and analyzed by Radio-GLC, in a 2 m x 4 mm column of 10% butanediol succinate polyester on chromosorb W(AW-DMCS, 60-80 mesh), at 185°C with argon as the

carrier gas inlet pressure (20 Kg/cm^2). The effluent gas was split into two streams, one half passing to a flame ionization detector (Pye Unicam, GLC), the other half passing through a tube containing CuO heated in a furnace at 700°C; the $^{14}CO_2$ formed from the [^{14}C]labelled peaks was then assayed by measurement of radioactivity in a proportional gas-flow counter (ESI Nuclear Radio Gas Chromatography detector). The flow rate through the radio-counter was set at 20 ml/min. The mass and the distribution of radioactivity were recorded simultaneously with a two-pen recorder (linear Instrument Corporation). The radioactivity of each peak was quantitated using a known amount/counts of [^{14}C]palmitate as internal standard.

XII. Gas Liquid Chromatography-Mass Spectrometry

The products of 18:1-CoA and 18:2-CoA desaturase activities were methylated and separated on 10% $AgNO_3$-SO_2 plates as described in Section C.IV and XIV.b. The components were scraped, eluted from the silica gel and rerun on washed precoated glass plates (silica gel, 250 µm; J.T. Baker, Chem. Co.,) in the solvent system petroleum ether: ethyl-ether: acetic acid (70:30:1 v/v). The bands corresponding to 18:1, 18:2, 18:3, B, C, and H ere carefully scraped, eluted from the silica gel with $CHCl_3$-CH_3OH and the eluates made to a known volume.

All esters were introduced into a mass spectrometer through a Dani Model 3800 gas chromatograph with a temperature programmer set at 200°C and designed to increase at a rate of 10°C/min. The gas chromatography was used with a 6 ft x 6 mm

glass column packed with 6% QV-17 on chromosorb W-HP. The column effluent was detected by a mass spectrometer (VG-7070E Mass Spectrometer equipped with a digital RL02 data system and printonix printer). Components in the effluent arriving at the mass spectrometer were detected by a total ionization monitor and mass spectra were taken at the apices of the peaks. The temperature at the ion source was set at 200°C and the ionization voltage at 70 EV with a source pressure of about 10^{-7} Torr.

Hydroxyl groups were silylated with trimethylsilane (Chromatographic Specialties Ltd.) in pyridine.

XIII. Analytical Procedures

1. Protein was measured by the procedure of Lowry et al., (231);
2. Acyl-CoA analysis was performed according to the spectrophotometric procedure of Peterson and Bloch (230).

XIV. Chromatography

a) Analytical TLC

Was carried out on 20 x 20 cm plates coated with 0.5 mm layer of silica gel G (E. Merck AG/Brinkmann), for non-polar and polar lipids, respectively. Samples were spotted under nitrogen on plates previously activated for 1 hour at 120°C. The solvent systems used for the development of the chromatograms were as follows:

1. Petroleum-ether:ethyl ether:acetic acid (70:30:1 v/v) for neutral lipids;
2. Chloroform-ethanol (100:2.5 vol.) for argentation TLC;
3. Two dimentional TLC was performed in chloroform-methanol-acetone-diethylamine-water (120:35:37.5:5:6:4.5 v/v) in the first dimension and chloroform-methanol-conc. ammonium hydroxide (65:25:5 v/v) in the second dimension.

Following the development of the chromatogram, spots were located either by autoradiography or by spraying the plate with the following general reagents:

1. Iodine;
2. 40% sulphuric acid in ethanol following by charring the plate;
3. 2',7'-dichlorofluorescein and visualization under u.v. light;

b) Silver Nitrate Impregnated Plates

Silver nitrate (3 g), silica gel G (E. Merck/Brinkmann, 30 g), and about 100 ml of water were mixed to give a fine slurry. After spreading, the plates were air-dried and subsequently activated at 120°C for 60 min. prior to utilization. These plates are light sensitive and were used immediately or shortly after preparation.

XV. Metabolism of 18:1-CoA and 18:2-CoA as a Function of Time

The assay system used for the kinetic studies was as described in section C.IV except that the substrate 18:1-CoA had a higher specific activity (2.0 μCi/μmole); all other reagents had final concentrations given in section C.IV. The reaction mixture was terminated by addition of 3.75 ml methanol-chloroform (2:1 v/v) at various time periods as indicated in the figure legends. A further addition of 1.25 ml of chloroform and 1.25 ml of water was required to make a biphasic system; the top methanol-water layer contained fatty acid-CoA and the bottom chloroform layer contained the complex lipids and free fatty acids. The tubes were mixed on a vortex mixer for a few seconds and subsequently centrifuged at 2,000 rpm for one minute. The bottom, chloroform phase, was transferred with a Pasteur pipette to a 15 ml glass-stoppered centrifuge tube; the water-methanol phase was washed three times with 2.0 ml $CHCl_3$. The combined chloroform phases were washed twice with 2.0 ml of upper phase and concentrated under a stream of nitrogen, with addition of benzene to remove traces of water, to about 2.0 ml and subsequently made up to a known volume (5.0 or 10.0 ml).

a) Analysis of Methanol-Water Phase (Acyl-CoA)

To the upper methanol-water phase (8.55 ml) containing the acyl-CoA's was added to 0.5 ml of 20% methanolic KOH and the mixture was saponified at 70°C for one hour. After cooling, 0.5 ml of concentrated HCl was added and a biphasic system was

formed by the addition of chloroform (5 ml) and methanol. The chloroform phase was transferred into a clean glass-stoppered centrifuge tube and the upper methanol-water phase was washed with chloroform (3-4 ml) 3 to 4 times. The combined chloroform extracts were concentrated under a stream of N_2 with addition of benzene to remove water, and made to a known volume (5.0 or 10.0 ml); and aliquot was taken for counting to check that all of the radioactivity, originally present in acyl-CoA was recovered as free fatty acid in the chloroform phase. The remaining solution was dried under a stream of N_2 and the residue methylated as described in section C.IV. The fatty acid methyl esters (FAME) were extracted and chromatographed on 10% $AgNO_3$ silica gel G plates as described in section C.XIV.b.

b) Analysis of Chloroform Phase (Acyl Lipids)

The chloroform phase containing the acyl-lipids was made up to 5.0 or 10.0 ml with chloroform-methanol (2:1) and 50 to 100 µl were taken for counting, for estimation of ^{14}C distribution among lipid compounds; the remainder of the chloroform solution was concentrated to 0.1 ml and spotted on TLC silica gel plates (0.25 mm thick), and developed in chloroform-methanol-acetone-diethylamine-water (120:35:37.5:6:4.5 v/v) in the first dimension and chloroform-methanol-conc. ammonium hydroxide (65:25:5 v/v) in the second dimension. Each lipid component was identified by running appropriate authentic standards. The ^{14}C spots were detected by autoradiography, scraped from the plate and counted as described in section C.XI.c.

XVI. Extraction of Membrane Lipids

Immediately after use in enzyme essays, the microsome and plastid suspensions were stored at -70°C until the lipid extractions were performed. The lipids were extracted by the method of Bligh and Dyer (225) modified as follows: to one volume of microsomal or plastid suspension, containing about 4-5 mg and 2-3 mg protein, respectively, was added 3.75 volumes of freshly prepared methanol-chloroform (2:1, v/v) to obtain a ratio of methanol-chloroform-water of (2:1:0.8 v/v). Extraction was carried out in 15 or 50 ml pyrex glass-stoppered centrifuge tubes; 150 µg and 250 µg of 17:0 internal standard fatty acid was added to the plastid and microsomes respectively and the mixture was vortexed vigorously (on a Vortex-Genie mixer, Scientific Industries) and allowed to stand for about 30 minutes before appropriate volumes of chloroform and water were added to give a final ratio of methanol-chloroform-H_2O of 1:1:0.9, v/v. The mixture was vortexed and allowed to stand for another 15-20 min. before centrifugation at 2,000 rpm (in a IEC HN-SII centrifuge) for 1-2 min. The bottom chloroform layer was removed to a separate tube and the upper phase was washed 3 times with 1-2 ml chloroform. The combined chloroform solutions were concentrated under a stream of N_2, after adding benzene to remove traces of water, to about 0.1 ml and the FAME (fatty acid methyl esters) were quantified as described under section C.IX.

D. RESULTS

I. Identification of Products of Enzyme Activities

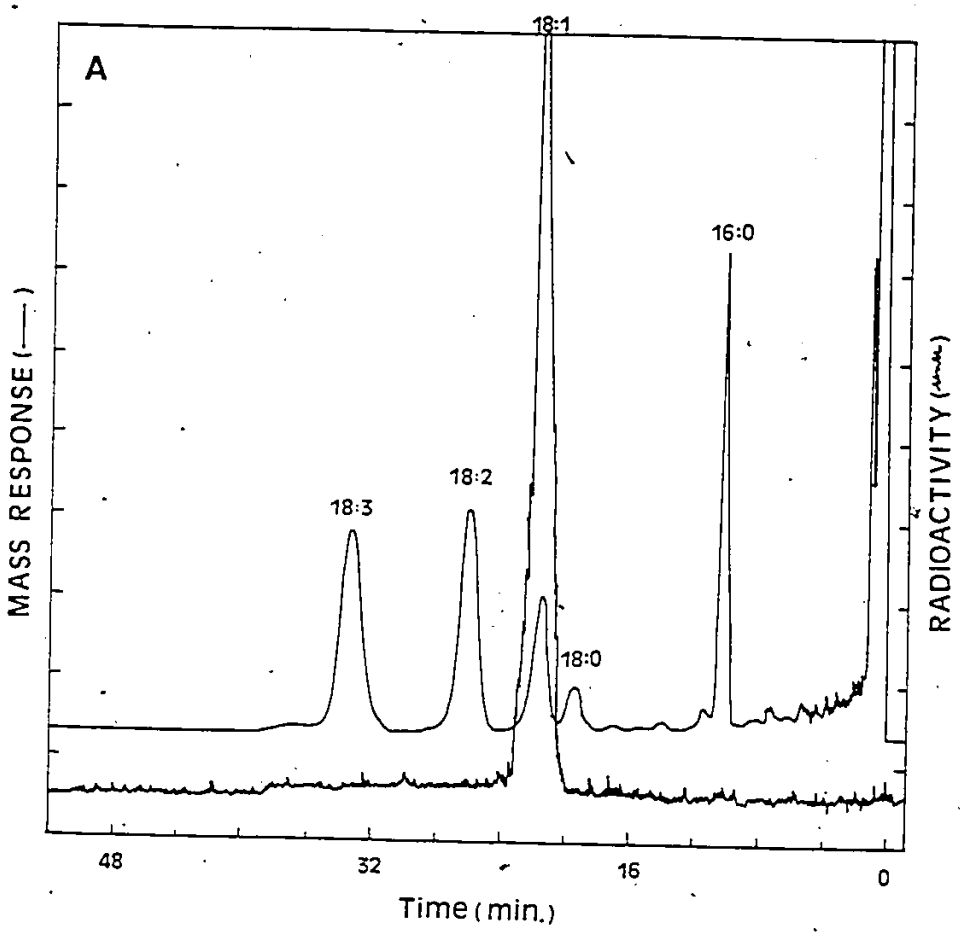
1. Products of 18:1-CoA Metabolism

a) TLC and GLC

On incubation of [^{14}C]oleoyl-CoA with either proplastid or microsomal fraction under the conditions described in materials and methods for the desaturase system, three products (A, B and C) were detected (Fig. 15 and 16B) instead of the one product (18:2) expected from Δ^{12}-desaturase activity. The distribution of ^{14}C among the products of 18:1-CoA metabolism was as given in Table 8. The expected desaturase product was only a relatively minor component (B) identified as [^{14}C] 18:2 on the basis of its co-migration with an authentic standard of 18:2 on $AgNO_3$ - SiO_2 TLC (Fig. 15) and on radio-GLC (Fig. 16B). The major product (C) of the enzyme system was in fact identified as a hydroxy-18:1 acid (HO-18:1) by its mobility on TLC (Table 8 and 9) and on radio-GLC (Fig. 16B and C, Table 9). Because of its long retention time, product C gave a shallow peak on radio-GLC which was not always detectable, particularly with the microsome system which formed relatively low levels of product C. The other major product A, was detected on radio-GLC (BDS column) (Fig. 16B) but not on $AgNO_3$-TLC, where it co-migrated with the 18:1 substrate (Fig. 15). These products were further identified by GLC before and after hydrogenation in the following experiments: In Exp. 1 (Table 10), a portion of the ^{14}C

FIGURE 15. Autoradiogram of a thin-layer chromatogram on 10% $AgNO_3$-silica gel G showing the formation of B (18:2) and C (3-OH-18:1) from 18:1-CoA. Incubation was performed with proplastids (PL) and microsomes (M) for 30 min. as described in Section C.IV.

FIGURE 16. Radio-GLC tracing on BDS at 185°C from 18:1-CoA incubation with proplastids as described in Section C, IV. A, 0 time (control) incubation, showing the major fatty acids (upper tracing, mass response) 16:0, 18:0, 18:1, 18:2 and 18:3; and [^{14}C]-18:1 (lower tracing, radioactivity): B, showing the formation of product B (18:2), A, (diunsaturated C_{18} fatty acid), and C (HO-18:1): (see Table 8 for ^{14}C distribution; C, after hydrogenation and methylation (see Table 10).


A
18:1
16:0
18:3
18:2
18:0
MASS RESPONSE (——)
RADIOACTIVITY (~~~)
48
32
16
0
Time (min.)

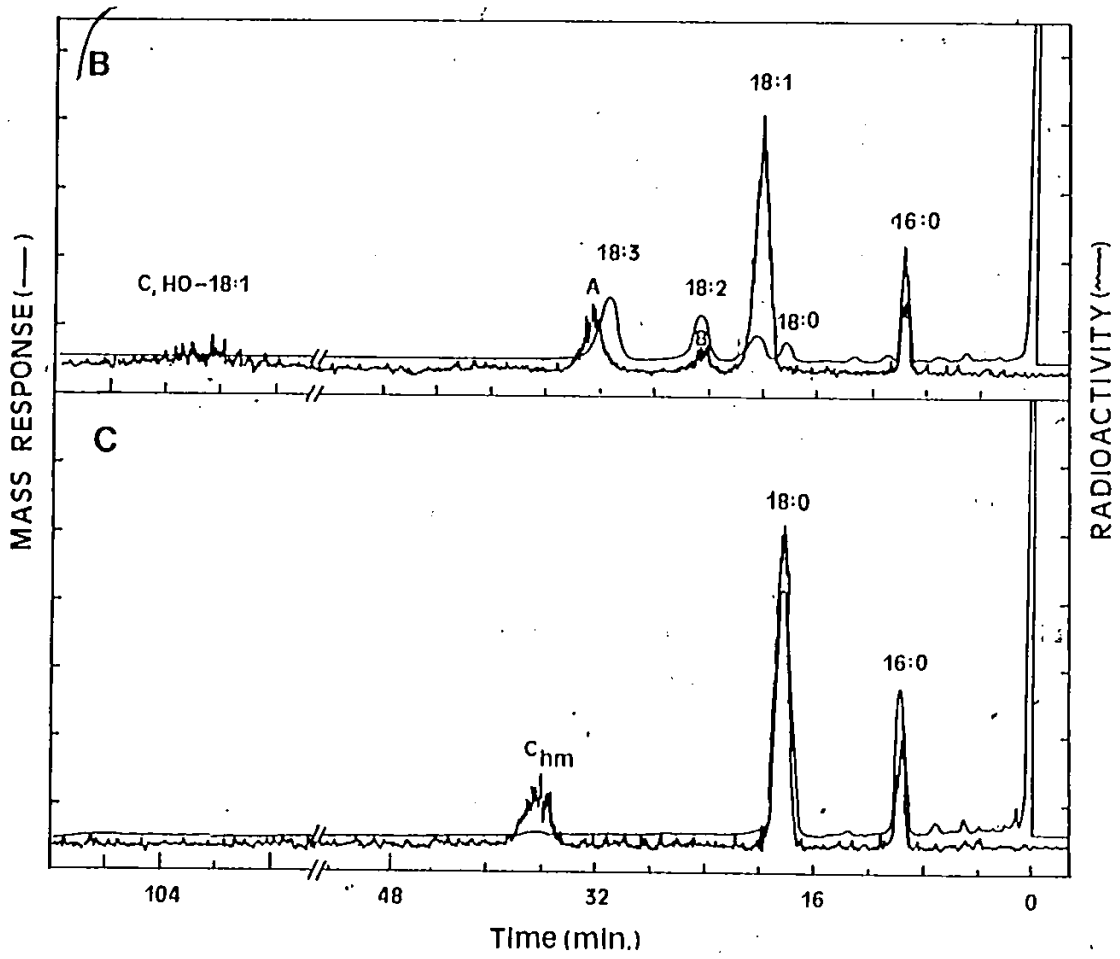

B
18:1
16:0
18:3
A
C, HO–18:1
18:2
B
18:0
C
18:0
16:0
C_{hm}
MASS RESPONSE (—)
RADIOACTIVITY (---)
104
48
32
16
0
Time (min.)

TABLE 8

Distribution of ^{14}C Among Products of 18:1-CoA Metabolism[a]

Products	Proplastids				Microsomes			
	TLC[b]		GLC[c]		TLC[b]		GLC[c]	
	R_f	%Dist.[n]	Rel Ret.	% Dist.[n]	R_f	% Dist.[n]	Rel Ret.	% Dist.[n]
Substrate (18:1)	0.70	-	2.1	74.9	0.70	-	2.1	91.4
		86.7		-		91.4		-
A	0.70	-	3.4	10.5	0.70	-	3.4	7.0
B	0.49	3.9	2.6	2.7	0.49	2.4	2.6	1.6
C	0.36	9.4	9.0	11.8	0.36	6.2	9.1	N.D.[d]

[a] Incubation was performed as described in section C.IV.

[b] TLC on 10% of $AgNO_3$-SiO_2 G in Chloroform:Ethanol (200:5 v/v): R_f of standards: 2(OH)-18:0; 0.56; 3(OH)-18:0, 0.54; product C_h and H_h, 0.54; 2(OCH_3)-18:0, 0.76; 3(OCH_3)-18:3, 0.73; 9(OH)-18:0, 0.55; 9(OCH_3)-18:0, 0.74; C_{hm} or H_{hm}, 0.73; 18:3 (Authentic standard) 0.49.

[c] GLC on BDS at 185°C; retention relative to 16:0 internal standard 9- and 12(OH)-18:0, 15.9; 9- and 12(OCH_3)-18:0, 4.6; C_{hm}, 3.8; 18:2 (Authentic standard), 2.6.

[d] N.D. = not detected, see explanation given in this section.

TABLE 9

Retention Time Relative to Methyl Palmitate of Standard Hydroxyacid Hydroxylated Metabolic Products and their Methoxy and TMS-ether Derivatives

Fatty Acid	GLC[a] Relative Retention			TLC[b] R_f	
	HO-Me ester	OCH_3-Me ester	OTMS-Me ester	HO-Me ester	OCH_3-Me ester
2-OH-18:0	5.7	3.1	2.2	0.41	0.63
3-OH-18:0	7.9	3.1	2.2	0.35	0.64
9-OH-18:0	10.4	4.0	3.2	0.25	0.60
12-OH-18:0	10.9	4.1	3.2	0.26	0.60
16-OH-18:0	14.2	3.6	-	0.13	0.53
18-OH-18:0	22.7	5.7	-	0.12	0.56
12-OH-18:1	11.1	4.9	3.4	-	-
Product HO-18:1 (C)	8.1	4.1	2.9	0.34	-
Product HO-18:0 (C_h)	7.9	3.1	-	0.35	0.64
Product HO-18:2 (H)	9.3	4.3	3.4	0.31	-
Product HO-18:0 (H_h)	7.9	3.1	-	0.35	0.63

[a] GLC on SP2330 at 205°C; retention relative to 16:0

[b] TLC on silica gel G in petroleum ether:ethylether:acetic acid (80:20:1 v/v).

TABLE 10

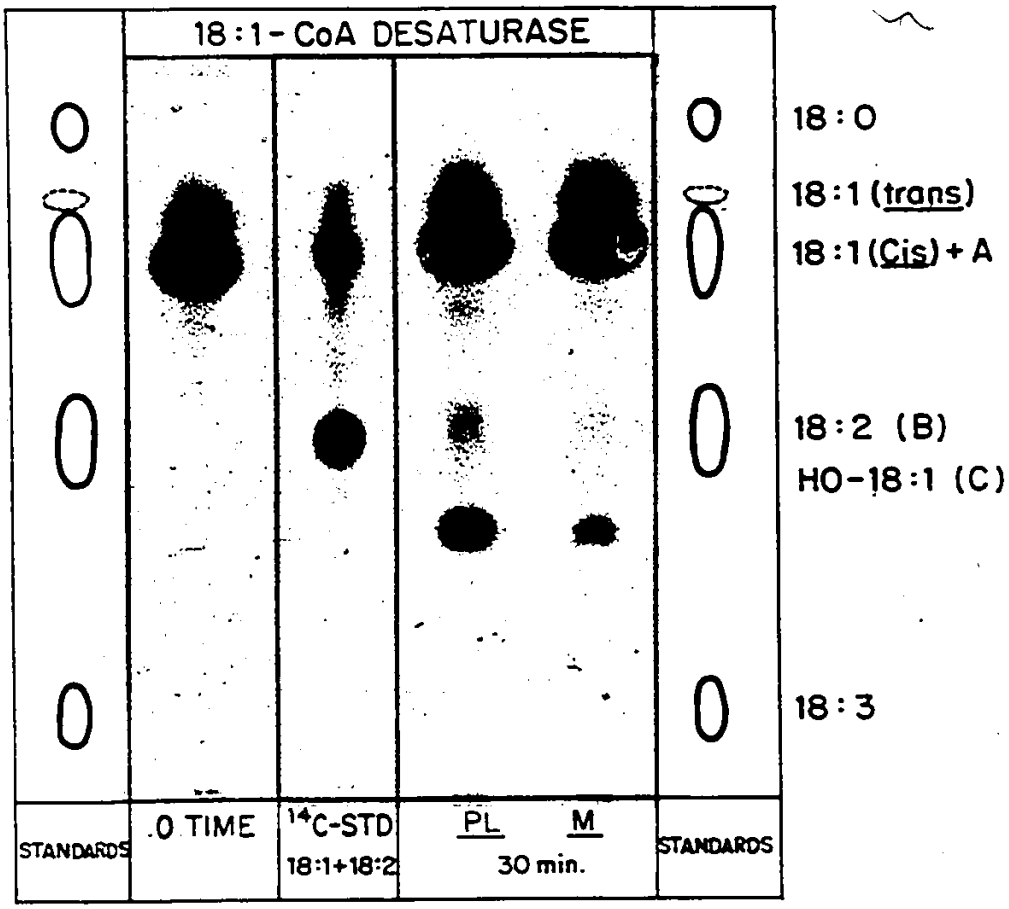
^{14}C Distribution Among 18:1-CoA Metabolic Products before and after Hydrogenation and Hydrogenation/Methylation

Products	Relative ^{14}C-distribution, mole Ratio[a]						Relative Ret. Time[b]
	Proplastids				Microsomes		
	Exp. 1		Exp. 2				
	Before Hydrog.	After Hydrog.	Before Hydrog./ Meth.	After Hydrog./ Meth.	Before Hydrog.	After Hydrog.	
Substrate (18:1)	3.3	-	5.4	-	4.03	-	2.1
A	0.46	-	1.76	-	.31	-	3.4
B (18:2)	0.12	-	0.33	-	.07	-	2.6
C	0.52	-	2.49	-	N.D.[c]		9.1
Hydrog. Products							
18:0	-	3.87	-	7.47		4.40	
Ch		N.D.[c]	-	-		N.D.[c]	-
Hydrog. and Methyl Product							
Chm				2.43			3.8
Totals	4.40	3.87	9.98	9.90	4.41	4.40	

[a]Relative to internal standard [^{14}C]16:0; GLC on BDS at 185°C.

[b]Relative to 16:0, GLC on BDS at 185°C.

[c]N.D. = not detected due to its long retention time.


18 : 1 - CoA DESATURASE
18 : 0
18 : 1 (trans)
18 : 1 (Cis) + A
18 : 2 (B)
HO-18 : 1 (C)
18 : 3
STANDARDS
0 TIME
14C-STD
18:1+18:2
PL
M
30 min.
STANDARDS

products was used to check their percent distribution relative to $[^{14}C]$16:0, as internal standard, while the rest was hydrogenated (see section C,VII) in the presence of hydrogen gas with platinum oxide as catalyst (H_2/Pt); and in Exp. 2 (Table 10), a portion of the $[^{14}C]$-products was again used to check their percent distribution relative to $[^{14}C]$16:0 and the remaining portion was first hydrogenated (Pt/H_2), and then methoxylated with methyl iodide in the presence of silver oxide (CH_3I/Ag_2O) (Section C, VIII).

The results showed that on hydrogenation products A and B formed with either proplastids or microsomes, were reduced to 18:0 but product C could not be detected on the GLC tracing due to its high retention time on the BDS column (Table 10). In Exp. 2 (Table 10) all products except C could be reduced to 18:0 on hydrogenation, as in Exp. 1, but after hydrogenation and methoxylation product C was converted to a compound having GLC retention similar to that of a methoxystearate. Comparison of GLC relative retention data of product C and its derivative (C_h and C_{hm}) with those of authentic hydroxyacid methylesters and their methoxy and TMS derivatives (Table 9) showed a close correspondence of C to 2-, or 3-hydroxy-18:0, indicating that the hydroxyl group in C was close to the carboxyl group. When Exp. 1 was repeated with isolated 18:1 + A mixture (see Section C, XII), the 18:0 formed on hydrogenation accounted for all of the ^{14}C present in 18:1 + A (Table 11). All of these experiments thus confirm that product B was 18:2, product C was a hydroxy-18:1

TABLE 11

Distribution of Purified[a] [^{14}C]18:1 Substrate + Product A Before and After Hydrogenation

^{14}C Products	Rel. ^{14}C-Distr. mole ratio[b]		Rel retention on SP-2330[b]	
	Before Hydrog.[n]	After Hydrog.[n]	Before Hydrog.[n]	After Hydrog.[n]
18:1	3.82	-	2.1	-
A	1.08	-	3.2	-
Hydrog.[n] Prod. 18:0	-	4.87	-	1.9
Totals	4.90	4.87		

[a]Purified as described in Section C.XII.

[b]Relative to internal standard [^{14}C]16:0; GLC on SP-2330 at 205°C.

[c]Relative retention to 16:0 internal standard on SP-2330 at 205°C; 18:0 authentic standard, 1.9.

fatty acid, and product A, was an unsaturated C_{18}-fatty acid with no other functional group in the molecule. Further information concerning the location of the double bonds in A and B, and the double bond and hydroxy group in C was obtained from the results of periodate permanganate oxidation of the products.

b. Permanganate-Periodate Oxidation of Fatty Acids

The reliability of this procedure (see section C, X) was first tested with authentic standard fatty acids: oleate (Δ^9-18:1), linoleate ($\Delta^{9,12}$-18:2) and linolenate ($\Delta^{9,12,15}$-18:3). Two main products were obtained from each acid: a C_9 dicarboxylic acid (azelaic acid) and a C_9, C_6 or C_3 monocarboxylic acid, respectively (Table 12). On the basis of relative retention data, the monocarboxylic and dicarboxylic acids produced by the oxidation of the 18:1 substrate were found to be nonanoic (C_9) and azelaic (C_9) acids, respectively, verifying that 18:1 substrate was Δ^9-octadecenoic acid (Table 12). Similarly, the oxidation products of product B were a C_6 monocarboxylic acid and C_9 dicarboxylic acid (Table 12) confirming that this product of Δ^{12}-desaturase reaction was indeed 9,12-octadecadienoic acid. Product C yielded a C_9 monocarboxylic acid, indicating the presence of a double bond at position-9, and a C_7 dicarboxylic acid indicating the presence of hydroxyl group at the C_3 position. Product A (in 18:1 + A) yielded a C_9 monocarboxylic acid and a C_7 dicarboxylic acid indicating the presence of one double bond at C_9 and the second double bond at C_2 to C_3 but the C_2 or C_3 diacids could not be detected because of its rapid decomposition to CO_2

TABLE 12

Permanganate-Periodate Oxidation of Products of 18:1-CoA Metabolism

Fatty Acid Product	$KMnO_4$-HIO_4 Oxidation Products			
	Monoacids		Diacids	
	GC-Rel.Ret.*	Identity	GC-Rel. Ret.*	Identity
Standard (18:1)	1.00	C_9	1.00	C_9
Standard (18:2)	0.20	C_6	0.99	C_9
Standard (18:3)	-	(C_3)	0.98	C_9
18:1 Substrate	0.99	C_9	0.99	C_9
Product B (18:2)	0.20	C_6	0.97	C_9
Product C (3-OH-18:1)	0.99	C_9	.36	C_7
Product A (18:1 + A)	0.99	C_9	.37	C_7

*GC on 10% butanediolsuccinate at 90°C for monocarboxylic acids or at 170°C for dicarboxylic acids; retentions are given relative to the C_9 monoacid (retention time 17.8 min.) or the C_9 diacid (retention time, 22.8 min.). Relative retention of standards diacids are: C_9, 1.0; C_8, 0.75; C_4, 0.19 while those of monocarboxylic acids are: C_{10}, 1.7; C_9, 1.0; C_8, 0.58; C_7, 0.33; C_6, 0.19.

Note that C_3 and C_2 dicarboxylic acid were not detected due to its ready oxidation by permanganate (227-228).

(227,228). The final identification of products A, B and C was achieved by GC-Mass Spectrometry, as described in the next section.

c) GC-Mass Spectrometry

i. Product A and B

The GC tracing of [^{14}C]18:1 (Fig. 17) obtained from [^{14}C]18:1-CoA substrate as described in section C, XII of Materials and Methods, showed methyl oleate as the major peak. Its mass spectrum (Fig. 18A) showed a molecular ion (M^+) peak at m/e 296 corresponding to the expected molecular weight and peaks at m/e 265 and 264 representing the elimination of a methoxy group (M-31) and methanol (M-32) respectively; fragment ions at m/e 74 (M-222) and 222(M-74) arise from the McLafferty rearrangement. The base peak at m/e 55 and other major fragment ions of low mass at m/e 41,69, 83 and 97 are typical ion fragments arising from hydrocarbon chains. The spectrum was in fact identical to that of authentic methyl oleate. The small peak which eluted before methyl oleate (Fig. 17) was, according to its MS fragmentation pattern, unlabelled methyl palmitate which co-migrated with methyl oleate on silver nitrate plates, and was probably derived from endogenous proplastid lipids.

The slow migrating peak corresponding to component A (Fig. 17) migrating close to methyl linolenate on a radio-GLC (Fig. 16B) and with methyl oleate on silver

FIGURE 17. GLC tracing of 18:1 + product A purified and analysed as described in Section C, XII. Mass response of 18:1, A and 16:0 as contaminant (see text for explanation). S = solvent ($CHCl_3$).

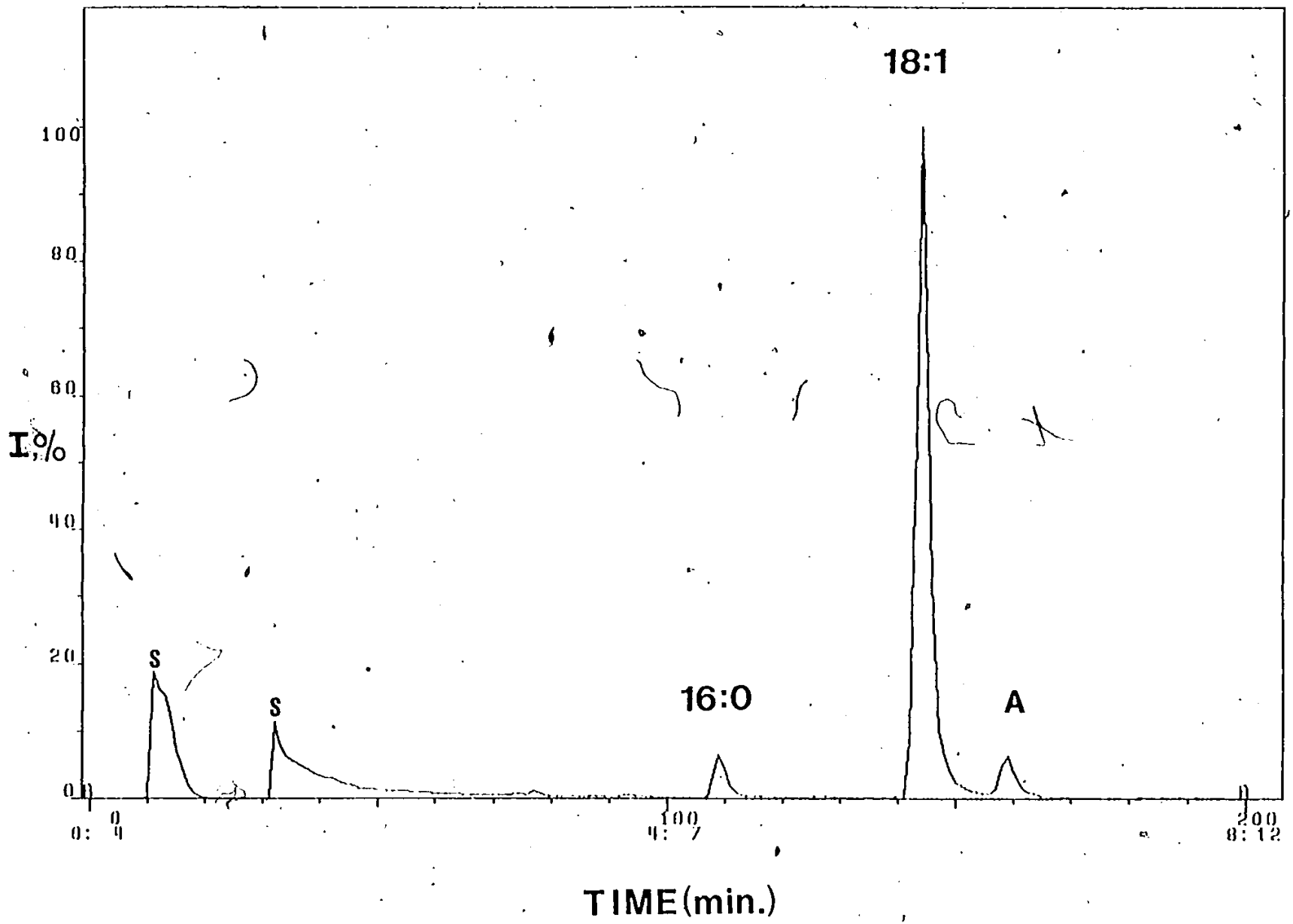
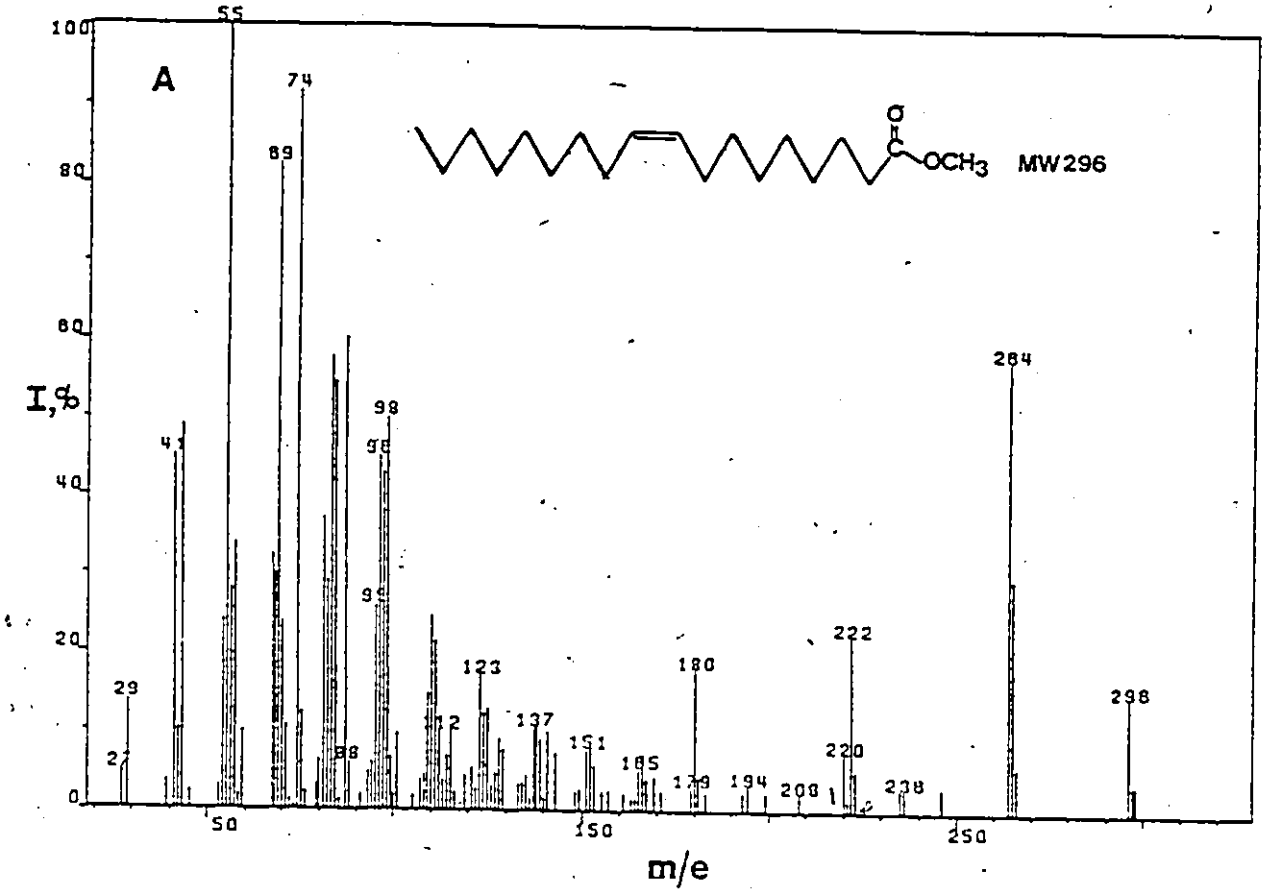
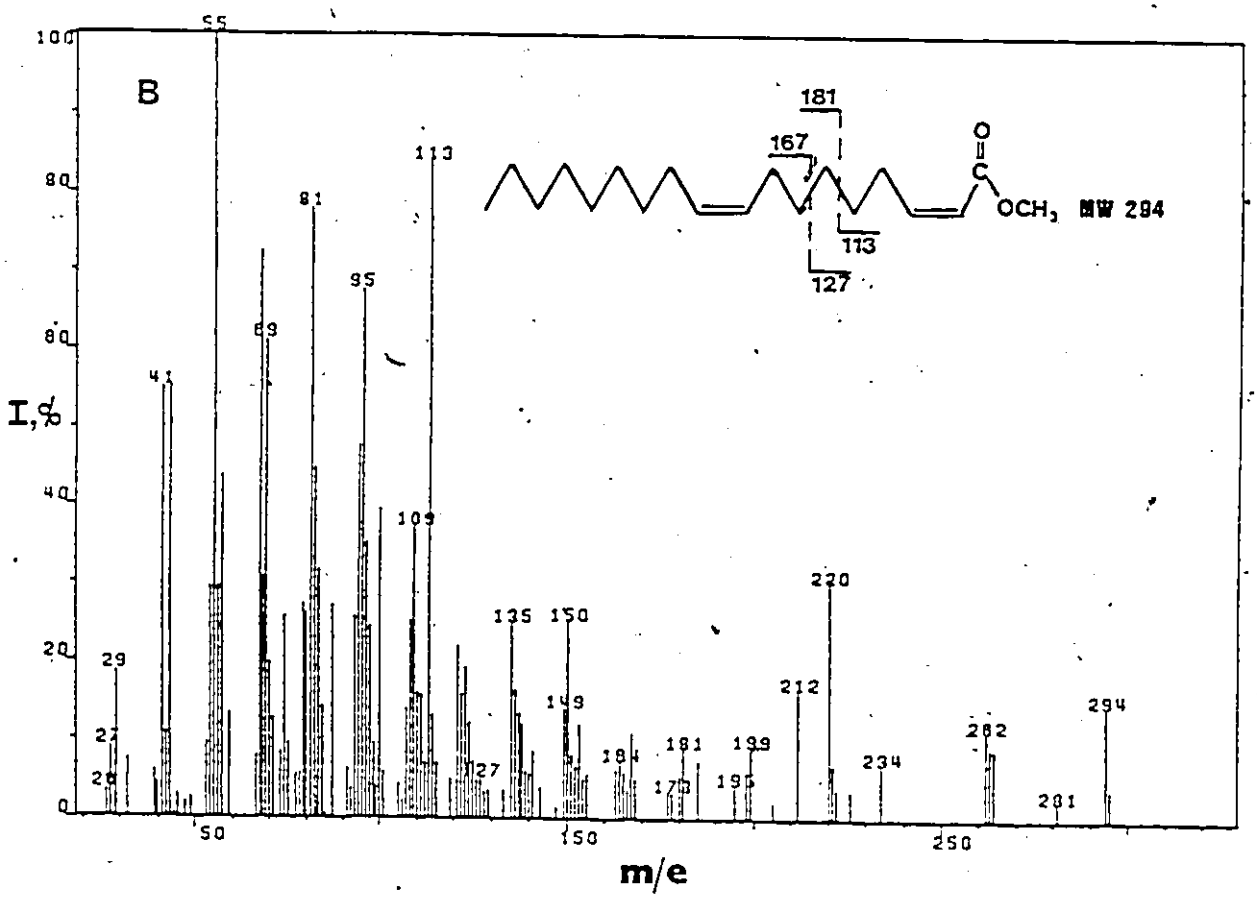

18:1
100
80
60
I,%
40
20
0
S
S
16:0
A
0
100
200
0: 0
4: 7
8: 12
TIME(min.)

FIGURE 18. Mass spectra of A, 18:1; B, product A from Fig. 17.

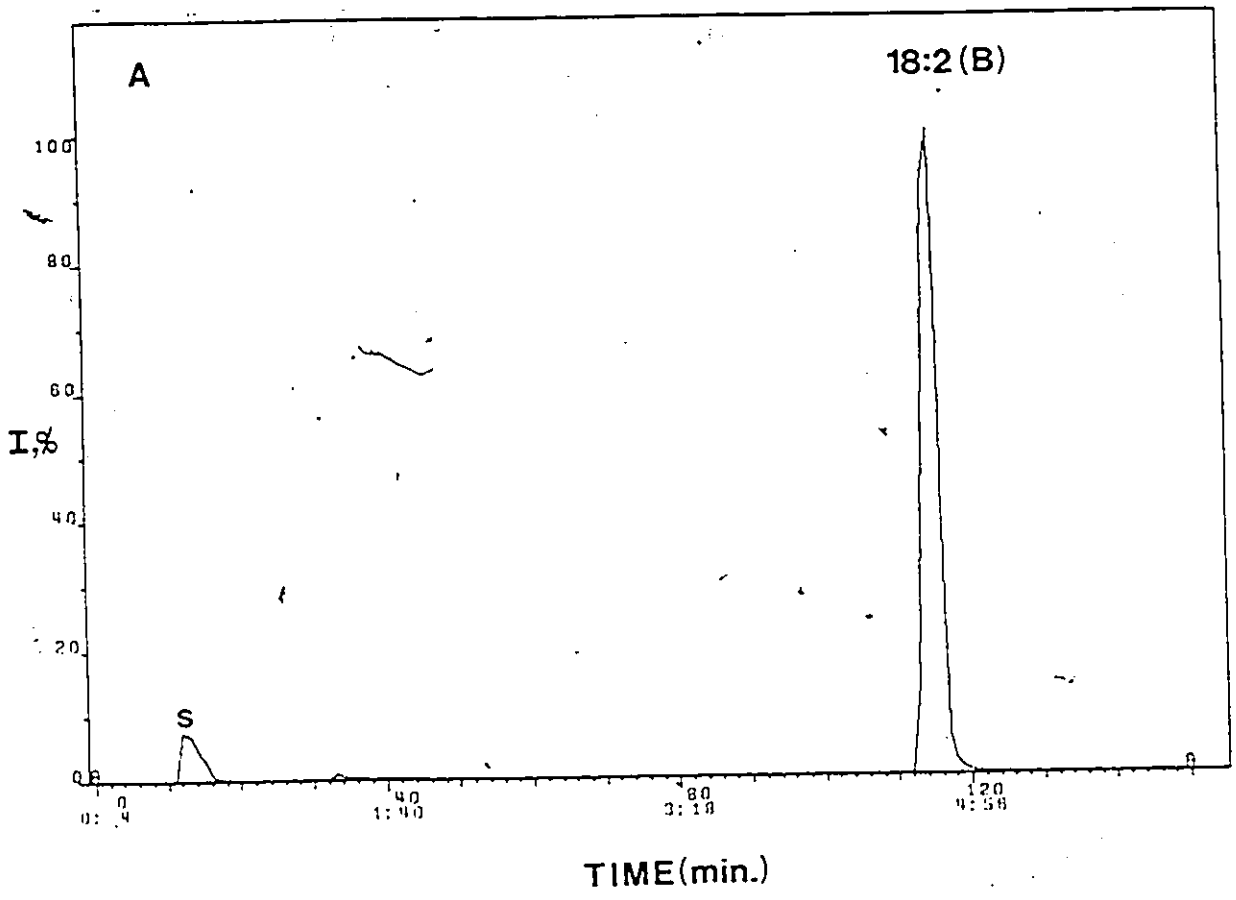

A
I,%
m/e
MW296
OCH3
55
74
89
41
98
29
123
180
222
264
298
50
150
250

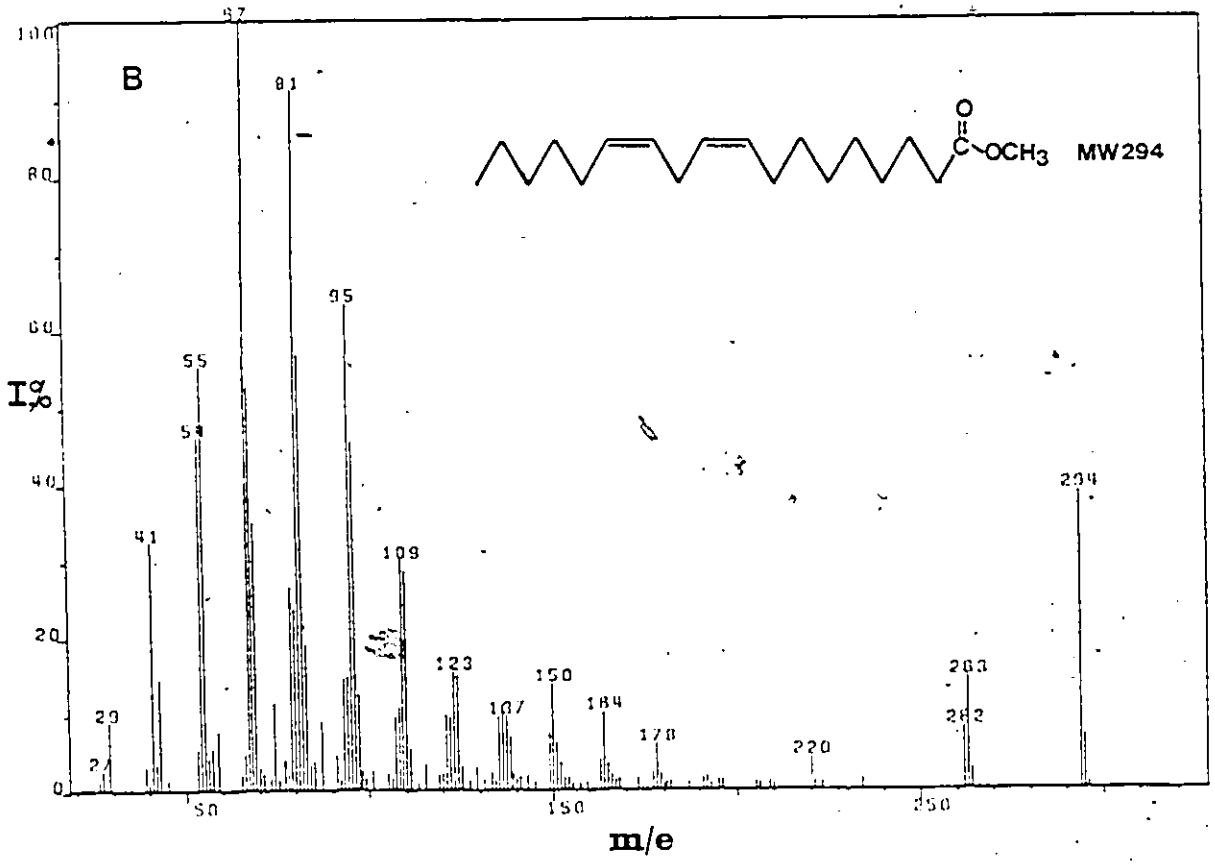

B
I,%
m/e
MW 284
OCH3
181
167
113
127
55
81
95
69
41
109
29
135
150
220
212
234
262
294
281
50
150
250

nitrate plates (Fig. 15) was one of the major metabolic products of 18:1-CoA with either proplastid or microsomal fraction. This product (A) gave a molecular ion peak at m/e 294 (M^+) (Fig. 18B) indicating the presence of a C_{18} diunsaturated acid. Its mass spectrum was similar to that of methyl linoleate (18:2, $\Delta^{9,12}$) with the major fragmentation ions at m/e 55 (base peak), 29,41,69, and 75 and other fragment ions at m/e 262 (M^+-32) and m/e 263 (M^+-31) arising from the elimination of methanol and methoxy groups while the ion peak at m/e 234 (M^+-32-28) arises from the loss of methanol and carbon monoxide. McLafferty rearrangement ions are at m/e 220 (M^+-74) and m/e 74 (M^+-220). However the spectrum differs from that of methyl linoleate in the appearance of the major diagnostic ion peaks at m/e 113 (M^+-181) and m/e 181 (M^+-113). This fragment occurs with splitting between C_5 and C_6 with the double bond α, β to the carboxyl group; ions at m/e 99 (M^+-195) and m/e 195 (M^+-99) may arise from fragmentation between C_4 and C_5 while splitting between C_6 and C_7 gives rise to ion peaks at m/e 167 (M^+-127) with the same location of the double bond. The presence of all these peaks strongly suggests the presence of the new double bond between C_2 and C_3.

The 18:2 formed by the Δ^{12}-desaturase system was GLC pure as shown in Fig. 19A, and its MS spectrum, taken at the apex of the peak, (Fig. 19B) gave a molecular ion (M^+) at m/e 294,

FIGURE 19. GC-MS of purified product B (18:2) from 18:1-CoA metabolism prepared as described in Section C,XII. A, GLC tracing; B, Mass spectrum. S = solvent ($CHCl_3$).


A
18:2 (B)
100
80
60
I,%
40
20
0
S
0
0: 0
40
1:40
80
3:18
120
4:56
TIME(min.)


B
97
81
95
55
54
41
109
123
150
107
184
178
220
293
282
294
20
27
100
80
60
I,%
40
20
0
50
150
250
m/e
O
C
OCH3
MW294

corresponding to the expected molecular weight. Fragmentation peaks at m/e 263 (M-31) and m/e 262 (M-32) represent the loss of methoxy and methanol groups respectively, while fragment ions at m/e 220 (M-74) and m/e 74 (M-220) may arise from McLafferty rearrangement. The base peak present at m/e 67 with the other fragment ions of low mass appearing at m/e 41, 55,81 and 95 are typical fragments arising from hydrocarbon chains. The fragment ions seen for product B, were identical to those of authentic linoleate, thus confirming our previous conclusions (see previous section) that product B is indeed 9,12-octadecadienoic acid, and is therefore a product of Δ^{12}-desaturase activity.

ii. Hydroxylated Products

The other major metabolic product, hydroxyoleate (C) was GLC pure (Fig. 20) and its mass spectrum gave a molecular ion (M^+) at m/e 312 indicating the presence of an oxygen atom in the C_{18} fatty acid molecule (Fig. 21A). The fragment ion at m/e 281 (M^+-31) represents the loss of a methoxy group while m/e 294 (M^+-18) is due to the loss of water. The ion at m/e 262 (M-32-18) may arise from the loss of water and methanol, while m/e 220 (M-18-74) and m/e 74 (M-18-220) represents first the loss of water followed by McLafferty rearrangement. The base peak at m/e 55 and the low molecular ion mass m/e 29, 41,43,69,81 and 95 are typical fragments arising from hydrocarbon chains. If the hydroxyl group is present on C_3,

FIGURE 20. GLC tracing of purified product C (HO-18:1) from 18:1-CoA metabolism prepared as described in Section C, XII. S = solvent ($CHCl_3$).

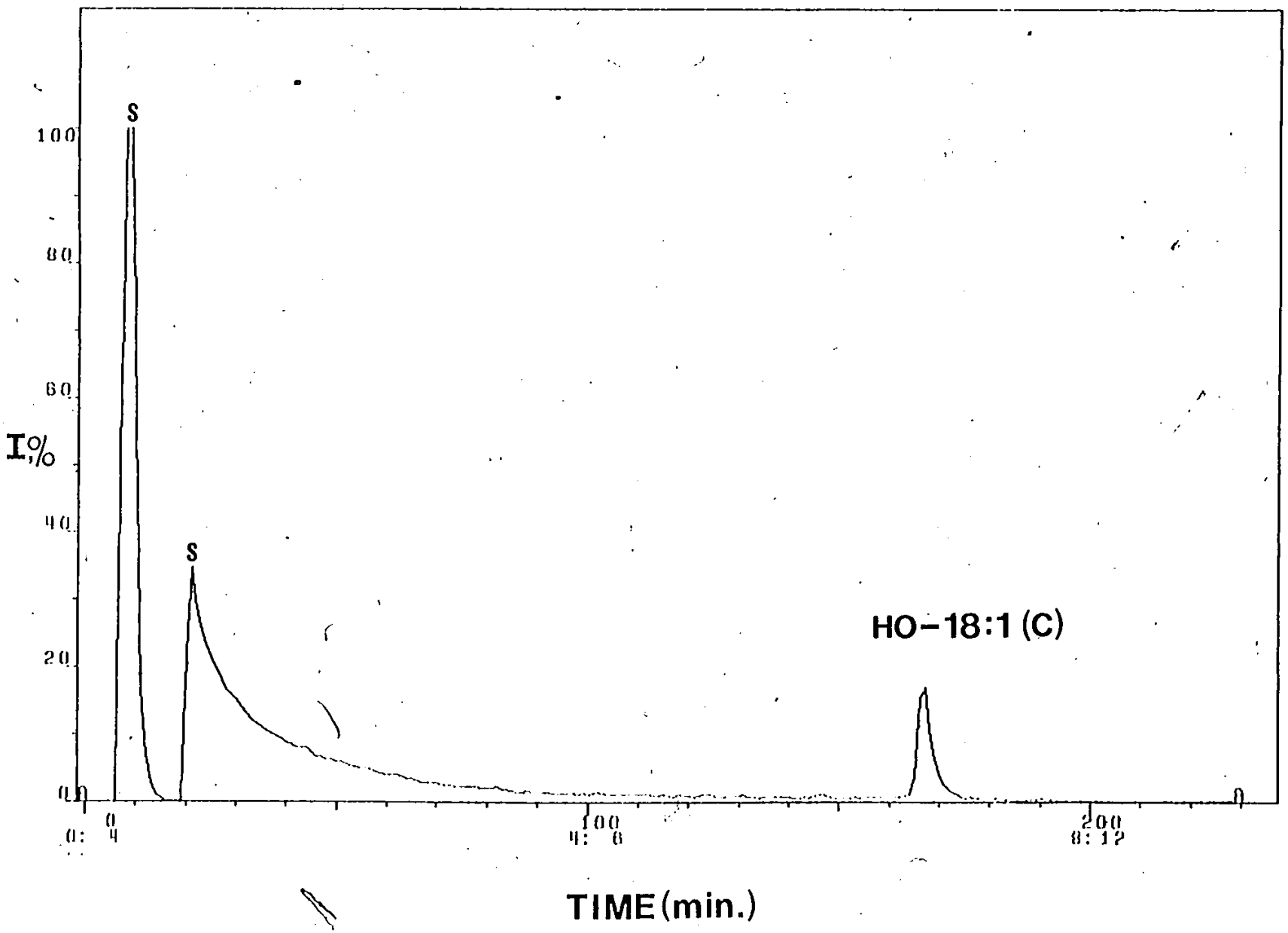
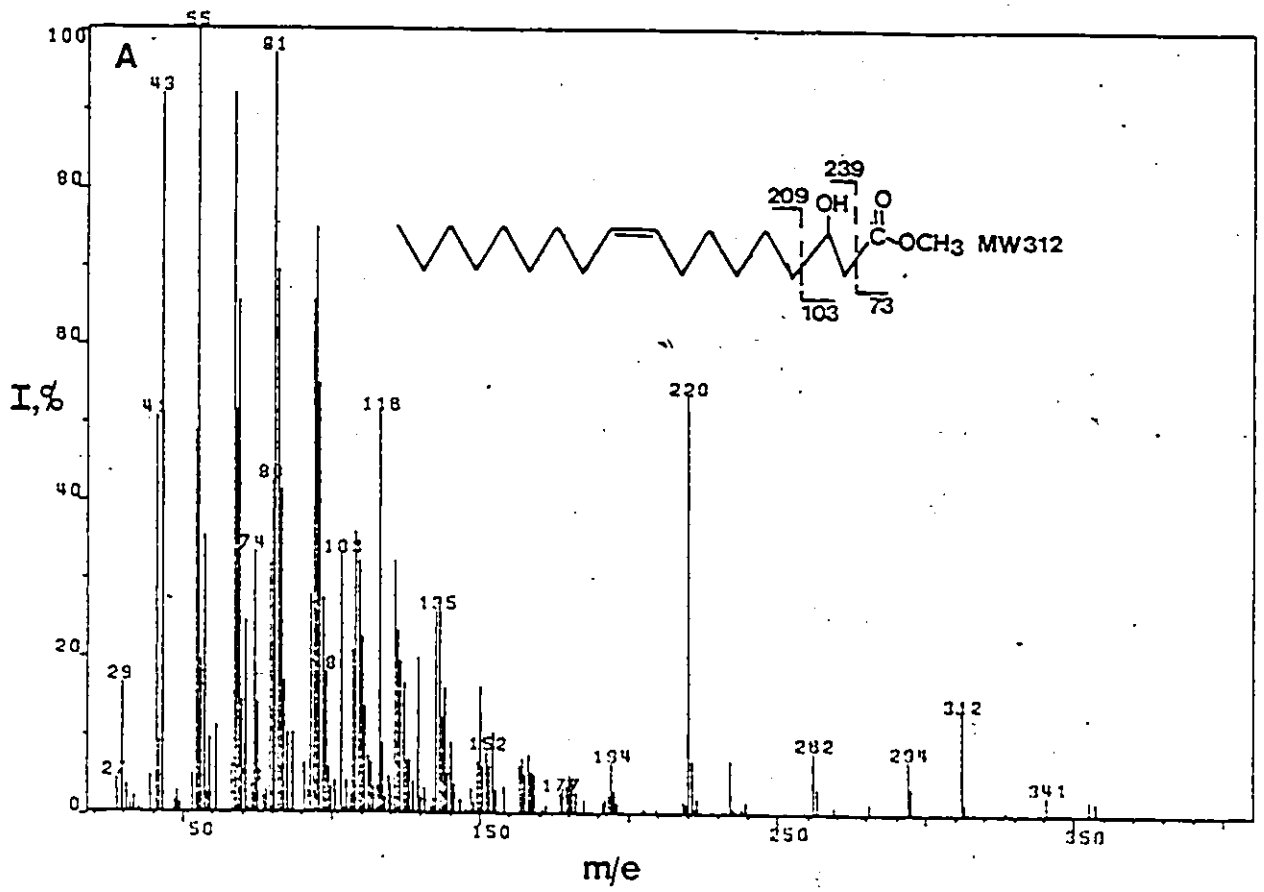
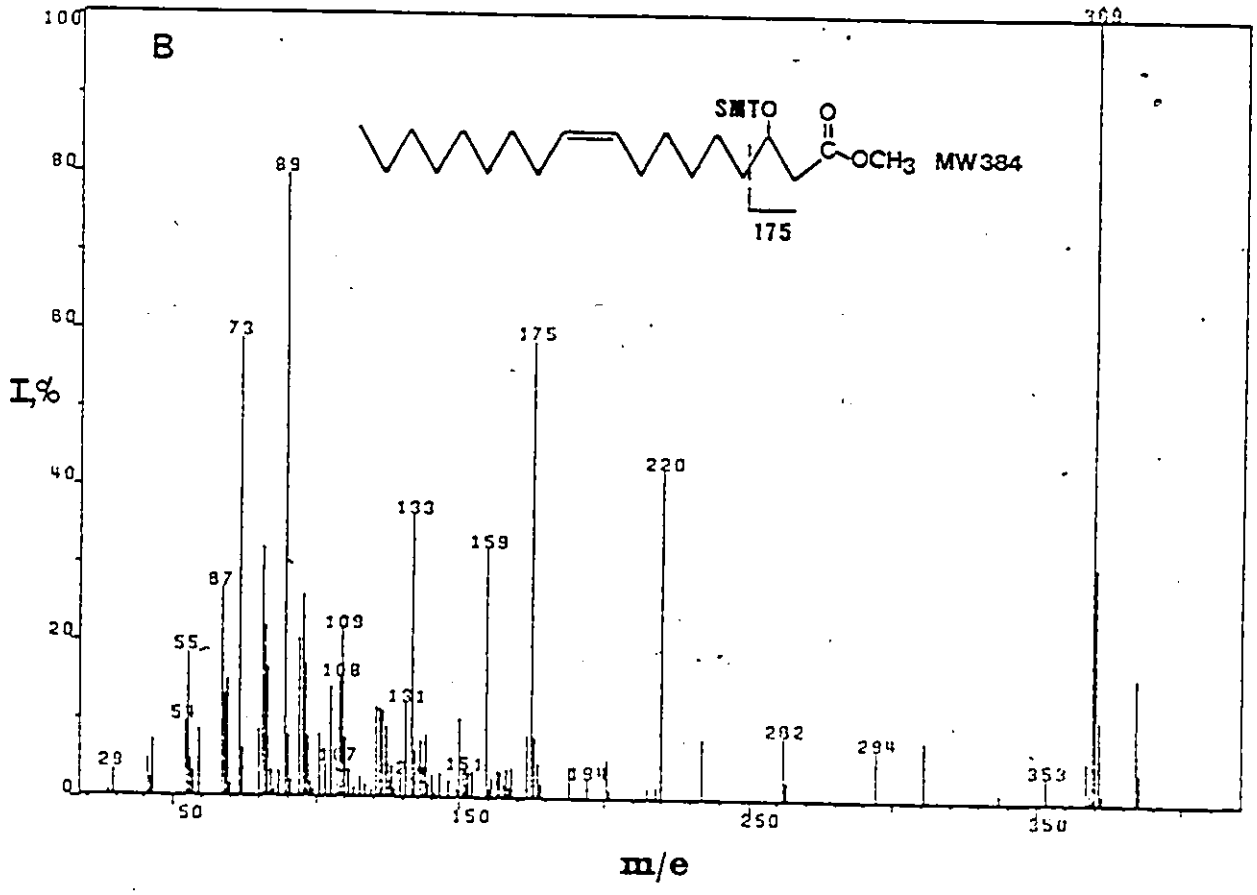

S
S
100
80
60
40
20
0
I,%
HO-18:1 (C)
0
0: 4
100
4: 8
200
8: 12
TIME(min.)

FIGURE 21. Mass spectra of A, product C (HO-18:1) and B, its TMS derivative (TMS-O-18:1) from 18:1-CoA metabolism prepared as described in Section C, XII (see text for explanation).

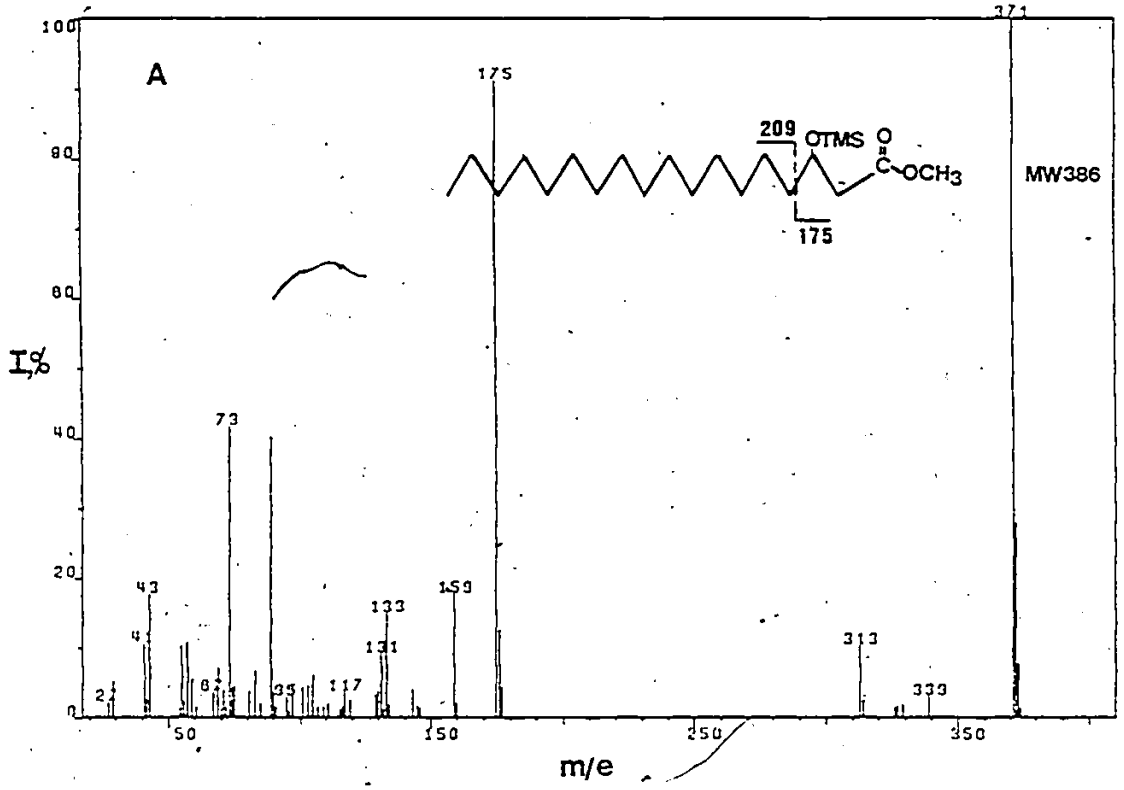

A
I,%
m/e
239
209
OH
O
C-OCH3 MW312
103
73

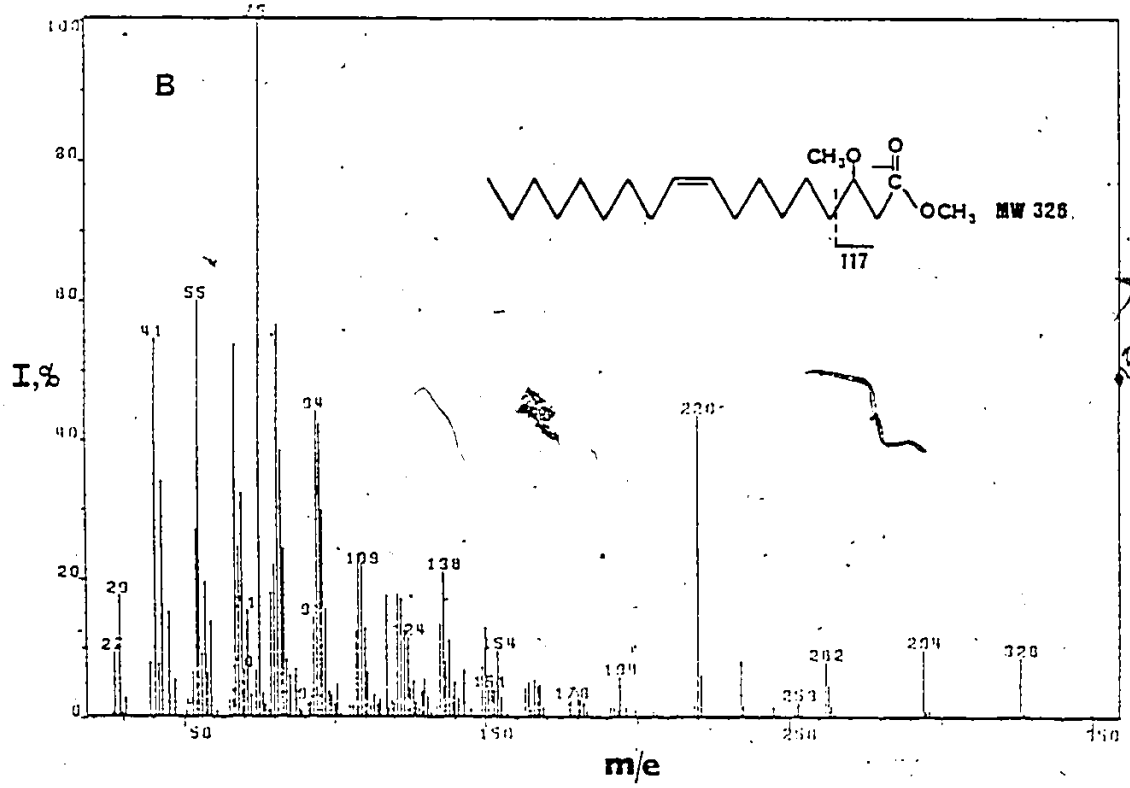

B
I,%
m/e
SMTO
O
C-OCH3 MW384
175

then splitting between C_3 and C_4 would give rise to ion peaks at m/e 103 (M^+-209) and m/e 209 (M^+-103) while splitting between C_2 to C_3 would give rise mainly to ion peak at 73 (M^+-239) and m/e 239 (M^+-73). The presence of all these peaks suggest that the hydroxyl group was on position C_3. The presence of an oxygen atom or hydroxyl group as an integral part of product B was further confirmed by analysis of the trimethylsilyl ether derivative which showed a molecular ion (M^+) at 384 (Fig. 21B). The base peak was now found at m/e 369 (M-15) with loss of a methyl group from the trimethyl silyl ether group. Fragmentation at m/e 353 (M-31) arises from the loss of a methoxy group while m/e 312 (M-72) and m/e 294 (M-90) may occur as result of the loss of trimethyl silyl ($Si(CH_3)_3$) and trimethylsilanol group ($Si(CH_3)OH$), respectively. Peaks at m/e 220 (M-90-74) and m/e 90 (M-90-220) arise from the loss of a trimethylsilanol group followed by McLafferty rearrangement. Fragments at m/e 29,55,67,73 and 159 arise from fragmentation of the hydrocarbon chain.

The formation of a strong ion peak at m/e 175 (M^+-209) is typical of 3-hydroxyacids as confirmed from the trimethyl silyl ether of authentic 3-hydroxy stearate (Fig. 22A and Appendix I), thus confirming the presence of an hydroxyl group at C_3; this ion can only arise if fragmentation occurs between C_3 and C_4 atoms. For further identification product C was methylated as described in section C,VIII, and the mass spectra were taken

FIGURE 22. Mass spectra of A, TMS derivative of authentic 3-OH-18:0 (TMS-O-18:0), and B, methoxy (OCH_3) derivative of product C prepared as described in section C, XII, (see text for explanation).


A
I,%
m/e
MW386
209
OTMS
175
371
73
43
133
159
131
313
333
117
35
41
50
150
250
350

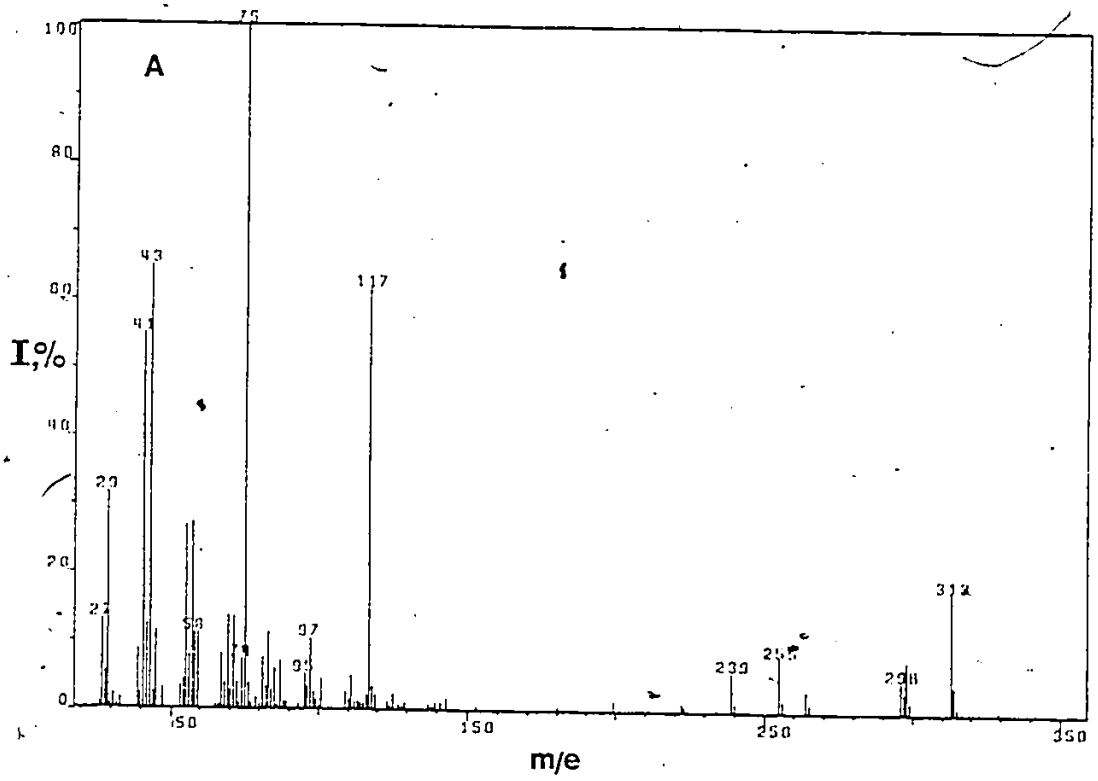

B
I,%
m/e
MW 326
117
55
41
69
220
109
138
29
154
262
294
326
50
150
250

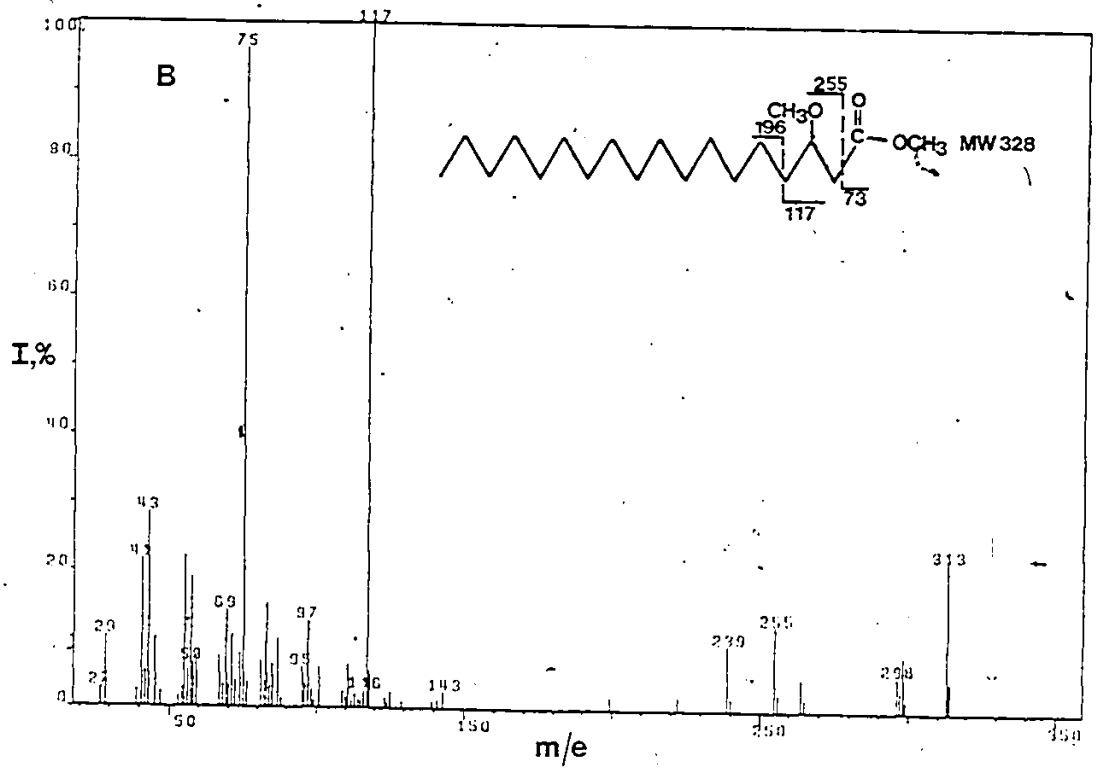
before and after hydrogenation of the respective components.

The mass spectrum of the methoxy derivative of C, methoxyoleate (Fig. 22B, Appendix II) gave a molecular ion (M^+) at m/e 326, corresponding to its expected molecular weight. The major ion fragments included the loss of methanol at m/e 294 (M^+-32) followed by McLafferty rearrangement ion at m/e 220 (M^+-32-74). The base peak at m/e 75 (M^+-31-220) may arise from the loss of a methoxy group followed by the McLafferty rearrangement. The fragment ion at m/e 262 (M^+-64) represents the loss of two methanol groups while the ion at m/e 234 (M^+-64-28) may arise from the loss of carbon monoxide from the ion fragment at m/e 262. The low mass ions at m/e 29, 41,55,81 and 94 are typical fragment ions of hydrocarbon chains and are similar to the low mass ions of oleate (Fig. 18A). The most important ions which are diagnostic in establishing the position of the hydroxyl group in the molecule were fragment ions at m/e 253 (M^+-59) or m/e 59 (M^+-253) and m/e 117 (M^+-209). These ions arise only if the functional methoxy group is present on C_3 with fragmentation occurring between C_2-C_3 to give rise to the ions at m/e 253 and m/e 59, between C_3-C_4 for the ion at m/e 117 (Fig. 22B).

On hydrogenation of methoxyoleate, the molecular ion (M^+) (m/e 328) corresponding to the molecular weight of methyoxystearate was very weak but a strong $(M-15)^+$ ion was observed (Fig. 23A, Appendix II). The diagnostic ion fragment

FIGURE 23. Mass spectra of A, hydrogenated and methylated product C from 18:1-CoA metabolism and B, methylated 3-OH-18:0 authentic standard.


A
I,%
m/e
75
117
43
41
97
313
239
255
298


B
I,%
m/e
117
75
255
CH3O
196
C
OCH3
MW328
73
313
239
255
298
143

at m/e 296 (M^+-32) represents the loss of methanol; McLafferty rearrangement of this ion gives ions at m/e 222 (M^+-32-222). Ion fragments at m/e 297 (M^+-31) and m/e 223 (M^+-31-74) arise from the loss of a methoxy group followed by McLafferty rearrangement, respectively, while the ion peak at m/e 298 (M^+-30) represent the loss of two methyl groups. The low abundance of the ions at m/e 297, m/e 223 and m/e 74 suggested that the main pathway for McLafferty rearrangement was preferentially from m/e 297 (M^+-31) to give rise to the base peak at m/e 75 (M^+-31-222). The ion fragment at m/e 264 (M^+-64) arises from the loss of the two molecules of methanol.

Of major diagnostic importance are the ions at m/e 255 (M^+-73) and m/e 73 (M^+-255) arising from the fragmentation between C_2-C_3 (Fig. 23A) amd m/e 117 (M^+-211) which arises from a fragmentation between C_3-C_4 (Appendix II). These results clearly suggest that the methoxy group is located in C_3; this was further confirmed by the fact that its mass spectrum is identical to that of authentic 3-methyoxystearate (Fig. 23B). All of the above results thus confirm that the major product of 18:1-CoA metabolism is in fact 3-hydroxyoleate.

2. Products of 18:2-CoA Metabolism

a) TLC and GLC

On incubation of [^{14}C]linoleoyl-CoA with proplastid fraction, under the conditions described in materials and Methods, for spots (E,F,G,H), apart from the derived from the 18:2, substrate

were observed on 10% $AgNO_3$-silica gel G plates with the assignments and percent distribution of [^{14}C] given in Fig. 24 and Table 13. However, when the analysis was performed on radio-GLC three peaks (D,E or G,H) apart from 18:2 were observed with the percent distribution of [^{14}C] as given in Table 13 (see also Fig. 24). Components F was not observed in subsequent experiments and will not be discussed further. The higher amount of [^{14}C] in 18:2 on TLC suggested the presence of another component (D) in the TLC spot, which appeared as a separate peak on radio-GLC (Fig. 25B, Table 13). Product G was not seen on radio-GC but was present on $AgNO_3$-SiO_2 plates. Thus component D probably migrates with the substrate (18:2) on $AgNO_3$-SiO_2 plates while component G appears to comigrate with component D on radio-GLC. Component E and H are detected on both TLC and GLC (Table 13,14 and Fig. 25B).

These products were further characterized as follows: After adding a known quantity of [^{14}C]16:0 as internal standard to the zero time incubation, as well as to proplastid reaction mixture (30 min. incubation), the products were examined by radio-GC. At zero time, all the radioactivity, was associated with [^{14}C]18:2 substrate (Fig. 25A), which on incubation gave products, D,E,G and H with the relative percent distribution given in (Table 14, Fig. 25B). After catalytic hydrogenation of the total products, the [^{14}C]stearate formed accounted for the sum of 18:2 substrate plus product D but may not include product E which is a minor component (Fig. 25C). Hydrogenated product H

FIGURE 24. Autoradiogram of a thin-layer chromatogram on 10% $AgNO_3$-silica gel G showing the formation of E,F,G, and H (HO-18:2) from 18:2-CoA metabolism. Incubation was performed with proplastids (PL) and microsomes (M) for one hour as described in Section C,IV. Note, product F was observed only in this experiment and will not be mentioned further in the course of this thesis.

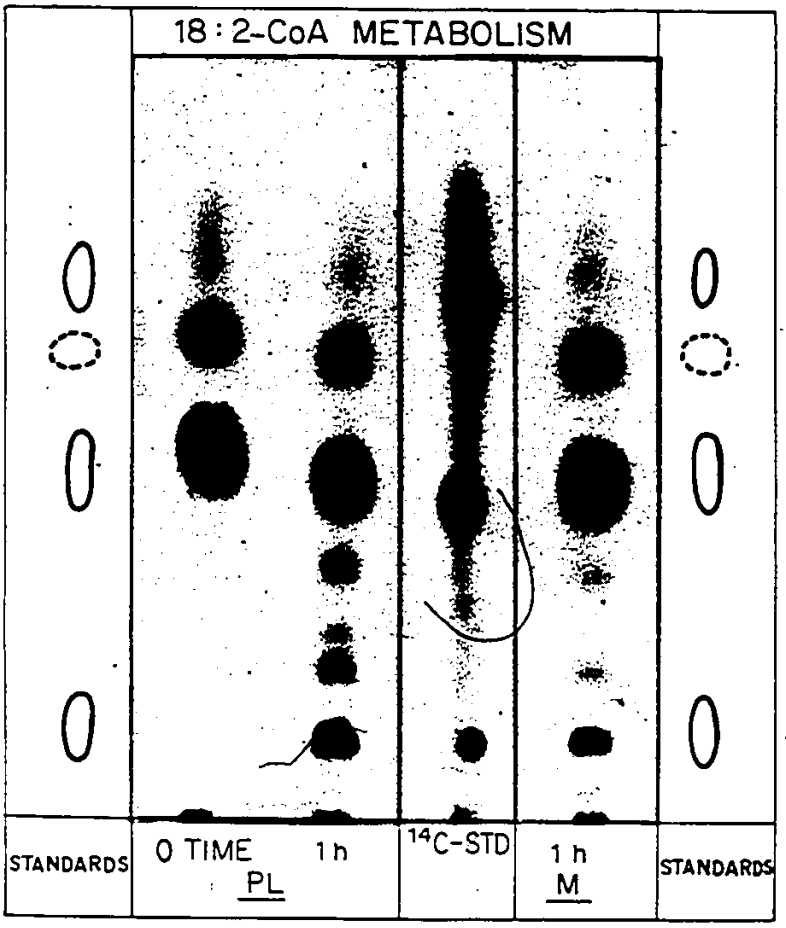

18 : 2-CoA METABOLISM
18:1
18:2 (trans)
18:2 (cis)+D
E
F
G
18:3; HO-18:2 (H)
STANDARDS
0 TIME
1 h
PL
14C-STD
1 h
M
STANDARDS

TABLE 13

Distribution of ^{14}C Among Products of 18:2-CoA Metabolism[a]

Product	TLC[b]		GLC[c]	
	R_f	^{14}C Dist.^{n} %	Ret^n rel. 16:0	^{14}C Dist.^{n} %
Substrate 18:2	0.48	57.8	2.8	33.2
Product D			4.4	31.9
Product E	0.32	3.4	3.3	3.9
Product G	0.20	5.2	-	N.D.[d]
Product H	0.11	31.3	16.7	31.0

[a]Incubation with the proplastid preparation as described in Materials and Methods section C.IV.

[b]TLC on 10% $AgNO_3$-SiO_2 plates in chloroform:ethanol (200:5 v/v). R_f values of standard: 2(OH)18:0, 0.56; 3(OH)18:0, 0.54; 2(OCH_3)18:0, 0.76; 3(OCH_3)-18:0, 0.73; 9(OH)-18:0, 0.55; 9(OCH_3)-18:0, 0.74; C_{hm} and H_{hm}, 0.73.

[c]GLC on SP-2330 at 170°C; ret. rel. to 16:0 of standards see Table 9.

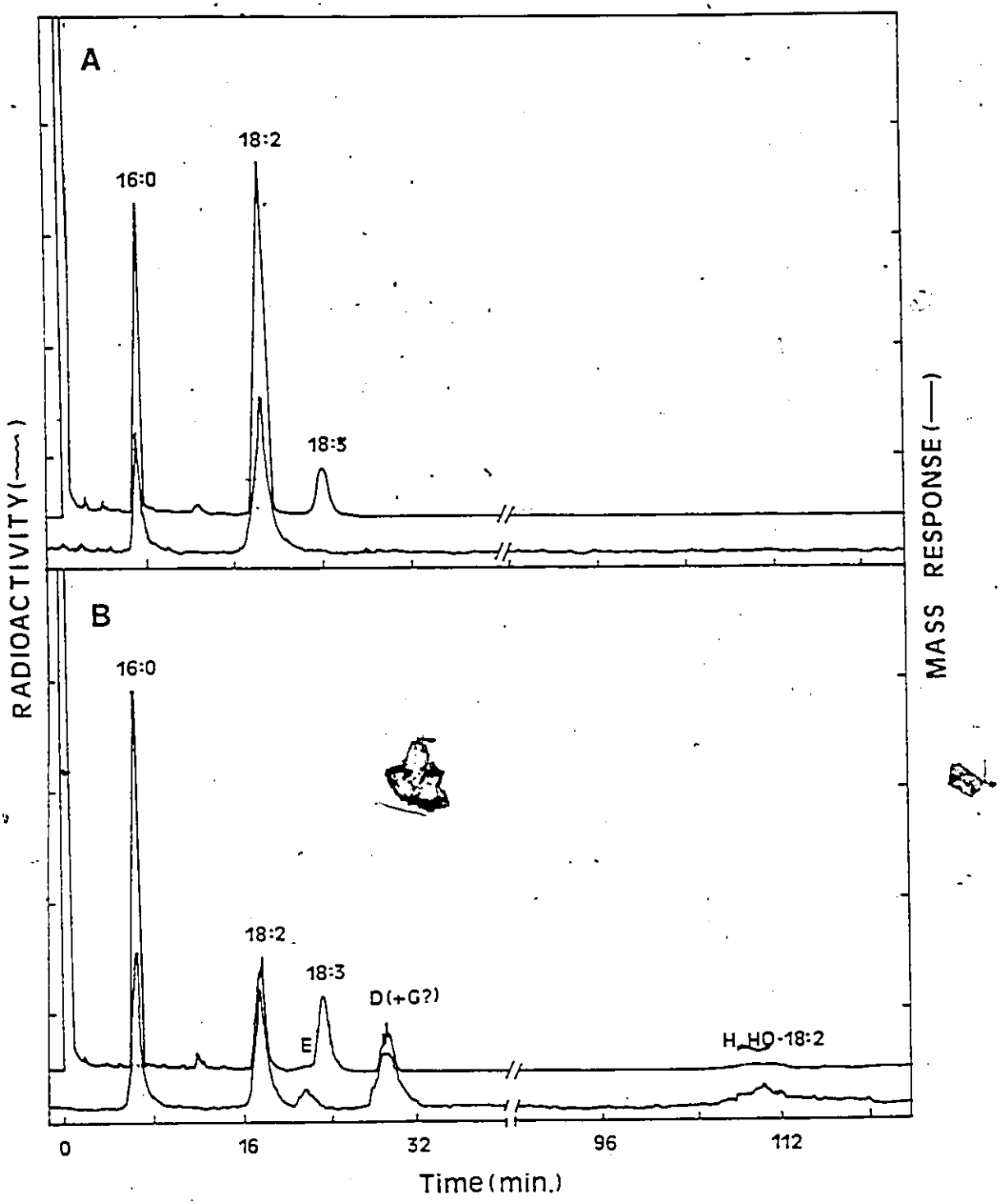
[d]N.D. = not detected; see explanation given in Section D.I.2.a.

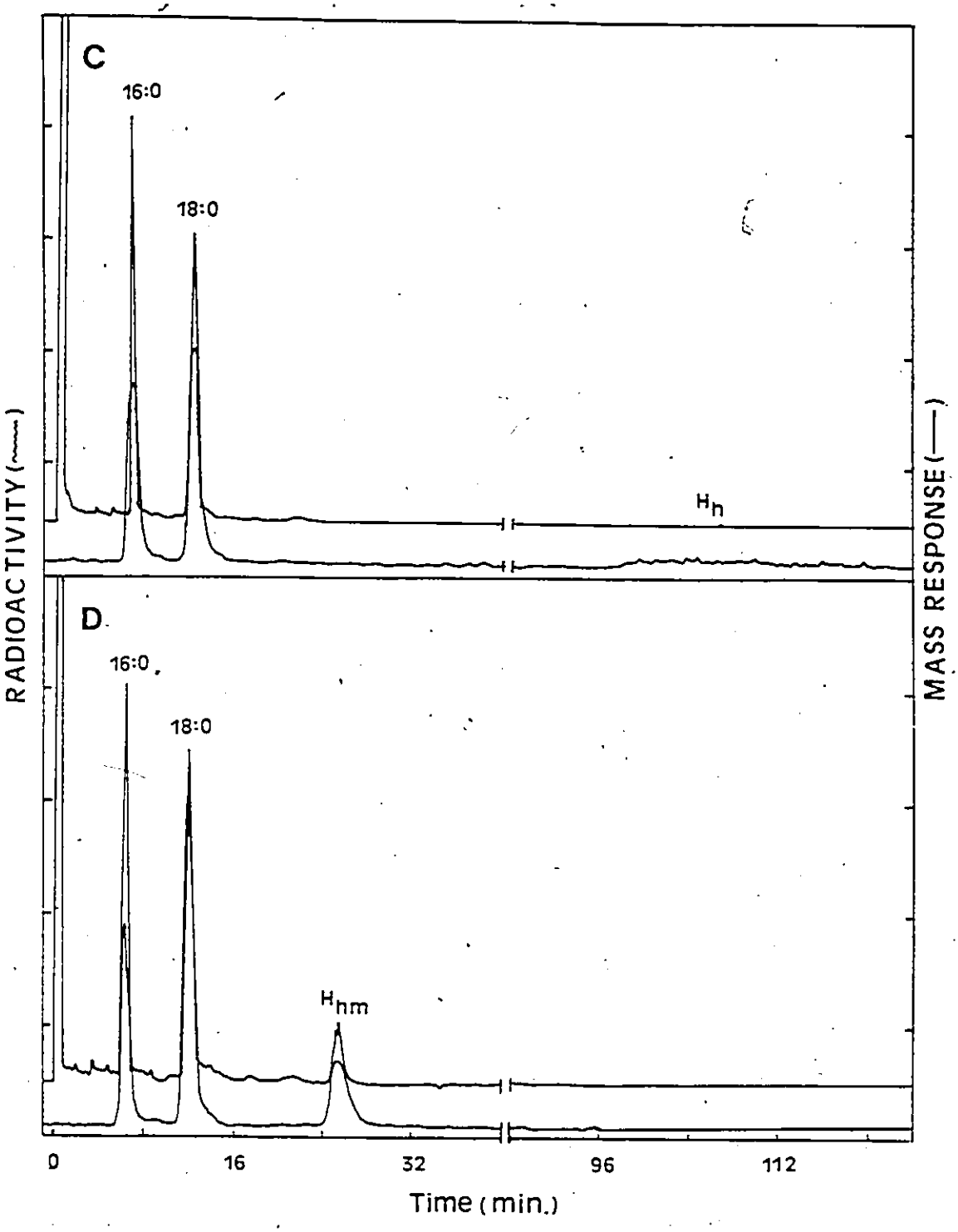
FIGURE 25. Radio-GLC tracing on SP-2330 at 170°C from 18:2-CoA incubation with proplastids (see Table 14).

Upper tracing: Mass response (——)

Lower tracing: Radioactivity (~~~)

A, control (0 time incubation); B, incubation for one hour as described in section C, IV, showing the distribution of ^{14}C 18:2 substrate, E, D (+G), and H (see Table 13); C, hydrogenated Fig. 25,B and Table 14; D, hydrogenated and methylated Fig. 25,C and Table 14.


A
16:0
18:2
18:3
B
16:0
18:2
18:3
E
D(+G?)
H-HO-18:2
0
16
32
96
112
Time (min.)
RADIOACTIVITY (——)
MASS RESPONSE (——)


C
16:0
18:0
H_h
D
16:0
18:0
H_{hm}
RADIOACTIVITY (~~~)
MASS RESPONSE (——)
0
16
32
96
112
Time (min.)

(H_h), with a shorter retention than H, was detected but its integration was subject to error because of the long retention time and skewed nature of its peak (Table 14, Fig. 25C). However, after hydrogenation followed by methoxylation (see section C, VII and VIII), the hydrogenated methoxylated product H_{hm} (Fig. 25D) gave a well-resolved and easily integrated peak, showing that product H was indeed an unsaturated ~~hydr~~oxyacid, with a significant shorter retention value than that of 9- or 12-methoxy stearate (Table 14). Thus the product H formed from 18:2-CoA by proplastids is an unsaturated hydroxyacid with the hydroxyl group located closer to the carboxyl group than C_9.

That product D migrated with the 18:2 substrate, was further confirmed by isolation and purification of 18:2 as described in Section C, XII; and on hydrogenation the 18:0 formed accounted for all the [^{14}C] present in 18:2 + D (Table 15). These experiments thus confirm that product D, migrating with the substrate, was an unsaturated C_{18} fatty acids with no other functional group in the molecule and product H was a hydroxy-18:2 fatty acid with the hydroxyl group being closer to the carboxyl end of the molecule than C_9 (Table 14). Products E and G were not identified further because of the low amount of conversion.

It should be noted that no 18:3 was detected among the products, showing that a Δ^{15}-desaturase system was not operative.

Further information concerning the location of the double bonds in D and the double bonds and hydroxy group in H was obtained from the results of GC-MS of these products.

b) Gas Liquid-Mass Spectrometry

i. Substrate and Product D

After incubation of 18:2-CoA with proplastids (see section C, IV), the [^{14}C] 18:1 isolated from $AgNO_2$-SiO_2 plates was found by GC-MS analysis to contain two minor components, palmitoleate and product D (Fig. 26A).

The fragmentation pattern of 18:2 substrate was identical to that of the authentic methyl linoleate standard and that of the methyl linoleate product of 18:1-CoA desaturase activity (Fig. 19B), described previously. The first minor component, eluting before 18:2, was identified as palmitoleate according to its mass spectrum, while the peak eluting after 18:2, product D, was present only after incubation with the proplastids, as described in section C,IV.

The mass spectrum of component D (Fig. 26B) gave a molecular ion (M^+) at m/e 292 corresponding to that of a trienoic-C_{18} acid. Fragment ions at m/e 261 (M^+-31) and 260 (M^+-32) represent the loss of a methoxy and methanol groups, respectively, while McLafferty rearrangement ions are at m/e 218 (M^+-74) and m/e 74 (M^+-218). Fragment ions at m/e 233 (M^+-31-28) and 232 (M^+-32-28) represent the elimination of methoxy or methanol group followed by the loss of carbon monoxide. Fragment ions at m/e 127 (M^+-165) and m/e 165 (M^+-127)

TABLE 14

^{14}C Distribution Among 18:2-CoA Metabolism Products Before and After Hydrogenation and Hydrogenation/Methylation

Product	Relative ^{14}C-Distribution, mole ratio[b]				
	Zero time Control	Before Hydrog.[n]	After Hydrog.[n]	After Hydrog./Methyl[n]	Relative Retention Time[c]
Substrate 18:2	2.46	0.76	-		
Product D	-	0.73	-		4.4
Product E	-	0.09	-		3.3
Product G	-	-	-		-
Product H	-	0.54-0.71[d]	-		16.7
Hydrog.[n] Products					
18:0	-	-	1.38	1.43	1.9
Product H_h	-	-	0.40[d]		15.7
Product H_{hm}	-			0.95	4.0
9- and 12-Hydroxystearate					20.2
9- and 12-Methoxystearate					5.0
Totals	2.46	2.12-2.29	1.78	2.38	

[a] Proplastid system as described in Materials and Methods

[b] Relative to internal standard [^{14}C] 16:0; [c] GLC on SP-2330 at 170°C: retention rel. to 16:0

[d] Integration of these peaks is subject to error because of their long retention times.

TABLE 15

Distribution of Purified[a] [^{14}C] 18:2 Substrate + Product D

Before and After Hydrogenation

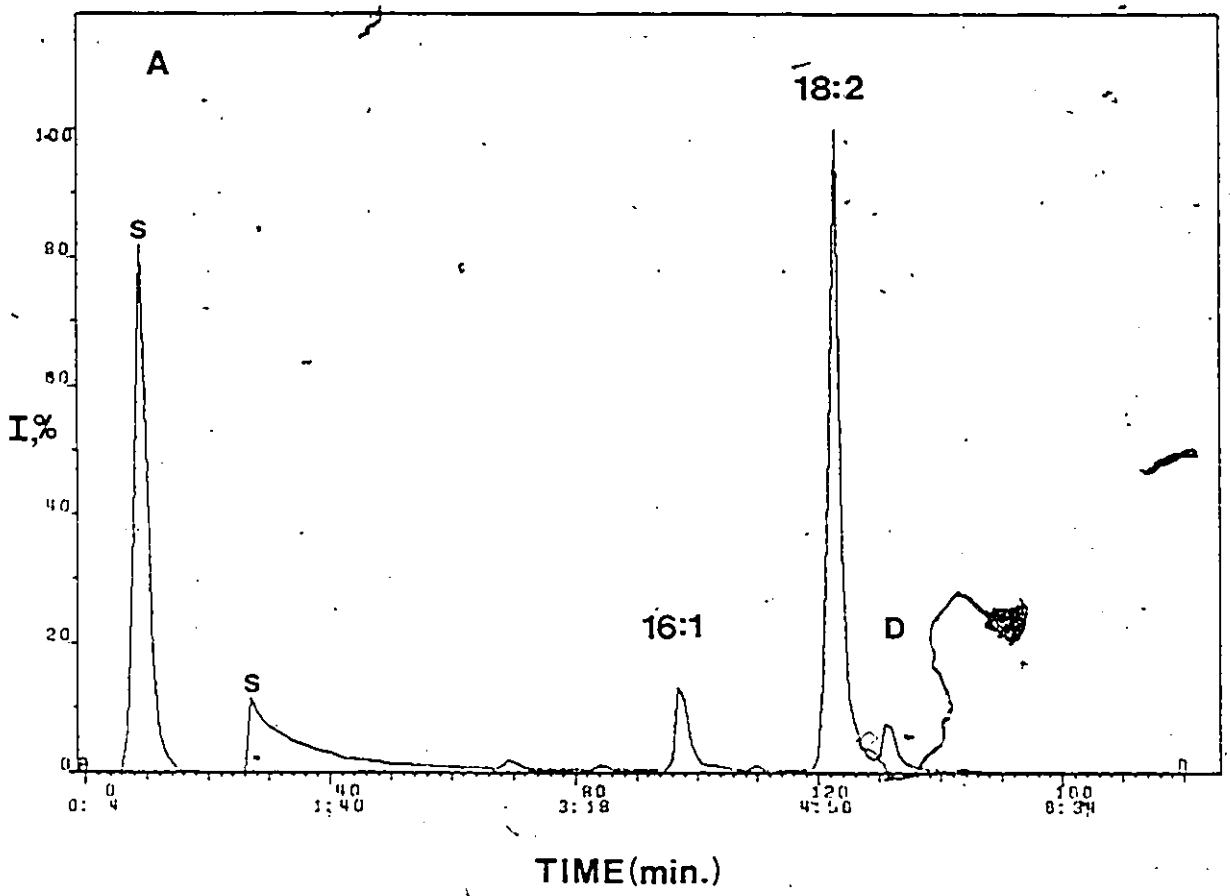
^{14}C Products	Rel. Distrib. of ^{14}C, mole ratio[b]		Rel. Retention on SP-2330[c]	
	Before Hydrog.^{n}	After Hydrog.^{n}	Before Hydrog.^{n}	After Hydrog.^{n}
18:2	4.26	-	2.5	-
D	1.02	-	3.9	-
Hydrog.^{n} Prod. 18:0	-	5.35	-	1.9
Totals	5.28	5.35		

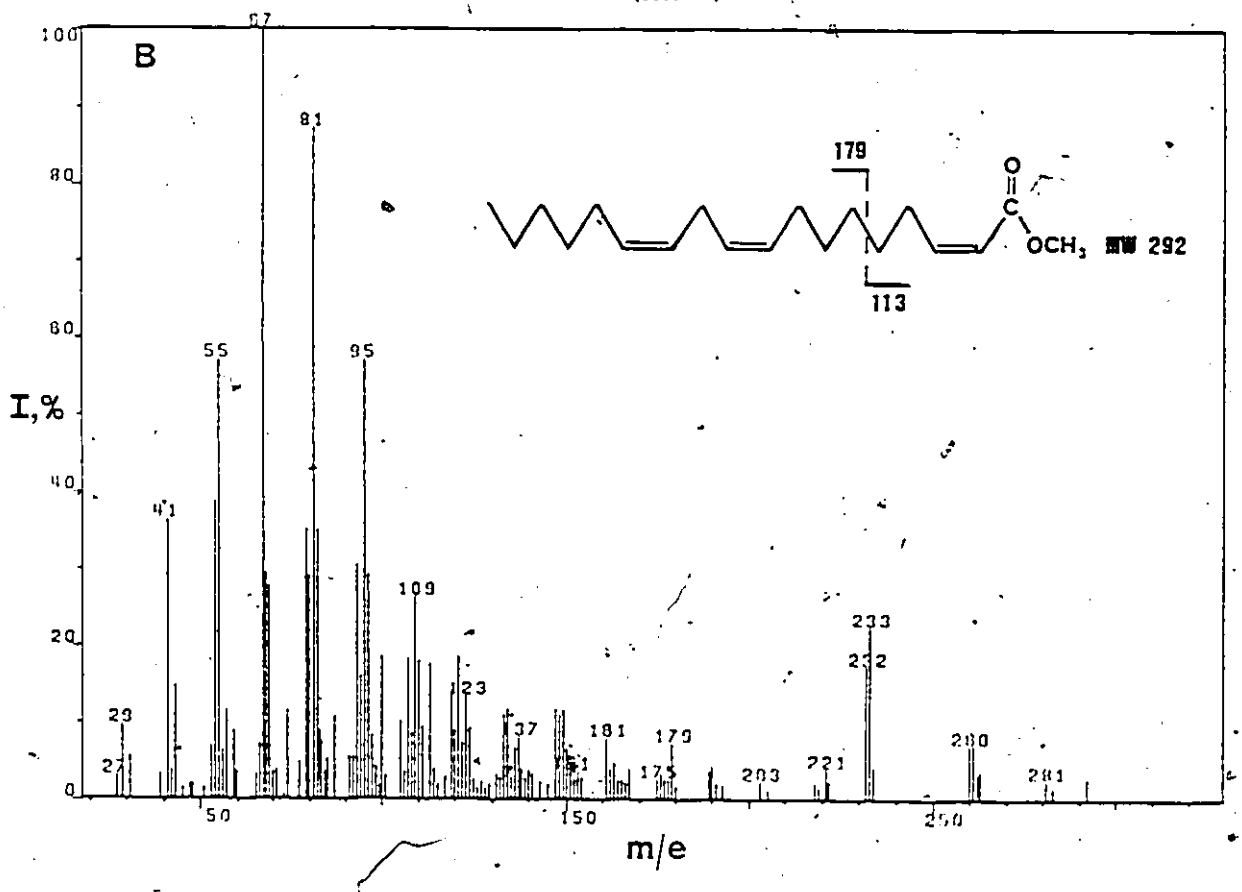
[a]Purified as described in section C.XII.

[b]Relative to internal standard [^{14}C] 16:0; GLC on SP-2330 at 205°C

[c]Relative retention to internal standard [^{14}C] 16:0 on SP-2330 at 205°C; 18:0 authentic standard, 1.9.

FIGURE 26. GLC tracing and mass spectrum of 18:2 substrate + product D prepared as described in Section C, XII. A, mass response showing 18:2, D and 16:1 as a contaminant; B, mass spectrum of product D (see text for explanation). S = solvent ($CHCl_3$).


A
18:2
S
S
16:1
D
I,%
100
80
60
40
20
0
0
0: 4
40
1:40
80
3:18
120
4:50
160
6:34
TIME(min.)

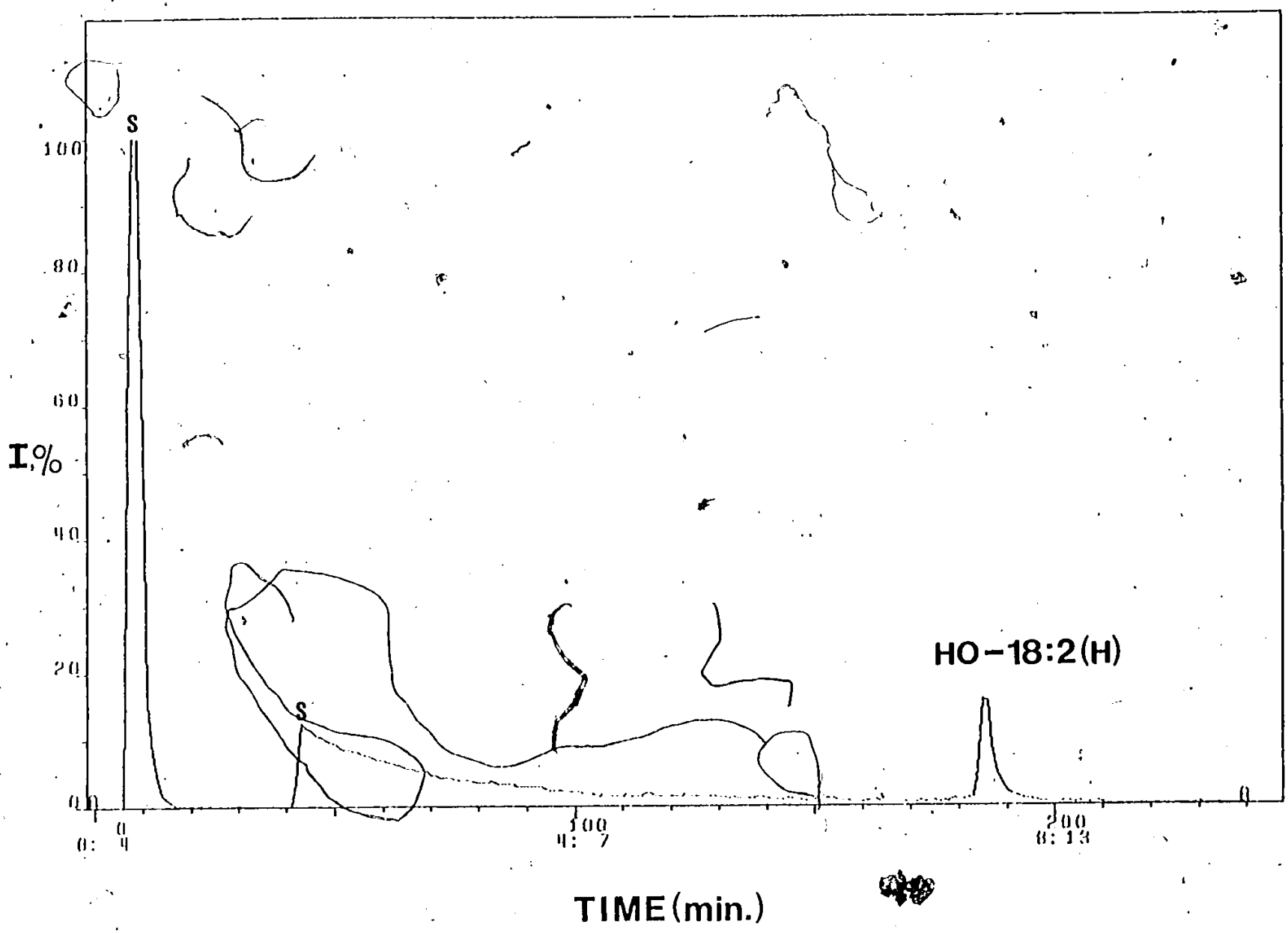

B
I,%
100
80
60
40
20
0
67
81
55
95
41
109
23
29
27
37
181
179
175
203
221
233
232
280
281
50
150
250
m/e
179
113
O
C
OCH3
MW 292

may arise from fragmentation between C_6 and C_7 while the ion peaks at m/e 151 (M^+-141) and m/e 141 (M^+-151) may arise from fragmentation between C_7 and C_8 of the molecule. Of major significance are the ions at m/e 99 (M^+-193) and m/e 193 (M^+-99) arising from fragmentation between C_4 and C_5 and m/e 113 (M^+-179) and m/e 179 (M^+-113) arising from fragmentation between C_5 and C_6. These ions occur only if a double bond exists between C_2 and C_3, suggesting that the third double bond is present at C_2. The base peak present at m/e 67 and the other low mass ions at m/e 29,41,55,81 and 95 represent hydrocarbon chain fragmentation ions.

ii. Hydroxylated Products

The GC tracing of hydroxylinoleate, product H, the other major component of 18:2 metabolism, showed the presence of a single peak (Fig. 27). The mass spectrum taken at its apex gave a molecular ion (M^+) at m/e 310 (Fig. 28A), corresponding to methyl linoleate plus an oxygen atom (hydroxylinoleate) analogous to product C (hydroxyoleate) of 18:1-CoA metabolism. Fragment ion at m/e 279 (M^+-31) represents the loss of a methoxy group from the ester group while m/e 292 (M^+-18) represents the elimination of water presumably from a hydroxyl group. The ion fragment at m/e 260 (M^+-32-18) arises from the loss of methanol and water respectively while the ions at m/e 236 (M^+-74) and m/e 74 (M^+-236) arise from the direct McLafferty rearrangement of the molecular ion (M^+) m/e 310; ion peak at m/e 218 (M^+-18-74)

FIGURE 27. GLC tracing of product H (HO-18:2) formed from 18:2-CoA metabolism prepared as described in Section C, XII. S = solvent ($CHCl_3$).

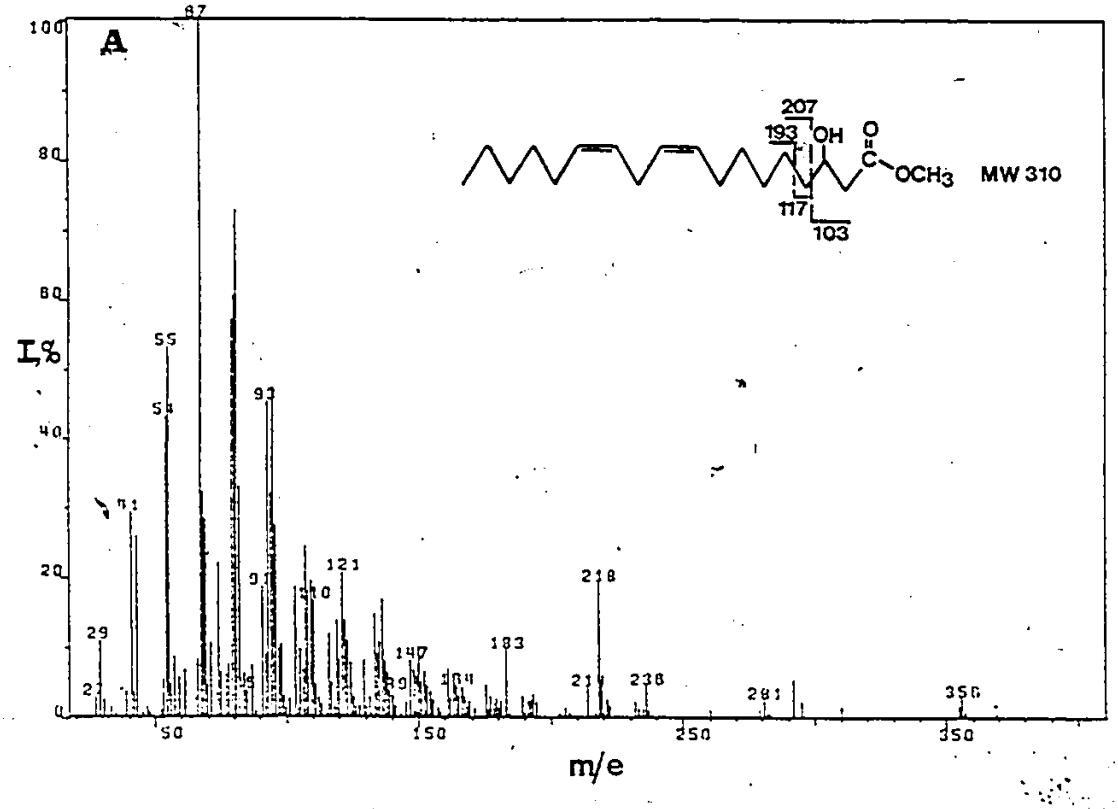

S
100
80
60
I,%
40
20
0
S
HO-18:2(H)
0
0: 4
100
4: 7
200
8: 13
TIME(min.)

represents the loss of water followed by the McLafferty rearrangement. The base peak at m/e 67 and the other low mass ions at 28,41,55 and 81 are typical fragments derived from hydrocarbon chains.

The presence of a hydroxyl group as an integral part of product H (hydroxylinoleate) was confirmed by analysis of the trimethylsilyl ether derivative which now gave a molecular ion (M^+) at m/e 382 (Fig. 28B and Appendix I). The loss of a methyl group from the trimethylsilyl ether gives rise to the peak at m/e 367 (M^+-15) while the appearance of athe diagnostic ion at m/e 292 (M^+-90) arises from the loss of trimethylsilanol moiety. The fragment ions at m/e 218 (M^+90-74) and m/e 74 (M^+-90-218) occur from the respective loss of the trimethylsilanol group followed by McLafferty rearrangement. The formation of a strong peak at m/e 175 (M^+-207) (Fig. 28B and Appendix I), typical of 3-hydroxyacids (Fig. 21B and 22A), confirms the presence of an hydroxyl group at C_3; this ion can arise if fragmentation occurs between C_3 and C_4.

For further identification product H was methylated as described in section C,VIII and the mass spectrum of the methoxy derivative was taken before and after hydrogenation. The molecular ion (M^+) was found at m/e 324 (Fig. 29A and Appendix II), corresponding to the molecular weight expected for methoxylinoleate. Ion fragments at m/e 293 and m/ 292

FIGURE 28. Mass spectra of A, product H (HO-18:2) and B, its TMS derivative (TMS-O-18:2) prepared as described in Section C,XII (see text for explanation).


A
I,%
m/e
MW 310
207
193
OH
OCH3
117
103
87
55
93
121
218
183
147
281
350

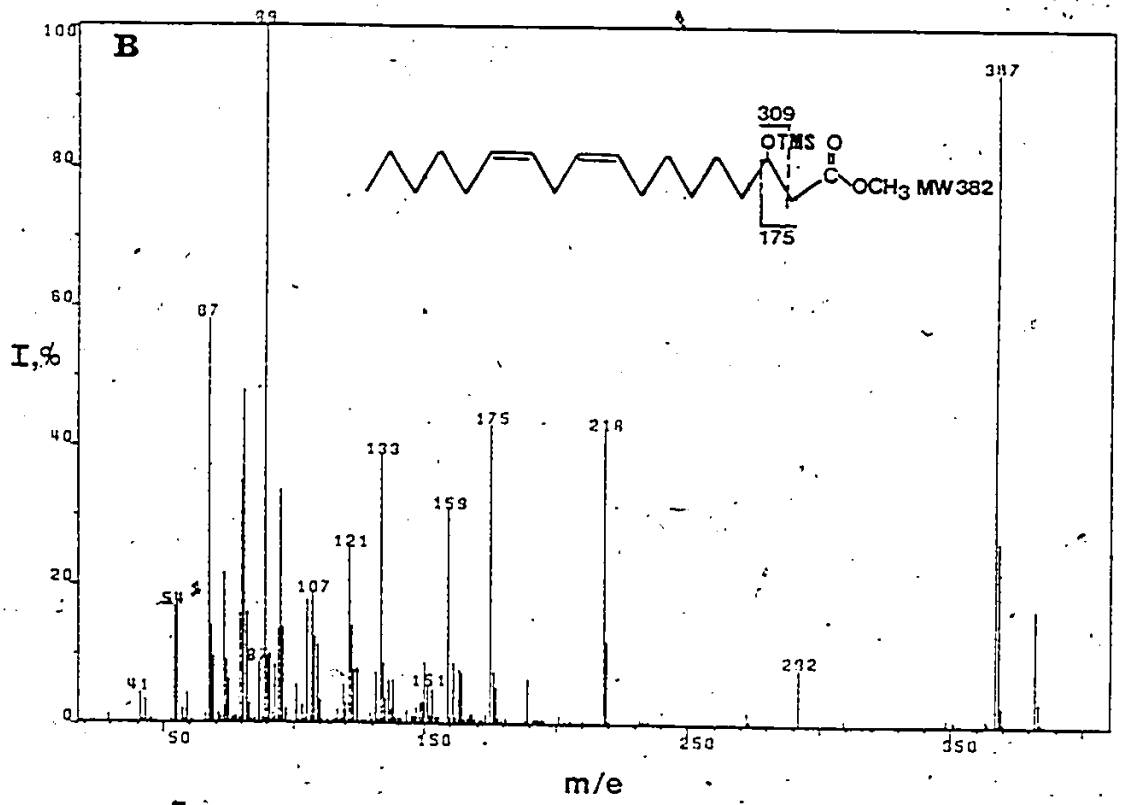
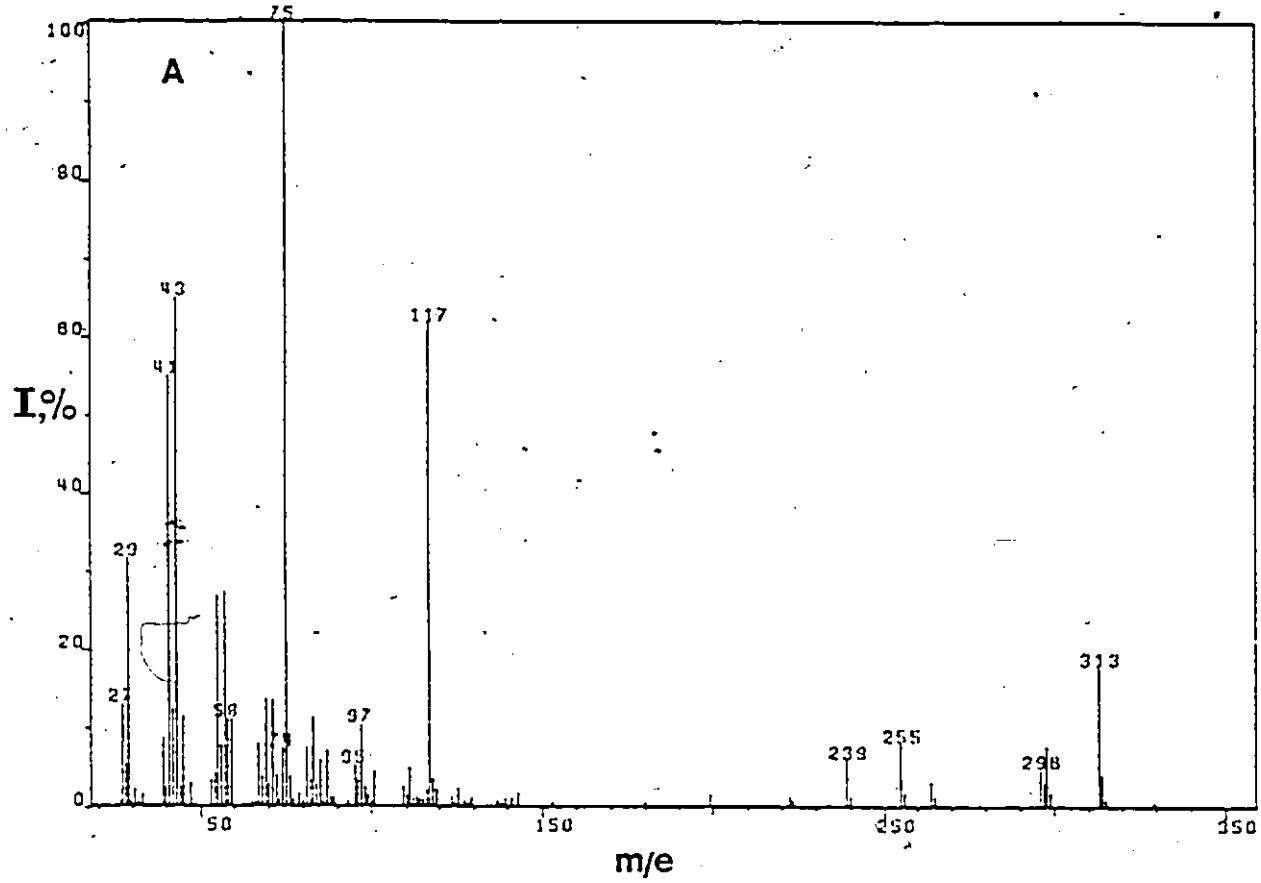
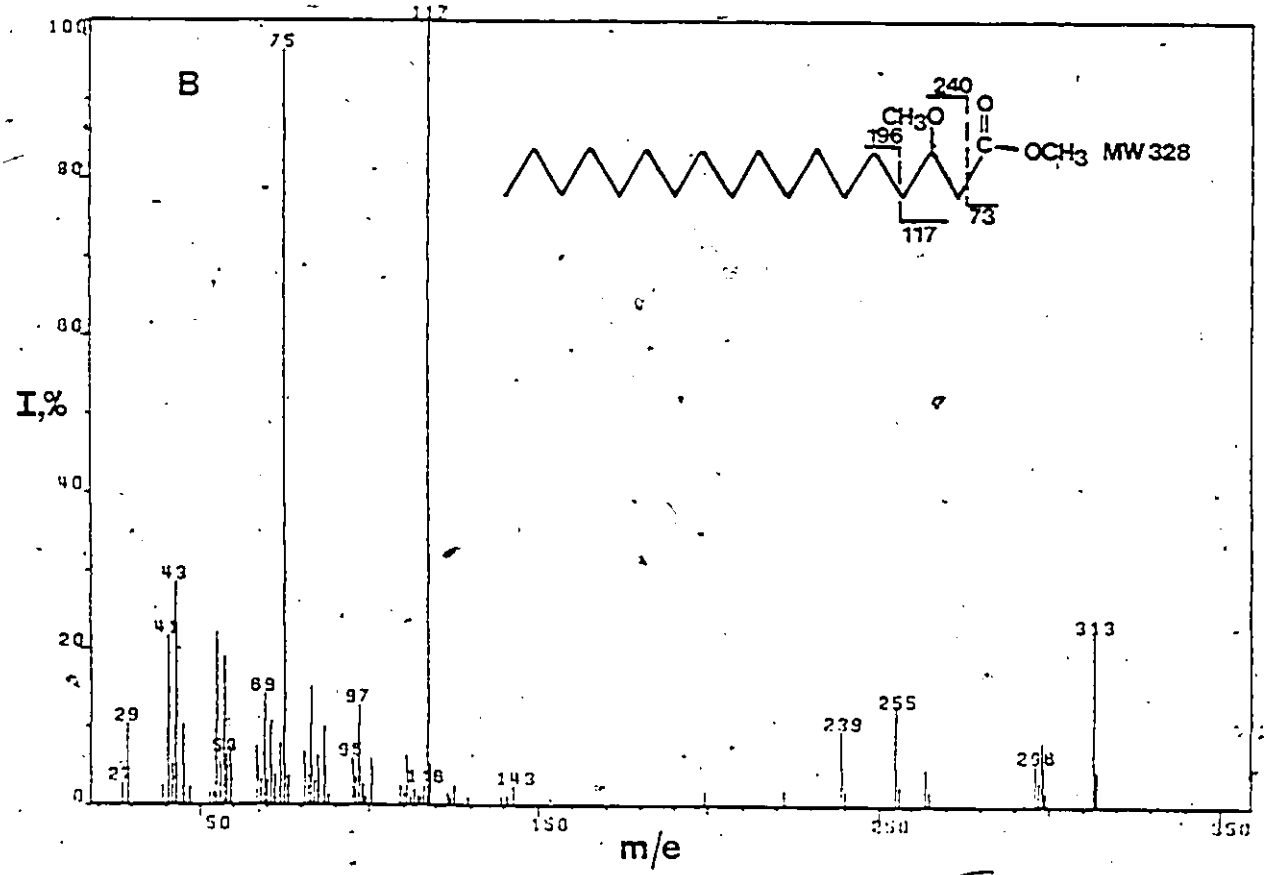

B
I,%
m/e
MW382
309
OTMS
OCH3
175
387
87
175
218
133
159
121
107
292

FIGURE 29. Mass spectra of methylated derivative of product H (OCH_3-18:2); A, before hydrogenation; B, after hydrogenation (see text for explanation).


A
I,%
m/e
75
117
43
41
29
313
255
239
298
97
50
150
250
350


B
I,%
m/e
117
75
43
41
29
69
97
313
255
239
298
143
50
150
250
350
240
CH3O
196
C
O
OCH3
MW328
117
73

represent the loss of methoxy and methanol groups, respectively, while those at m/e 218 (M -32-74), m/e 219 (M -31-74), and m/e 74 (M -32-218 or M -31-219) arise from the loss of methoxy and methanol groups, respectively, followed by McLafferty rearrangment. The fragment ion at m/e 117 (M -207) could occur only from fragmentation between C_3 to C_4 of the molecule.

The mass spectrum of the hydrogenated and methylated product H (Fig. 29B and Appendix II) was identical to that of authentic 3-methoxystearate (Fig. 22A). Thus component H has the structure of 3-hydroxylinoleate.

II. Metabolism of 18:1-CoA Substrate

a) Properties of Δ^{12}-Desaturase and Hydroxylating Enzyme System

1. Intracellular Location

Differential centrifugation of the total homogenate of the soybean cell suspension culture, revealed that most of the protein (83%) was recovered in the high speed supernatant (HSS) with only minor amounts being recovered in the proplastid (7%), mitochondrial (5%) (Fig. 30) and microsomal (5%) (Fig. 31) fractions (Table 16). In contrast, the bulk of the 18:1-CoA desaturase was recovered mostly in the proplastid and microsomal fractions with lesser activity in the mitochondrial fraction. The bulk of

FIGURE 30. Electron micrograph of mitochondrial fraction obtained by differential centrifugation at 19,000 x g as described in Fig. 13 (x 31,920). M, mitochondria; C = cristae; arrow indicating mitochondrial ribosomes; V = Vacuole.


M
V

FIGURE 31. Electron micrograph of microsomal fraction obtained by differential centrifugation at 150,000 x g (see Fig. 31) (x 20,000).

TABLE 16

Soybean Cell Fractionation by Differential Centrifugation

Cell Fraction	Protein % of Total	18:1-CoA Desaturase pmoles/min/mg	HO-18:1 Formation nmoles/min/mg	UDP-gal Transferase nmoles/h/mg	Catalase nmoles/min/mg	Succinate Dehydrogenase nmoles/min/mg
Total Homogenate	100	42	0.06	2.4	1.7	4.5
Proplastids	7	188	1.06	8.9	-	-
Mitochondria	5	110	0.19	12.3	11.3	19.0
Microsomes	5	162	0.50	10.2	3.0	7.4
Supernatant	83	20	0	1.2	0.6	1.3

hydroxylase activity was also found present in the proplastid fraction and to a lesser extent in microsomes. Further fractionation of the proplastid fraction on a discontinuous sucrose gradient (2.0 - 0.75M) resulted in the formation of five bands (Fig. 14) plus a pellet. Since the top bands were very faint and low in yield of protein, they were combined and designated as band E. After sucrose density gradient centrifugation of the crude proplastid fraction, the Δ^{12}-desaturase activity, which was originally concentrated mostly in the crude proplastid fraction, was found to be distributed among all of the sucrose density layers, but its specific activity appeared to be highest in band C (Table 17). Band C also contained the highest specific activity for UDP-gal transferase, which is a marker for plastids, while band B had the highest catalase specific activity, the marker for peroxisomes, and band D had the highest succinate dehydrogenase activity, the marker for mitochondria (Table 17). The highest specific activity for the hydroxylase system was also associated with band C.

Electron microscopy studies of these bands (B-E) confirmed the assignment of each of the bands as studied with marker enzymes and the known specific gravity of sucrose density gradients (232). Band B contained peroxisomes as well as some mitochondria and proplastids with a considerable contamination by cell wall fragments (Fig. 32A). Band C, corresponding to

TABLE 17

Further Soybean Cell Fractionation of Proplastid Fraction

by Sucrose Density Gradient Centrifugation

Sucrose Density Fractions[a]	Protein % of Total	18:1-CoA Desaturase pmoles/min/mg	HO-18:1 Formation nmoles/ming/mg	UDP-gal Transferase nmoles/h/mg	Catalase nmoles/min/mg	Succinate Dehydrogenase pmoles/min/mg
A	3.6	112	0.3	1.1	3.5	4.4
B	0.6	132	0.3	8.9	7.4	8.3
C	0.9	239	0.8	20.0	3.3	16.3
D	1.4	161	0.4	14.0	3.1	43.0
E	0.5	127	0.4	17.0	2.8	9.8

[a]Proplastid fraction, run on sucrose gradients, gave fractions: A, 2.0M, pellet; B, 2.0-1.75M, interface band; C, 1.75-1.5M, band; D, 1.5-1.0M band; E, 1.0-0.75M band.

FIGURE 32. Electron micrograph of Band B and C obtained from sucrose gradients as described in Section C, III. A, band B showing many peroxisomes (Pe) and cell wall fragments (CW) (X 15,960) with the insert showing a typical peroxisome of soybean cells (31,920 X); B, band C containing many intact plastids (P) showing thylakoid membranes (L) and few lipid droplets (Ld) (X 15,960) with minor contaminations by mitochondria (M) and vesicles (V).


B
V

the plastidial fraction, containing in addition few metochondria and vesicles originating from the tonoplast and plasma membranes (Fig. 32C). The mitochondrial enriched fraction band D, was enriched in mitochondria (Fig. 33A) but also contained vesicles derived perhaps from the plasma membrane. The upper band E, consists of a mixture of small membrane vesicles derived from tonoplast membrane by a normal process of budding off (see Fig. 33B).

The fatty acid composition of these bands (A-E) revealed that the highest proportions of 18:3 and highest 18:3/18:2 mole ratios were associated with bands B and C (Table 18); the latter band was enriched in proplastids, consistent with the observations that 18:3 is mainly associated with the plastid membranes (223,234).

2. Effect of ATP and $MgCl_2$ on Desaturation

In the absence of ATP-Mg^{2+} in the incubation mixture a time course experiment revealed that 18:1-CoA was rapidly hydrolysed to the free oleic acid and coenzyme A (Table 19). This rapid hydrolysis could be reversed by the addition of increasing amounts of ATP-Mg^{2+}. It was found that the use of 40 mM of ATP and Mg^{2+} was sufficient to completely inhibit the hydrolysis of 18:1-CoA (Table 19). However, the increased concentration of the substrate (18:1-CoA) in both proplastids and microsomes in the presence of 40 mM ATP-Mg^{2+}, resulted in only a slightly increased incorporation of [^{14}C]oleic acid

FIGURE 33. Electron micrograph of band D and E obtained from sucrose gradients as described in Section C, III. A, band D showing intact mitochondria and the presence of many vesicles (V) and perhaps broken plastids (P) (X 15,960); B, band E showing membrane vesicles originating either from plasma membranes (V) or from budding-off of tonoplast membrane (Ve) as illustrated in Fig. 11 (X 12,540).

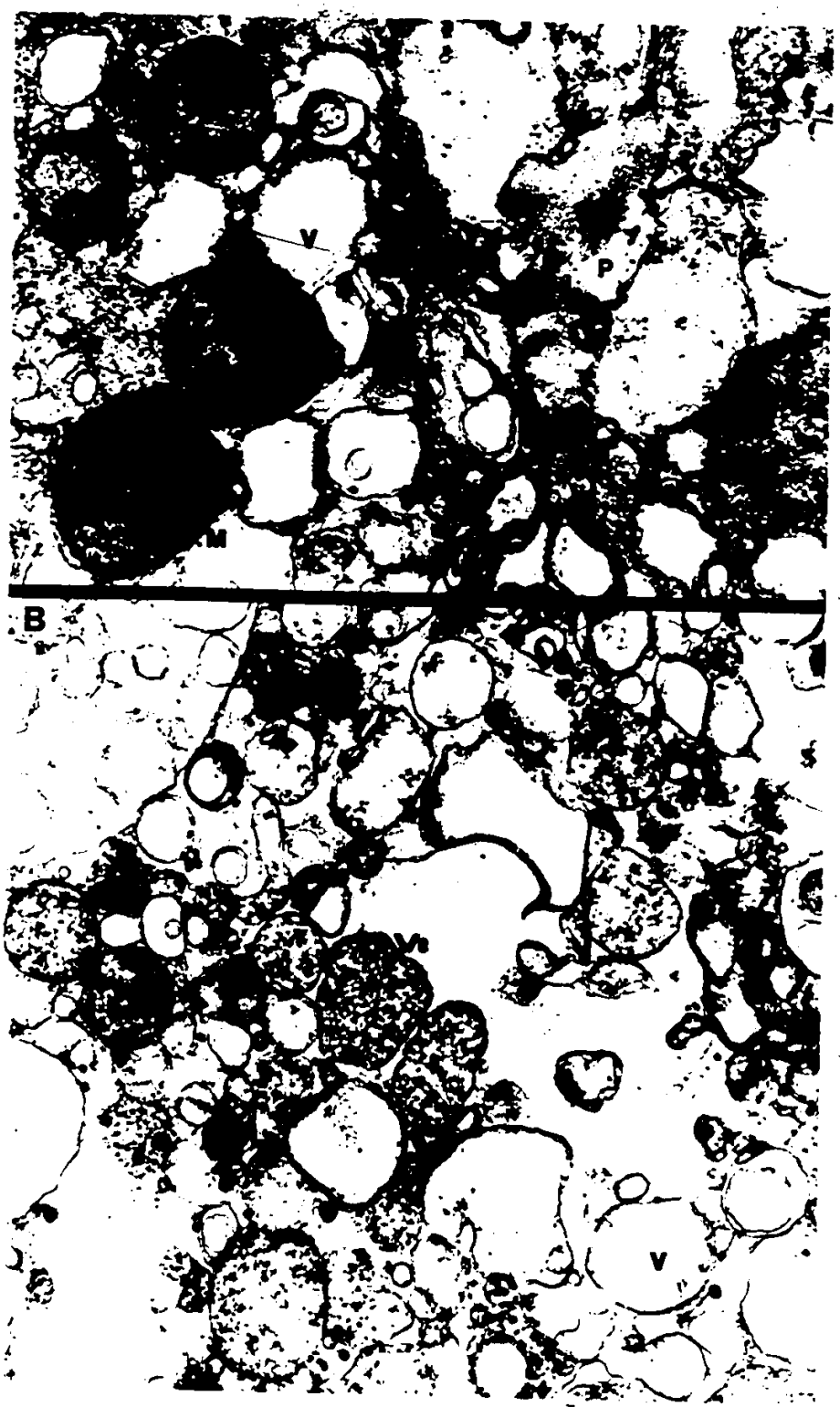

V
P
M
B
V

TABLE 18

Fatty Acid Composition (%) of Cellular Organelles Prepared From Sucrose Gradients

Fraction	Fatty Acids (%)						Mole Ratio	
	16:0	16:1	18:0	18:1	18:2	18:3	18:3/18:2	Δ/mole*
A	23	2	8	5	33	28	0.85	0.57
B	20	2	7	5	27	36	1.33	0.61
C	19	2	6	4	28	39	1.39	0.65
D	17	1	5	3	40	33	0.83	0.66
E	22	2	9	6	32	27	0.84	0.55

*Number of double bonds per mol fatty acid (Δ/mole).

TABLE 19

Effect of ATP + Mg^{2+} on Hydrolysis of 18:1-CoA by Thiolase of Plastid Microsome Fractions

ATP mM	Mg^{2+} mM	Distribution of ^{14}C from [^{14}C]18:1-CoA, % of total ^{14}C					
		Proplastids			Microsomes		
		Free Fatty Acids	18:1-CoA	Polar + Neut. Lipids	Free Fatty Acids	18:1-CoA	Polar + Neut. Lipids
0	0	40	17	43	35	33	32
10	10	1	54	45	3	65	32
20	20	0.4	60	39	1	69	30
40	40	0	55	45	1	64	35
80	80	0	49	51	0.6	69	30

Incubation system (1 ml): tricine buffer (pH 8) 100 mM, [^{14}C]18:1-CoA 44 nmoles (0.049 µCi), 0.5 mg protein; incubation for 15 min. Reaction mixtures were partitioned according to Bligh-Dyer; 18:1-CoA counted in methanol-water phase and lipids in $CHCl_3$ phase were chromatographed on silica gel H in $CHCl_3$-acetone-MeOH-HAc-H_2O (10:4:2:2:1) and radioactive spots were counted.

into the polar and neutral lipids (Table 19). All subsequent assays of desaturatase activity were carried out with an incubation medium containing 40 mM ATP and 40 mM $MgCl_2$.

3. Influence of Protein Concentration

Desaturase activity of both proplastid and microsomes fractions was found to be proportional to protein concentration with either the proplastid or microsomal fraction, up to 0.2 mg/ml; with either fraction maximum desaturation was obtained at a concentration of 0.5 mg/ml (Fig. 34). We have routinely used 0.20 mg/ml, unless otherwise specified. Maximum hydroylase activity, with either proplastids or microsomes, was also obtained at a concentration of 0.5 mg/ml.

4. Influence of pH

The pH optimum for Δ^{12}-desaturase activity for either proplastids or microsomes was in the range of 7.5 to 8.0 (Fig. 35). The optimum pH for the hydroxylase system was also in the range of 7.5 to 8.0.

5. Time Course

With either proplastid or microsomal fraction the rate of desaturation was found to be rapid and linear with the time of incubation up to 15 min., reaching a plateau after 30 min. of incubation, (Fig. 36).

The rate of hydroxylation, with either the microsomal or proplastid fraction, was also linear up to 15 min., reaching a plateau after 30 min. incubation.

FIGURE 34. Influence of proplastid (O-O) and microsomal (Δ-Δ) protein on Δ^{12}-desaturase activity. Incubation system (1.0 ml): Tricine buffer (pH 8), 100 mM; ATP, 40 mM; $MgCl_2$, 40 mM; CoA, 0.1 mM; NADPH, 200 µM; [^{14}C]18:1-CoA, 49 µM; incubation at 29°C for 30 min. with increasing concentration of proplastid and microsomal protein.

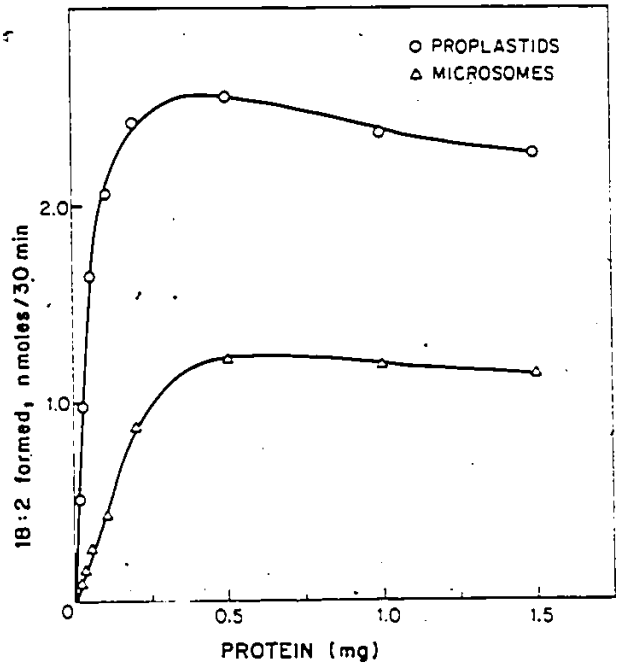

O PROPLASTIDS
Δ MICROSOMES
18:2 formed, nmoles/30 min
2.0
1.0
0
0.5
1.0
1.5
PROTEIN (mg)

FIGURE 35. Influence of pH on the proplastid (O-O) and microsomal (Δ-Δ) Δ^{12}-desaturase activity. Incubation system (1.0 ml): Buffer (acetate, phosphate or tricine) (pH 5.5-9.5), 100 mM; ATP, 40 mM; $MgCl_2$, 40 mM; CoA, 0.1 mM; NADPH, 200 µM; [^{14}C]18:1-CoA, 59 µM; Protein, 150 mg; Incubation at 29°C for 30 min.

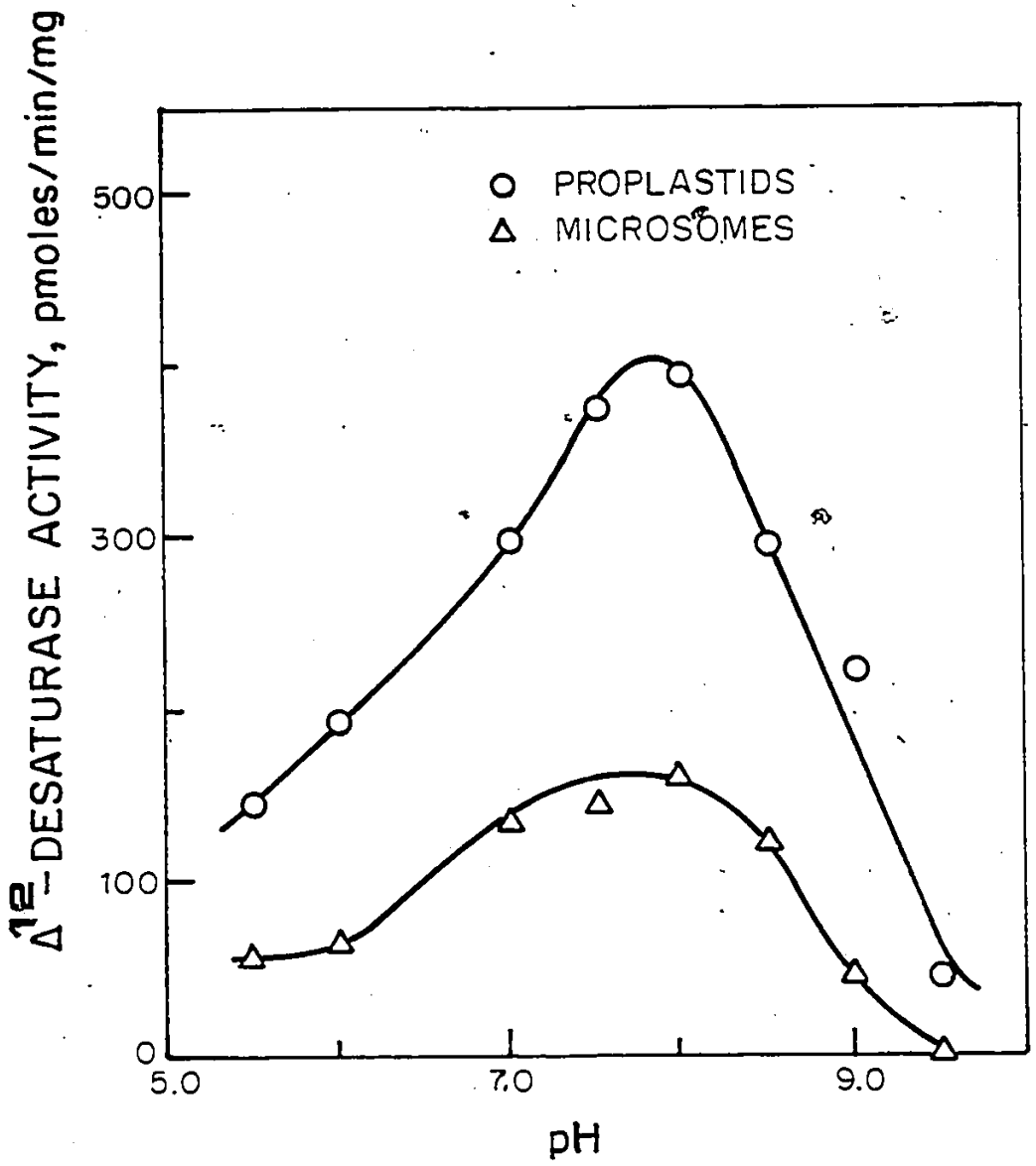
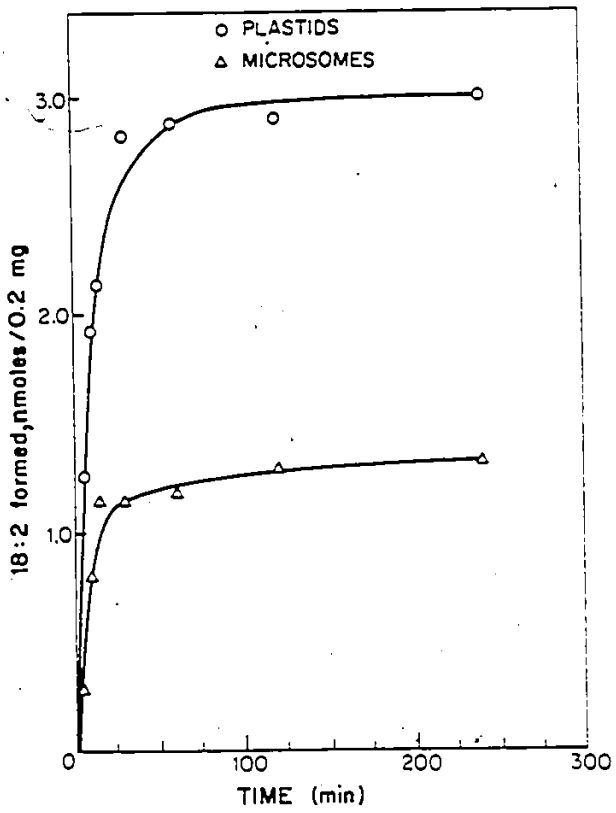

PROPLASTIDS
MICROSOMES
Δ12-DESATURASE ACTIVITY, pmoles/min/mg
500
300
100
0
5.0
7.0
9.0
pH

FIGURE 36. Desaturation of oleoyl-CoA by proplastids (O-O) and microsomes (Δ-Δ) prepared from soybean cell suspension cultures grown for seven days at 29°C as a function of time. Incubation system (1.0 ml): tricine buffer (pH 8), 100 mM; ATP, 40 mM; $MgCl_2$, 40 mM; CoA, 0.1 mM; NADPH, 200 μM; [^{14}C]oleoyl-CoA, 49 μM; protein, 200 μg; incubation at 29°C for various time periods.


PLASTIDS
MICROSOMES
3.0
2.0
1.0
0
100
200
300
18:2 formed, nmoles/0.2 mg
TIME (min)

6. Influence of Temperature

The optimum assay temperature of Δ^{12}-desaturase activity was found to range between 20 to 30°C with a maximum at 25°C for both proplastids and microsomes (Fig. 37). The enzyme is inactivated above 50°C.

The optimum temperature of hydroxylase activity, with either proplastids or microsomes, was also in the range between 20 to 30°C.

7. Influence of Substrate (18:1-CoA) Concentration on Δ^{12}-Desaturase and Hydroxylase

The rate of oleoyl-CoA desaturation was found to be linear up to a 18:1-CoA concentration of 100 µM, thereafter levelling off from linearity and reaching saturation above 300 µM (Fig. 38A). Lineweaver-Burk plot (Fig. 38,B) indicating an apparent Km of 435 µM and Vmax 2.9 µmoles/min./mg. The rate of hydroxyoleate formation was also linear up to a 18:1-CoA concentration of 100 µM, thereafter levelling off from linearity and reaching a plateau above 300 µM (Fig. 39A). Lineweaver-Burk plot (Fig. 39B) indicated an apparent Km of 250 µM and Vmax of 11 µmoles/min./mg.

8. Effect of Reduced Pyridine Nucleotides and Other Cofactors

Of the reduced pyridine nucleotides tested, NADPH produced a stimulation of Δ^{12}-desaturase and an inhibition of hydroxylase activity while NADH was somewhat inhibitory for both activities (Table 20). However, ferredoxin, an electron

FIGURE 37. Desaturation of oleoyl-CoA by proplastids (O-O) and microsomes (Δ-Δ) prepared from soybean cell suspension cultures grown for seven days at 29°C as a function of temperature. Incubation system (1.0 ml): tricine buffer (pH 8), 100 mM; ATP, 40 mM; $MgCl_2$, 40 mM; CoA 0.1 mM; NADPH, 200 µM; [^{14}C] oleoyl-CoA, 49 µM; protein, 200 µg; incubated for 30 min. at various temperatures.

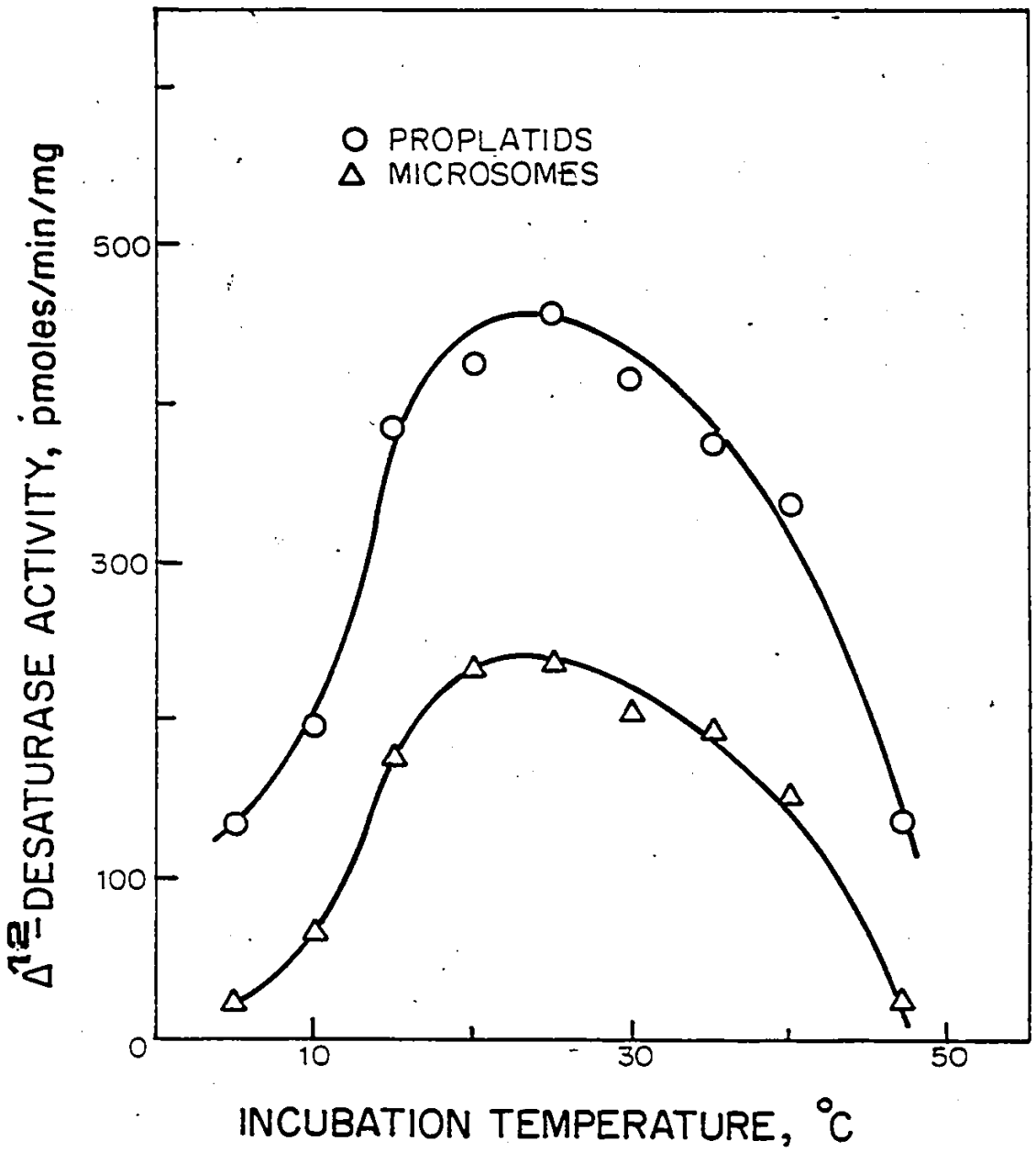

PROPLATIDS
MICROSOMES
500
300
100
0
10
30
50
Δ12 DESATURASE ACTIVITY, pmoles/min/mg
INCUBATION TEMPERATURE, °C

FIGURE 38. Oleoyl-CoA desaturase activity of proplastid fraction prepared from soybean cell suspension cultures grown for seven days at 29°C as a function of oleoyl-CoA concentration. A, plot of V against S; B, double-reciprocal plot (Lineweaver-Burk). Incubation mixture was described in Fig. 37, for 30 min. at various concentrations of oleoyl-CoA.

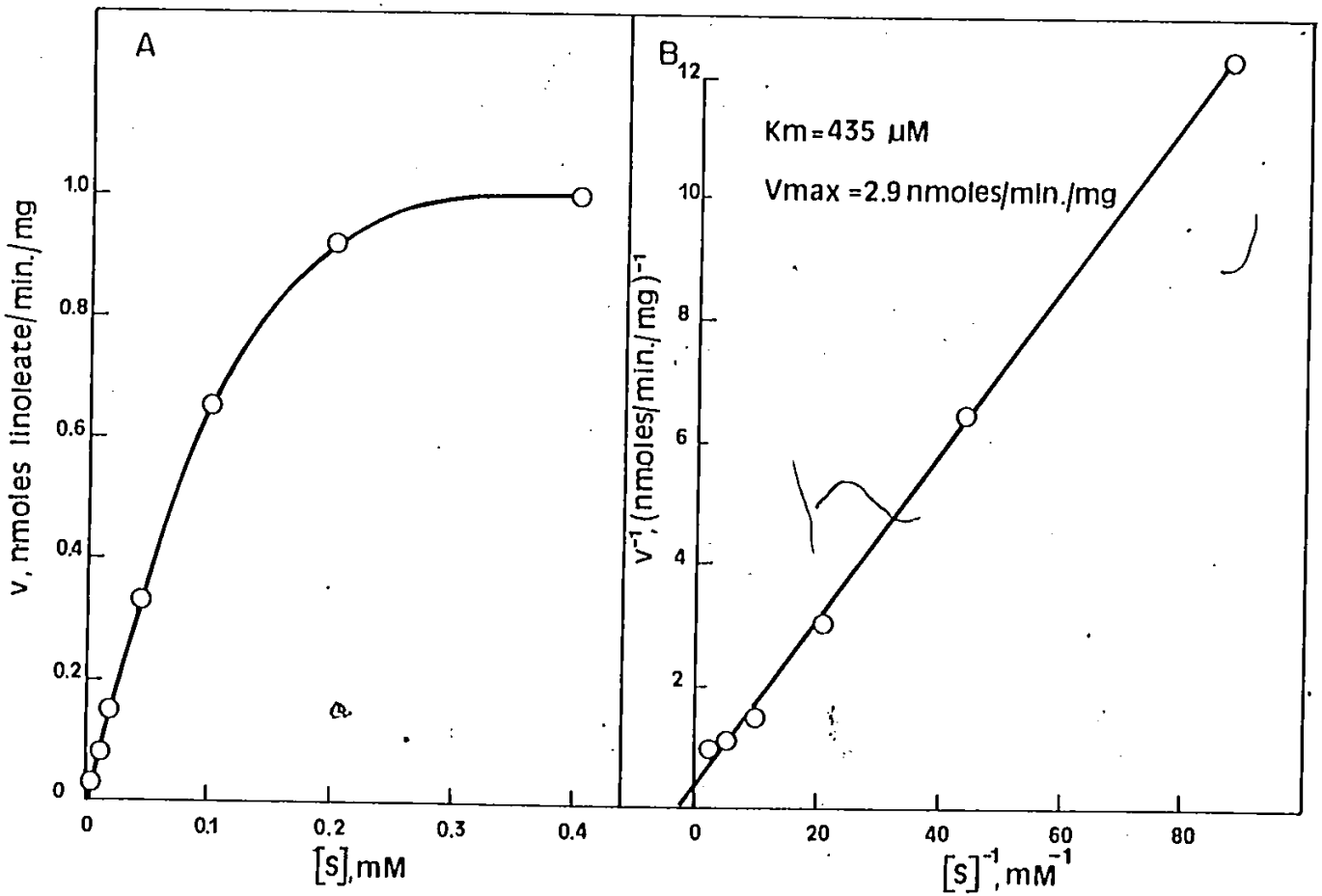

A
B12
Km=435 µM
Vmax =2.9 nmoles/min./mg
v, nmoles linoleate/min./mg
v⁻¹, (nmoles/min./mg)⁻¹
[S], mM
[S]⁻¹, mM⁻¹
0
0.2
0.4
0.6
0.8
1.0
2
4
6
8
10
0.1
0.3
20
40
60
80

FIGURE 39. Oleoyl-CoA hydroxylase activity of proplastids prepared from soybean cell suspension cultures grown for seven days at 29°C as a function of oleoyl-CoA concentration. A, plot of V against S; B, double-reciprocal plot (Lineweaver-Burk). Incubation was as described in Fig. 37.

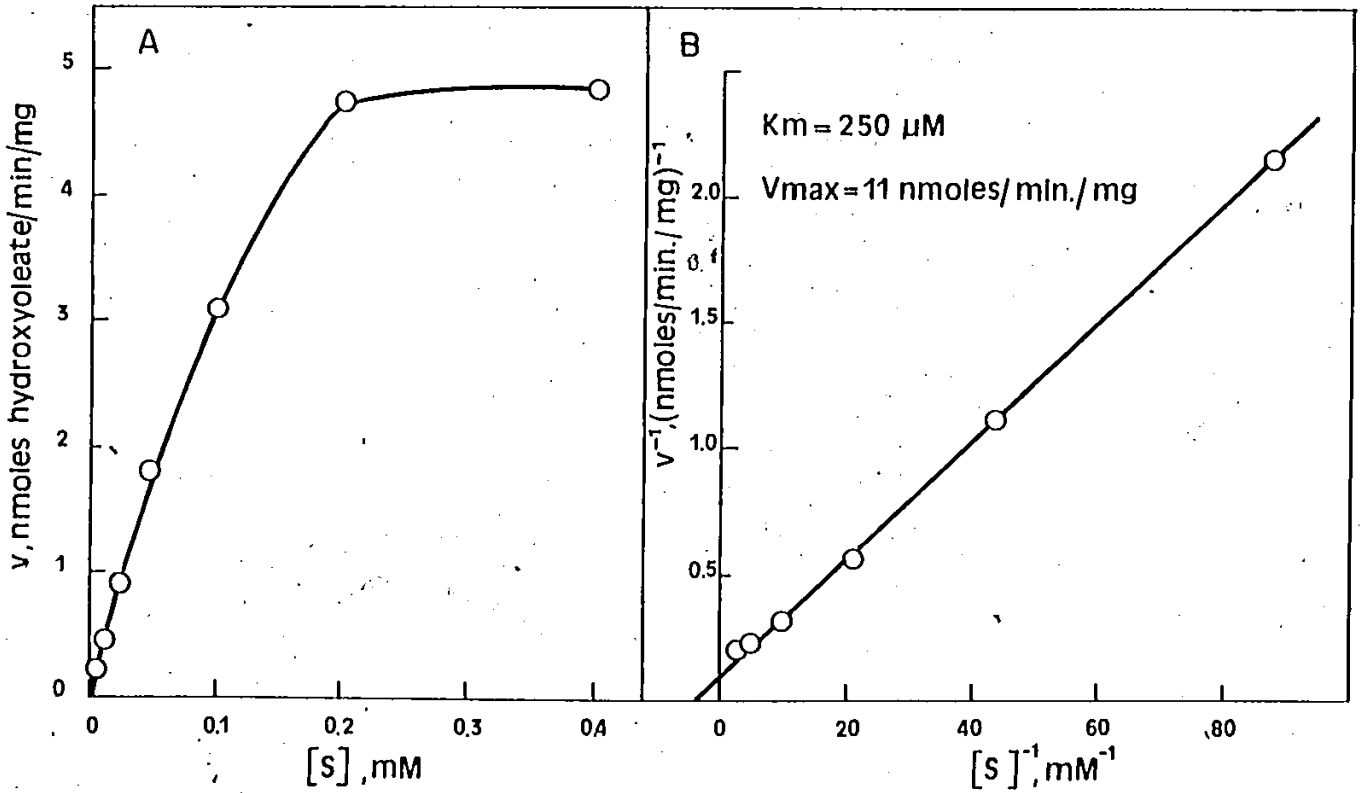

A
B
v, nmoles hydroxyoleate/min/mg
[S], mM
v^{-1}, (nmoles/min./mg)$^{-1}$
$[S]^{-1}$, mM^{-1}
Km = 250 µM
Vmax = 11 nmoles/ min./ mg
0
1
2
3
4
5
0.1
0.2
0.3
0.4
0.5
1.0
1.5
2.0
20
40
60
80

TABLE 20

Effect of Pyridine Nucleotides, and other Cofactors on Metabolism of 18:1-CoA in Soybean Proplastids

Conditions	Activity, % of Control* 18:2 Product	HO-18:1 Product
Complete system + O_2	100	100
Complete system + N_2	7	2
Complete system + O_2 + NADH (200 µM)	81	59
Complete system + O_2 + NADPH (200 µM)	109-136	81
Complete system + O_2 + Ferredoxin (10-60 µg)	100-102	91-100

*Control: complete system (1 ml) contained tricine buffer (pH 8), 100 mM; $MgCl_2$, 40 mM; ATP, 40 mM; CoA, 0.1 mM; [^{14}C] 18:1-CoA, 47-49 µM; protein, 200 µg; incubation 30 min at 29°C; activity of "complete system", 11.3 nmoles 18:2/30 min/mg for Δ^{12} desaturase and 37.5 µmoles/30 min/mg for hydroxylase activity.

donor in plants, in the range 10-60 µg had no effect on the desaturase and hydroxylase activities. These results seem to indicate that the proplastid desaturase and hydroxylase systems were already saturated with endogenous electron donors of either NADPH or ferredoxin. To test the possibility that the proplastid preparation does not require electron donors for desaturation, as has been demonstrated in the psychrophilic bacterium Micrococcus cryophilus (122), this fraction was sonicated at 0°C (on ice) in a sonifier with a microtip (Quigley-Rochester Inc.) operated at an output of 2A for 15 sec, with 15 sec. cooling intervals, up to a maximum of 2 min. The assay of Δ^{12}-desaturase activity was performed in the presence and absence of reduced nucleotides on the whole sonicated fraction as well as on pellets and supernatants obtained by centrifugation at the speeds indicated in Table 21. These results show that NADPH is not absolutely required for the formation of 18:2 or HO-18:1. Desaturation activities in the the presence of NADPH were alsways lower than in its absence. It should be noted that these enzyme activities are associate with the lamellar membranes as indicated by the high enzyme activity of the 150,000 x g pellet, containing chlorophyll, and virtual absence of activity in the supernatant which contained the soluble proteins. Since NADPH produced a slight stimulation with the crude enzyme preparation (Table 20), the desaturation reaction was routinely carried out in the presence of NADPH in the incubation mixture.

TABLE 21

Effect of Sonication on 18:1-CoA Metabolism in Soybean Proplastids

Conditions	NADPH	Activity, % of control* 18:2 product	HO-18:1 product
Control	-	100	100
	+	100	100
Sonicated	-	133	106
	+	84	114
Pellet 5,000 x g	-	154	107
	+	97	87
Super 5,000 x g	-	168	101
	+	114	82
Pellet 150,000 x g (green)	-	166	100
	+	104	88
Supernatant 150,000 x g	-	59	0.10
	+	37	0.10

*Control: complete system (1.0 ml): tricine buffer (pH 8.0), 100 mM; $MgCl_2$, 40 mM; ATP, 40 mM; CoA, 0.1 mM, [1-^{14}C]oleoyl-CoA, 100 µM; protein, 0.2 mg; incubation at 29°C for 30 min. Activity of complete system was 8.5 nmoles/30 min/mg (-NADPH) and 13.5 nmoles/30 min/mg (+ NADPH) for Δ^{12}-desaturase and 99 µmoles/30 min/mg (-NADPH) and 104 nmoles/30 min/mg (+ NADPH) for hydroxylase activity.

9. Effect of Inhibitors on Δ^{12}-Desaturase and Hydroxylase Systems

Relatively little or no inhibition was observed with the electron transport inhibitor, azide, in contrast to the strong inhibitory effect reported for the Δ^{9}-desaturase in rat liver microsomes (235) and the microsomal midchain hydroxyating system of Vicia faba (198,199). However, inhibition by cyanide was observed at high concentration (Table 22), indicating the probable involvement of a cyanide sensitive factor. Carbon monoxide (CO), a specific inhibitor of cyrochrome P-450, had little effect, indicating that this cytochrome may not be involved. That the products of desaturation are indeed enzymatic products was shown by the complete lack of activity on boiling the enzyme system. Replacement of oxygen, by bubbling nitrogen in the reaction mixture, almost completely abolished the Δ^{12}-desaturase and hydroxylase enzymes; demonstrating the absolute requirement of oxygen for both enzyme activities.

While the desaturase and hydroxylase activities were not affected by EDTA (20 mM), a general chelating agent, o-phenanthroline (Table 23) and α,α-dipyridyl (Table 22), which specifically bind Fe^{2+}, reduced the desaturase activity by 37 and 83% and hydroxylase activity by 53 and 91% respectively, indicating the possible involvement of heme- and/or non-heme iron proteins.

TABLE 22

Effect of Inhibitors on 18:1-CoA Desaturase and Hydroxylase Activity

Conditions	Activity, % of Control*	
	18:2	HO-18:1
Complete system + O_2	100	100
Complete system + O_2 boiled	7	1
Complete system + O_2 + azide (0.1 mM-1.0 mM)	100-92	99-91
Complete system + O_2 + cyanide (40 mM)	67	56
Complete system + O_2 + cyanide (80 mM)	40	18
Complete system + O_2 + $CO/O_2/N_2$ (bubbled) (31:3:66)	76	64
Mercaptoethanol (1.0 mM)	96	100
Iodoacetamide (1.0 mM)	100	94
N-ethylmaleimide (1.0 mM)	9	4
5,5'-dithiobis-2-nitrobenzoate (1.0 mM)	5	3
EDTA (20 mM)	98	99
α,α'-dipyridyl (1.0 mM)	17	9
BHT (1.0 mM)	25	13
Triton X-100 (0.05% - 0.1%)	2	0

*Control: complete system (1.0 ml) contained: tricine buffer (pH 8), 100 mM; $MgCl_2$, 40 mM; ATP, 40 mM; CoA, 0.1 mM; [^{14}C]18:1-CoA, 59 μM; protein, 0.5 mg; incubation at 29°C for 30 min; activity of "complete system", 4.56 nmoles/30 min/mg protein.

TABLE 23

Effect of Other Inhibitors on 18:1-CoA Desaturase and Hydroxylase Activity

Conditions		Activity, %, of Control* 18:2	HO-18:1
Control		100	100
SAN 6706**	1.0 mM	63	62
	5.0 mM	7	4
SAN 9785**	1.0 mM	63	100
	5.0 mM	26	18
Destun**	.013 mM	0	1
	.026 mM	0	0
Atebrine	1.0 mM	63	38
	5.0 mM	26	26
Dithiothreitol	1.0 mM	63	100
	5.0 mM	48	52
o-phenanthroline	1.0 mM	63	47
	5.0 mM	7	4

*Control: complete system (1.0 ml) contained: tricine buffer (pH 8), 100 mM; ATP, 40 mM; $MgCl_2$, 40 mM; CoA, 0.1 mM; [1-^{14}C] oleoyl-CoA, 100 µM; protein 0.2 mg; incubation at 29°C for 30 min. Activity of "complete system" was 13.5 nmoles/30 min/mg. for Δ^{12}-desaturase and 104 mmoles/30 min/mg for hydroxylase activity.

**See abbreviations, page XV.

The thiol-directed reagents N-ethylmaleimide and dithiobis-2-nitrobenzoate almost completely inhibited the desaturase and hydroxylase reactions, (Table 22), but iodoacetamide had no significant effect; of the thiol-protecting agents, mercaptoethanol had no effect but dithiothreitol was inhibitory at high concentrations. On the basis of the inhibition by N-ethylmaleimide and dithiobis-2-nitrobenzoate, it would appear that sulfhydryl groups might be required for both the desaturase and hydroxylase reactions, but the lack of inhibition by iodoacetamide and the inhibition by dithiothreitol is puzzling. The non-ionic detergent, Triton X-100, and butylated hydroxytoluene (antioxidant) almost completely abolished the desaturase and hydroxylase activities as might be expected for membrane-bound enzyme systems.

The pyridazinone (San 6706 and San 9785) and Destun herbicides were strong inhibitors both of 18:2 formation as well as on the formation of hydroxyoleic acid (HO-18:1) (Table 23). It is interesting to note that San 9785 at 1.0 mM showed preferential inhibition of 18:2 formation, as did 1.0 mM dithiothreitol. This preferential inhibition suggests that the enzymes involved in the formation of the two products are separate enzyme systems. Atebrine, an antimalarial agent which has been reported to inhibit acyltransferase activity (236), at 1.0 mM concentration inhibited the formation of

hydroxyoleic acid (HO-18:1) more strongly than the formation of 18:2. This preferential inhibitory effect lends further support on the independence of the two enzyme systems.

b) Influence of Storage on Enzyme Stability

Proplastid and microsomal fractions were stored at -70°C for various periods of time (Fig. 40). The Δ^{12}-desaturase after 8 day storage lost about 25% or 29% of the initial activity in the proplastids and microsomes respectively; after 32 days storage, the losses were 34% and 43% respectively (Fig. 30A). The loss of hydroxylase activity on storage followed the same pattern as the desaturase activity with either proplastids or microsomes (Fig. 40B) except that the hydroxylase activity of the microsomal fraction appears to become stable after 8 hours.

c) Influence of Growth Phase

Maximum Δ^{12}-desaturase was obtained with proplastids and microsomes derived from cells grown for a period of 7 days (end of log phase) (Fig. 41). The extent of hydroxyoleate formation was also maximmal on the seventh day, (Fig. 41). Note that the rate of formation of 18:2 and hydroxyoleate by the proplastid fraction is always higher than that produced by the microsomes. After seven days (stationary phase) the desaturase activity in both plastids and microsomes decreased while the hydroxylase activity remained essentially constant.

FIGURE 40. Oleoyl-CoA desaturase and hydroxylase activity of proplastids and microsomes prepared from soybean cell suspension cultures grown for seven days at 29°C as a function of storage at -70°C. A, Δ^{12}-desaturase activity (o-o proplastids; Δ-Δ microsomes): B, hydroxylase activity (□—□ proplastids; ▽-▽ microsomes). The enzyme assay was as described in section C, IV.

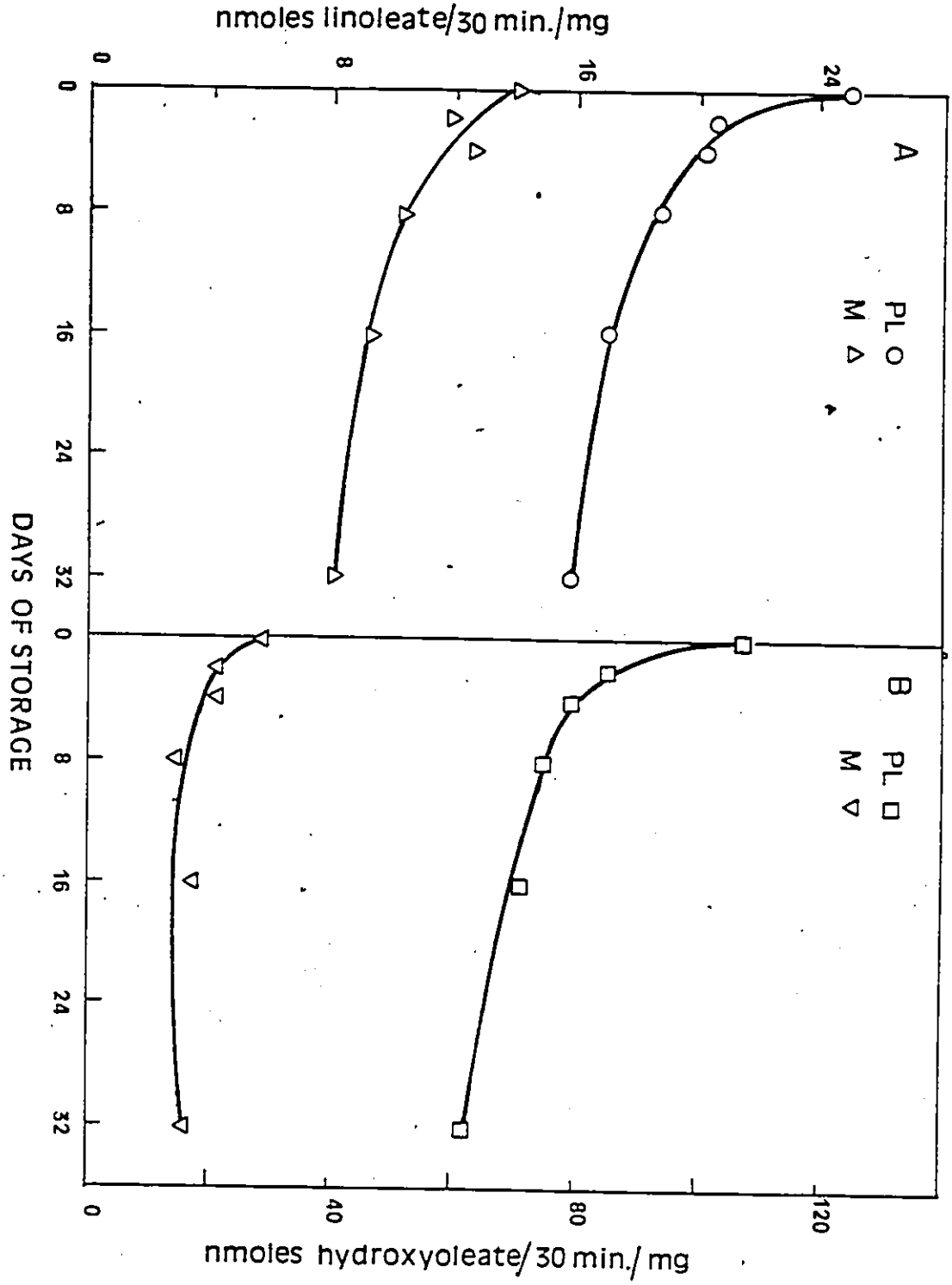
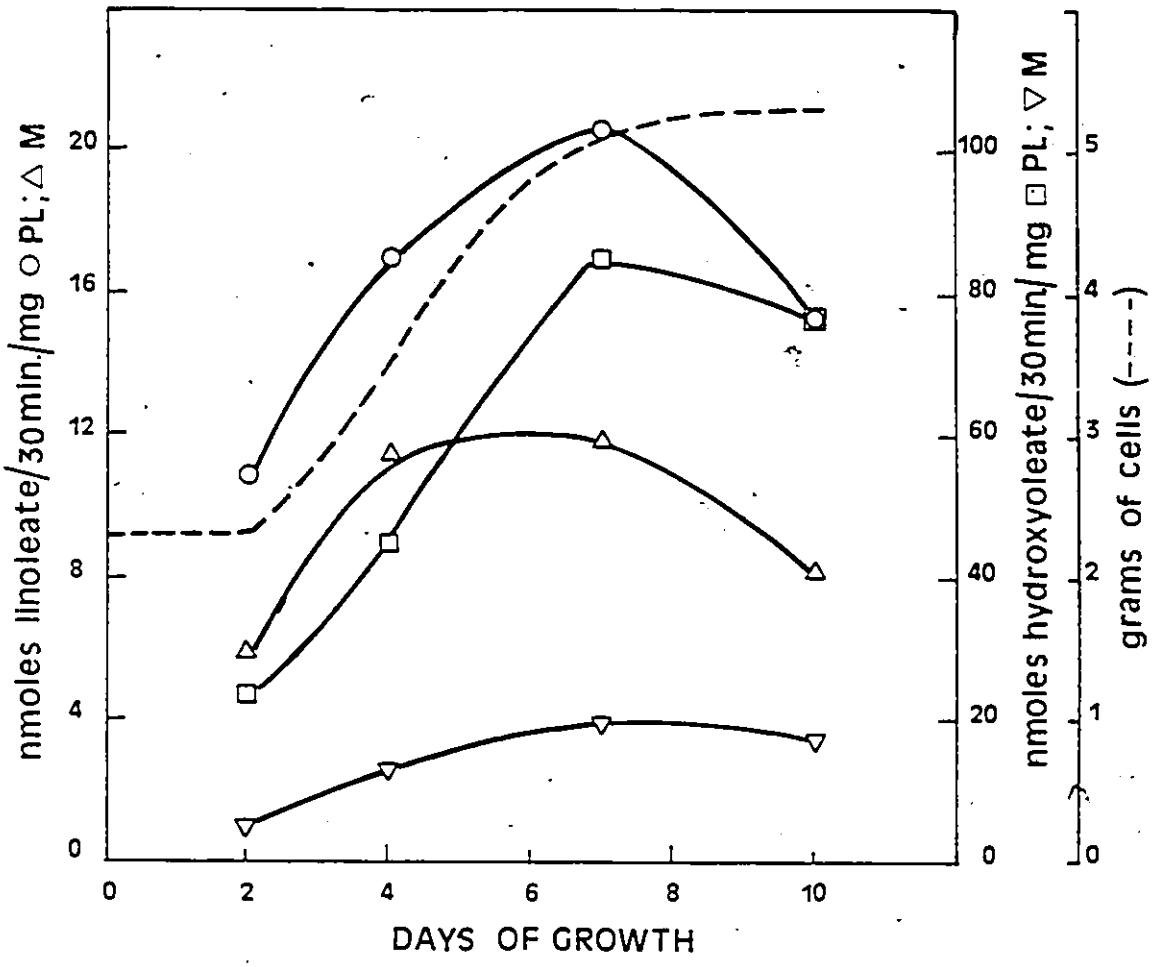

nmoles linoleate/30 min./mg
0
8
16
24
A
PL O
M △
B
PL □
M ▽
DAYS OF STORAGE
0
8
16
24
32
0
40
80
120
nmoles hydroxyoleate/30 min./mg

FIGURE 41. Oleoyl-CoA desaturase and hydroxylase activity of proplastid and microsomes prepared from soybean cell suspension cultures grown in the light for seven days at 29°C as a function of growth (-·-·-). Δ^{12}-desaturase activity (o-o proplastids; Δ-Δ microsomes); hydroxylase activity (□—□ proplastids; ∇-∇ microsomes). The enzyme assay was as described in section C, IV.


nmoles linoleate/30min./mg ○ PL; △ M
nmoles hydroxyoleate/30min/mg □ PL; ▽ M
grams of cells (----)
DAYS OF GROWTH
0
2
4
6
8
10
0
4
8
12
16
20
0
20
40
60
80
100
0
1
2
3
4
5

The major fatty acids of proplastids and microsomes prepared from soybean cell suspension cultures are palmitic, stearic, oleic, linoleic and linolenic acids; minor proportions of myristic, pentadecanoic, palmitoleic and eicosanoic acids are also present (Table 24,25). Up to seven days of growth, the fatty acid composition of proplastids or microsomes showed little change, but growth of cells for longer periods (10 days), resulted in higher proportions of 18:3 at the expense of 18:2 fatty acid with a doubling of the 18:3/18:2 mole ratio with no change in the number of double bonds (Table 24,25).

In absolute amounts, the total fatty acid content (nmoles/mg protein) with either proplastids or microsomes was highest at 2 days of growth and decreased continuously thereafter. The content of 18:2 and 18:3 in particular, decreased markedly while the more saturated fatty acids remained virtually unchanged up to four days; thereafter, while 18:2 continued to decrease to a low value at 10 days of growth, the 18:3 content increased to higher levels at 10 days of growth thereby suggesting the possible conversion of 18:2 to 18:3 during this period (Table 24). It should be noted that at all times during growth, the total amount of lipid per mg of protein found associated with the proplastid fraction was higher than that of microsomes.

TABLE 24

Influence of Growth-Phase on the Fatty Acid

Composition of Proplastids Prepared from 29°C Grown Cells[a]

Days of Growth	Fatty Acid Content (nmoles/mg protein)[b]						Fatty Acid Proportions (moles %)[b]					Ratio	Ratio	Δ/mole[c]
	16:0	18:0	18:1	18:2	18:3	Total	16:0	18:0	18:1	18:2	18:3	18:3/18:2	unsat./sat.	
2	190	50	40	310	210	800	24	6	5	39	26	0.68	2.54	0.56
4	190	60	50	250	140	690	28	9	7	36	20	0.56	1.87	0.51
7	130	40	40	230	150	590	22	7	7	39	25	0.65	2.53	0.58
10	120	40	30	140	160	490	25	8	6	29	32	1.14	2.12	0.58

[a]Results are averages of two analyses with deviation from the means ± <1%.

[b]All samples contained <1% of 14:0, 15:0, 16:1, 20:0 fatty acids.

[c]Number of double bonds per mol fatty acid (Δ/mole).

TABLE 25

Influence of Growth-Phase on the Fatty Acid

Composition of Microsomes Prepared from 29°C Grown Cells

Days of Growth	Fatty Acid Content (nmoles/mg protein)[a]						Fatty Acid Proportion (moles %)[a]					Ratio	Ratio	Δ/mole[c]
	16:0	18:0	18:1	18:2	18:3	Total	16:0	18:0	18:1	18:2	18:3	18:3/18:2	unsat./sat.	
2	160	40	40	230	180	650	25	6	6	35	28	0.78	2.34	0.57
4	200	50	70	200	130	650	31	8	11	31	20	0.65	1.76	0.48
7	100	30	20	150	100	400	25	8	5	38	25	0.67	2.22	0.56
10	100	30	10	110	110	360	28	8	3	31	31	1.00	1.99	0.56

[a]Results are averages of two analysis with deviations from the means of ± <1%

[b]All samples contained <1% of 14:0, 15:0, 16:1 and 20:0 fatty acids.

[c]Number of double bonds per mol fatty acid (Δ/mole).

d) Influence of Light

Light has been reported to influence the development (237) and fatty acid composition (238) of plastids and microsomes. Changes in fatty acid contents of proplastids and microsomes, prepared from soybean cell suspension cultures, grown in the presence or absence of light was studied and the results are presented in Table 26. The total fatty acid content of either proplastids or microsomes increased by 16 and 26% respectively when the cells were grown in the presence of light. Of the proplastid fatty acids, the contents of 18:0, 18:1, 18:2 and 18:3 remained essentially constant when the cells were grown in the presence of light but the most significant change occurred with 16:0 acid which increased by 158%. This change was responsible for the increased content of total fatty acids and most likely results from an increased de novo fatty acid synthesis (Table 26).

In contrast, microsomes prepared under the same conditions, showed increases in the content of all fatty acids, resulting in little change in the DBI.

The specific activity of the Δ^{12}-desaturase and hydroxylase enzymes did not change on exposure to light (Table 27), thus reflecting the constant amount of 18:2 acid observed in proplastid membranes; the rate of formation of hydroxyoleic (C) acid also did not change significantly. In contrast microsomal Δ^{12}-desaturase on exposure to light increased by about 50%, reflecting the increased levels of

TABLE 26

Effect of Light/Dark Regime on the Content of Proplastid and Microsomal Fatty Acids[a]

Fatty Acid	Microsomes (nmoles/mg protein)[b]		Proplastids (nmoles/mg protein)[b]	
	Light	Dark	Light	Dark
16:0	89	75	123	48
16:1	7	4	8	9
18:0	40	26	54	43
18:1	10	8	16	16
18:2	134	110	227	229
18:3	133	105	224	221
Total	413	328	652	566
Δ/mole[c]	1.98	1.96	4.12	4.11

[a]Results are averages of two analysis with deviations from the means of ± ≤1%.

[b]All samples contained <1% of 14:0, 15:0 and 20:0 fatty acids.

[c]Number of double bonds per mol fatty acid (Δ/mole).

Table 27

Effect of Light/Dark Regime on the Metabolism of 18:1-CoA and 18:2-CoA of Proplastid and Microsomal Fractions Prepared from Soybean Cell Suspension Cultures Grown at 29°C

Regime	18:1-CoA Metabolism (nmoles/mg/30 min)		18:2-CoA Metabolism (nmoles/mg/h)		
	18:2	HO-18:1 (C)	HO-18:2 (H)	E	G
		Proplastids			
Dark	11	121	49	7	4
Light*	11	126	50	8	4
		Microsomes			
Dark	4	28	10	1	1
Light*	6	31	14	2	1

*Approx. 2000 lux

18:2 in microsomal membranes (Table 27). The rate of hydroxyoleic acid (C) formation was also increased to some extent.

The formation of hydroxylinoleic acid (product H) together with the minor products (E,G), was not affected by light when the assay was performed with the proplastid fraction (Table 27); however, when the assay was performed with the microsomal fraction, while the formation of the minor components E and G remained virtually unchanged, the formation of hydroxylinoleic acid increased by about 40%.

e) Formation of Products of [^{14}C]18:1-CoA Metabolism with Time

Under the optimum incubation conditions described in section C,IV, [^{14}C] 18:1-CoA was slowly incorporated into phospholipids and neutral lipids but not glycolipids both in the presence or absence of reduced pyridine nucleotides (Table 28). The rate of acyl transfer thus appeared to be independent of the addition of reduced nucleotides. After 60 min of incubation, only about 10% of the [^{14}C] substrate was associated with the acyl lipids (phospholipids and neutral lipids) (Table 28) while the rest remained in the form of acyl-CoA's. A typical two-dimentional TLC plate and autoradiogram of the [^{14}C] acyl lipids is shown in Fig. 42).

The major radioactive phospholipid component in both proplastids and microsomes was the unidentified phospholipid "PX", which followed an inverse relationship with PG in

TABLE 28

Distribution of [^{14}C] with Time Using [1-^{14}C]18:1-CoA as Substrate when Incubated with Either Proplastids or Microsomes[a]

Time min.	Proplastids						Microsomes					
	Acyl-CoA (nmoles)[b]				Acyl Lipids[c] Total (nmoles)	% Rec	Acyl-CoA (nmoles)[b]				Acyl Lipid[c] Total (nmoles)	% Rec.
	18:1	18:2 (B)	HO-18:1 (C)	Total nmoles			18:1	18:2 (B)	HO-18:1 (C)	Total nmoles		
0	102.9	0	0	102.9	0	100	102.9	0	0	102.9	0	100
5	90.0	0.8	8.9	99.7	3.2	100	101.7	0.1	0.9	102.7	0.2	100
10	81.8	1.1	14.9	97.8	5.0	99.9	95.7	0.3	2.1	98.1	4.9	100.1
15	75.9	1.2	19.6	96.7	6.2	100	92.8	0.5	4.0	97.4	5.5	100
30	68.8	1.3	24.3	94.4	8.4	99.9	87.5	0.7	6.2	94.3	8.6	100
60	64.3	1.6	27.6	93.5	9.4	100	82.3	0.8	8.1	91.2	11.7	100

[a]Incubation mixture was as described in section C,XV.

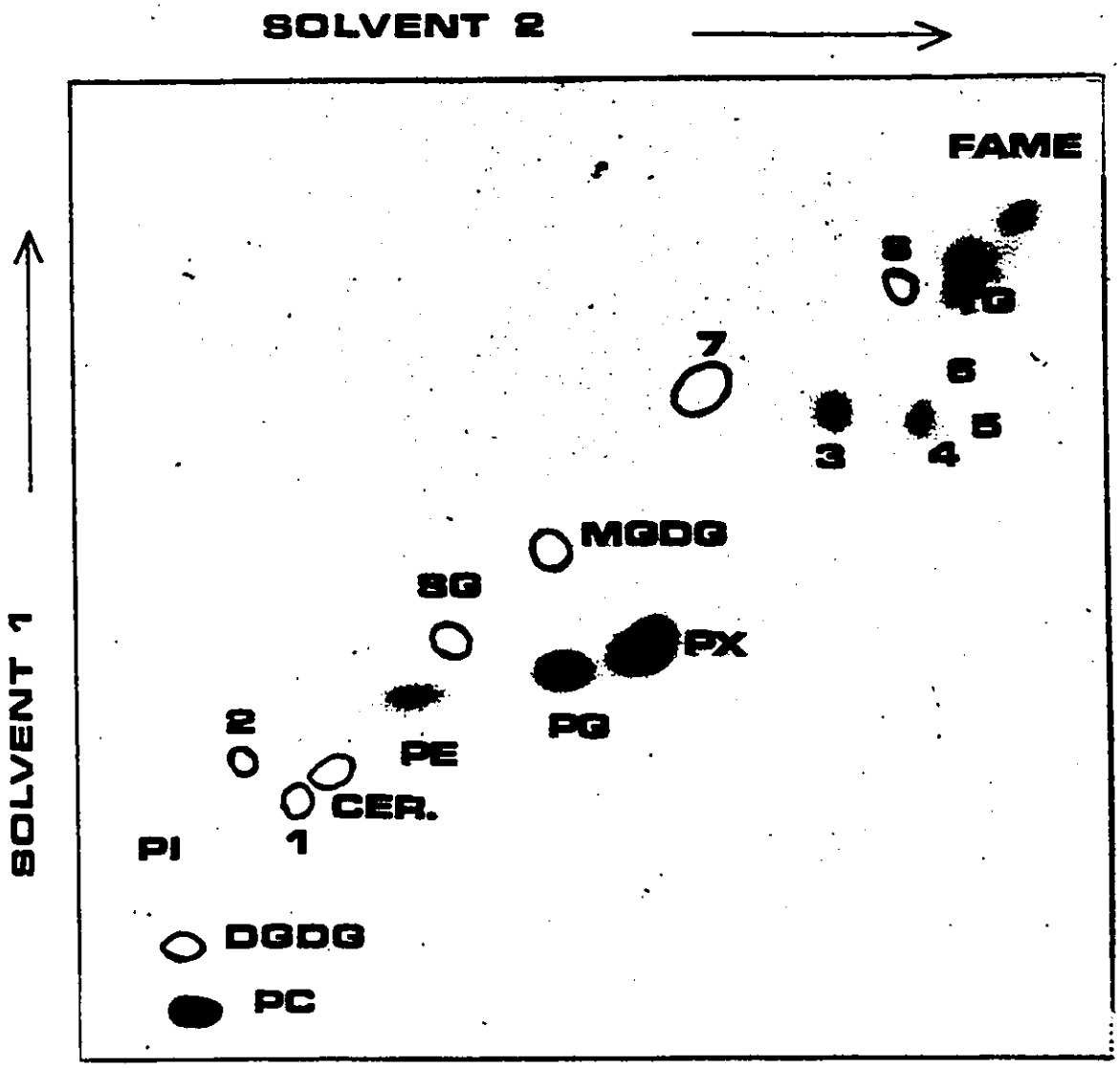
[b]Methanol-water soluble components (acyl-CoA); see section C, XVa.

[c]Chloroform soluble lipids as described in section C, XVb.

FIGURE 42. Autoradiogram of thin-layer chromatogram on silica gel G showing the distribution of ^{14}C among acyl lipids; Incubation was performed, as described in section C,XV, with the proplastid fraction. Cer., Ceramide; PX unidentified phospholipid; 1,2 and 7, undidentified lipids; 3,4,5 and 6, grouped with the neutral lipid (NL) fraction; for other abbreviations see page XV.

Solvent System

1. Chloroform-methanol-acetone-diethylamine-water (120:35:37.5:6:4.5 v/v)
2. Chloroform-methanol-conc. ammonium hydroxide (65:25:5 v/v).


SOLVENT 2
SOLVENT 1
FAME
8
TG
7
6
5
4
3
MGDG
SG
PX
PG
2
PE
CER.
1
PI
DGDG
PC

proplastids but not in microsomes where it continued to accumulate (Table 29). The ^{14}C associated with phosphatidylcholine was highest in the early incubation time while decreasing thereafter in both proplastids and microsomes. Note that the ^{14}C associated with PC of microsomal origin was at all times higher than that of proplastid PC. The ^{14}C found associated with PI and PE, though higher in microsomes than proplastids, was significantly lower than the ^{14}C incorporated into neutral lipids and the other phospholipids.

The NL fraction accounted for about 15 and 20% of ^{14}C incorporated into acyl lipids of microsomes and proplastids, respectively after 60 min. incubation; in both cases the ^{14}C incorporation in NL increased slightly up to 60 min. of incubation(Table 29). The neutral fraction consisted of at least 4-7 components, mainly fatty acid methyl esters, triglycerides, acylated sterols and unidentified components (Fig. 42). In regard to the acyl-CoA's formed after 60 min. of incubation, only small amounts of [^{14}C]18:2-CoA (1.5% and 0.80%) were found respectively in the proplastids and microsomes while the formation of hydroxylated (C) was much higher (27% and 7.9% respectively). Note that all of the newly formed hydroxyacid was in the acyl-CoA form. A plot of Δ^{12}-desaturase or hydroxylase activity versus time (Fig. 43) revealed that the reactions were almost complete after 30 min. incubation with either proplastids or microsomes. It should be noted that all of the [^{14}C]18:2 formed was recovered from the acyl-CoA fraction

Table 29

% Distribution of ^{14}C Among Acyl-Lipids and Neutral Lipid Classes with 18:1-CoA as Substrate

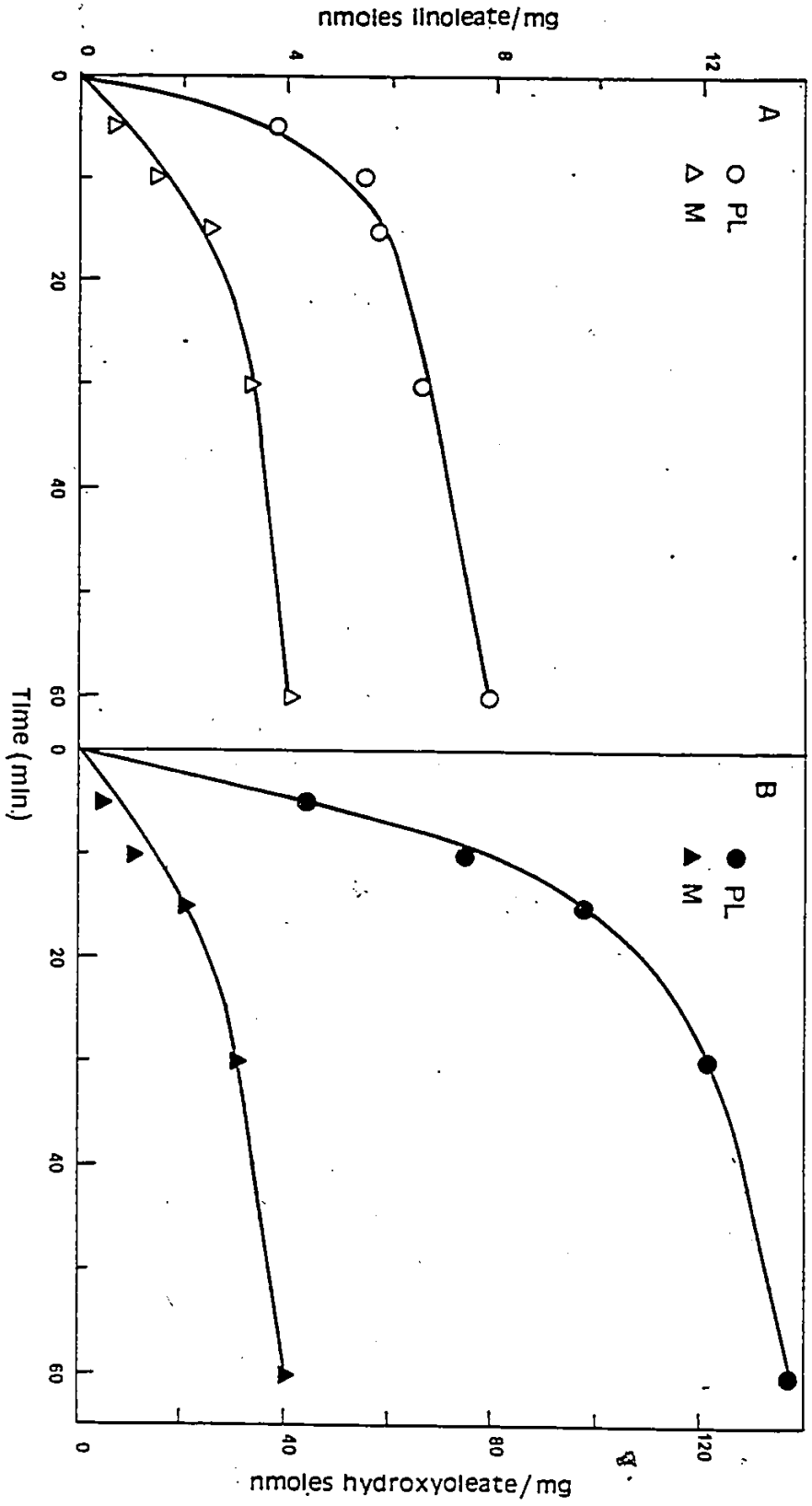
Time min.	PROPLASTIDS[a]							MICROSOMES[a]						
	PI	PC	PE	PG	NL[b]	PX[c]	Total.	PI	PC	PE	PG	NL[b]	PX[c]	Total
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	15.9	2.3	6.4	16.8	58.7	100.1	5.5	56.4	3.6	0	9.1	25.5	100.1
10	0	15.2	2.5	12.3	19.7	50.4	100.1	2.8	22.5	2.6	0.01	18.4	52.8	99.1
15	0	10.8	2.0	19.3	18.7	49.1	100	2.8	22.8	2.4	1.1	15.2	55.7	100.0
30	.01	9.5	1.9	27.7	18.5	41.5	99.1	1.4	15.6	2.3	0.01	14.4	65.5	99.2
60	.01	8.5	2.5	36.2	18.5	29.1	99.1	1.2	12.2	2.7	0.01	15.2	67.9	99.2

[a]No glycolipids were detected on autoradiogram of the two dimentional TLC plates (Fig. 42).

[b]NL = total [^{14}C] neutral lipids is made up of 4-7 components (see Fig. 42).

[c]PX = unidentified phospholipid.

FIGURE 43. Time course of Δ^{12}-desaturase and hydroxylase activities of proplastids and microsomes prepared from soybean cell suspension cultures grown for seven days at 29°C. A, desaturase activity (O-O proplastids; Δ-Δ microsomes); B, hydroxylase activity (●--● proplastids; ▲-▲ microsomes). Incubation mixture was as described in section C, XV.


nmoles linoleate/mg
0
4
8
12
A
O PL
Δ M
0
20
40
60
Time (min)
0
B
● PL
▲ M
20
40
60
0
40
80
120
nmoles hydroxyoleate/mg

and none was detected in the acyl lipids (Table 28); in addition, it was interesting to note that the 18:2 formed by the proplastids fraction was at all times greater than that produced by the microsomes (Fig. 43A). The formation of hydroxyoleic acid was also almost complete after 30 min. incubation and its formation was higher in the proplastid than the microsomal fraction (Fig. 43B).

III. Metabolism of 18:2-CoA Substrate

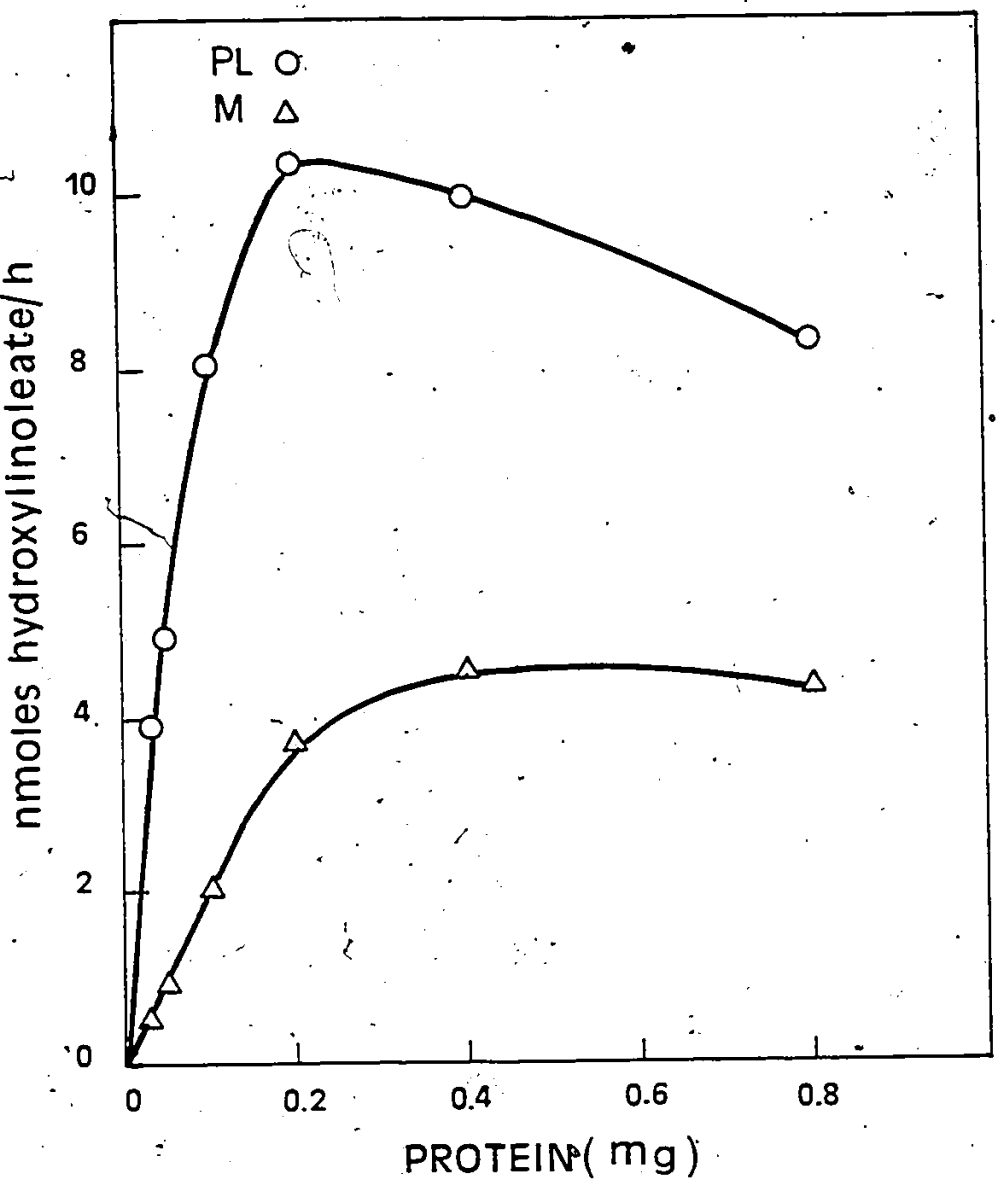
As shown in section D,I,2a, the products of [^{14}C] linoleoyl-CoA metabolism by proplastids or microsomes were: product (H) identified as hydroxylinoleic acid; component (D), a trienoic acid and two unidentified minor components E and G. Since no 18:3 was detected, studies were then carried out on the formation of hydroxylinoleate and related products in order to gain more information on this enzyme system and to perhaps explain why 18:2-CoA is not desaturated *in vitro* while the membrane lipids contain 18:3 in abundance.

a) Properties of Hydroxylating Enzyme System

1. Infuence of Protein Concentration

The rate of hydroxylinoleic acid formation was linear with proplastid or microsomal protein concentration up to 200 µg but some inhibition was observed at higher protein concentrations, being more pronounced with proplastid than microsomal protein (Fig. 44).

FIGURE 44. Influence of proplastid (O-O) and microsomal (Δ-Δ) protein on hydroxylase activity. Incubation mixture (1.0 ml) contained: tricine buffer (pH 8), 100 mM; ATP, 40 mM; $MgCl_2$, 40 mM; CoA, 0.1 mM; NADPH, 200 μM; [^{14}C]18:2-CoA, 32 μM; Incubation was at 29°C for one hour with varying amounts of protein.


PL ○
M △
nmoles hydroxylinoleate/h
0
2
4
6
8
10
0
0.2
0.4
0.6
0.8
PROTEIN(mg)

2. Time Course

Under optimum conditions, the formation of HO-18:2 by proplastid system was fairly rapid up to 40 min. of incubation thereafter reaching a plateau at 80 min. (see Fig. 45). Formation of minor products E and G was relatively slow and was essentially complete by 80 min. and no lag phase was apparent for the formation of either product.

3. Influence of Substrate (18:2-CoA) Concentration

The proplastid hydroxylating enzyme system reached an apparent saturation at a relatively low substrate concentration (32 μM) while displaying considerable substrate inhibition at higher concentrations. (Fig. 46). The formation of products E and G also appeared to reach saturation at low substrate concentrations and, be slightly inhibited at higher concentrations.

4. Influence of Pyridine Nucleotides, and Other Cofactors

Of the reduced pyridine nucleotides, NADPH displayed a slight stimulation, but NADH was found to inhibit the formation of HO-18:2 and related products (Table 30). Since metabolism of 18:2-CoA occurred even in the absence of reducing substrates, the results suggested the presence of an endogenous source of electron donors. The system required oxygen and was inhibited by high concentrations of cyanide. Carbon monoxide, an inhibitor of cytochrome P-450, had only a slight inhibitory effect while complete inactivation of the

FIGURE 45. Linoleoyl-CoA hydroxylase activity of proplastids prepared from soybean cell suspension cultures grown for seven days at 29°C as a function of incubation time. Incubation mixture was as described in Fig. 44, except for [^{14}C]linoleoyl-CoA 63 µM; and 0.5 mg protein with incubation at 29°C performed at various time periods.

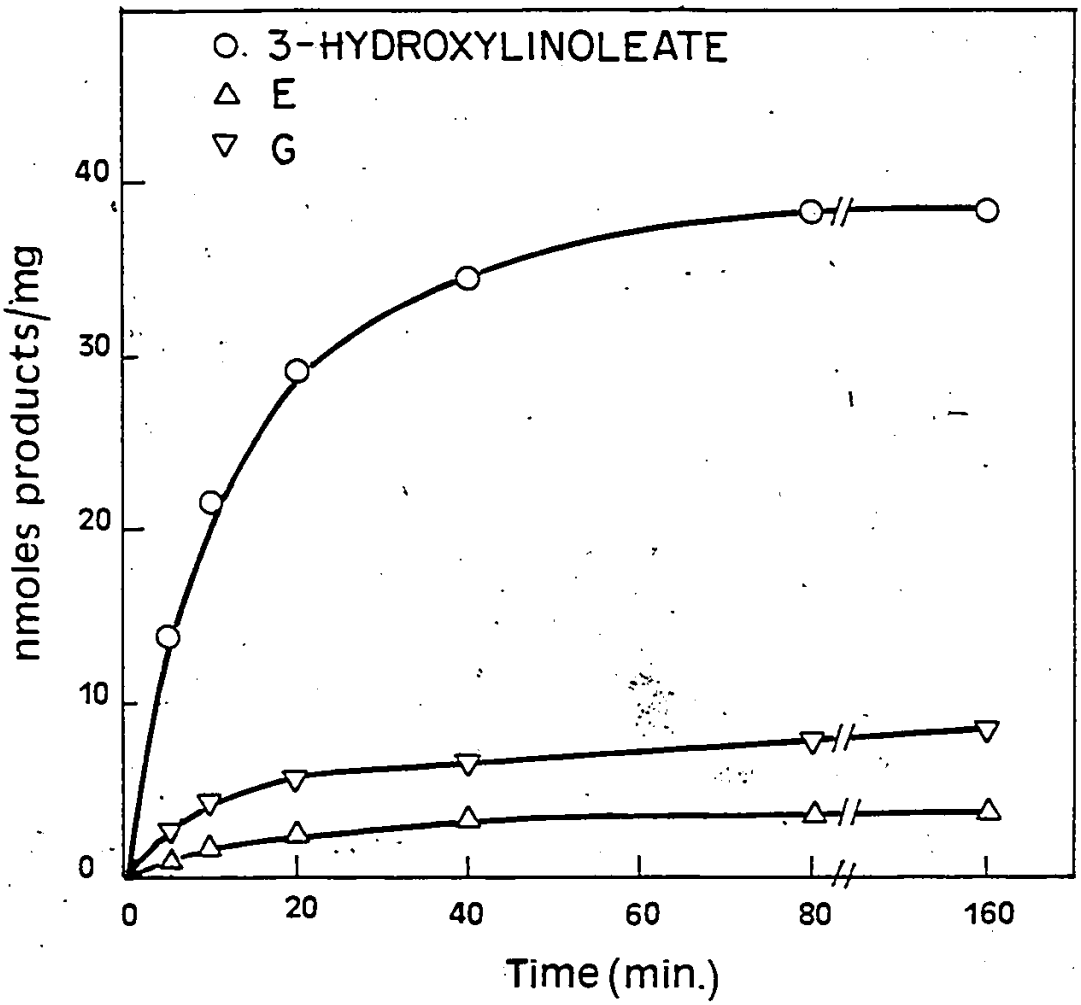

○ 3-HYDROXYLINOLEATE
△ E
▽ G
nmoles products/mg
0
10
20
30
40
0
20
40
60
80
160
Time (min.)

FIGURE 46. Linoleoyl-CoA hydroxylase activity of proplastids prepared from soybean cell suspension cultures grown for seven days at 29°C as a function of linoleoyl-CoA concentration. Incubation mixture was as described in Fig. 44, with 200 µg protein and varying concentrations of linoleoyl-CoA.

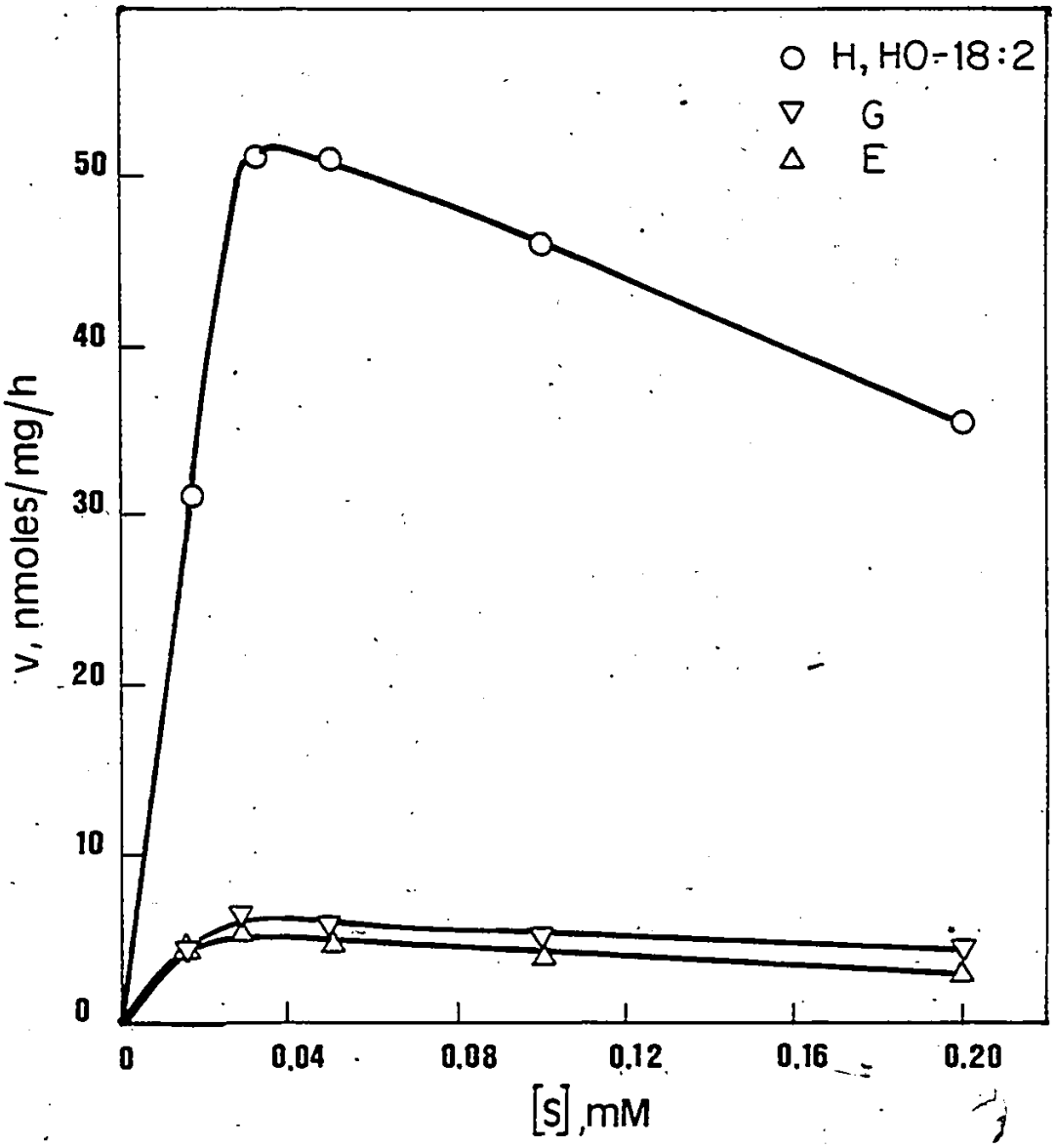

○ H, HO-18:2
▽ G
△ E
v, nmoles/mg/h
50
40
30
20
10
0
0
0.04
0.08
0.12
0.16
0.20
[S], mM

TABLE 30

Effect of Pyridine Nucleotides and Other Cofactors, on 18:2-CoA metabolism of soybean proplastids[a,b]

System	HO-18:2 (%)
complete system + O_2	100
complete system + N_2	10
complete system + O_2 + NADPH (200 µM)	104
complete system + O_2 + NADH (200 µM)	22
boiled enzyme	0
complete system + O_2 + CN^- (40 mM)	64
complete system + O_2 + CN^- (80 mM)	25
$CO/O_2/N_2$ (31:3:66)	83

[a]Complete system (1.0 ml): Tricine buffer (pH 8), 100 mM; $MgCl_2$, 40 mM; ATP, 40 mM; CoA, 0.1 mM; [^{14}C]18:2-CoA, 32 µM; protein, 200 µg; Incubation 60 min at 29°C; activity of the complete system, 10 nmoles/mg/h.

[b]The minor components E and G were found to be affected analogously to HO-18:2.

proplastid enzyme activity, resulted after boiling (Table 30).

b) Influence of Growth Phase on 18:2-CoA Metabolism

Maximum formation of HO-18:2 was obtained with proplastids or microsomes derived from cells grown for a period of seven days (Fig. 47). The proplastid hydroxylase activity decreased considerably (35%) between 7-10 days of growth, while the decrease in the microsomal hydroxylase activity was much less (16%).

c) Influence of Storage

The rate of HO-18:2 acid formation decreased on storage of either proplastids and microsomal fraction at -70°C, to the extent of about 30% and 50% with proplastids and microsomes, respectively over a period of 32 days (Fig. 48).

d) Formation of Products of ^{14}C 18:2-CoA Metabolism with Time

Under optimum incubation conditions, as described in section C, IV, [^{14}C]18:2-CoA was slowly incorporated into phospholipids and neutral lipids but not glycolipids (Table 31), both in the presence or absence of reduced pyridine nucleotides. After 60 min. of incubation with either proplastids or microsomes only about 2% of the [^{14}C]18:2 was associated with the acyl lipids (Table 31) while the remaining ^{14}C was present in acyl-CoA's. It should be noted that the acyl-CoA fraction contained all products (HO-18:2 (H), E,G) of the hydroxylase system and none of these products were detected in the acyl lipids (Table 31). In addition, the rate of product formation

FIGURE 47. Linoleoyl-CoA hydroxylase activity of proplastids (O-O) and microsomes (Δ-Δ) prepared from soybean cell suspension cultures grown for seven days at 29°C as a function of growth (---). Incubation mixture was as described in section C,IV.

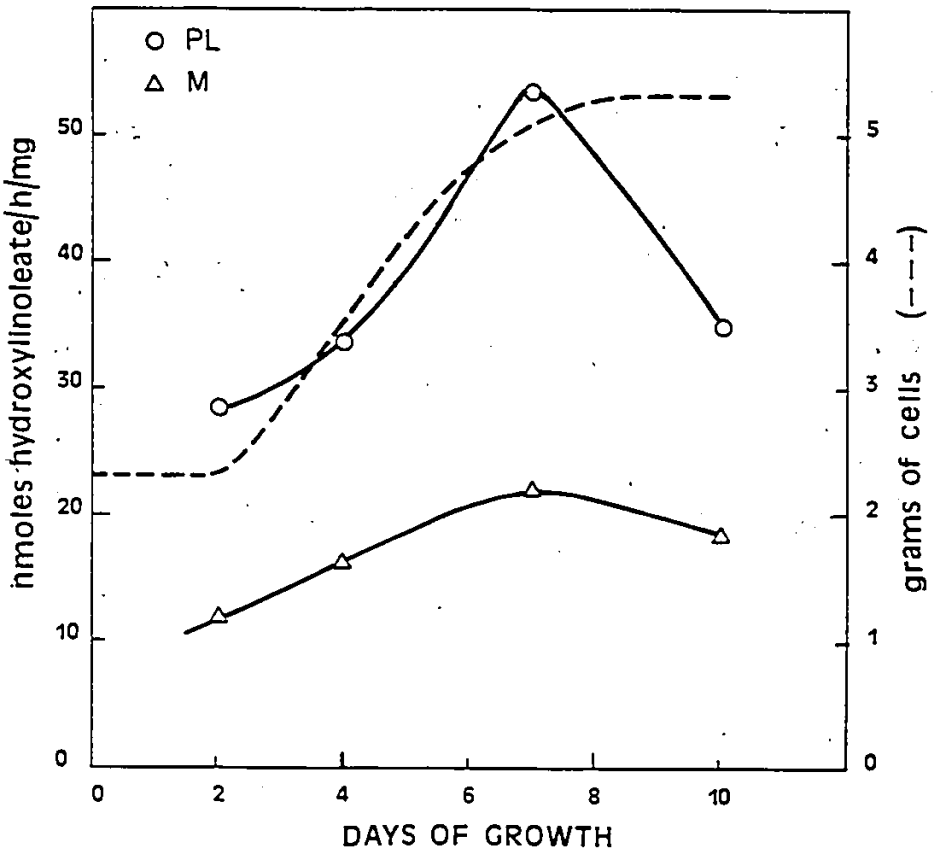

PL
M
nmoles hydroxyllnoleate/h/mg
grams of cells (— — —)
DAYS OF GROWTH
0
10
20
30
40
50
1
2
3
4
5
0
2
4
6
8
10

FIGURE 48. Linoleoyl-CoA hydroxylase activity of proplastids (◇-◇) and microsomes (Δ-Δ) prepared from soybean cell suspension cultures grown for seven days at 29°C as a function of storage at -70°C. Incubation was as described in section C,IV.

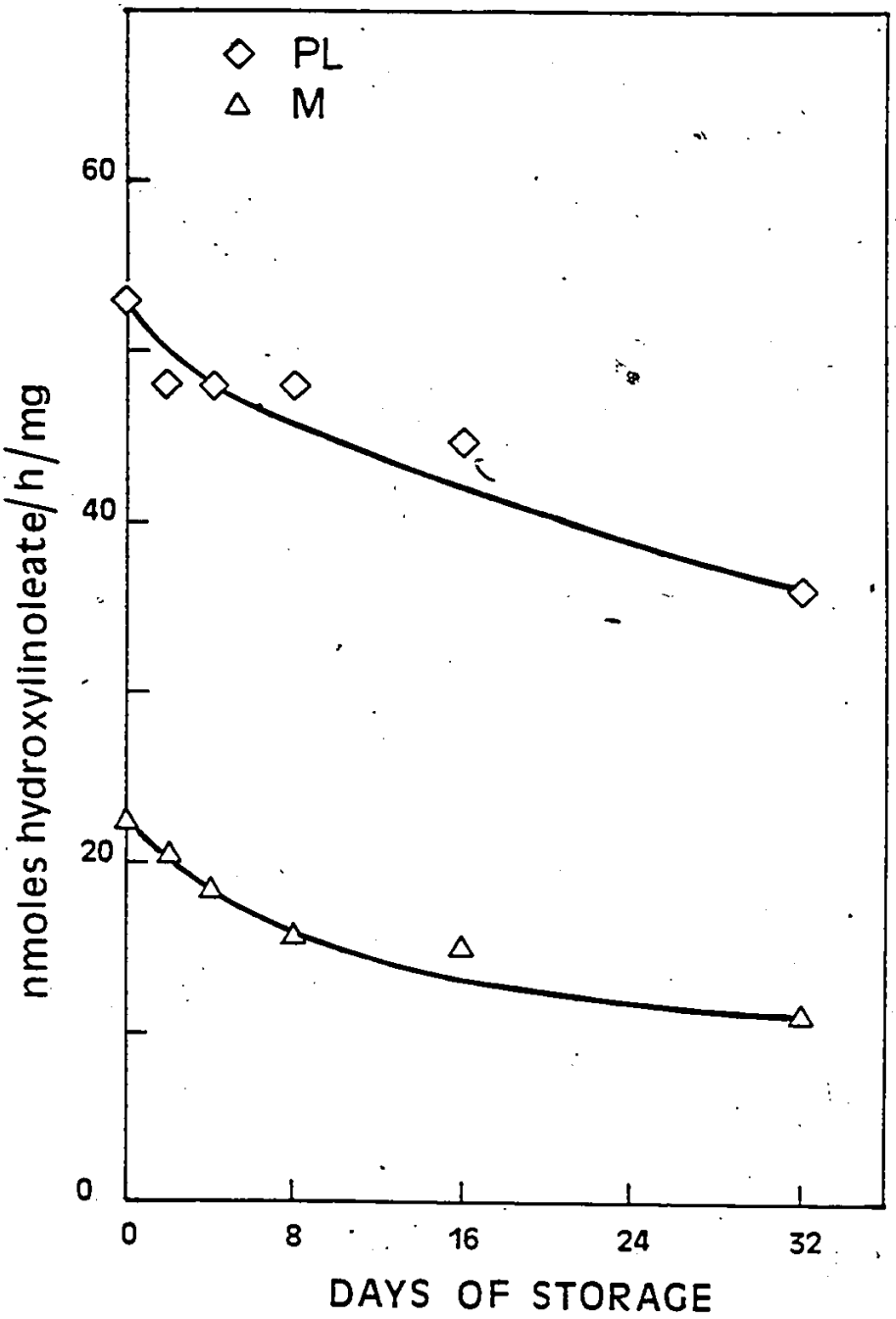

PL
M
60
40
20
0
nmoles hydroxylinoleate/h/mg
0
8
16
24
32
DAYS OF STORAGE

TABLE 31

Distribution of [^{14}C] with Time, Using [1-^{14}C]18:2-CoA as Substrate when Incubated with Either Proplastids or Microsomes[a]

	Proplastids							Microsomes						
Time min.	Acyl-CoA (nmoles)[b]					Acyl Lipids[c] Total	% Recovery	Acyl-CoA (nmoles)[b]					Acyl Lipids[c] Total	% Recovery
	18:2	E	G	HO-18:2 (H)	Total nmoles			18:2	E	G	HO-18:2 (H)	Total nmoles		
0	33.3	0	0	0	33.3	0	100	33.3	0	0	0	33.3	0	100
5	28.2	0.4	0.4	3.6	32.6	0.7	100	33.8	0.1	0.1	0.3	33.1	0.2	100
10	24.9	0.7	0.8	6.0	32.4	0.8	99.7	32.4	0.1	0.3	0.5	32.4	0.8	99.7
20	20.9	1.1	1.0	8.9	31.9	1.3	99.7	31.4	0.1	0.4	1.3	31.9	1.3	99.7
40	17.6	1.4	1.2	10.6	31.6	1.7	100	29.9	0.3	0.5	2.6	30.9	2.4	100
80	17.5	1.5	1.5	10.7	31.3	2.0	100	28.1	0.5	0.8	3.9	30.6	2.6	99.7

[a]Incubation was as described in section C,XV.

[b]Methanol-water soluble components (acyl-CoA); see section C, XVa.

[c]Chloroform soluble lipids as described in section C, XVb.

in the acyl-CoA fraction, was always higher with proplastids than microsomes and was almost complete after 40 min. incubation (Fig. 49).

In regard to the acyl lipids, the major radioactive phospholipid, in both proplastids and microsomes, was the unidentified phospholipid "PX", which displayed a reciprocal relationship with PG in proplastids but not in microsomes where "PX" continued to increase (Table 32). The [^{14}C]18:2 associated with proplastid PC was at all times higher than that of microsomal PC and its value decreased with time in both fractions (Table 32). The [^{14}C]18:2 associated with PI and PE were considerably lower than those of neutral lipids and other phospholipids in both proplastid and microsomal fractions. The NL fraction accounted for 34 and 26% of the ^{14}C incorporated into acyl lipids after 60 min. of incubation with proplastids and microsomes, respectively. It should be mentioned that the NL fraction consisted of at least 4-7 components comprising mainly fatty acid methyl esters, triglycerides, acylated sterols and other unidentified components as with 18:1-CoA as substrate. In both cases, the ^{14}C incorporated in NL remained virtually unchanged over the entire time course.

FIGURE 49. Time course of linoleoyl-CoA hydroxylase activity of proplastid and microsomes prepared from soybean cell suspension cultures grown for seven days at 29°C. A, proplastids; B, microsomes. Incubation was as described in section C,XV.

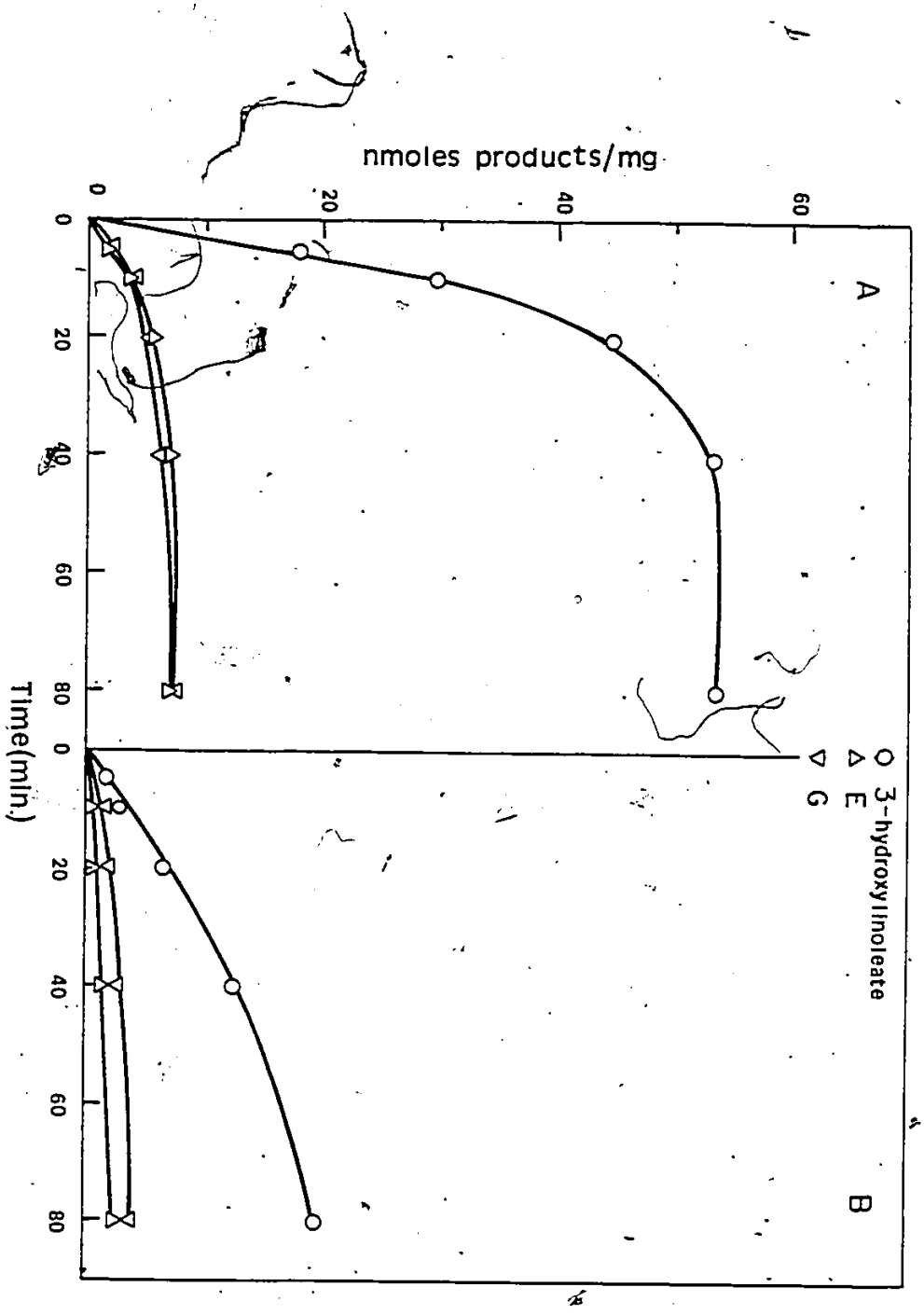

nmoles products/mg
0
20
40
60
A
B
Time(min.)
0
20
40
60
80
○ 3-hydroxylinoleate
△ E
▽ G

TABLE 32

% Distribution of ^{14}C Among Acyl-lipids and Neutral Lipid Classes with 18:2-CoA as Substrate

Time min.	PROPLASTIDS[a]								MICROSOMES[a]						
	PI	PC	PE	PG	NL	PX[c]	Other[d]	Total	PI	PC	PE	PG	NL	PX[c]	Total
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	26.1	4.1	4.1	29.2	36.5	0	100	6.4	14.7	2.8	0	25.8	48.9	98.6
10	3.7	14.5	3.7	7.6	24.7	45.7	0	99.9	3.3	9.3	1.6	0	33.1	52.3	99.6
20	2.1	10.0	2.8	11.8	31.2	40.5	1.6	100.0	3.4	7.7	2.3	1.1	29.9	55.4	99.8
40	3.2	11.6	4.8	24.5	43.5	8.4	3.9	99.9	3.1	4.9	2.1	2.1	21.5	66.0	99.7
80	2.3	12.3	5.4	40.1	33.7	3.1	3.1	100	1.9	5.3	2.6	2.7	25.7	61.7	99.9

[a]No glycolipids were detected on autoradiogram of the two dimentional TLC plates.

[b]NL = total [^{14}C] neutral lipids was made up of 4-7 components.

[c]PX = unidentified phospholipids

[d]Unidentified lipi component found only in proplastids.

E. DISCUSSION

I. Identification of Metabolic Products of [^{14}C]18:1-CoA

On the basis of the results presented, the three products formed from 18:1-CoA metabolism were identified as follows: component A, a C_{18} dienoic acid; component B, 18:2 (product of Δ^{12}-desaturase), and component C, 3-hydroxyoleate.

The dienoic acid (A) was not identical to the Δ^{12}-desaturase product 9,12-octadecadienoic acid. However, one double bond must be located at C_9 because of the formation of C_9 monocarboxic acid on oxidation with permanganate-periodate (Table 12), and MS-fragmentation pattern (Fig. 18B) indicated that the newly formed double bond must be located close to the carboxyl group of the molecule probably at C_2 because of the presence of a major fragmentation ion at m/e 113 and minor diagnostic ions at m/e 99, 181 and 195 which are typical for α,β-unsaturated fatty acids (239). This structure still requires further confirmation by U.V. analysis of component A, which should give an absorbance band at about 250 nm for a conjugated double bond system and by infrared and NMR spectral analysis in comparison with authentic synthetic standards. The latter two methods would also be useful in establishing conclusively the stereochemistry of the newly formed double bond (cis or trans). We were unable to carry out all these studies because of insufficient material at our disposal considering the rather high amounts required

for measurements of these spectra.

Product A might have arisen from the 3-hydroxyoleate (product C) by a specific enzymatic dehydration, in which case the newly formed double bond would be either at C_2 or C_3 depending on the specificity of the enzyme. Alternatively A could have arisen by the action of an α,β-dehydrogenase on the substrate 18:1-CoA (see section E,V. for further discussion).

Product C was conclusively identified as 3-hydroxyoleate on the basis of results presented in (Table 9, 10, 12 and Fig. 21, A and B; 22, B and 23, A). However, it would be of interest to determine the optical activity (D or L) of this product in order to pinpoint its biosynthetic pathway; while the D-hydroxyacids are intermediates of fatty acid synthesis, the L-isomers are believed to be intermediates in fatty acid oxidation. It must be kept in mind, however, that the oxidation of long and short chain fatty acids is considered to proceed directly to acetate without the accumulation of intermediates (240). Similarly, in the biosynthetic system only the short chain intermediates have been detected and not the long chain intermediates (14).

Davidoff and Korn (15) have observed the accumulation of 3-hydroxy fatty acids from saturated substrates, in the order myristoyl-CoA > palmitoyl-CoA > stearoyl-CoA by a soluble preparation from slime mold Dictyostelium discoideum. These authors also conclusively demonstrated that the formation of the 3-hydroxyacid occurred via the α,β-unsaturated fatty acid

derivates as precursor. A similar pathway was also demonstrated in a mitochondrial fraction of guinea pig liver incubated with palmitoyl-CoA (241). The formation of 3-hydroxyacids have also been reported in high-speed supernatant fraction from maturing avocado mesocarp (242) and to a much lesser extent in safflower supernatants (242) both of which act on short and medium chain fatty acids such as octanoic, decanoic and dodecanoic acids. These enzyme systems were soluble preparations in contrast to the proplastid enzyme system described in this thesis, which is most likely bound to lamellar membranes.

Identification of product B as 9,12-octadecadienoic acid shows that a Δ^{12}-desaturase system is present in both proplastids and microsomes of soybean cell suspension cultures. The desaturation of 18:1, in both systems, occurs at the level of acyl-CoA, in contrast to desaturation in differentiated plant systems such as leaves (172,243) or developing seeds (173,174,244) where desaturation of 18:1 → 18:2 occurs exclusively in microsomes at the level of phospholipids, mainly PC, and not in the chloroplast (163). Thus the Δ^{12}-desaturase system from the undifferentiated soybean cells behave quite differently than that of fully differentiated plant cells, with respect to substrate used.

II. Identification of Metabolic Products of 18:2-CoA

The two major products formed from 18:2-CoA metabolism were identified as follows: component D, a C_{18}

trienoic acid and component H, 3-hydroxylinoleate. The minor components E and G were not identified because the small amounts of products formed were not enough for any structural studies.

The C_{18} trienoic acid (Table 14,15; Fig. 26A and B) was not identical to 9,12,15-octadecatrienoic acid, product of Δ^{15}-desaturase activity. However, two of the three double bonds are retained in $\Delta^{9,12}$-position since the ion fragments produced from this part of the molecule are identical to those of methyl linoleate (Fig. 19,B). The major differences between the MS of methyl linolenate and product D, is in the appearance of the molecular ion at m/e 292 which indicated the presence of a third double bond, and the ion fragments at m/e 113, 179 with splitting between C_5 and C_6, and at m/e 99 and 193 with splitting between C_4 and C_5. These ion peaks occur only if the third double bond is present between C_2 and C_3. The α,β-unsaturated acids have recently been shown to give rise to the ion peak at m/e 113 (240) which is therefore typical of unsaturation between C_2 to C_5. The structure of product D, presumably $\Delta^{2,9,12}$-octadecatrienoic acid was not further confirmed by U.V., IR and NMR studies, because of insufficient material.

This trienoic acid could be formed either by a specific enzymatic dehydration of 3-hydroxylinoleate or by action of a specific α,β-dehydrogenase on 18:2-CoA, as suggested for the formation of product A, (see section E, I and V for further

discussion). On the basis of their mobilities on $AgNO_3$-TLC and on GLC, products E and G might be stereochemical isomers (cis or trans) of the α,β-unsaturated acid (D) or isomers of the β,γ-unsaturated fatty acid arising from isomerization of the α,β-unsaturated acid (Fig. 50B); such an isomerization has been demonstrated in slime mold (15).

Product H, was conclusively identified as 3-hydroxy-linoleate on the basis of results presented in Table 9 and 14 and Fig. 28 (A and B), 29 (A and B). It may arise either from direct hydroxylation by a mixed function oxidase or from a α,β-unsaturated (e.g. $\Delta^{2,9,12}$-octadecatrienoic) acid by a specific hydratase; for further comments see section E,V.

III. Enzyme Systems of 18:1-CoA and 18:2-CoA Metabolism

a) General Properties and Subcellular Location

The concentration of protein (Fig. 34), optimum pH (Fig. 35), optimum temperature (Fig. 37), and time of incubation (Fig. 36) required for optimum conversion of 18:1 → 18:2, were similar to the values obtained for Δ^{12}-desaturase systems in other plants, such as pea leaves (245) potato tubers (246) and soya bean cotyledons (246). However, the proplastid Δ^{12}-desaturase had a much lower substrate affintiy (Km, 0.4 mM) than previously reported in other plant systems (245,246). The association of a Δ^{12}-desaturase largely with the proplastids and to lesser extent with microsomal fractions (Table 16,17) is in contrast to other plant Δ^{12}-desaturases which are largely associated with the microsomes

(169, 245-247). It should be noted also that the proplastid hydroxylating system had the same optimum pH as reported for other plant hydroxylating systems (198,199,242) but it differed in its cellular location, being associated largely with the lamellar membranes (Table 21), while other plant hydroxylating systems are either of microsomal origin (198,199) or are soluble (242). The differences in Km and subcellular location of Δ^{12}-desaturase as well as the location of the 18:1-CoA hydroxylating system, indicate that these enzymes in undifferentiated plant systems, such as soybean cell cultures, have different properties than those of fully differentiated plants (169,198,199,242,245-247).

Under the optimum conditions required for desaturation in plants, no Δ^{15}-desaturase activity was observed with either proplastids or microsomes; however, a very active hydroxylating system was observed with the properties reported in Fig. 44,45, and 46. The lack of 18:2 → 18:3 desaturation may in part be due to this very active hydroxylating system which rapidly utilises the substrate (18:2-CoA) and converts it to products which are not substrates of desaturation. The substrate inhibition observed at higher concentrations of 18:2-CoA (Fig. 46), toward the hydroxylase activity, may be due to the higher detergent effect of the 18:2-CoA substrate and is different than the observations reported with 18:1-CoA as the substrate (Fig. 39A). The hydroxylating enzyme with 18:2-CoA

as the substrate was also mainly associated with the proplastid fraction (Fig. 49).

b) Effect of Pyridine Nucleotides, Cofactors and Inhibitors

The proplastid Δ^{12}-desaturase system displayed an absolute oxygen requirement (Table 20,22) as for other microsomal Δ^{12}-desaturase systems from leaves (169,245), potato tubers (246) and seeds (248). However, no absolute requirement for reduced pyridine nucleotides could be demonstrated; while NADPH stimulated desaturation only to a small extent, NADH was found to be inhibitory (Table 20,21). Ferredoxin, an electron donor in plants did not affect the level of 18:2 produced by the proplastids (Table 20). The lack of a pronounced stimulation by these electron donors, usually observed with plant systems (245-247), would suggest either the presence of tightly bound electron donors or cofactors (Table 21), or a self-regenerating system for electron donors as suggested for other microsomal plant Δ^{12}-desaturases (169,245,246). Slack et al., (169) have reported that microsomes prepared from pea leaves did not require reduced pyridine nucleotides for maximum 19:1 → 18:2 desaturation; however, on washing or gel filtration of the microsomes, the Δ^{12}-desaturase activity was decreased but not eliminated when the assay was done in the absence of reduced pyridine nucleotides. Full enzyme activity was re-established when NADPH or the supernatant or sephadex-treated supernatant was added back to the assay mixture as an

electron donor. These authors suggested from these studies that a membrane bound high and/or low molecular weight component acting in a manner similar to NADPH was present in microsomes (169). Similar observations have been reported for the Δ^{12}-desaturase of yeast (142). In addition, Foot et al., (122) observed that a microsomal preparation from the psychrophilic bacterium Micrococcus cryophilus did not absolutely require electron donors for maximum Δ^{9}-desaturation. Our findings are therefore similar to previous observations that reduced pyridine nucleotides are not absolutely required for maximum Δ^{12}-desaturase activity in certain plant systems. The function of reduced pyridine nucleotides can be substituted by a membrane bound component (Table 21) which acts in a manner similar to NADPH. This membrane bound component can either be an existing membrane-pool of reduced electron donors (e.g., cyt. b_5, Ferredoxin) or some unknown component yet to be identified. More studies are required to shed more light on the mechanism of Δ^{12}-desaturation in soybean cell suspension cultures and in particular on the role of NADPH as electron donor. Further investigation is also required to demonstrate the mechanism of NADH inhibition of Δ^{12}-desaturase in soybean cell cultures. NADH can be converted to NAD^{+} and it can act as an inhibitor as was demonstrated for the hydroxylating system of avocado mesocarp (242).

Azide, an inhibitor of electron transport, which has been shown to inhibit the Δ^9-desaturase in rat liver microsomes (235) as well as the ω- and midchain hydroxylases of microsomes prepared from Vicia faba (198,199), had no effect on either enzyme systems (Table 22). Cyanide a typical desaturase inhibitor at low concentration (1 mM) (248) inhibited the Δ^{12}-desaturase as well as the hydroxylating system only at high concentrations (40 mM) (Table 22). Since a cyanide concentration greater than 1 mM can lead to nonspecific secondary reactions (248), the significance of inhibition at this very high cyanide concentration, with regard to a cyanide-sensitive heme system, is not clear; at best it may indicate the presence of a cyanide sensitive factor which may be involved with either the Δ^{12}-desaturation or the hydroxylation system.

The inhibitory effects observed with either the more specific heavy metal chelators or the thiol directed reagents (Table 22, 23) indicate the possible involvement of heme- and/or non-heme iron proteins as well as the involvement of sulfhydryl groups in the process of desaturation and hydroxylation reactions. Similar observations have been made with regard to other plant Δ^{12}-desaturases (248) as well as plant hydroxylating systems (198,199).

Carbon monoxide, a known specific inhibitor of cytochrome P-450, inhibited the formation of 18:2 and hydroxylase activity only slightly (Table 22). This partial inhibition by

CO may have resulted from a partial denaturation of proteins resulting from the bubbling effect of CO in the incubation mixture and not by the known effects of CO. In conclusion, cyt P-450 is not involved in the process of desaturation and may not be involved in the hydroxylating mechanism as well. With regard to the hydroxylating mechanism, the non-involvement of cyt P-450 as stated above, is contrary to the probable involvement of cyt P-450 in the formation of 3-hydroxyacids in avocado mesocarp (242), and its certain involvement in the ω- and midchain hydroxylation in plants (198,199) but it is similar to the mixed function oxidase involved in the formation of ricinoleic acid (197) which does not involve P-450. Thus plants may use more than one mechanism of hydroxylation (CO sensitive or insensitive) depending on whether the tissue studied is differentiated or not.

The desaturase and hydroxylase systems are also sensitive to detergents and antioxidants (Table 22) as is expected of membrane bound enzyme systems. The pyridazinone herbicides (San 6706 and San 9765) and Destun, are strong inhibitors of the desaturation reaction (Table 23) as demonstrated *in vivo* studies in other plant systems such as broad bean (*Vicia faba*) (249), spinach and pea leaves (250), linseed and wheat (250) and in maize seedlings and sunflower cotyledons (251). Atebrine, a known inhibitor of acyltransferase activity (236), also inhibited both Δ^{12}-desaturase and

hydroxylase activities (Table 23). It should be noted that San 9785 at 1 mM had a partial inhibiting effect on 18:2 formation but not hydroxyoleate formation while atebrine inhibited the formation of hydroxyoleate more so than that 18:2 formation, thus indicating the possible involvement of separate desaturase and hydroxylase. The mechanism of inhibition by the herbicides, Destun and Atebrine are not known and will require further study.

The proplastid hydroxylase system acting on 18:2-CoA exhibited the same general properties toward oxygen requirement, reduced pyridine nucleotides, cyanide, inactivation of enzyme on boiling, and cyt. P-450 (Table 30) as with 18:1-CoA as substrate. All these observations suggest the presence of a single proplastid hydroxylating system acting preferentially on 18:1-CoA and to a lesser extend on 18:2-CoA.

IV. Formation of Products of 18:1-CoA and 18:2-CoA Metabolism with Time

On incubation of [^{14}C]18:1-CoA with proplastid or microsomal fractions only about 10% of the [^{14}C] were transferred to acyl lipids (chiefly phospholipids but not glycolipids) while the remainder was associated with acyl-CoA's including the product of Δ^{12}-desaturase, 18:2, and that of the hydroxylase enzyme, hydroxyoleate (Table 28). Thus, both the desaturase and hydroxylase reaction utilize 18:1- CoA as substrate and neither reaction occurs at the level of phospholipids or

glycolipids. This result is in contrast, at least as far as the desaturase is concerned, to the findings in most plant systems in which the actual substrate for desaturation of 18:1 → 18:2 takes place at the level of phospholipids, principally PC (163,171). In regard to the [^{14}C] incorporated into the acyl lipids, the unidentified phospholipid "PX" was at all times the most heavily labelled acyl component, displaying an inverse relationship with PG of proplastids but not with microsomal PG. While "PX" contained the bulk of [^{14}C] in the early stage of incubation and decreased thereafter, PG was low initially but became more progressively labelled with time (Table 29). These results suggests that "PX" acts as a donor of [^{14}C] acyl groups of DAG backbone to PG. The unidentified phospholipid "PX" may be bis-phosphatidic acid (bis-PA) previously detected in soybean suspension cultures (34) and implicated in the process of desaturation by Wilson et al., (35). However, its R_f value on TLC appear to be closer to those for a lyso-bis-PA. PC was also heavily labelled during the early stages of incubation, in microsomes more than proplastids, and decreased thereafter, suggesting that it too could act as a donor of DAG backbone to the other phospholipids as summarized in Fig. 7, (section A,VIc). In conclusion, proplastids and microsomes prepared from heterotrophic cell suspension cultures seem to have a very active hydroxylating system acting on 18:1-CoA as substrate and a less active Δ^{12}-desaturase system which also acts on 18:1-CoA as substrate contrary to previous observations that this desaturase in fully

differentiated plant cells acted exclusively on phospholipid substrates, mainly PC, of microsomal origin (163). It is possible that heterotrophic cell cultures have a unique Δ^{12}-desaturase and hydroxylating enzymes concentrated in the proplastids and acting exclusively on 18:1-CoA. In order to study the mechanism of desaturation in heterotrophic soybean cell suspension cultures more effectively, it seems mandatory to find a way to inhibit the very active hydroxylating system which interferes with the mechanism of desaturation by removing the substrate. For comparison one might employ phototrophic cell cultures which have been shown to desaturate exogenous 18:1-CoA in vitro without the reported formation of hydroxyacids (243).

In regard to the hydroxylase enzyme, the enzyme acts on 18:1-CoA as substrate as concluded above, and in this respect is similar to the hydroxylating system reported in slime mold (15) and maturing avocado mesocarp (242).

With [^{14}C]18:2-CoA as substrate with proplastids or microsomal fractions even less (2%) of the [^{14}C] was transferred into acyl lipids again mostly phospholipids but not glycolipids while the remainder was associated with acyl-CoA's containing all the products of 18:2-CoA metabolism (Table 31). It should be emphasized that no 18:3 ($\Delta^{9,12,15}$-octadecatrienoic acid), the expected product of Δ^{15}-desaturase, was observed; instead only hydroxyacids and trienoic acids were formed in both proplastids and microsomes. Thus, the

question still remains as to what mechanism not involving glycolipid intermediates could account for the in vivo synthesis of 18:3, a major fatty acid of membranes from soybean cell cultures. To solve this problem, it would be helpful to find a way of inhibiting the active hydroxylating system, without inhibiting the desaturase system. Perhaps the differential inhibition observed with Atebrine might be exploited for this purpose.

With regard to the [^{14}C]18:2 acylated lipids, "PX" was again the most heavily labelled component, the [^{14}C] level of which also displayed a reciprocal relationship with that of proplastid PG but not microsomal PG (Table 32).

In the early stages of incubation with [^{14}C]18:2-CoA, a preferential acylation of proplastid PC (Table 32), relative to microsomal PC was observed which decreased on prolonged incubation. These results are in contrast to those observed with [^{14}C]18:1-CoA substrate where the acylation of microsomal PC in the early stages of incubation, was greater than that of proplastid PC (Table 29). This preferential acylation of microsomal PC by 18:1-CoA and of proplastid PC by 18:2-CoA suggests that these organelles in vivo may play different roles with respect to the biosynthesis of polyunsaturated fatty acids.

VII. Physiological Significance

The degree of fatty acid unsaturation has a large effect on its physical properties, such as melting or boiling point; the greater the degree of unsaturation the lower the melting and boiling points and therefore, the greater the fluidity. These properties will be reflected in those of the membrane phospholipids and glycolipids to which the fatty acids are esterified. Thus the greater the degree of unsaturation in phospholipid and glycolipid fatty acids, the greater the fluidity and hence the permeability of the membrane. In addition, the activity of several membrane bound enzyme seems to be directly affected by the nature of the polar head group as well as the fluidity of the acyl chains of boundary polar lipids. Thus, since the desaturases are directly involved in regulating the properties of acyl chains in membrane lipids, it is of interest in plants which contain 18:1, 18:2 and 18:3, fatty acids in abundance, to study and elucidate the mechanism of 18:1 → 18:2 and 18:2 → 18:3 desaturation.

In the present study, we were able to demonstrate 18:1 → 18:2 desaturation in proplastids, and to a lesser extent in microsomes, of undifferentiated heterotrophic cell suspension cultures, but the desaturation of 18:2 → 18:3 was not observed in either cell fraction. These studies were complicated by the very active hydroxylating system which was found to be present in both proplastids and microsomes from the above mentioned cells. The use of phototrophic cell suspension cultures would be more

advantageous since they seem to be able to desaturate 18:1 → 18:2 → 18:3 effectively without the formation of interfering products (252).

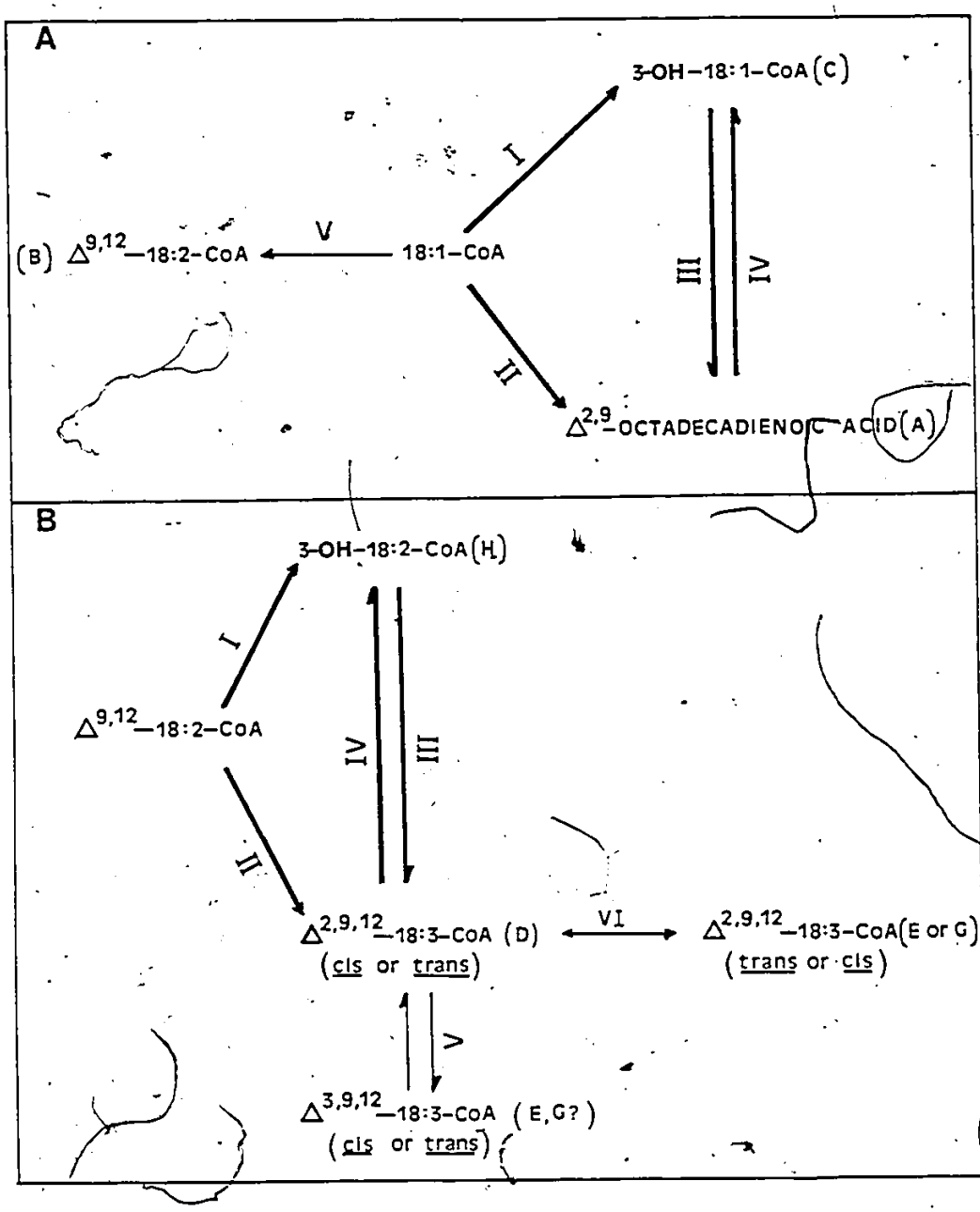
With regard to the hydroxylated and other products of the enzyme preparation originating either from 18:1-CoA or 18:2-CoA, their metabolic role is not obvious; the 3-hydroxyacids may be intermediates in fatty acid oxidation or synthesis or both, or they may be part of a novel pathway yet to be determined. The data cannot be consistently interpreted to favor any of the first three possibilities for the following reasons: (a) our enzyme system is strongly inhibited by pyridine nucleotides, which are essential cofactors for both β-oxidation and fatty acid synthesis and (b) the hydroxylated and unsaturated intermediates in the proplastid or microsomes (products A and C, D and H) show time dependent accumulation while the intermediated of β-oxidation and fatty acid synthesis do not accumulate. Thus, the hydroxylase system is probably a separate system operating independently of either the fatty acid synthetase or β-oxidation systems; it is also different from other plant hydroxylases, e.g. the ω-, mid-chain and 3-hydroxylase systems (198,199,242), and may represent a novel pathway characteristic of undifferentiated cells.

On the basis of the results obtained, the pathways for the formation of the hydroxylated and unsaturated products are summarized in Fig. 50:

FIGURE 50.. Proposed pathways for the synthesis of A, 18:1-CoA metabolites and B, 18:2-CoA metabolites [linoleic acid (B), 3-hydroxyacids (C and H), and α,β-unsaturated fatty acids (A and D)] in proplastids or microsomes of soybean cell suspension cultures.

A. pathway I, hydroxylase; pathway II, α,β-dehydrogenase; pathway III, dehydrase; pathway IV, hydrase.

B. pathway I - IV as in A; pathway V, isomerase; pathway VI, isomerase.


A
3-OH-18:1-CoA (C)
I
(B) Δ9,12-18:2-CoA
V
18:1-CoA
III
IV
II
Δ2,9-OCTADECADIENOIC ACID (A)
B
3-OH-18:2-CoA (H)
I
Δ9,12-18:2-CoA
IV
III
II
Δ2,9,12-18:3-CoA (D)
(cis or trans)
VI
Δ2,9,12-18:3-CoA (E or G)
(trans or cis)
V
Δ3,9,12-18:3-CoA (E,G?)
(cis or trans)

The 18:1-CoA can be either hydroxylated first to form 3-hydroxyoleate (Fig. 50A, pathway I) followed by a dehydrase enzyme (pathway III) to produce $\Delta^{2,9}$-octadecadienoic acid or the 18:1-CoA can be first acted on by specific α,β-dehydrogenase enzyme (pathway II) to form the $\Delta^{2,9}$-octadecadienoic acid, followed by a hydratase enzyme (pathway IV) to produce 3-OH-18:1-CoA. The formation of $\Delta^{9,12}$-octadecadienoic acid by the Δ^{12}-desaturase system, is shown in pathway V, and it should be emphasized that this pathway is only a minor one when compared to pathways I - IV.

The pathways for the two main products formed from 18:2-CoA metabolism are shown in Fig. 50B, and the steps involved are similar to those described for 18:1-CoA as substrate (Fig. 50, pathway I - IV).

The minor products E and G may arise from either isomerization of the α,β-unsaturated acid to the β,Y-unsaturated fatty acid (Fig. 50B, pathway V) or represents the cis or trans counterpart of product D (Fig. 50B, pathway VI). It should be possible to demonstrate the main biosynthetic pathways (I - IV) for the formation of A or D and C or H by a time course experiment showing the substrate to product relationship.

The function of the 3-hydroxy and α,β-unsaturated fatty acids in soybean cell suspension culture is not clear. The 3-hydroxyacids cannot be constituents of cerebrosides which have been identified in soybean cell cultures (35) since these probably contain the 2-hydroxyacids as has been reported for

runner-bean leaves (10). In other systems 3-hydroxyacids have a variety of functions such as: constituents of extracellular glycolipids in the red yeast Rhodotorula graminis (203); as components of depsipeptides in fungi (253); as constituents of an antibiotic in Bacillus mesentericus (254); constituent of an agent causing hemorrhage and regression of mouse tumor in E. coli (255).

Whether or not 3-hydroxyacids of soybean cell cultures have similar functions, it is not known, and further studies are required to settle this point.

F. CLAIMS TO ORIGINAL RESEARCH

1. In vitro studies have shown that proplastids and to a lesser extent microsomes from undifferentiated soybean cell suspension cultures can carry out 18:1 → 18:2 but not 18:2 → 18:3 desaturation.

2. Contrary to the general belief that 18:1 → 18:2 desaturation in plants occurs only in microsomes, our system clearly demonstrates the existance of a Δ^{12}-desaturase of proplastid as well as a microsomal origin. The proplastid Δ^{12}-desaturase may be associated with the lamellar membranes.

3. The Δ^{12}-desaturase of both microsomes and proplastids was found to behave differently than the microsomal Δ^{12}-desaturase of other plant systems with respect to the requirement for reduced pyridine nucleotides and response to CN.

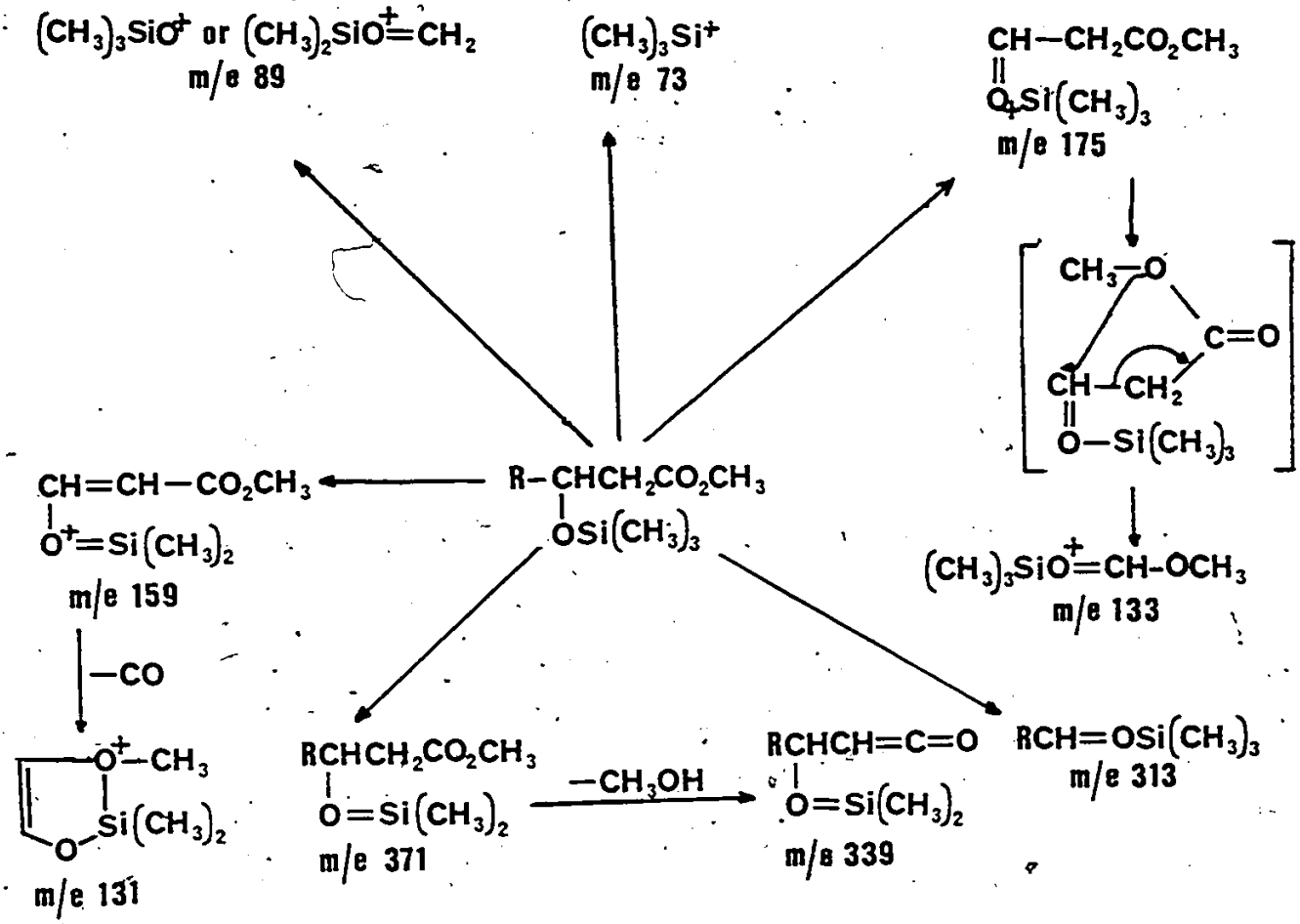
4. Contrary to previous reports which indicated that Δ^{12}-desaturation in plants occurs at the level of phospholipids, this enzyme system was found to act only on the acyl-CoA derivative and not on phospholipids.

5. Metabolism of 18:1-CoA by proplastids or microsomes resulted in the formation two major components identified as $\Delta^{2,9}$-octadecadienoic acid and 3-hydroxyoleate and a minor component identified as $\Delta^{9,12}$-octadecadienoic acid. The

hydroxylase was also found to act only on the acyl-CoA of 18:1 or 18:2 and not on phospholipids or glycolipids as substrate.

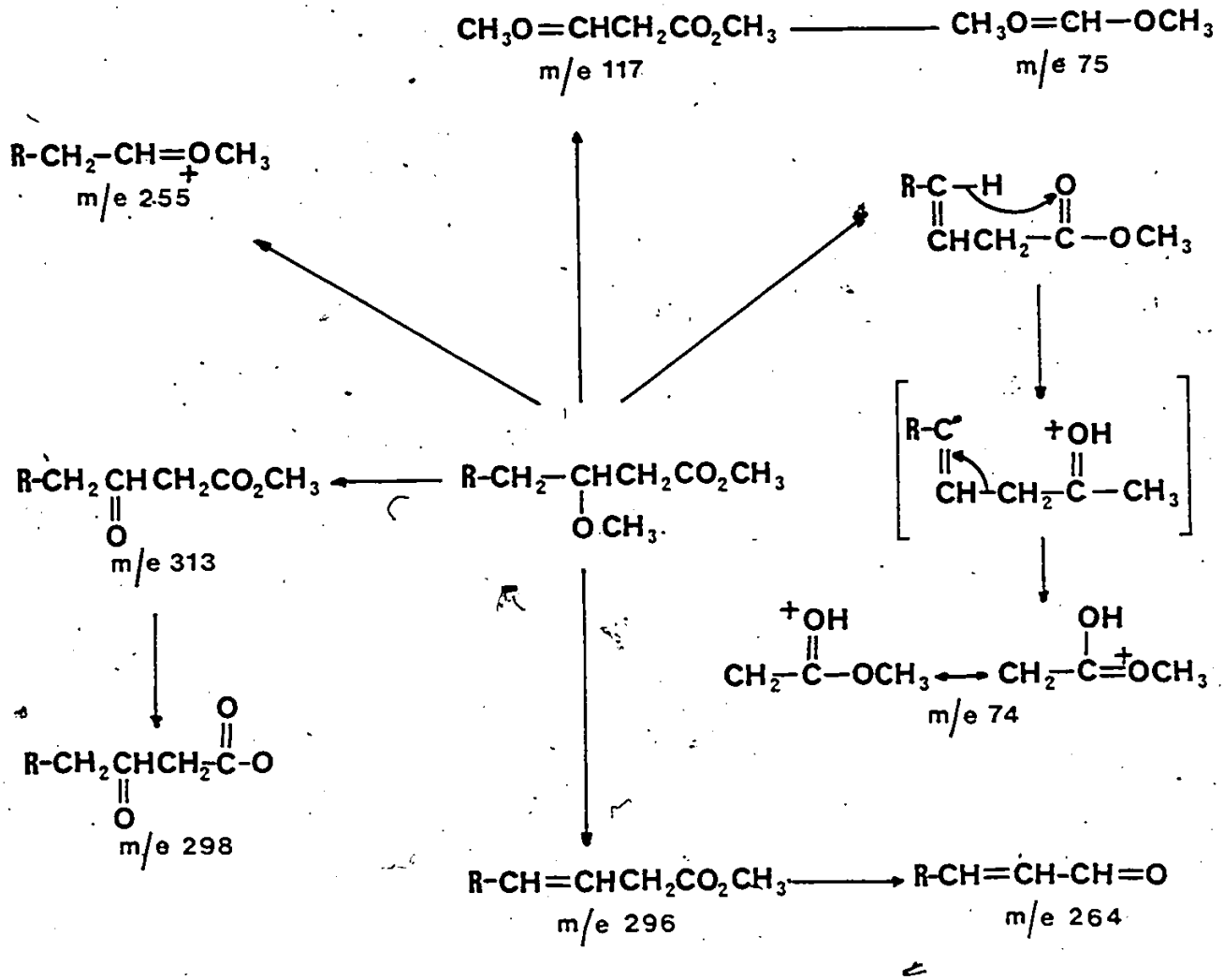
6. Metabolism of 18:2-CoA by either proplastids or microsomes resulted in the formation of two major components identified as $\Delta^{2,9,12}$-octadecatrienoic acid and 3-hydroxylinoleate and two unidentified minor components. No Δ^{15}-desaturase activity was observed. The hydroxylating enzyme system was found present mainly in proplastids and to a lesser extent in microsomes of soybean cell cultures.

APPENDIX I



Summary of the major diagnostic ion peaks arising from the fragmentation of the trimethyl silyl ether derivative of products C, H and authentic 3-hydroxystearate.

APPENDIX II



Summary of the major diagnostic ion peaks arising from the fragmentation of the methoxy derivative of products C, H and authentic 3-methoxystearate.

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