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ABBREVIATIONS

ATP	Adenosine 5'-Triphosphate
ADP	Adenosine 5'-Disphosphate
ACP	Acyl Carrier Protein
BCCP	Biotin Carboxyl Carrier Protein
POPOP	1,4-Bis-[2(5-phenyloxazolyl)]-benzene
CN	Cyanide
Ci	Curie
CoA	Coenzyme A
3-Decynoyl-NAC	3-Decynoyl-N-Acetylcysteamine
PPO	2,5-Diphenyloxazole
DG	Diglyceride
FAS	Fatty Acid Synthetase
FFA	Free Fatty Acids
FAME	Fatty Acid Methyl Esters
GLC	Gas Liquid Chromatography
HSS	High Speed Supernatant
Pi	Inorganic Phosphate
18:2	Linoleic Acid
18:2-CoA	Linoleoyl-CoA
NADH	β-Nicotinamide Adenine Dinucleotide, Reduced Form
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NL	Neutral Lipids

18:1	Oleic Acid
18:1-CoA	Oleoyl-CoA
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PI	Phosphatidylinositol
18:0	Stearic Acid
18:0-CoA	Stearoyl-CoA
SDS	Sodium Dodecyl Sulfate
TG	Triglyceride
TLC	Thin Layer Chromatography

ABSTRACT

Detailed studies were carried out on the process of lipid desaturation by microsomal membranes prepared from cells of Candida lipolytica grown at 10°C and 25°C and harvested at various stages in the growth cycle. The following results were obtained:

1) A reciprocal relationship between oleic (18:1) and linoleic (18:2) acids occurred during the early log phase of growth of the cells at both temperatures;
2) Fatty acids were more unsaturated at 10°C than 25°C and unsaturation of the fatty acids was maintained at a higher level later in the growth phase at 10°C than at 25°C;
3) 18:0-CoA, 18:1-CoA and PC desaturase specific activities in microsomes of cells grown at 25°C were maximal at the mid-log phase and were correlated with the maximum absolute content of 18:2 fatty acid in the microsomes. In contrast microsomal membranes from 10°C cells, had maximum 18:0-CoA, 18:1-CoA and PC desaturase activities in the early log-phase of growth, coinciding with maximum and minimum proportions of 18:2 and 18:1 acids respectively, but not precisely with the maximum absolute content of 18:2; while maximum 18:1-CoA activity in 10°C cells was double the maximum activity in 25°C cells the activities of the 18:0-CoA and PC desaturases were considerably lower in 10°C cells;

4) Increase in aeration rate (shaking speed) resulted in increased percentages of 18:2 acid and in increased 18:1-CoA desaturase activity but did not affect the lecithin desaturase or the 18:0-CoA desaturase activities;

5) Kinetic studies of 18:1-CoA desaturase using microsomes prepared from cells at 25°C to mid-log phase, in the presence of ATP, $MgCl_2$ and CoA and both in the presence and absence of NADH, showed that a relatively low hydrolysis rate of acyl-CoA (10% in 30 min.) and a rapid transacylation of ^{14}C-acyl groups into phospholipids (31% in 30 min.) and triglycerides (10% in 30 min.) had occurred. In the presence of NADH, formation of linoleic acid occurred in the phospholipids more rapidly (2.4% in 2 min.) than in the neutral lipids (0.2% in 2 min.) or in the acyl-CoA (0.7% in 2 min.). After 2 minutes the [^{14}C]-18:2 acid in the phospholipids accounted for 74% of the total newly formed [^{14}C]-18:2, while the acyl-CoA accounted for 20% and the neutral lipids for 6%. The newly formed 18:2 acid was distributed among the phospholipids after 2 min. and 30 min., respectively, as follows:

PC, 66%, 47%; PE, 15%, 34%; and PS and PI 19%, 19%. These results suggest that a considerable portion of 18:2 acid present in microsomes of Candida lipolytica is formed by the action of the phospholipid desaturase. Although these studies cannot completely rule out the action of an 18:1-CoA desaturase, the recent studies by Stymne and

Glad (1981), suggest that even the low level of desaturation observed with 18:1-CoA (Table IX, Fig. 14) may have resulted from back-exchange between [^{14}C] phospholipids and the acyl-CoA, and therefore it may be that most of the observed desaturation occurred by the activity of a phospholipid desaturase.

INTRODUCTION

I. General

Biological membranes are essential to life. In addition to providing a physical barrier which separate cells and subcellular organelles from their respective environments, membranes are intimately involved in essential processes such as facilitation and control of transport of metabolites between the environments they separate. In eukaryotic cells they are involved in the process of oxidative phosphorylation, protein and lipid synthesis as well as other cell functions, while in photosynthetic organisms the lamellae membranes contain the molecular apparatus of photosynthesis (Branton, 1969; Korn, 1969).

The study of membranes is one of the most exciting fields of modern biochemistry. Of particular interest is the problem of control of membrane lipid composition. For example, knowledge of the control of unsaturated fatty acid biosynthesis would be helpful in understanding the mechanism of control of membrane fluidity. Specifically our aim was to obtain knowledge of the biosynthetic pathways for 18:1 and 18:2 acids and on the control and regulation of these pathways. The organism chosen for the present study was Candida lipolytica, primarily because it synthesizes oleic and linoleic acids in large quantities but not higher poly-unsaturated fatty acids, and secondly, the content of these acids can be manipulated quite readily by change in growth

temperature or phase of growth. This thesis will present the results of a study of the changes in desaturase activity in Candida lipolytica as a function of growth phase and growth temperature as correlated with corresponding changes in fatty acid composition.

II. The Organism

Yeasts may be classified into three different thermal categories:

a) the obligate psychrophilic yeasts e.g., Torulopsis psychrophila, Leucosporidium gelideum, and L. nivalis, which grow in the temperature range -2° to 20°C;

b) the obligate thermophilic yeasts, e.g., Torulopsis bovina, Candida sloofii, and Saccharomyces telluris which are facultative anaerobes with temperature limits of 25° to 45°C; and

c) the mesophilic yeasts, e.g., Candida lipolytica, Torulopsis candida, and Saccharomyces cerevisiae, which comprise the majority of yeasts having temperature limits of 5° to 35°C (Arthur and Watson, 1976).

Previous studies have established that a characteristic differentiating property between these three classes is the content of polyunsaturated fatty acids (Kates and Baxter, 1962; Arthur and Watson, 1976); thus psychrophilic yeasts have the highest content, mesophiles are next and thermophiles have the lowest. Conversely the thermophiles and mesophiles are rich in monounsaturated fatty acids. These characteristics

are summarized in Table I.

The most notable features of the genus Candida are the presence of pseudomycelia and in some species, including C. lipolytica, the presence of a true mycelial structure. Members of this genus are widely distributed in nature, usually existing as saprophytes in association with both plants and animals. Some species, most notably C. albicans, are pathogenic. In industry, a variety of Candida species are found as contaminants in beverages, dairy products, wood pulp and stock yeast cultures. Certain strains of C. lipolytica appear to be associated with meat spoilage and contamination of refrigerated grape juice, and other strains have been implicated in infections of skin and nails.

C. lipolytica is found often associated with fatty substances such as margarine, butter, and olives. This association is due to its ability to produce lipases, a factor which permits its growth on lipid material.

III. Lipid Composition of C. lipolytica

1. Fatty Acids

Kates and Baxter (1962) have shown that the major fatty acids of C. lipolytica when grown on glucose medium are palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1) and linoleic (18:2) acids. Small amounts (<1%) of shorter chains as well as odd numbered chains (15:0 and 17:0) acids were also found. The total fatty acid composition and that of the phospholipids varies with a) age of the

TABLE I

Differentiating Properties of Psychrophilic, Thermophilic and Mesophilic Yeast[a]

Yeast	Temp. Limits for Growth °C	Anaerobic Growth	Unsaturated Fatty Acids		
			18:1	18:2	18:3
Psychrophilic	-2 to 20	-	++	++	++
Mesphilic	5 to 35	+	+	++	-b
Thermophilic	25 to 45	+	++	-	-

[a]Data taken from Arthur and Watson (1976), Watson (1980), and Kates and Baxter (1962).

[b]Some mesophilic yeasts e.g., Torulopsis candida contain 18:3 fatty acid.

culture and b) growth temperature. The changes in fatty acid composition as a function of growth temperature may represent a mechanism allowing the organism to adapt to a particular environment.

2. Phosphatides

Kates and Baxter (1962) and Kates and Paradis (1973) analysed the lipids of C. lipolytica and found the following components: Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), diglycerides, free fatty acids and sterols. Phosphatides comprised 80% of the total lipids, with phosphatidylcholine as major component (35-40% of total phospholipids), followed by phosphatidylethanolamine (20-30%) and phosphatidylserine plus phosphatidylinositol (33%). These analyses are quite similar to the phosphatide compositions reported for other species of Candida (Letters, 1968).

3. The Effect of Temperature and Growth Phase on Lipid Composition

The three thermal categories of yeasts, namely psychrophilic, mesophilic and thermophilic yeasts, have different temperature limits for growth, namely -2° to 20°C, 5° to 35°C, and 25° to 45°C respectively (Table I). The lipid composition of these three groups reveal that the lower the temperature for maximum growth the greater the degree of lipid unsaturation. For example, the membrane lipid composition of mesophilic and psychrophilic yeasts shows particularly high contents of unsaturated fatty acids

(80-90%) and low contents of saturated fatty acids (10-20%), as compared to thermophilic yeasts which have a reduced content of unsaturated fatty acids (60-80%) with a corresponding increase in more saturated fatty acids (20-40%).

Kates and Baxter (1962) demonstrated that mesophilic and psychrophilic strains of yeast had higher levels of unsaturated fatty acids at 10°C than 25°C. Meyer and Bloch (1963) using cell-free extracts of Torulopsis utilis were able to demonstrate a higher conversion of acyl-CoA to unsaturated derivatives at 19°C than at 30°C. They showed that this phenomenon could easily be explained by the increase in activity of the desaturating enzymes of the particulate phase of their cell-free system. Kates and Paradis (1973), looking at the fatty acid composition and degree of unsaturation during the growth of Candida lipolytica at both 25°C and 10°C found that at both temperatures, a marked increase in the proportion of linoleic acid and a corresponding decrease in the proportion of oleic acid, relative to the inoculum, occurred very early in the log phase. Linoleic and oleic acids reached a maximum and minimum, respectively, at the beginning of the active growth phase. Thereafter, the proportions of these acids were restored to the value of the inoculum when the stationary phase was reached at 25°C, but at 10°C this process was slower and the original inoculum values were not attained even in stationary

phase. As result, the proportion of linoleic acid and therefore the degree of unsaturation, was much higher in cells grown at 10°C than in cells grown at 25°C. Closer examination of the changes in fatty acids in the individual phospholipids and neutral lipids revealed that at both growth temperatures the reciprocal changes in linoleic and oleic acids occur in all phospholipids, being most pronounced in the lecithin and PE components (Fig. 1). While the changes in PC occur in the fatty acids at both the α- and β-positions, in PE the reciprocal changes occur only at the β-position (Kates and Paradis, 1973).

IV. Fatty Acid Synthesis

1. Saturated Fatty Acid Biosynthesis

The pathway for saturated fatty acid biosynthesis is essentially the same for all organisms so far examined. The primary substrate for saturated fatty acid synthesis is acetyl-CoA. In eukaryotic cells the availability of acetyl-CoA is controlled by ATP-citrate lyase more commonly known as citrate cleavage enzyme. Citrate transported from the mitochondria to the cytoplasm is cleaved by the enzyme, citrate lyase to oxaloacetate and acetyl-CoA as illustrated in Scheme I. One of the first steps in the biosynthesis of saturated fatty acids is the activation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase enzyme (Wakil, 1958). The latter enzyme occupies a central role in the biosynthesis pathway and is regarded as a key regulatory enzyme. In yeasts

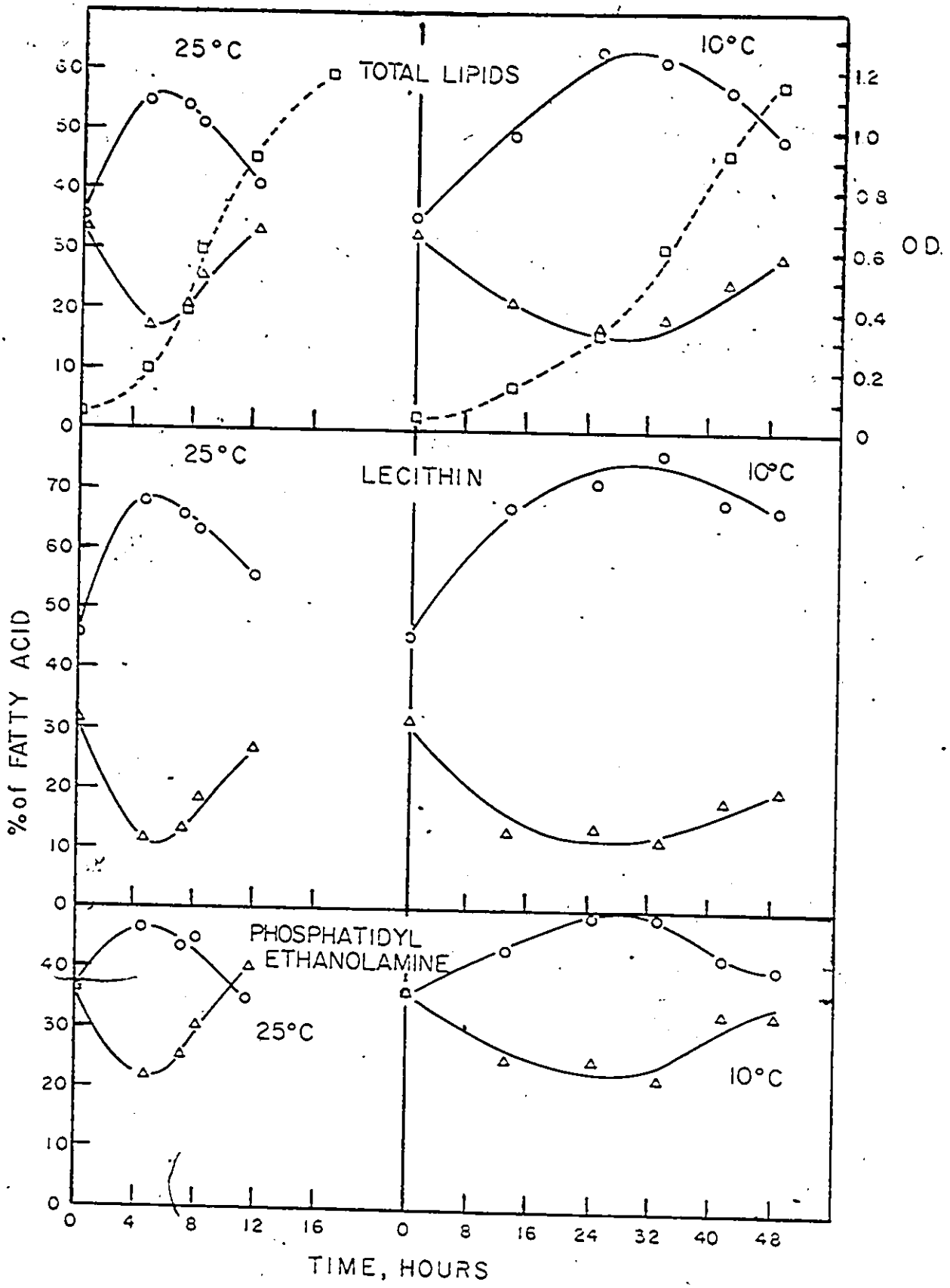
FIGURE 1. (Kates and Paradis 1973). Changes in fatty acid composition of total lipids, lecithin and phosphatidylethanolamine at 25°C and 10°C.

Growth curves □——□

Linoleic acid o-o-o

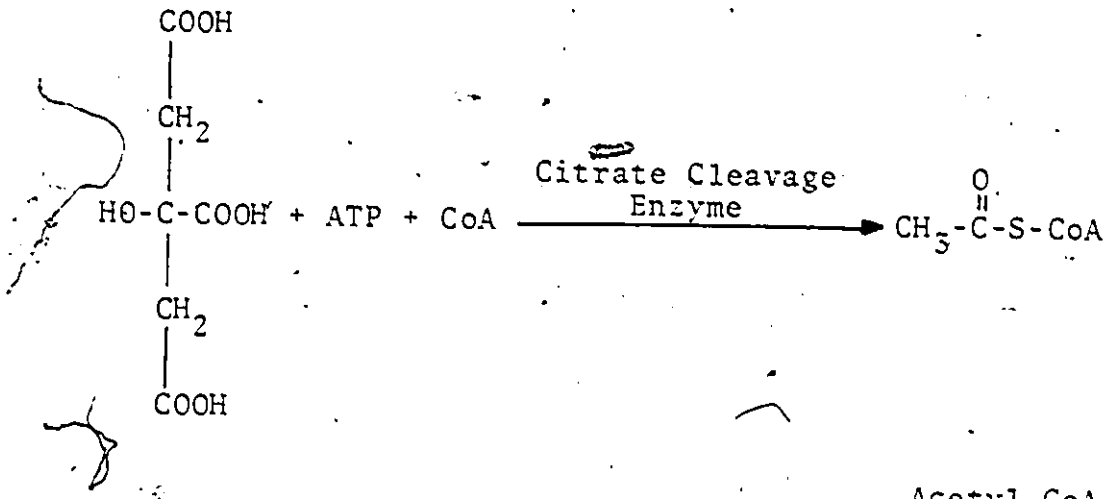
Oleic acid Δ-Δ-Δ

Fig. 1


25°C
TOTAL LIPIDS
10°C
O.D.
25°C
LECITHIN
10°C
% of FATTY ACID
PHOSPHATIDYL
ETHANOLAMINE
25°C
10°C
TIME, HOURS

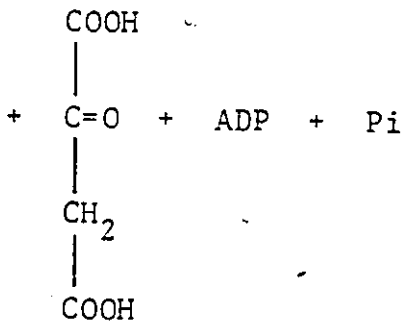
SCHEME I

Transport of Acetate from the Mitochondria (Thomspon, 1980)



Citrate

Acetyl CoA



Oxaloacetate

and animals, the monomeric form of this enzyme, which is enzymatically inactive, is made up of four subunits: i) biotin carboxyl carrier protein ii) biotin carboxylase, iii) acetyl-malonyl-CoA transcarboxylase and iv) citrate binding protein. The binding of citrate to the fourth subunit leads to a conformational change of the enzyme and a shift in the equilibrium from the monomeric form to the polymeric form, consisting on the average of 20 monomeric units, which is the active form of the enzyme complex. The reactions carried out by this complex are illustrated in Scheme II.

Malonyl-CoA is utilized by an enzyme complex known as fatty acid synthetase (FAS). The bacterial fatty acid synthetase, from which much of our knowledge originates, is an easily dissociable multienzyme complex of six enzymes and a low molecular weight heat-stable protein having a 4'-phosphopantetheine group, known as "acyl carrier protein" (ACP). The reactions are schematically represented in Fig. 2 and summarized in a stepwise fashion in scheme III. From the initial step of the overall reaction, the substrates and products remain covalently bound to the sulfhydryl group or the "parking" -SH group of the acyl carrier protein (ACP) until a chain length of sixteen or eighteen carbons is reached after which palmitate or stearate is transferred to CoA in the cytosol. Termination of the process is apparently controlled by the β-ketoacyl-ACP synthetase component. In E. coli palmitoyl-CoA was found to bind to this enzyme less

SCHEME II

Activation of Acetyl-CoA to Malonyl-CoA (Thompson, 1980)

i. $ATP + HCO_3^- + BCCP \xrightleftharpoons{\text{Biotin Carboxylase}} CO_2^- - BCCP + ADP + Pi$

ii. $CO_2^- - BCCP + CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} \xrightarrow{\text{Carboxyl Transferase}} HO\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-S-CoA} + BCCP$

OVERALL $CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} + HCO_3^- + ATP \xrightarrow{\text{Acetyl-CoA Carboxylase}} HO\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} + ADP + Pi$

Acetyl CoA

Malonyl-CoA

SCHEME III

General Pathway for Fatty Acid Biosynthesis

(Thompson, 1980)

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

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

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

$$CH_3\text{-CO-S-synthase} + \begin{matrix}COOH\\|\\CH_2\end{matrix}\text{-CO-S-ACP} \xrightarrow{\text{β-ketoacyl-ACP synthase (condensing enzyme)}} CH_3\text{-CO-}CH_2\text{-CO-S-ACP} + \text{synthase-SH} + CO_2$$

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

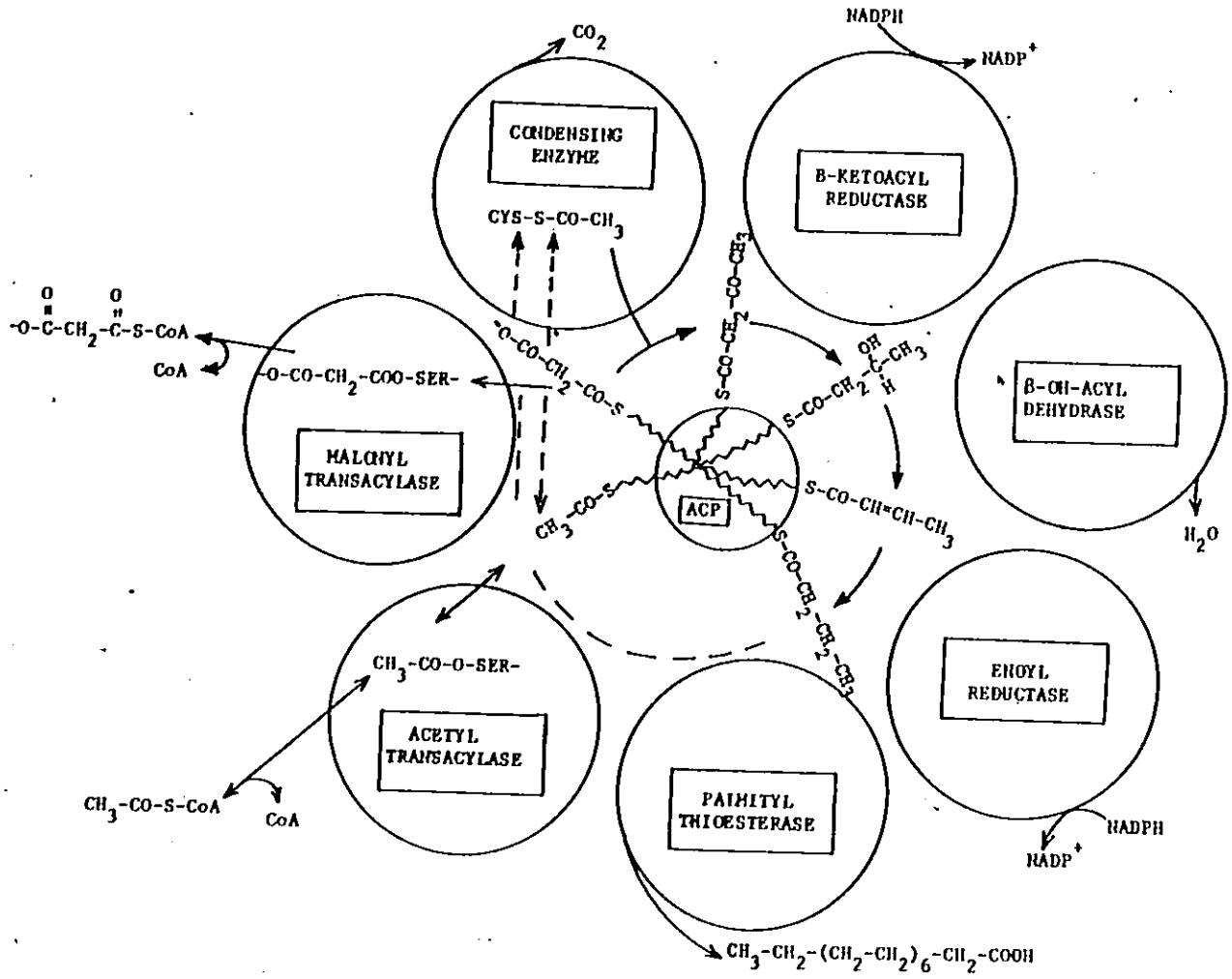
$$CH_3\text{-}\begin{matrix}OH\\|\\CH\end{matrix}\text{-}CH_2\text{-CO-S-ACP} \xrightleftharpoons{\text{β-hydroxyacyl-ACP dehydratase}} CH_3\text{-CH=CH-CO-S-ACP} + H_2O$$

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

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.

FIGURE 2. Fatty acid synthetase (FAS) of bacteria, taken from Stoops et al., 1977.

FIGURE 2


CO2
NADPH
NADP+
CONDENSING ENZYME
B-KETOACYL REDUCTASE
CYS-S-CO-CH3
-O-C-CH2-C-S-CoA
CoA
-O-CO-CH2-COO-SER-
MALONYL TRANSACYLASE
-O-CO-CH2-CO-S
S-CO-CH2-CO-CH3
S-CO-CH2-CH-CH3
OH
H
β-OH-ACYL DEHYDRASE
H2O
CH3-CO-S
ACP
S-CO-CH=CH-CH3
S-CO-CH2-CH2-CH3
CH3-CO-O-SER-
ACETYL TRANSACYLASE
CH3-CO-S-CoA
CoA
PALMITYL THIOESTERASE
ENOYL REDUCTASE
NADPH
NADP+
CH3-CH2-(CH2-CH2)6-CH2-COOH

tightly than acetyl-CoA, and the binding of acyl-ACP substrates decreased when the chain length exceeded C_{10}. Hexadecanoyl-ACP, the main fatty acid of E. coli, shows no affinity for the enzyme (Greenspan et al., 1970).

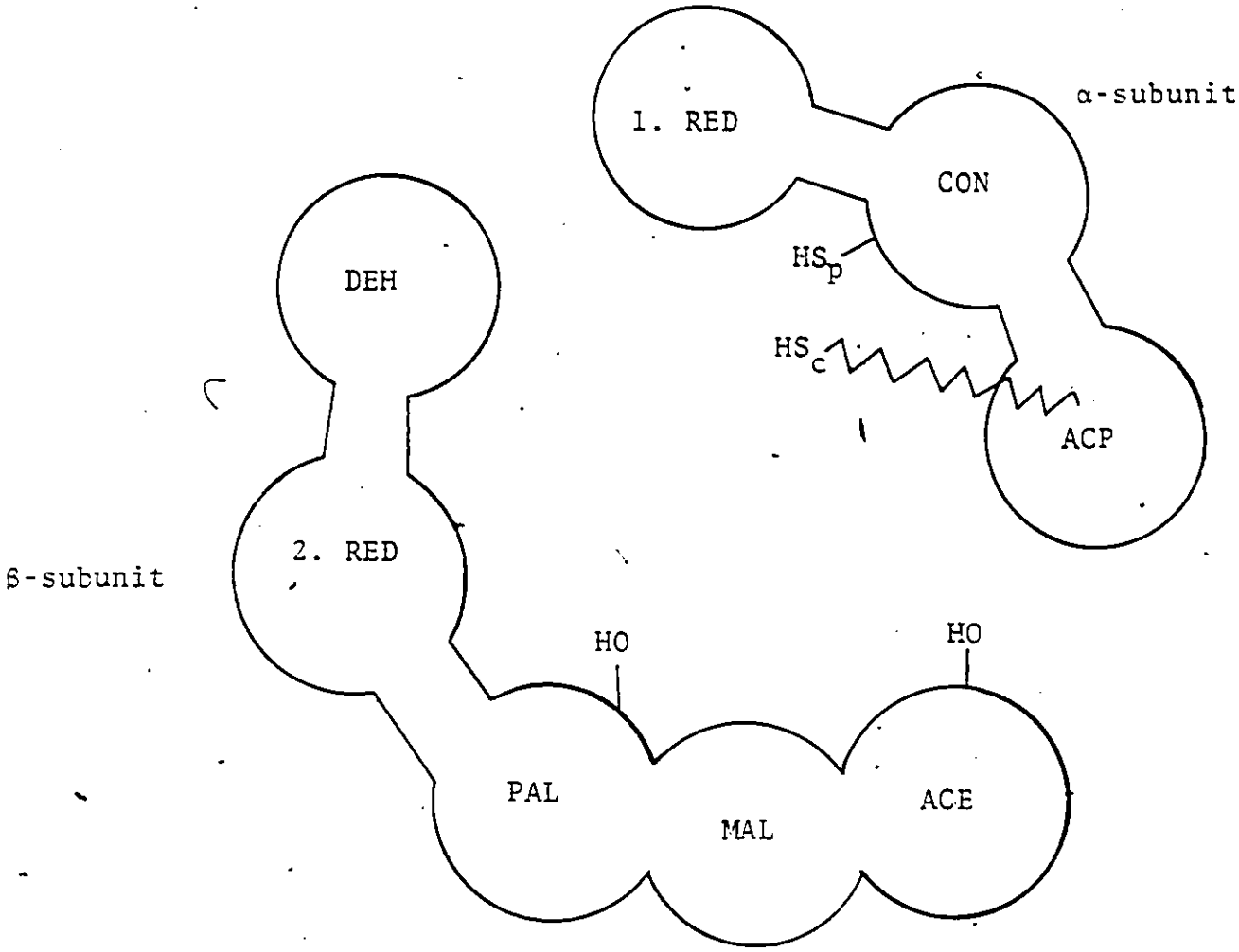
In yeast the completed fatty acid (palmitate or stearate) is transferred from the FAS complex to CoA. A still different product is obtained in animal systems, where an active thiolesterase gives rise to free fatty acids. The fatty acid chain length in animal systems, as studied in the mammary gland, is strongly influenced by a soluble protein; this protein appears to interact with the FAS causing it to terminate its fatty acids at chain lengths of C_8 to C_{12} rather than C_{16}. It should be pointed out that fatty acyl-CoA derivatives such as propionyl-CoA may substitute for acetyl-CoA in the priming reaction as described above, leading to the biosynthesis of odd-numbered fatty acids.

In contrast to the bacterial FAS, the fatty acid synthetase from eukaryotic organisms, animals and yeasts, are stable multienzyme complexes of the same enzymes and ACP as in bacterial FAS. So far, neither their dissociation into individual enzymatically active component enzymes nor the isolation of a distinctive acyl carrier protein from them has been unequivocally demonstrated. Although the pathway of fatty acid biosynthesis, as shown above, has been elucidated in detail and appears to be essentially the same in all systems so far studied, the structural and functional

organization of the eukaryotic FAS complex is only now being elucidated. A recent genetic investigation by Schweizer et al., (1973) of the FAS complex in yeast indicated that the FAS complex was encoded by two polycistronic and genetically unlinked gene loci which they called fas-1 and fas-2. End group analysis of the yeast fatty acid synthetase (Schwietz et al., 1976) was consistent with the presence of two nonidentical polypeptide chains in the FAS complex. Later Stoops et al., (1978) were able to isolate the intact FAS complex by the use of protease inhibitors to eliminate proteolytic degradation of the polypeptide chains during the isolation procedure. Under these conditions, only two bands (α and β) appeared on SDS gel electrophoresis of the FAS complex, compared to several bands when protease inhibitors were omitted. The α and β components, of almost equal molecular weight, when isolated in the absence of substrates (acetyl-CoA, malonyl-CoA, and NADPH) existed predominantly in the oligomeric inactive form $\alpha_2\beta_2$ at low protein concentration with very little of the polymeric active form $\alpha_6\beta_6$. In the presence of the substrates, the polymeric form $\alpha_6\beta_6$ predominated over the smaller oligomeric form $\alpha_2\beta_2$ or $\alpha_4\beta_4$ (Stoops et al., 1978). The simplest form of the FAS complex (α β) in the yeast is represented in Fig. 3. Note that the ACP is on the α- chain together with the condensing enzyme and β-ketoacyl reductase, while the remaining enzymes are on the β-chain.

FIGURE 3. Active sites on the α and β subunits of yeast fatty synthetase: α subunit: 1. RED, β-ketoacy-ACP reductase; CON, condensing enzyme (HS_p, parking site); ACP, acyl-carrier protein (HSc, carrier site).
β subunit: PAL, MAL, ACE - ACP-palmitoyl-, malonyl-, and acetyl-transacylase, respectively; Thompson, 1980.

FIGURE 3


1. RED
α-subunit
CON
HS$_p$
DEH
HS$_c$
ACP
2. RED
β-subunit
HO
HO
PAL
MAL
ACE

The FAS complex of animal tissues have been shown to occur as dimers of two identical subunits (Stoops et al., 1979), indicating that the domains for all catalytic activities and acyl carrier protein involved in fatty acid synthesis exist within one large multifunctional polypeptide chain. A more recent study by Zendra et al., (1980) presented evidence that the FAS from the goose uropygial gland contains a large mRNA synthetase that is translated as a complete unit. This confirmed the large size of the synthetase subunit and its multifunctional nature.

V. Biosynthesis of Unsaturated Fatty Acids

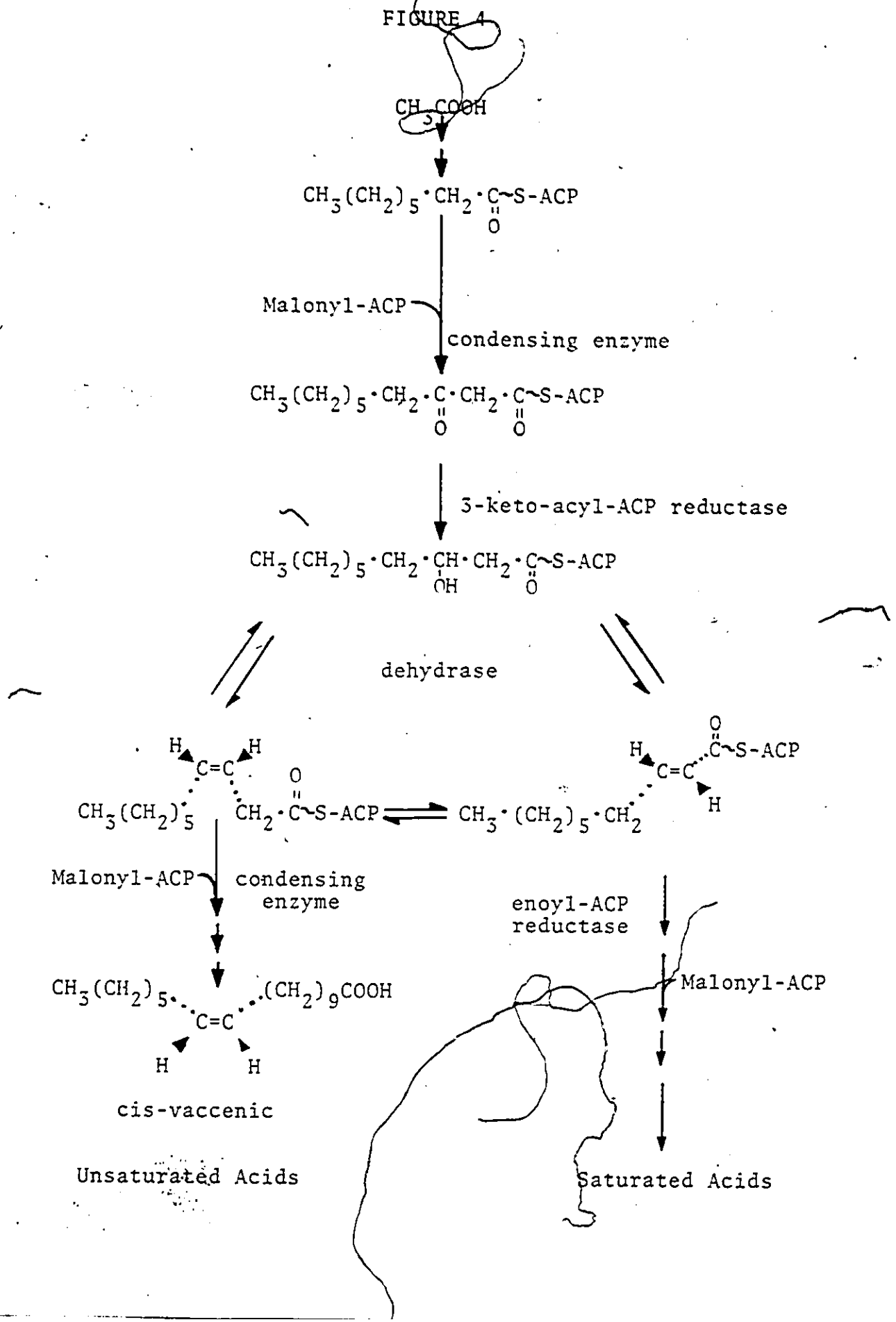
1. Anaerobic Mechanism of Fatty Acid Desaturation in Prokaryots

Anaerobic desaturation occurs only in prokaryots and involves the retention of a 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 (1962) who were studying 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: Δ7- and Δ9-hexadecenoic and Δ9- and Δ11-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 shown in Fig. 4. At these branch points,

FIGURE 4. The anaerobic pathway for synthesis of monounsaturated fatty acids; Gurr and James (1971).

FIGURE 4


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 CH2·C~S-ACP
O
H C=C O C~S-ACP H
CH3·(CH2)5·CH2
Malonyl-ACP
condensing enzyme
enoyl-ACP reductase
Malonyl-ACP
CH3(CH2)5 (CH2)9COOH
C=C
H H
cis-vaccenic
Unsaturated Acids
Saturated Acids

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 (Kass and Bloch, 1967) 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 (Norris et al., 1964 and Brock et al., 1967) 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.

Using a specific inhibitor (3-decynoyl-N-acetylsteamine) Kass and Bloch (1967) 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. 4). This idea was confirmed by the finding of Silbert and Vagelos (1967) 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-hydroxydecanoyl thioester dehydrase has the ability to catalyse both dehydration and isomerization reactions (Helmkamp and Bloch 1969) 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-NAC 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 (Mizugaki et al., 1968), one dehydratase specific for the dehydration of β-hydroxydecanoyl-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 (Pugh et al., 1966). It seems, therefore, that although a specific dehydratase is essential for the

synthesis of long 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.

2. Oxidative Mechanism of Fatty Acid Desaturation in Eukaryots

The most important pathway of unsaturation in eukaryots is by an oxidative mechanism in which the double bond is introduced directly into the preformed long chain saturated fatty acid with oxygen and NADH or NADPH as cofactors. This pathway is almost universal and is used by yeasts, higher animals, protozoa, algae and some bacteria. Thus far, no organism has been found that contains both pathways of desaturation. With the exception of some bacteria and plants (Gurr and James, 1980) the oxidative pathway exhibits a remarkable positional specificity for the 9,10-position, most monoenoic fatty acids produced having a Δ9-cis double bond. 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 vitro studies. Work by Oshino et al., (1971) and Holloway and Katz (1972), led to the identification of three microsomal bound proteins: 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., (1974) 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. 5. 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. Evidence provided by Schroepfer and Bloch (1965) and confirmed by Morris (1967, 1970) and Morris et al., (1968) showed that the stereospecificity of the hydrogen removal at one position of each double bond was absolute for the D-configuration. The Morris group (1968) proceeded then to established 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

FIGURE 5

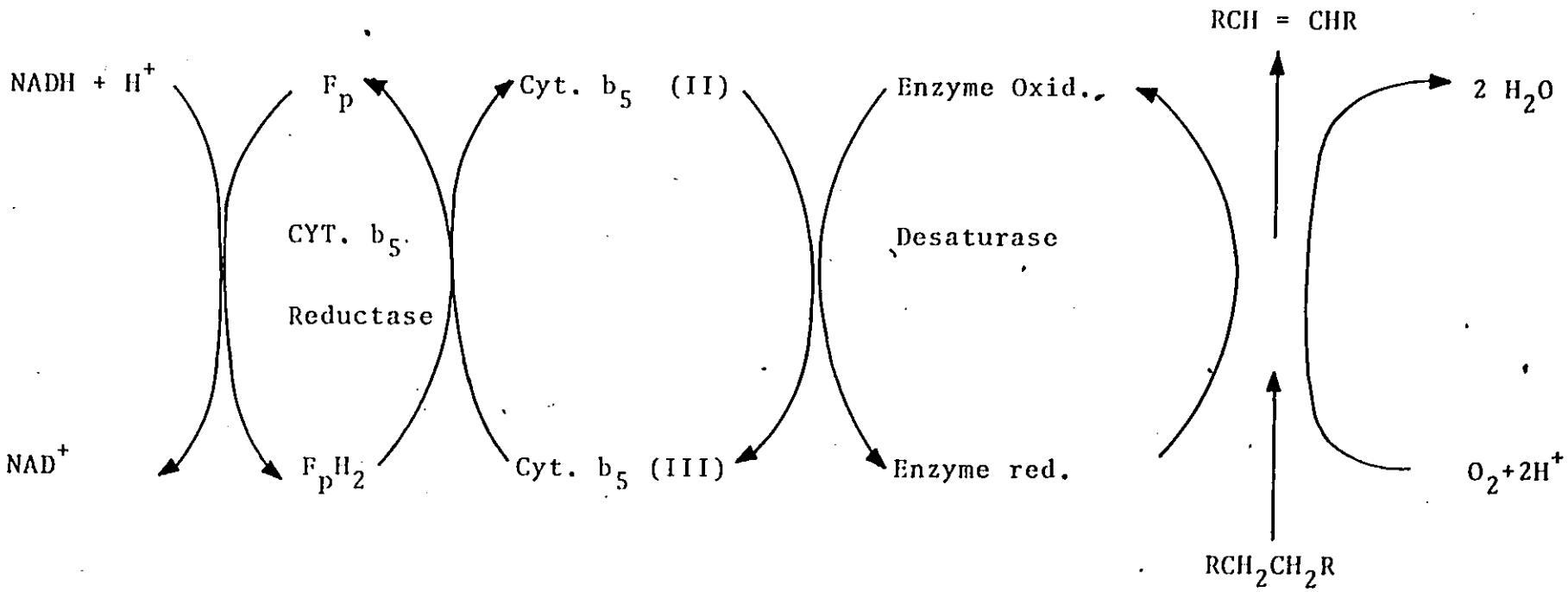


Fig. 5. Microsomal-bound lipid desaturase system. (Oshino et al 1971)

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.

3. Substrates for Desaturase Activity

Desaturation of acyl chains can occur in the form of CoA or ACP thiolesters. The substrates for Δ9 desaturation of saturated acids has been shown to be the acyl-CoA in yeasts (Bloomfield and Bloch 1960), seed tissues (Vijay and Stumpf 1972), bacteria (Fulco and Bloch 1962), and animal tissues (Holloway and Holloway, 1973; Marsh and James, 1962). As far as photosynthetic tissue is concerned, the desaturase that converts oleic acid into linoleic acid was shown by Harris and James (1965) to have similar requirements, but many attemps to demonstrate the conversion of stearoyl-CoA into oleoyl-CoA failed. The problem was resolved by Nagai and Bloch (1965, 1966, 1968) when they found that a soluble preparation 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 the ACP derivative was a substrate.

Regarding the synthesis of dienoic acids, evidence reported by Gurr et al (1969) with Chlorella, showed that Δ12 desaturation of oleic acid might occur on the phospholipids, particularly with PC, as had been previously postulated by Nichols et al (1967). These findings were corroborated by Baker and Lynen (1971) while studying Δ12 desaturases of the fungus Neurospora crassa. More direct evidence was obtained by Talamo et al (1973), 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 (1973), in attempting to explain the reciprocal changes of 18:1 → 18:2 in Candida lipolytica (see section III) 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 (1973) 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 or 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. Using labelled lecithins, Pugh and Kates (1975) then characterized the membrane bound phospholipid desaturase in comparison with the acyl-CoA desaturase. They observed the following: a) Both desaturases showed the same requirements for cytochrome b_5 reductase, cyt b_5 (lack of CO inhibition), and the terminal desaturase (cyanide sensitivity); b) Membranes prepared in the mid-log phase at 10°C contained 50% linoleic and 30% oleic acids (molar ratio 18:2/18:1, 1.7), whereas those grown at 25°C contained 40% linoleic and 40% oleic acids (molar ratio 18:2/18:1, 1.0); phospholipid desaturase activity was found to be lower (20-25 pmoles/min/mg) in membranes from cells grown at 10°C than that 50-80 pmole/min/mg from cells grown at 25°C; c) In contrast, the oleoyl-CoA desaturase was found to be higher in membrane from cells grown at 10°C than that cells grown at 25°C; d) Arrhenius plots of phospholipid desaturase activity for the two membranes not only showed that the activity of the 10°C membrane was lower than that of the 25°C membrane but it also showed that plots to be linear with both membrane preparations over the temperature range of 10-37°C, no clear-cut evidence for a change in slope or phase transition was observed. The activation energy on the other hand was

higher at 25°C than 10°C. The increase in the phospholipid desaturase activity at 25°C was due to an increase in Vmax of the enzyme and not to a change in Km for the substrate.

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 (Kates and Pugh, 1980). A similar conclusion has been reached by Skriver and Thompson (1979) for the palmitoyl-CoA desaturase of Tetrahymena and by Riordan (1979) for the Mg^{2+}-ATPase of rat liver plasma membrane. Thus the activity of the membrane bound phospholipid desaturase may be considered as a self-regulating mechanism 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 and suboptimal interaction or conformation occurring in a membrane of high 18:2/18:1 ratio or high fluidity Fig. 18 (see p. 112).

4. Does a True Acyl-CoA Desaturase Exist?

Using rat microsomes Kates and Pugh (1980) demonstrated that 20:3-CoA, substrate, was rapidly hydrolyzed and transacylated into phospholipids, and that the 20:3-phospholipid was desaturated to 20:4-phospholipid at a greater rate than desaturation of 20:3-CoA. Similar results have been reported for the 18:1-CoA desaturase activity of sunflower seed microsomes by (Stymne and Appelqvist, 1978, 1979) and on microsomes of Fusarium oxysporum by Wilson et al

(1980). Therefore the question arises whether specific acyl-CoA desaturases actually exist or whether the observed activity is accounted for by a combination of transacylase and phospholipid desaturase activities. These questions will be considered in detail in this thesis.

VI. Aim of the Research

The purpose of the research undertaken and reported in this thesis was to examine in detail the process of lipid desaturase in C. lipolytica at both 25°C and 10°C. Specifically it was my purpose to determine: a) whether the activity of the 18:1-desaturase system is correlated with the reciprocal changes of oleic and linoleic acids occurring during the cell cycle; b) whether the formation of high amounts of linoleic acid in response to low-temperature is due to the increased activity of the desaturase enzyme; and c) whether acyl-CoA or phospholipid or both are the actual substrates for the desaturase enzymes. The final aim of these studies was to shed light on the mechanism of control of oleic and linoleic acid synthesis in the yeast C. lipolytica.

MATERIALS AND METHODS

I. MATERIALS

A. Yeast

The organism used was the mesophilic yeast, C. lipolytica strain NRRLY 1094. This is the same strain which was first used by Lawrence et al (1959) and studied by Kates and Baxter (1962), Kates and Paradis (1973) and Pugh and Kates (1973, 1975). The organism was first obtained from the National Research Council of Canada in a lyophilized form and maintained on agar slants at 4°C.

B. Chemicals

All solvents were glass distilled before use. All other chemicals used were of 'Reagent Grade' quality unless otherwise specified. NADH, ATP, CoA, Triton X-100, oleoyl-CoA and stearoyl-CoA were purchased from Sigma Chemical Co., St. Louis, MO. [1-^{14}C]stearoyl-CoA, [1-^{14}C]oleoyl-CoA and 1,2-Di[^{14}C]oleoyl-sn-3-glycerophosphorylcholine were prepared as described in section J. [1-^{14}C]oleoyl-CoA (57.5 Ci/mmole) was also obtained from New England Nuclear, Lachine, P.Q. and was adjusted to a specific activity of 6.85 μCi/μmole.

II. METHODS

A. Growth of the Organism

1. Growth Medium

The medium used for the growth of the organism was first

used by Lawrence et al (1959) in the initial isolation of the organism and by Kates and Baxter (1962) and Kates and Paradis (1973) in their studies of the lipids of C. lipolytica.

The medium was slightly modified by Ohno and Kates (1978) and their medium is used in the present studies. The composition of this liquid medium is as follows: 2% glucose, 0.1% yeast extract, 0.1% NH_4Cl, 1.36% KH_2PO_4; the pH was adjusted to 6.0 with 0.1 N KOH. Agar slants contained 15 g of nutrient agar per 100 ml of medium.

2. Culture of Cells

A subculture was prepared by loop inoculating 100 ml of fresh autoclaved medium in a 500 ml Erlenmeyer flask with sealed-on side-arm and the flask was incubated for 40 hours at 25°C and a shaking speed of 120 rpm; the final optical density which ranged from 1.3 to 1.4 was measured at 680 nm in a Coleman junior spectrophotometer by tipping the flask so as to fill the sidearm with the culture. The instrument was set to read 100% T with an uninoculated culture medium incubated in a similar flask as described above. The large cultures were grown in 1 liter of liquid medium in a 4 liter Erlenmeyer flask; a ratio of 1:4 for medium volume to flask volume was used throughout in order to maintain a constant degree of aeration in all cultures, at a given shaking rate. The 40 hour subculture was used as an inoculum to initiate growth of large cultures (50 ml

inoculum to 950 ml of fresh medium) to be grown in a Controlled Environment Incubator-Shaker (New Brunswick Co. Inc.) at 10°C or 25°C and a shaking speed of 120 rpm. The culture flasks were harvested at different time periods.

3. Growth Curves

To follow the growth of the cultures, optical densities were determined periodically on 10 ml aliquots of the culture removed aseptically into 18 x 100 nm cuvettes as described above, using an appropriate blank. Growth was followed at 10°C and 25°C to an O.D. of about 1.0, the beginning of the stationary phase.

4. Harvesting of Cells

Cells were harvested at various stages in the growth cycle. When cultures reached the appropriate optical density, cells were harvested by centrifugation in 250 ml plastic buckets in a Sorval RC2-B automatic refrigerated centrifuge for 10 min. at 4,000 xg. The pellet of packed cells was washed once with cold water and recentrifuged at 4,000 xg. Yield of wet cells per liter culture was: 2.7 g at O.D. 0.22; 13.4 g at O.D. 0.84; and 17.2 g at O.D. 1.00.

B. Preparation of Cell Free Extract

The cell free extracts were prepared as follows: the cells were suspended in 25 ml of potassium phosphate buffer (pH 7.2) containing 0.25 M sucrose (1 g wet cells/ml of suspending medium) and broken by shaking in a heavy walled

glass bottle (70 ml total volume), with ballotini glass beads (0.45-0.50 mm in diameter; 33 g / 25 ml of suspension medium) in a high speed, CO_2-cooled shaking apparatus (Braun shaker, Bronwell Scientific, Inc.) for 30 sec. The total contents of the bottle were transferred to a centrifuge tube and subjected to differential centrifugation as illustrated in Fig. 6. All preparations were carried out at 4°C and fractions were kept on ice. For preparation of the microsomal fractions the final high speed (160,000 xg) supernatant was poured off, and the microsomal pellet was suspended in a hand operated glass "Potter" homogenizer (loose-fitting pestle) in 5 to 10 ml of 0.1 M phosphate buffer (pH 7.2) to a concentration of 15 to 20 mg protein/ml; the suspension was used immediately. Protein concentration was measured by the procedure of Lowry et al (1951).

C. Preparation of Lipid Dispersions

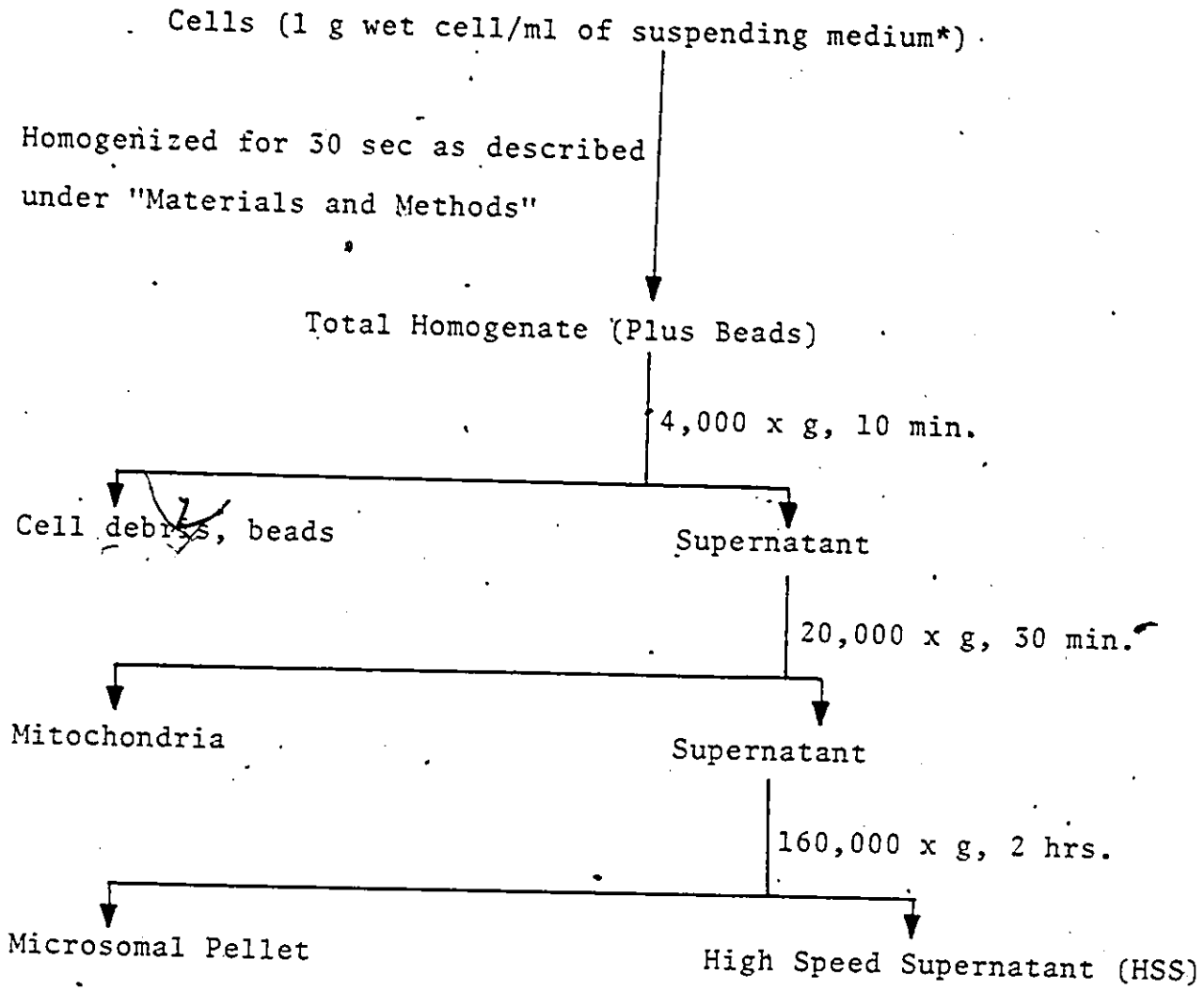
About 0.45 µCi (10^6 dpm) of [^{14}C]-dioleoyl-phosphatidyl-choline (171 nmoles) was dispersed in 0.5 ml of 0.1 M phosphate buffer (pH 7.2) by sonication at 0°C (ice bath), under an atmosphere of N_2 in a sonifier with microtip (Quigley-Rochester, Inc.) operated at an output of 2A for 15 sec. intervals with 15 sec. cooling intervals, up to a maximum of 2 min. or until the dispersion cleared.

D. Assay of Desaturase Activity

The stearoyl-CoA and oleoyl-CoA desaturase activities of

FIGURE 6. Cell Fractionation Procedure for Candida lipolytica

FIGURE 6



*0.1M phosphate buffer (pH 7.2) in 0.25M sucrose.

C. lipolytica microsomes were determined in a reaction mixture (0.5 ml) containing 100 mM phosphate buffer (pH 7.2), 10 mM NADH, 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM CoA, 27 nmoles (0.4 µCi) of stearoyl-CoA or 54 nmoles (0.06 µCi) of oleoyl-CoA, and 1.0 mg of microsomal protein.

Desaturation of the phospholipid substrate was assayed in a reaction mixture (0.5 ml) containing 100 mM phosphate buffer (pH 7.2), 10 mM NADH, 0.1% Triton X-100, 26 nmoles (0.07 µCi) of the [^{14}C]-dioleoyl-phosphatidylcholine and 2.0 mg microsomal protein.

The reactions were initiated by adding the microsomes, and incubations were performed in open test tubes with shaking for 15 min. in a water-bath shaker (120 rpm) at 25°C. Reactions were terminated by adding 0.3 ml of 40% aqueous KOH. The mixture was saponified at 70°C for 1-2 hours followed by acidification with 0.5 ml concentrated HCl and addition of 2.5 ml of methanol and 2.5 ml of chloroform plus 1.0 ml of H_2O to form a biphasic system. The mixture was vortexed and centrifuged at 2,000 xg 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 (1-2 ml) 3 to 4 times. The combined chloroform extracts were concentrated under a stream of N_2, with addition of benzene to remove any water, and the residue was diluted with 2.0 ml of 2.5% methanolic-

HCl; and heated under reflux for 2 hours at 75°C. After cooling 0.2 ml of water was added and the fatty acid methyl esters (FAME) were extracted with petroleum ether (B.p. 30 - 60, with 2.0 ml 4 times). The combined pet-ether extracts were brought to dryness in 15 ml glass stoppered centrifuge-tubes under a stream of N_2 using benzene to remove any water, and the residue was dissolved in 100 µl of chloroform-methanol (2:1 v/v). The fatty acid methyl esters obtained were separated according to their degree of unsaturation on 10% $AgNO_3$-impregnated silica gel G (0.5 mm thick) plates together with authentic standards of 18:0, 18:1, and 18:2 methyl esters. The solvent used was chloroform-ethanol (200:3 v/v). The spots were detected with either 2',7'-dichlorofluorescein (see section II.I) or by autoradiography (section II.J.3), scraped off into counting vials and the radioactivity measured as described in section II.J. 2. The specific activity of the desaturase was expressed as pmoles desaturated product formed per minute per mg of microsomal protein.

When isolation of the lipids was desired, the reaction was stopped by addition of 3.75 volumes of methanol-chloroform (2:1 v/v) followed by 1.25 ml chloroform and 1.25 ml water to make the biphasic system. The separation of lipid components was achieved as described in section II.K. Acyltransferase activity and acyl-CoA hydrolase were measured using the standard desaturase assay procedure with [1-^{14}C]-oleoyl-CoA as substrate except that NADH was omitted (Jones

et al 1969).

E. Extraction of Microsomal Lipids

Immediately after use in enzyme essays, the microsomal suspensions were stored at -70°C until the lipid extractions were performed. The lipids were extracted by the method of Bligh and Dyer (1959) modified as follows: to one volume of microsomal suspension (about 20 mg protein) 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. 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: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 minutes before centrifugation at 2,000 rpm in a IEC HN-SII centrifuge for 1-2 minutes. 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 1 ml and made to a known volume (5.0 ml). A known aliquot was used for fatty acid quantitation as described under section II.F

F. Methanolysis Procedure

An aliquot of the chloroform solution of lipids (containing about 15 mg of lipids) was pipetted into a 20 ml glass-stoppered test tube and to that was added a known quantity of internal standard (C_{17}) and the solvent evaporated under a stream of nitrogen; 2.0 ml of 2.5% HCl in methanol (made by dissolving 2.5 g of hydrogen chloride gas in 100 ml of methanol) was added; the tube was stoppered and heated for 1 hour at 70-75°C in a temperature-controlled block heater (lab-line Instruments Inc., Melrose Park, Ill.); 0.2 ml of water was added and the fatty acid methyl esters (FAME) were extracted 4 times with 2 ml portions of petroleum ether (B.p. 30-60°C); the ether extract was concentrated under a stream of N_2 and the residue dissolved in a known volume of chloroform.

The FAME were either analyzed by TLC on a silica gel G impregnated with 10% $AgNO_3$ or by gas liquid chromatography (GLC).

G. Analysis of Fatty Acids by Gas-Liquid Chromatography

Fatty acid methyl esters (FAME) prepared as described above, were analysed by GLC on a column, (2 mm x 0.4 mm) of 10% butanediol succinate polyester on Chromosorb W (AW - DMCS, 60 - 80 mesh) at 185°C using a Carlo Erba gas chromatograph. Inlet carrier gas (N_2) pressure was 0.7 Kg/cm^2; flowrate 30-40 ml/min. The gases fed to the detector were hydrogen (0.5 Kg/cm^2) and compressed air (1.5 Kg/cm^2).

Electrometer attenuation was set at 1600. Peak areas were measured by the retention time x peak height method of Carroll (1961).

H. Analytical Procedures

Protein was measured by the procedure of Lowry et al (1951); and phosphorus (P) was determined by the procedure of Bartlett as modified by Kates (1972). Acyl-CoA analysis was performed according to the spectrophotometric procedure of Peterson and Bloch (1977).

I. Chromatography

1. Analytical TLC was carried out on 20 x 20 cm plates coated with 0.5 mm layer of silica gel H or 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:

A. Pet-ether-ethyl ether-acetic acid (81:15:1 v/v). for neutral lipids;

B. Chloroform-ethanol (100:1.5 vol.) for argentation TLC;

C. Chloroform-methanol-acetic acid-water (65:25:8:4 vol.) for polar lipids;

D. Chloroform-methanol-acetic acid water (25:15:4:2 vol.) for polar lipids;

E. Chloroform-methanol-water (65:35:5 vol.) for polar

lipids;

F. Chloroform-methanol-water (45:45:15 vol.) for polar lipids;

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

a. Iodine;

b. 40% sulphuric acid in ethanol following by charring the plate;

c. 2',7'-dichlorofluorescein and visualization under u.v. light;

d. Copper-phosphoric acid spray followed by charring;

e. Rhodamine 6G and visualization under u.v. light.

The molybdate reagent, as described by Hack and Ferrans (1959), was used as a specific phospholipid stain.

2. Oxalate-Silica Gel Thin-layer System

The oxalate plates were made with potassium oxalate (1.75 g), silica gel H (E. Merck/Brinkmann, 88 g), water (175 ml), and methanol (1 ml) as described by Ullman and Radin (1972), the coatings on the plate were 0.5 mm thick. After spreading, the plates were air-dried and activated at 120°C for 60 min. before use.

3. 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.

4. Paper Chromatography

Analytical paper chromatography was carried out on Whatman No. 1 filter paper with ascending development in butanol-glacial acetic acid-water (5:2:3 v/v). The lipids were detected by staining with Rhodamine 6 G: the chromatogram was immersed in 0.0012% aqueous solution of Rhodamine 6 G for 1-3 min. and immediately viewed under the UV light (366 nm).

J. Radioisotopic Procedures

1. Labelled Compounds

a) Commercially Available

[1-^{14}C]oleic acid (50 mCi/mmole) and [1-^{14}C]stearic acid (50 mCi/mmole) were purchased from New England Nuclear Corporation. [1-^{14}C]oleoyl CoA and [1-^{14}C]stearoyl-CoA were synthesized as described below.

b) Preparation of Labelled Substrates

i) Chemical Synthesis of 1,2[^{14}C]dioleoylphosphatidylcholine

1,2-[^{14}C]dioleoylphosphatidylcholine was prepared by the method of Warner and Benson (1977). In this procedure fatty acids are first converted to fatty acid imidazolide with carbonyldiimidazole and then reacted with the $CdCl_2$ complex

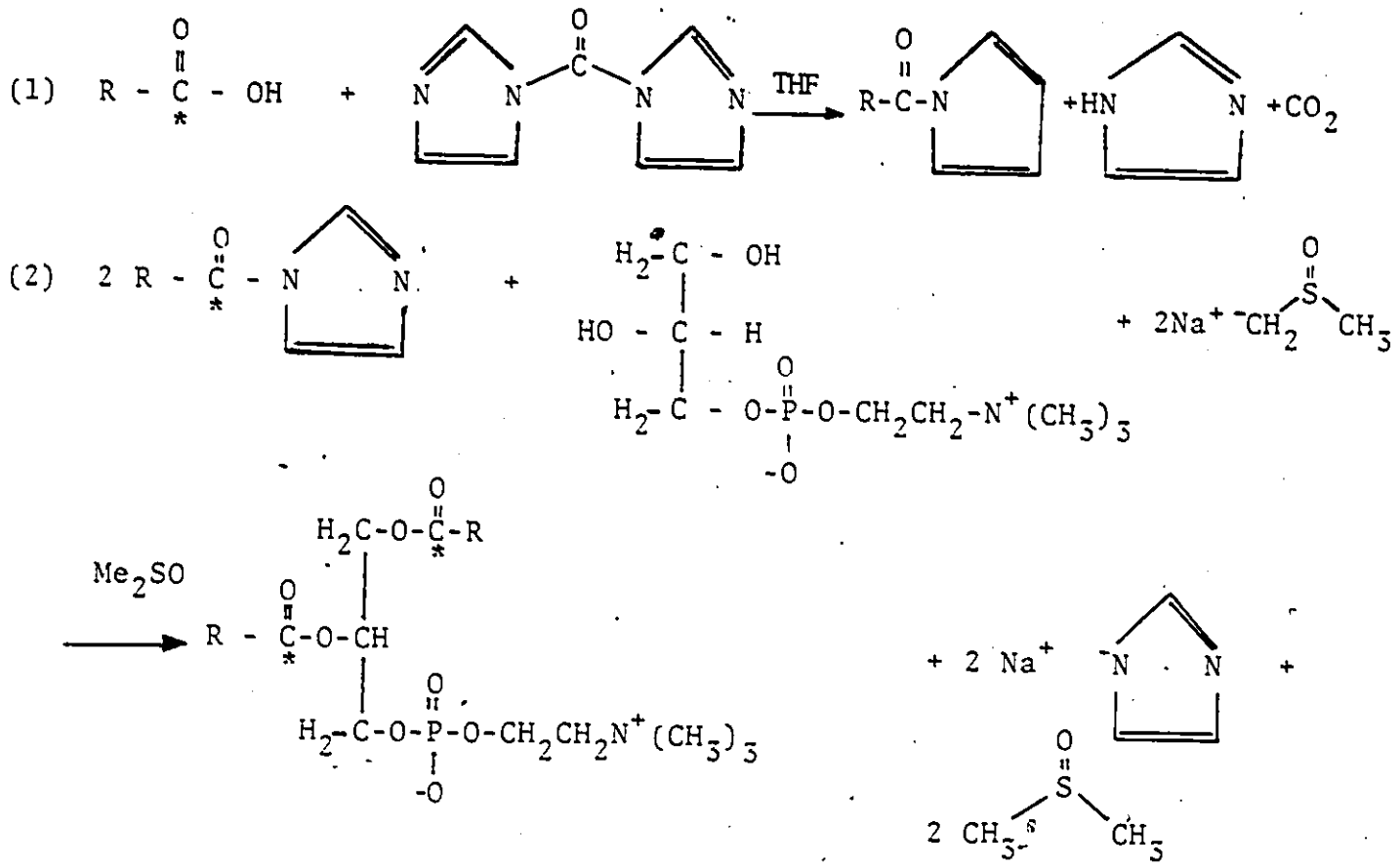
of sn-glycero-3-phosphorylcholine and sodium methylsulfinylmethide in dimethylsulfoxide solvent. A reaction mechanism for this procedure is shown in Scheme IV.

To 40 μmoles of labelled fatty acids was added 49 μmoles of carbonyldiimidazole in 1.0 ml of dry tetrahydrofuran (freshly distilled over $LiAlH_4$). The reaction was allowed to proceed for 45 min. at room temperature under nitrogen with occasional shaking. Anhydrous conditions were maintained throughout the preparation by purging the reaction vessel with dry nitrogen. Upon completion of the reaction the solvent was removed with a stream of nitrogen and the resulting fatty acid imidazolide was dissolved in 1.0 ml of freshly distilled dimethylsulfoxide.

The cadmium chloride complex of sn-glycerophosphorylcholine [$(GPC)_2\ (CdCl_2)_3$, 10.5 μmoles] in 1.0 ml of dimethylsulfoxide was added to the fatty acid imidazolide, followed by the addition of another 1.0 ml dimethylsulfoxide and 1.5 ml of sodium methylsulfinylmethide solution (prepared as a stock solution by reacting 0.37 mmole metallic sodium with 6.9 ml of dimethylsulfoxide; under nitrogen) was slowly added to the rapidly mixing reactants.

The reaction was allowed to proceed at 25°C for 5 min. under nitrogen and was terminated by rapidly immersing the reaction vessel in an ice bath followed by addition of 1.0 ml of 0.4 N HCl to neutralize the sodium. The pH was adjusted to 1.0 with the addition of 5.0 ml of 0.2 N HCl.

SCHEME IV

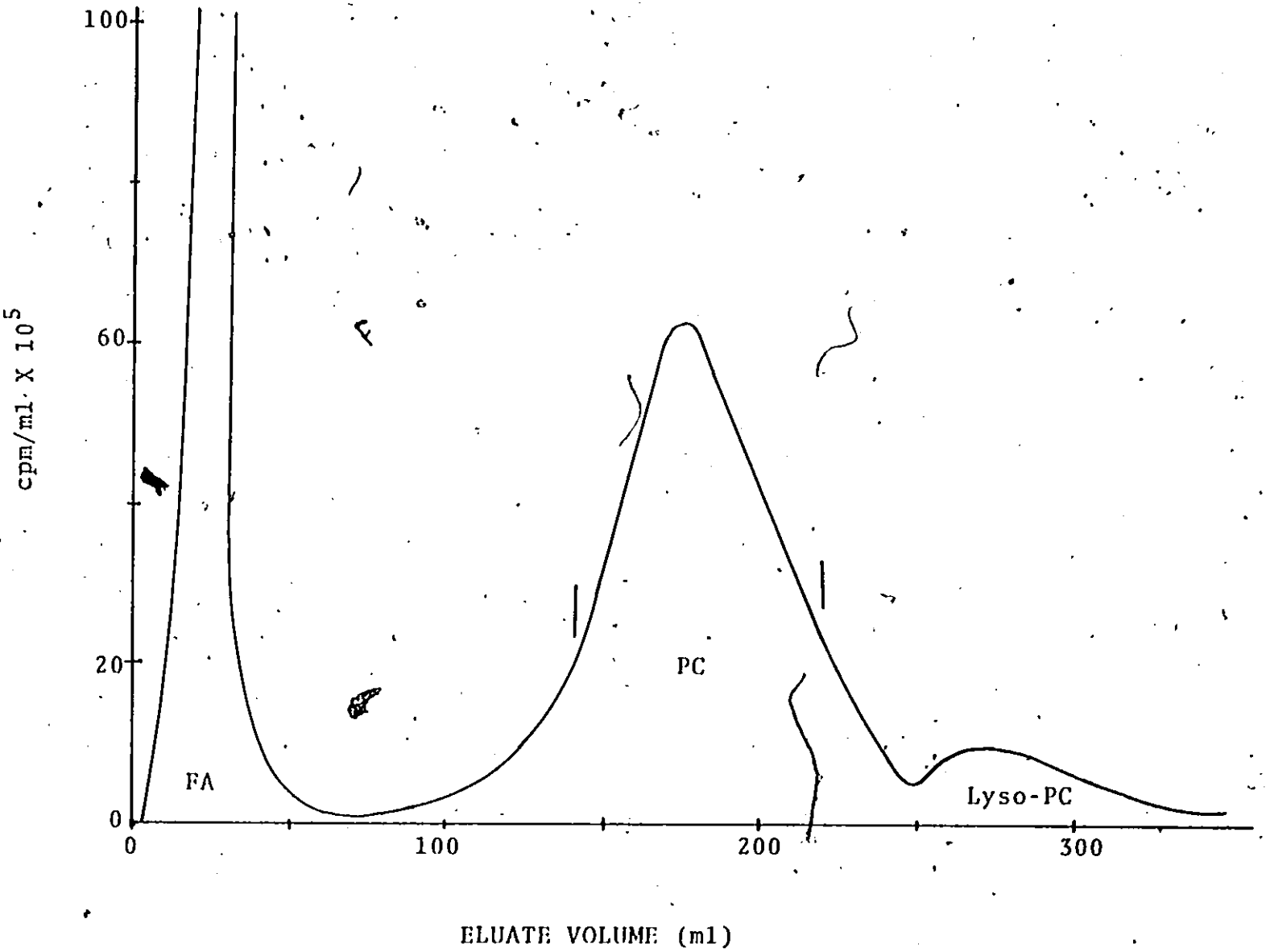


Reaction scheme for the preparation of dioleoyl phosphatidylcholine. Reaction (1), formation of fatty acid imidazolide from carbonyl-diimidazole and fatty acid in tetrahydrofuran (THF). Reaction (2), fatty acid imidazolide is reacted without purification, with the cadmium complex of sn-glycero-3-phosphorylcholine and sodium methyl-sulfinyl-methide in dimethylsulfoxide, (Me_2SO). Experimentally, sodium methylsulfinylmethide in excess of the theoretical amount is required due to the presence of the imidazole that is formed in reaction (1).

The acidified reaction mixture (10.5 ml) was extracted 4 times with 15 ml of $CHCl_3$ and the combined chloroform extracts were concentrated under nitrogen to 1-2 ml. The residual lipid mixture was applied to a small column containing silicic acid (30 g silicic acid/1g lipid) preequilibrated with chloroform. Elution of the column was monitored by determining the radioactivity present in each fraction; the elution profile of the column is shown in Fig. 7. Fractions 1-10 eluting with chloroform and chloroform-methanol (9:1) contained free acids; fractions 28-42 containing 1,2-dioleoyl-phosphatidylcholine were pooled and concentrated under reduced pressure and made to a known volume. Fractions 43-70 were found to contain lysophosphatidylcholine. The yield of 1,2-di[^{14}C]oleoylphosphatidylcholine based on GPC was found to be 60% (4.0 mg of 1,2-dioleoyl-sn-glycero-3-phosphorylcholine; 4.73 µmoles by phosphorus analysis). The material was analyzed by thin-layer chromatography using the solvent system chloroform-methanol-water (65:25:4 v/v) with highly purified egg phosphatidylcholine as a standard. The lipid areas on the plate were visualized with iodine vapor, or the lipid phosphorus spray [procedure of Hack and Ferrans (1959) as described by Kates 1972] and by radioautography. The lipid areas detected by radio-autography were scraped from the plate and the ^{14}C counted in a scintillation counter. The phosphatidylcholine spot was found to contain 99% of the radioactivity and less than

FIGURE 7. Elution profile of 1,2-di[^{14}C]oleoylphosphatidylcholine from a silicic acid column.

FIGURE 7


100
60
20
0
cpm/ml X 10^5
FA
PC
Lyso-PC
0
100
200
300
ELUATE VOLUME (ml)

1% of the ^{14}C was associated with a slower-moving minor component detected only on the autoradiogram.

The estimated specific activity of the 1,2[^{14}C]dioleoyl-phosphatidyl choline obtained was found to be 2.63 μCi/μmole.

ii) Synthesis of [1-^{14}C] 18:1 and [1-^{14}C] 18:0-CoA

The acyl-CoA esters of the above fatty acids were prepared by the method of Al-Arif and Blecher (1969), with slight modification.

The overall reaction is shown in Scheme V.

a. N-hydroxysuccinimide Ester of Stearic or Oleic Acid

50 μmoles of either [1-^{14}C]oleic or [1-^{14}C]stearic acids in chloroform were transferred to a 15 ml stoppered centrifuge tube and the chloroform removed under a nitrogen stream. To the residue was added N-hydroxysuccinimide (50 μmoles) and dicyclohexylcarbodiimide (50 μmoles) and the mixture was flushed with nitrogen, stoppered, and left overnight at room temperature. After removal of the white crystals of dicyclohexylurea by centrifugation at 2,000 xg for 2 min., the supernatant was transferred into 50 ml round bottom flask and the solvent was evaporated under reduced pressure. The N-hydroxysuccinimide esters of fatty acids were obtained as stable crystalline compounds in good yield. The crystals were dissolved in 4.0 ml of tetrahydrofuran (freshly distilled over $LiAlH_4$) and used immediately.

SCHEME V

A. $R - COOH + HON\langle CO-CH_2 \rangle + C_6H_{11}N = C = NC_6H_{11} \longrightarrow R - COO - N\langle CO-CH_2 \rangle + C_6H_{11}NH - CO-NHC_6H_{11}$

I II III IV V

The fatty acid (I) is condensed with N-hydroxysuccinimide (II) in the presence of dicylohexylcarbodiimide (III). Dicyclohexylurea,(V), which precipitates out, is removed by centrifugation at 2,000 xg for 1-2 min., and the N-hydroxysuccinimide esters of fatty acids (IV) are obtained as stable crystalline compounds in good yields.

B. $R - COO - N\langle CO - CH_2 \rangle + CoA - SH \xrightarrow{NaHCO_3} CoA-S-\overset{O}{\overset{\|}{C}}CR + HN\langle CO - CH_2 \rangle$

IV VI VII VIII

N-hydroxysuccinimide esters of fatty acids (IV) are reacted with reduced CoA (VI) in the presence of bicarbonate to produce the acyl-CoA (VII) and succinimide (VIII).

b. Reduction of Coenzyme A With Sodium Amalgam

To a solution of coenzyme A (30 μmoles) in a 15.0 ml centrifuge tube was added 0.5 ml of 0.5% sodium amalgam (0.5 g of sodium metal in 100 g of mercury). The onset of the reaction was marked by a rapid evolution of hydrogen gas and it was allowed to proceed at room temperature for 10 min. The pH was maintained near neutrality by adding a few grains of the H^+ - form of dowex-50; upon completion of the reduction, the coenzyme A solution was removed and made to a volume of 2.0 ml (with water).

c. Synthesis of Acyl-CoA's

Sodium bicarbonate (84 mg) was added to the solution (2.0 ml) of the reduced coenzyme A and the mixture was added to the 4.0 ml of tetrahydrofuran containing the N-hydroxy succinimide esters of the fatty acids. The one-phased mixture was agitated by a stream of nitrogen gas and the reaction allowed to proceed for 4 hours. Since it is imperative that the reaction mixture remain a single phase throughout the reaction in order to get the product in reasonable yield, it was necessary to add additional tetrahydrofuran to maintain homogeneity.

After the reaction was complete 6.0 ml of 5% $HClO_4$ was added to precipitate the acyl-CoA, excess N-hydrosuccinimide esters and the free fatty acids. To achieve complete precipitation of these compounds, the THF was removed under reduced pressure in a rotary evaporator and the tube, flushed

with N_2 and stoppered, was left on ice for 30 min. The precipitate was collected by centrifugation (at 2,000 x g for 2 min) and washed 3 times with 5.0 ml of 0.8% $HClO_4$, 4 times with 5.0 ml of acetone and 4 times with 5.0 ml of ethyl ether. The residue was dried under nitrogen and extracted twice with 2.5 ml of phosphate buffer (pH 5.9).

The product had the following properties:

a) UV spectra were identical with those of corresponding commercial standards.

b) The UV spectrum measured against a reference solution of coenzyme A showed a peak at 232 µm, which disappeared after treatment with methanolic NaOH, indicating hydrolysis of the thiol ester bond.

c) Chromatography on Whatman No. 1 filter paper in butanol glacial acid-water 5:2:3 v/v revealed a single spot which co-migrated with authentic standards.

d) Ratio of absorbances, A_{232}/A_{257} was 0.6 (theoretical ratio 0.55); assuming an E_{max} 15.4 mM^{-1}, the yield was found to be 30%, based on CoA.

e) Autoradiography of the thin-layer chromatogram on oxalate-impregated silica gel H revealed the presence of a single spot which co-migrated with authentic standards and accounted for 97% of the ^{14}C; 3% of the total radioactivity was found to be associated with the trans-isomer of the respective fatty acid.

2. 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 obtained from 10% $AgNO_3$ impregnated TLC plates and visualized with 2',7'-dichlorofluorescein under U.V. light, were scraped directly into scintillation fluid prepared without the solubilizer and counted as described above.

3. 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; 2 days for 1,000 dpm per spot on the TLC plates.

The film was developed and fixed by standard photographic

procedures.

K. Kinetic Studies of 18:1-CoA Desaturase Activity

The assay system used for the kinetic studies was as described in section II D, except that the total volume of the reaction mixture was increased to 1.0 ml and the substrate 18:1-CoA (58.5 nmoles) had a higher specific activity (6.85 μCi/μmole); all other reagents had final concentrations given in section II.D. 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 vortexer for a few seconds and subsequently centrifuged at 2,000 x g 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 once with 1.9 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.

a) Analysis of Methanol-Water Phase

To the upper phase (6.0 ml) was added 0.3 ml of 40% aqueous KOH and the mixture was saponified at 70°C for one

hour. After cooling 0.5 ml of concentrated HCl was added a biphasic system was formed by the addition of chloroform (5 ml) and methanol (0.9 or 1.0 ml). The chloroform phase was transferred into clean glass-stoppered centrifuge tubes and the upper methanol-water phase was washed with chloroform (3-4 ml) 3 to 4 times. The combined chloroform extracts, was concentrated under a stream of N_2 with addition of benzene to remove water, and made to a known volume: an aliquot was taken for counting which showed that all of the radioactivity, originally present in acyl-CoA was recovered as free fatty acid in the chloroform phase. The remaining solution was concentrated under a stream of N_2 and the residue was heated under reflux in 2 ml of 2.5% methanolic HCl for 2 hours at 70-75°C. The fatty acid methyl esters (FAME) were extracted and chromatographed on 10% $AgNO_3$ silica gel G plates as described in section II.I.3

b) Analysis of Chloroform Phase

The chloroform phase was made up to 5.0 or 10.0 ml with chloroform-methanol (2:1) and 50 to 100 µl were taken for counting. The remainder of the chloroform solution was divided into three portions: a) For estimation of ^{14}C distribution among lipid compounds, 1.0 ml was concentrated to 0.1 ml and spotted on TLC silica gel H plates (0.5 mm thick) and which were developed in chloroform-methanol-water (65:35:5 v/v) alongside authentic standards (PC, PE, PS, PI, FAME, FFA) (Fig. 13); the spots were detected by autoradio-

graphy, scraped from the plate and counted. b) For determination of total desaturation, 2.0 ml of chloroform phase was concentrated under a stream of N_2 and the residue was heated under reflux in 2.0 ml of 2.5% methanolic-HCl for one hour at 70-75°C; the FAME were extracted and analysed on 10% $AgNO_3$ silica gel G plates as described in section II.F. c) For estimation of [^{14}C]18:2 in each lipid class, the remainder of the chloroform phase was concentrated to 0.1 ml and spotted on silica gel H, 0.5 mm thick, developed as described in a) (see Fig. 14); the phospholipid spots were scraped into glass-stoppered tubes and heated under reflux in 2.0 ml of 2.5% methanolic-HCl for one hour at 70-75°C; the FAME were extracted and analysed on 10% $AgNO_3$ silica gel G plates as described in section II.F. The neutral lipids, running with the solvent front, were immediately scraped into 15.0 ml glass-stoppered centrifuge tubes and extracted by the Bligh and Dyer procedure (1959); the chloroform extracts were concentrated to 0.1 ml and spotted on TLC silica gel G (0.5 mm thick) and re-developed in petroleum ether: ethyl ether:acetic acid (81:15:1 v/v) along with authentic standards (FAME, TG, DG, FFA). The radioactive spots FAME + FA and TG + DG were scraped into glass-stoppered tubes and heated under reflux in 2.0 ml of 2.5% methanolic-HCl for one hour at 70-75°C; the fatty acid methyl esters were extracted and analysed on 10% $AgNO_3$ silica gel G plates as described above.

Results

I. General Properties of 18:1-CoA Desaturase

a) Localization of Desaturase System

Preliminary studies revealed that the desaturase activity was distributed between the 105,000 x g pellet (heavy microsomal fraction, 12% of total activity) and the 105,000 xg supernatant fluid still containing a considerable amount (94%) of particle-bound enzyme activity (Table IIA) which could be sedimented on further centrifugation at 160,000 x g for 2 hours (Table IIB). The 160,000 x g fraction was found to contain a higher proportion (33%) of total 18:1-CoA desaturase activity, and the highest specific activity (Table IIB). The 160,000 x g supernatant still contained a high proportion of activity (62%), but with a low specific activity; the 160,000 x g supernatant activity could not be sedimented by further centrifugation at 200,000 x g.

In all subsequent studies the final centrifugation step was performed at 160,000 x g rather than 105,000 x g, and the pellet obtained was used as the enzyme preparation.

b) Assay Conditions

With the above enzyme preparation the rate of desaturase of 18:1-CoA was found to be linear up to 1.0 mg (Fig. 8). Further, the rate of desaturation was linear with increasing substrate (18:1-CoA) concentrations up to 60 µM without an apparent saturation being reached (Fig. 9A). Lineweaver-Burk

TABLE II

Sub-cellular Distribution of 18:1-CoA Desaturase Activity in the Mesophilic Yeast C. lipolytica

A.

Cell Fraction	Protein		Synthesis of Linoleic Acid		
	mg	%	Activity nmoles/min	%	Specific Activity pmoles/min/mg
A. EXPERIMENT 1					
Cell Free Homogenate	273.3	100	189.5	100	693.4
Mitochondria	32.8	12.0	1.1	0.6	33.5
Microsomal Pellet (105,000 x g)	70.1	25.7	23.1	12.2	329.5
Supernatant at 105,000 x g	172.5	63.1	179.5	94.7	1,040.6[a]
% Recovery		100.8		107.5	
B. EXPERIMENT 2					
Cell Free Homogenate	461	100	300.1	100	651.0
Mitochondria	93.9	20.4	3.7	1.2	39.4
Microsomal Pellet 160,000 x g	57.1	12.4	100	33.3	1,751.3
Supernatant at 160,000 x g	310.0	67.3	186.4	62.1	601.3[a]
% Recovery		102.1		96.6	

[a]Specific activity of supernatant fractions are not considered valid because of the high content of soluble protein.

FIGURE 8. Desaturation of oleoyl-CoA by C. lipolytica microsomes as a function of microsomal protein concentration. The incubation mixture contained per 0.5 ml: 100 mM phosphate buffer (pH 7.2), 10 mM NADH, 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM CoA, 54.3 µmoles 18:1-CoA and increasing concentration of microsomal protein. Incubation time, 15 min.

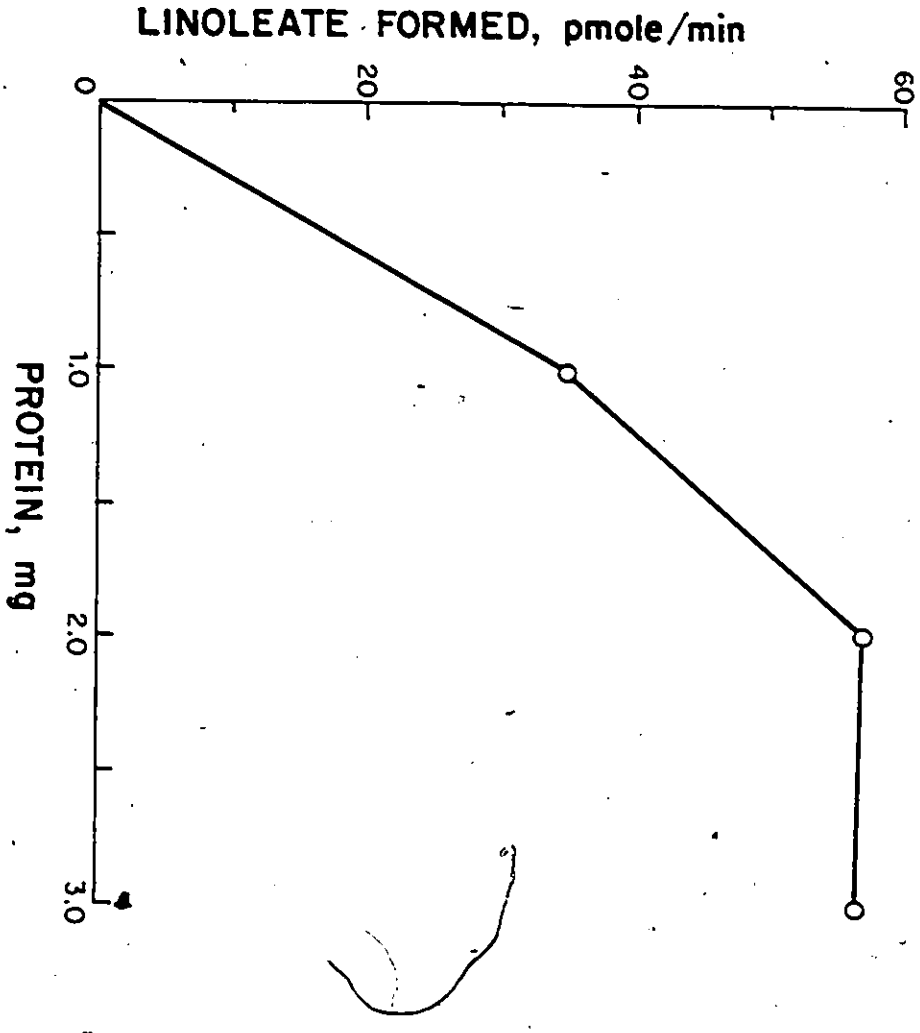
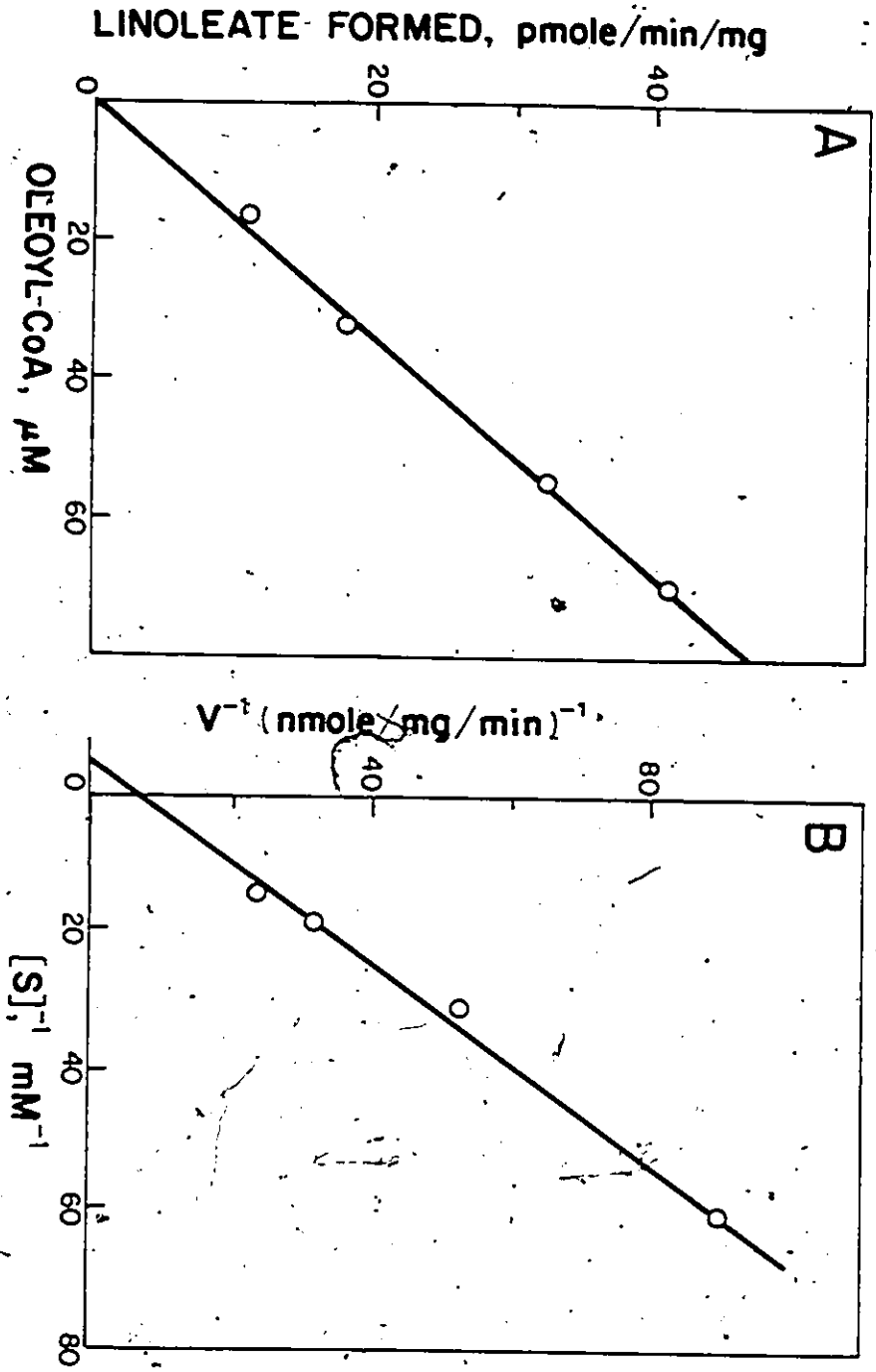

LINOLEATE FORMED, pmole/min
0
20
40
60
PROTEIN, mg
1.0
2.0
3.0

FIGURE 9. Oleoyl-CoA desaturase activity of microsomal membranes of C. lipolytica grown at 25°C as a function of oleoyl-CoA concentration. A, plot of V against S; B, Double-reciprocal plot (Lineweaver-Burk). Incubation mixture was as described in Fig. 8 using 1.0 mg protein.


LINOLEATE FORMED, pmole/min/mg
0
20
40
A
OLEOYL-CoA, μM
20
40
60
V⁻¹ (nmole/mg/min)⁻¹
0
40
80
B
[S]⁻¹, mM⁻¹
20
40
60
80

plot indicated an apparent Km of 250 μM, a value similar to that previously reported by Pugh and Kates (1975) for 1-acyl-2-oleoyl-phosphatidylcholine as substrate.

c) Optimal Assay Conditions

Throughout these studies, except were otherwise indicated, 1.0 mg of microsomal protein and about 50 nmole of substrate were used in the assay mixture as described under section II.D.

II. Effect of Cell Aeration on Desaturase Activities

The percentage of the relative amount of linoleic acid was found to incrase by 14.5% on increasing the rate of aeration of the cell culture (shaking speed, 70 rpm to 130 rmp) while that of oleic acid decreased by 11.2% (Table III) corresponding to a doubling of the molar ratio of 18:2/18:1 from 0.81 at 70 rpm to 1.62 at 130 rpm. The 18:0-CoA desaturase activity showed no significant change with change in shaking speed, (Table IV) while the lecithin desaturase activity showed a smaller increase (22%) with increased shaking speed from 120 rpm to 130 rpm. The 18:1-CoA desaturase activity in contrast showed a pronounced increase with increased shaking speed paralleling the increase in 18:2/18:1 ratio.

III. Changes in Fatty Acid Composition During Growth at 10° and 25°C

Microsomes from cells grown at 10° were found to have a higher fatty acid content than that from cells grown at 25°C.

TABLE III

Fatty acid composition of microsomal lipids from C. lipolytica grown at different aeration rates. A 40 hour subculture was inoculated into 1 litre fresh medium in a 4 litre flask. The ratio of inoculum volume to fresh medium volume was 1:100. Cells were grown at 25°C and harvested at 0.46 - 0.47 O.D.

Shaking Speed	Time	O.D. 680	Fatty acid composition (%)							Molar ratio
rpm	h		16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:2/18:1
70	13	0.46	14.0	9.7	0.7	1.0	2.5	40.0	32.2	0.81
120	12	0.47	12.6	7.4	0.6	1.1	2.5	36.4	39.5	1.10
130	10	0.47	13.0	7.7	0.9	0.9	2.1	28.8	46.7	1.62

TABLE IV

Desaturase activities of microsomes from C. lipolytica grown at different aeration rates

Shaking speed (rpm)	Growth (hr.)	O.D., 680 nm	Molar ratio 18:2/18:1	SPECIFIC ACTIVITY (pmoles/min/mg		
				18:0-CoA Desaturase	18:1-CoA Desaturase	dioleoyl-PC Desaturase
70	13	0.46	0.81	1,077	83	32
120	12	0.47	1.10	1,043	109	32
130	10	0.47	1.62	1,085	140	41

(Table V). At both temperatures the major fatty acids identified were linoleic (18:2), oleic (18:1), palmitic (16:0), and palmitoleic (16:1); smaller amounts of stearic (18:0), heptadecenoic (17:1), and trace amounts (<1%) of myristic (14:0), heptadecanoic (17:0) and pentadecanoic (15:0) Acid were also present (Table III,V). Microsomes prepared from cells grown at 10°C had an overall higher degree of fatty acid unsaturation than microsomes whose cells had been grown at 25°C. The level of 18:2 acid increased from the inoculum value of 27% to a maximum of 55% early in the growth phase (4.5 hr.) while a concurrent decrease in 18:1 acid from 41.5% to a minimum of 19% occurred (Fig. 10A). After 4.5 hr. linoleic acid decreased slowly to 26.5% (32 hr.) while oleic acid increased proportionately to 41.8% (32 hr.). No other significant changes in fatty acids were observed other than a relatively small increase in palmitic and stearic acids. At 10°C the same general pattern was observed except that the maximum of linoleic acid was 5% higher at 10° than 25°C and higher levels of 18:2 acid were maintained throughout the growth phase (Fig. 10B). The double bond index, usually used as a measure of membrane unsaturation, changes from an early high value of 9.7 at 4.5 hr. to a low 5.2 at 32 hr. (25°C); (Table V). The double bond index, from 10°C-microsomal membranes, changed from an early growth high of 14.3 at 37 hr. to a low of 12.1 at 72 hr. (Table VI). In absolute amounts (nmoles per mg protein) both 18:1 and 18:2 acids in 25°C microsomal membranes showed simultaneous increases in the

TABLE V

Fatty acid composition of microsomes from 25°C grown cells

Optical Density[a]	Time Hrs.	Fatty Acid Content (nmoles/mg protein)[b]						Fatty Acid Proportions[b] moles %						
		16:0	16:1	17:1	18:0	18:1	18:2	16:0	16:1	17:1	18:0	18:1	18:2	DBI[c]
0.22	4.5	39	31	3.3	4	57	163	13.1	10.4	1.1	1.3	19.1	55.0	9.7
0.32[b]	8	59	41	4.5	9	151	217	12.3	8.4	1.0	1.8	31.1	45.1	9.3
0.40	10	82	54	4.5	11	195	257	13.5	9.0	0.8	1.8	32.4	42.6	8.3
0.47[b]	12	98	63	6.7	11	273	300	13.1	8.3	0.9	1.4	36.3	39.9	8.6
0.62	16	108	66	7.1	18	317	260	13.9	8.5	0.9	2.3	40.8	33.6	7.3
0.81[b]	22	119	82	6.7	19	336	255	14.6	10.0	0.8	2.3	41.1	31.2	6.8
0.93	28	114	81	6.3	19	309	239	14.9	10.5	0.8	2.4	40.3	31.3	6.6
1.00	32	121	76	5.6	25	300	190	16.9	10.6	0.8	3.4	41.8	26.5	5.2
1.30	40	144	122	12.3	35	414	272	14.5	12.2	1.2	3.5	41.5	27.1	6.1

[a]Optical densities of samples taken at various points in the growth cycle were reproducible to ± 0.01 to 0.02 OD units.

[b]Values are given as averages of 2 determinations; deviations from the means were <1%. All samples also contained <1% of 14:0, 15:0 and 17:0 Fatty Acids.

[c]DBI = Double Bond Index = moles of double bonds: moles of saturated fatty acids.

TABLE VI

Fatty Acid Composition of Microsomes from 10° Grown Cells

Optical Density	Time Hrs.	Fatty Acid Content (nmoles/mg protein)[b]						Fatty Acid Proportions[b] moles %						
		16:0	16:1	17:1	18:0	18:1	18:2	16:0	16:1	17:1	18:0	18:1	18:2	DBI[c]
0.17	37	91	57	2.6	11	222	598	9.3	5.8	0.3	1.1	22.6	60.9	14.3
0.29	42	102	66	3.7	10	222	603	10.1	6.6	0.4	1.0	22.1	59.9	13.4
0.40	47	117	76	3.0	10	260	638	10.6	6.9	0.3	0.9	23.5	57.8	12.7
0.55[d]	50	77	53	3.0	11	180	447	10.0	6.9	0.4	1.4	23.3	58.0	12.9
0.60	53	121	81	3.4	11	282	658	10.4	7.1	0.3	0.9	24.4	56.9	12.8
0.76	59	131	109	4.1	10	359	648	10.4	8.6	0.3	0.8	28.5	51.4	12.6
1.05	72	158	153	5.6	8	498	679	10.5	10.2	0.4	0.6	33.1	45.2	12.1

[a] Optical densities of samples taken at various points in the growth cycle were reproducible to ± 0.01 to 0.02 OD units.

[b] Values are given as averages of 2 determinations; deviations from the means were <1%; all samples also contained <1% of 14:0, 15:0, and 17:0 fatty acids.

[c] DBI = Double Bond Index = Moles of Doubles: Moles of saturated fat.

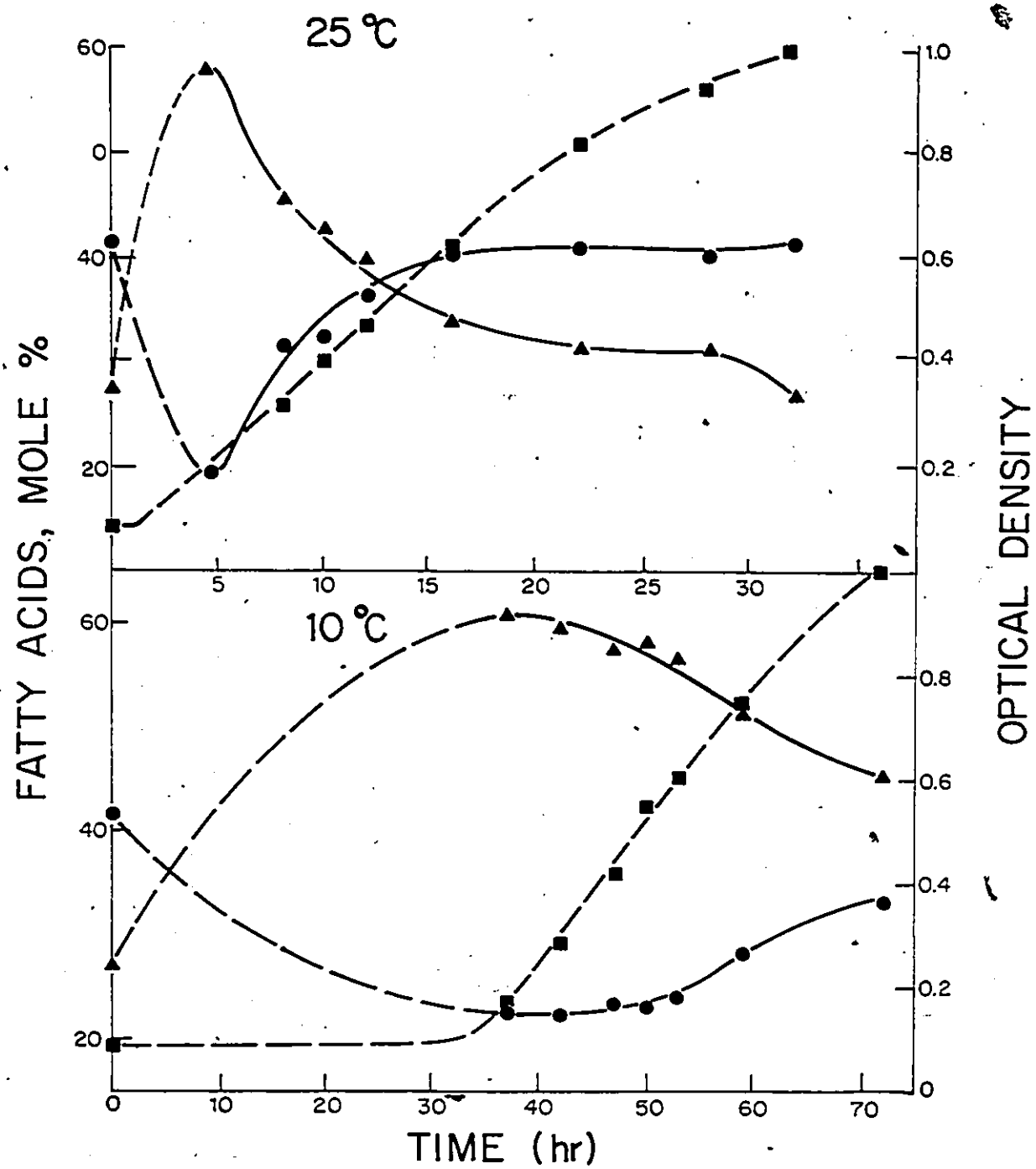
[d] Values for this sample are not considered to be reliable.

FIGURE 10. Changes in 18:1 and 18:2 Acid Percentage Composition of Microsomal Membranes as a Function of Growth of Candida lipolytica at (A), 25°C and (B), 10°C. Culture conditions as given in the text (Material and Methods, II.2).

■-----■ Growth Curve

▲——▲ Linoleic Acid

●——● Oleic Acid


25 °C
10 °C
FATTY ACIDS, MOLE %
OPTICAL DENSITY
TIME (hr)
60
0
40
20
1.0
0.8
0.6
0.4
0.2
5
10
15
20
25
30
0
10
20
30
40
50
60
70

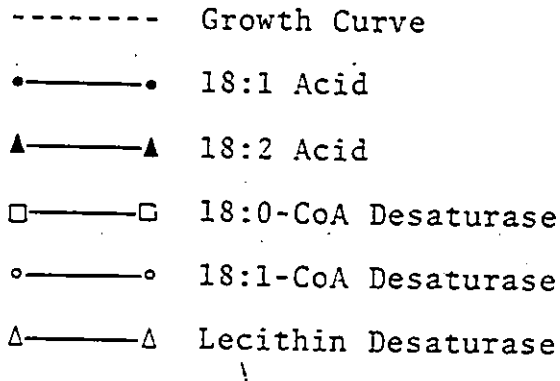
early stages of growth (Fig. 11A), but the amounts of 18:2 were always greater than 18:1 up to 12 hr of growth; Thereafter 18:1 continued to increase to a constant level at 22 hr while 18:2 decreased to a reciprocally constant lower level at 22 hours.

The absolute amounts of 18:2 and 18:1 in 10°C-microsomal membranes showed extensive reciprocal change with respect to the inoculum values (Fig. 12A) in early log-phase (up to 37 hours); thus, 18:2 increased by 53% while 18:1 decreased by 46% during this period. After 37 hours, the absolute amounts of 18:2 acid increased very slowly to a maximum level in the stationary phase, while 18:1 increased much more rapidly giving a 18:2/18:1 ratio of 1.4 compared to 2.7 at 37 hours. Overall, the degree of unsaturation is higher at 10° than 25°C and the decrease in the double bond with time is much slower at 10°C than 25°C.

IV. Effect of Temperature on Desaturase Activity

Since the proportions of oleic and linoleic acid in microsomes of C. lipolytica change reciprocally in the log-phase, a study was undertaken to determine whether these changes were related to changes in activities of desaturases during growth. Microsomes were prepared from cells grown for 4 to 32 hours at 25°C and from 24 to 80 hours at 10°C, at a shaking speed of 120 rpm and the specific activities of stearoyl-CoA, oleoyl-CoA, and 1,2-[^{14}C]dioleoylphosphatidyl choline, desaturases were determined. In microsomes from

FIGURE 11. Changes in A, content of 18:1 and 18:2 acids and B, desaturase activities, as a function of growth in microsomal membranes prepared from cells grown at 25°C.



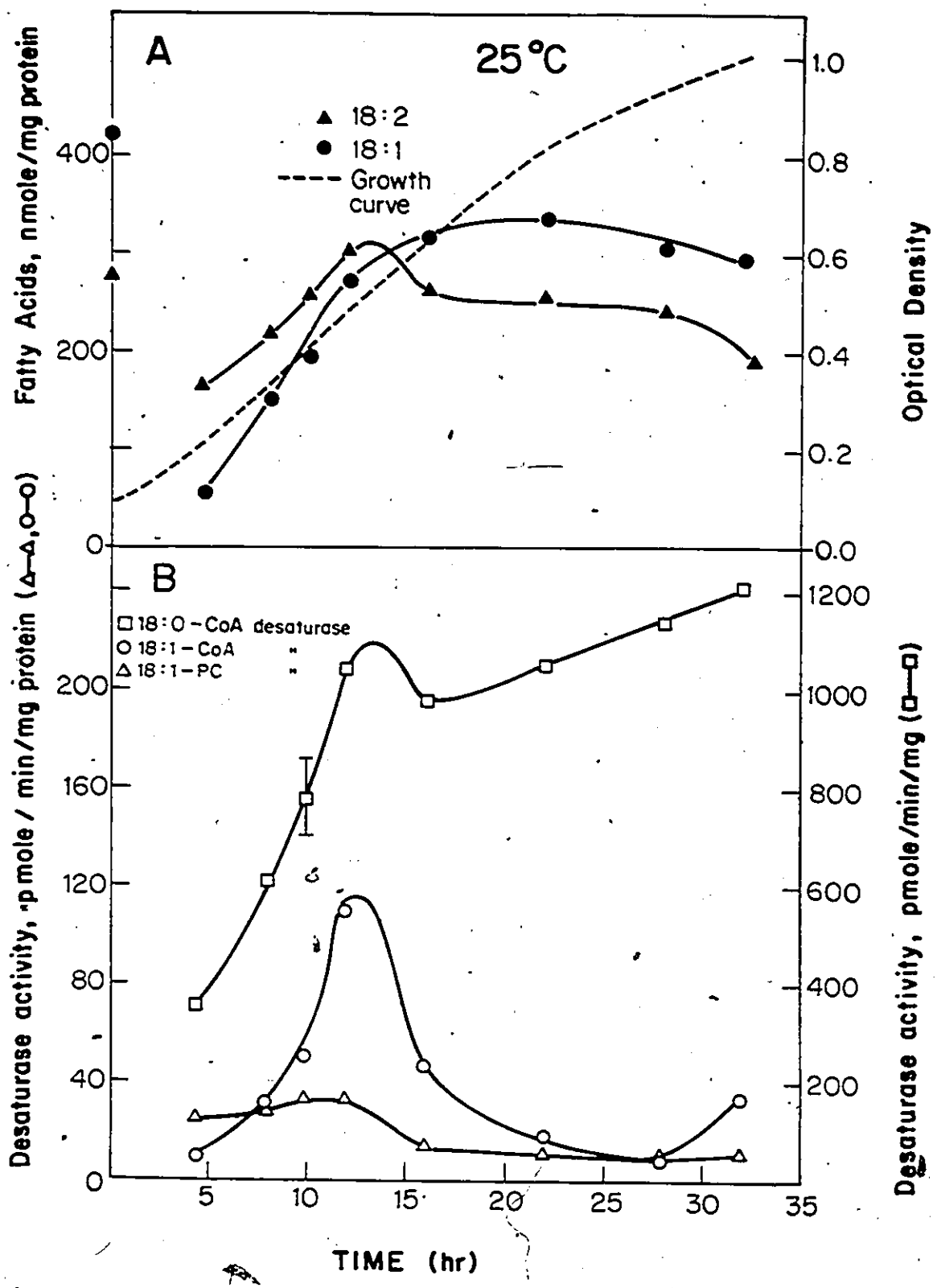

A
25°C
18:2
18:1
Growth curve
Fatty Acids, nmole/mg protein
Optical Density
400
200
0
1.0
0.8
0.6
0.4
0.2
0.0
B
18:0-CoA desaturase
18:1-CoA "
18:1-PC "
Desaturase activity, pmole/min/mg protein (Δ—Δ, O—O)
Desaturase activity, pmole/min/mg (□—□)
200
160
120
80
40
0
1200
1000
800
600
400
200
5
10
15
20
25
30
35
TIME (hr)

FIGURE 12. Changes in A, content of 18:1 and 18:2 acids and B, desaturase activities, as a function of growth in microsomal membranes prepared from cells grown at 10°C.

----- Growth Curve

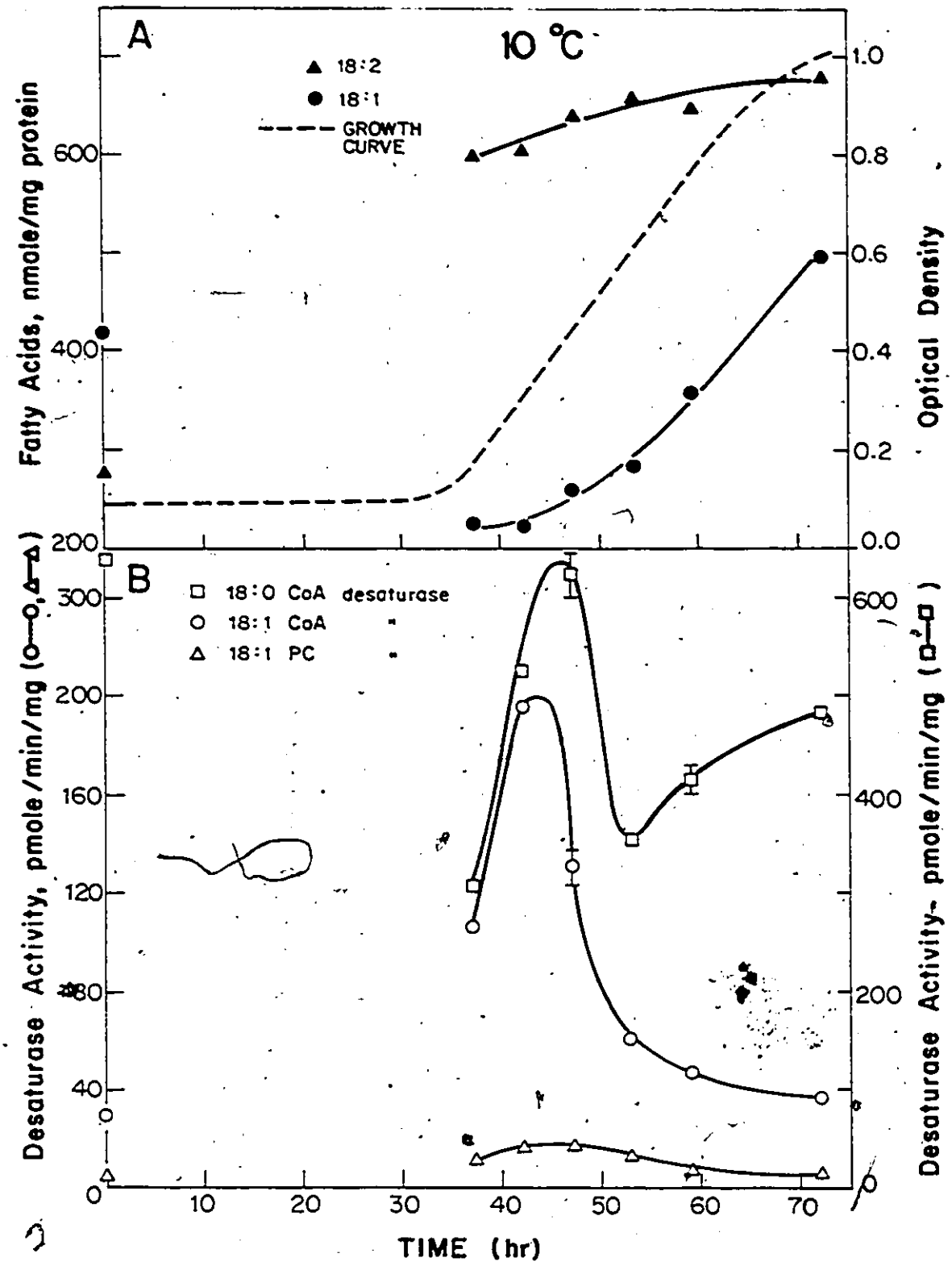
•—• 18:1 Acid

▲—▲ 18:2 Acid

□—□ 18:0-CoA Desaturase

○—○ 18:1-CoA Desaturase

Δ—Δ Lecithin Desaturase


A
10 °C
▲ 18:2
● 18:1
GROWTH CURVE
Fatty Acids, nmole/mg protein
Optical Density
600
400
200
1.0
0.8
0.6
0.4
0.2
0.0
B
□ 18:0 CoA desaturase
○ 18:1 CoA "
△ 18:1 PC "
Desaturase Activity, pmole/min/mg (○—○, △—△)
Desaturase Activity- pmole/min/mg (□—□)
300
200
160
120
80
40
0
600
400
200
10
20
30
40
50
60
70
TIME (hr)

25° cells, specific activity of 18:0-CoA desaturase increased abruptly from a low value of 358 pmoles/min/mg very early in log-phase (4.5 hours) to a plateau of 1,043 pmoles/min/mg at mid-log phase (12 hours) and then gradually increased further to 1,214 pmoles/min/mg at stationary phase 32 hours (Fig. 11B). In microsomes of 10° cells, the specific activity of the 18:0-CoA desaturase showed a maximum in early log-phase (47 hours, Fig. 12B) which was 50% lower than that in 25° cells, then reached a minimum at 53 hours and then gradually increased again at the stationary phase. The generally high activity of the 18:0-CoA desaturase during the growth cycle explained the low levels of stearic acid found in C. lipolytica at 25°C or at 10°C.

The 18:1-CoA desaturase in microsomes of 25° cells undergoes an increase in specific activity from an early low value of 8.7 pmoles/min/mg in early log phase 4.5 hours to a maximum of 109 pmoles/min/mg in mid-log phase (12 hours) and decreasing to low value in stationary phase (Table VII, Fig. 11B).

In microsomes of 10° Cells changes in 18:1-CoA desaturase activity were similar to those in 25° microsomes except that the maximum activity was double (200 pmoles/min/mg) that of the 25°C microsomes (109 pmoles/min/mg) and occurred earlier in the growth phase. The lecithin desaturase in 25° microsomes reached its maximum specific activity somewhat earlier in the growth curve than the 18:0-CoA and 18:1-CoA desaturase and the maximum activity of the lecithin desaturase was twice that in 10° microsomes.

TABLE VII

Summary of data on changes in 18:1 and 18:2 levels and on 18:1-CoA, 18:0-CoA and PC-desaturase activities as a function of growth of C. lipolytica at 25°C

Time Hrs.	Optical Density	Fatty Acid mole %		Specific Activity (pmoles/min/mg)[a]		
		18:1	18:2	18:1-CoA	18:0-CoA	18:1 - PC
4.5	0.22	57	163	9	358	24
8	0.32	151	217	31 ± 0.2	609 ± 5.0	27 ± 0.3
10	0.40	195	257	51 ± 2.1	790 ± 79.5	32 ± 0.8
12	0.47	273	300	109	1,043	32
16	0.62	317	260	47	980	13
22	0.83	336	255	18 ± 0.5	1,054 ± 11.0	10 ± 0.2
28	0.93	309	239	9	1,141	12
32	1.00	300	190	34	1,214	11
40	1.30	414	271	28	700	2

[a]Where indicated data given are averages ± deviation from means of 2 independent assays all other values are for single determinations.

TABLE VIII

Summary of data on changes in 18:1 and 18:2 levels and on 18:1-CoA, 18:0-CoA and PC-desaturase activities as a function of growth of C. lipolytica at 10°C

Time Hrs.	Optical Density	Fatty Acid mole %		Specific Activity (pmoles/min/mg)[a]		
		18:1	18:2	18:1-CoA	18:0-CoA	18:1 - PC
37	0.17	22.6	60.9	107.3	308.2	11.0
42	0.29	22.1	59.9	195.8	524.7	15.6
47	0.41	23.5	57.8	131 ± 9	625.3 ± 29.1	17.4 ± 1.3
50[b]	0.55	23.3	58.0	61.2	372.5	16.1
53	0.60	24.4	56.9	60.9	355.4	12.8
59	0.76	28.5	51.4	46.9 ± 3.2	416.0 ± 14.4	7.3 ± 0.7
72	1.05	33.1	45.2	35.8	482.6	7.1

[a]Where indicated data given are averages ± deviation of means of 2 independent assays all other values are for single determinations.

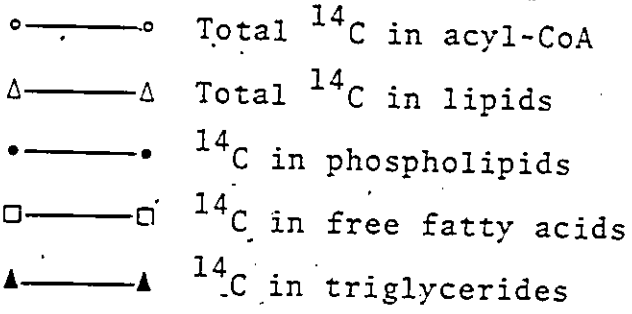
[b]Values for this sample were considered to be unreliable.

V. Kinetics of 18:1-CoA Desaturase Activity

To investigate the possibility that desaturation of 18:1 occurred after transacylation from 18:1-CoA into phospholipids, we studied the distribution of 18:1 among the lipid components and its desaturation as a function of time in microsomes of Candida lipolytica. In these experiments, after incubation of microsomes with [^{14}C] oleoyl-CoA, the mixtures were extracted with a biphasic system according to Bligh and Dyer (1959), in which the upper methanol-water phase, contained all of the (water-soluble) acyl-CoA present while the lower chloroform phase contained the complex lipids and free fatty acids. By following the distribution of ^{14}C in each of the phases, it was found that oleoyl-CoA was rapidly converted to free fatty acids in absence of ATP and Mg^{2+} due to the action of an acyl-CoA thiolase and only about 14% of the initial substrate was incorporated into total phospholipids in 30 min. (Fig. 13).

The circumvent the thiolase reaction, ATP, $MgCl_2$, and CoA were added to the reaction mixture and this resulted in a decrease in the extent of acyl-CoA hydrolysis from 70% to about 10% in 30 min., while the extent of ^{14}C incorporated into the phospholipids increased from 14% to 31% in 30 min. (Fig. 14 and 15). Note that the free fatty acids were released almost entirely in the first 2 minutes of the reaction and thereafter remained essentially constant (Fig. 14) in contrast to the rapid and continuous release of fatty acids in absence of ATP and Mg^{2+} (Fig. 13). In the

FIGURE 13. Distribution of [^{14}C] in acyl-CoA and acyl lipids during incubation of [1-^{14}C]oleoyl-CoA with microsomes of C. lipolytica grown in the mid-log phase at 25°C. Incubations were performed in the presence of NADH as described in section II.K. with the exception that ATP, $MgCl_2$ and CoA were omitted.



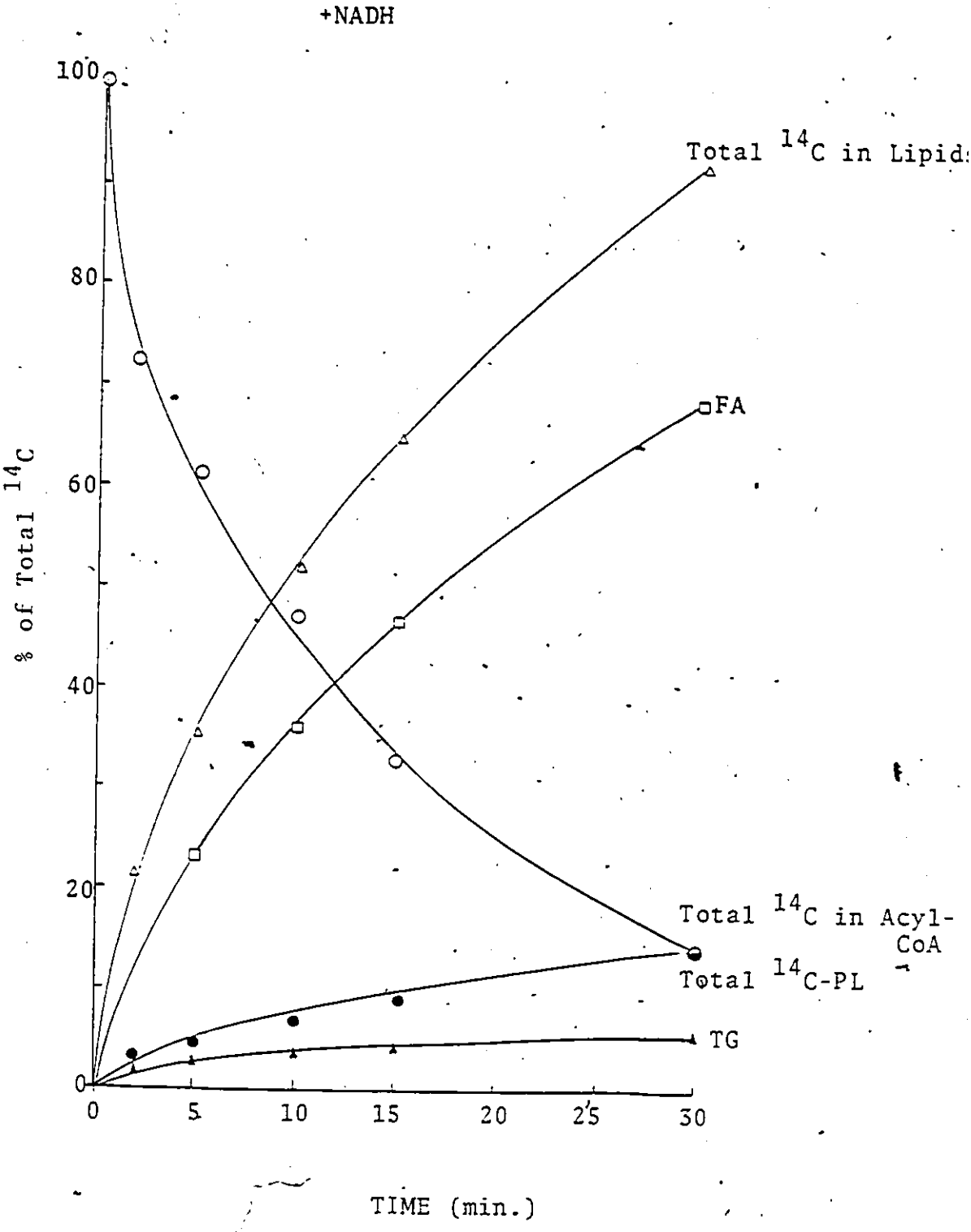

+NADH
100
80
60
40
20
0
% of Total ^{14}C
Total ^{14}C in Lipid
FA
Total ^{14}C in Acyl-CoA
Total ^{14}C-PL
TG
0
5
10
15
20
25
30
TIME (min.)

FIGURE 14. Autoradiogram of [^{14}C]acyl lipids during incubation of [1-^{14}C]oleoyl-CoA with microsomes of Candida lipolytica. The X band, consisting of <3% of total [^{14}C] after 30 min., was not investigated further. Chromatography was performed on silica gel H plate (0.5 mm) and developed in chloroform:methanol:water (65:35:5 v/v).

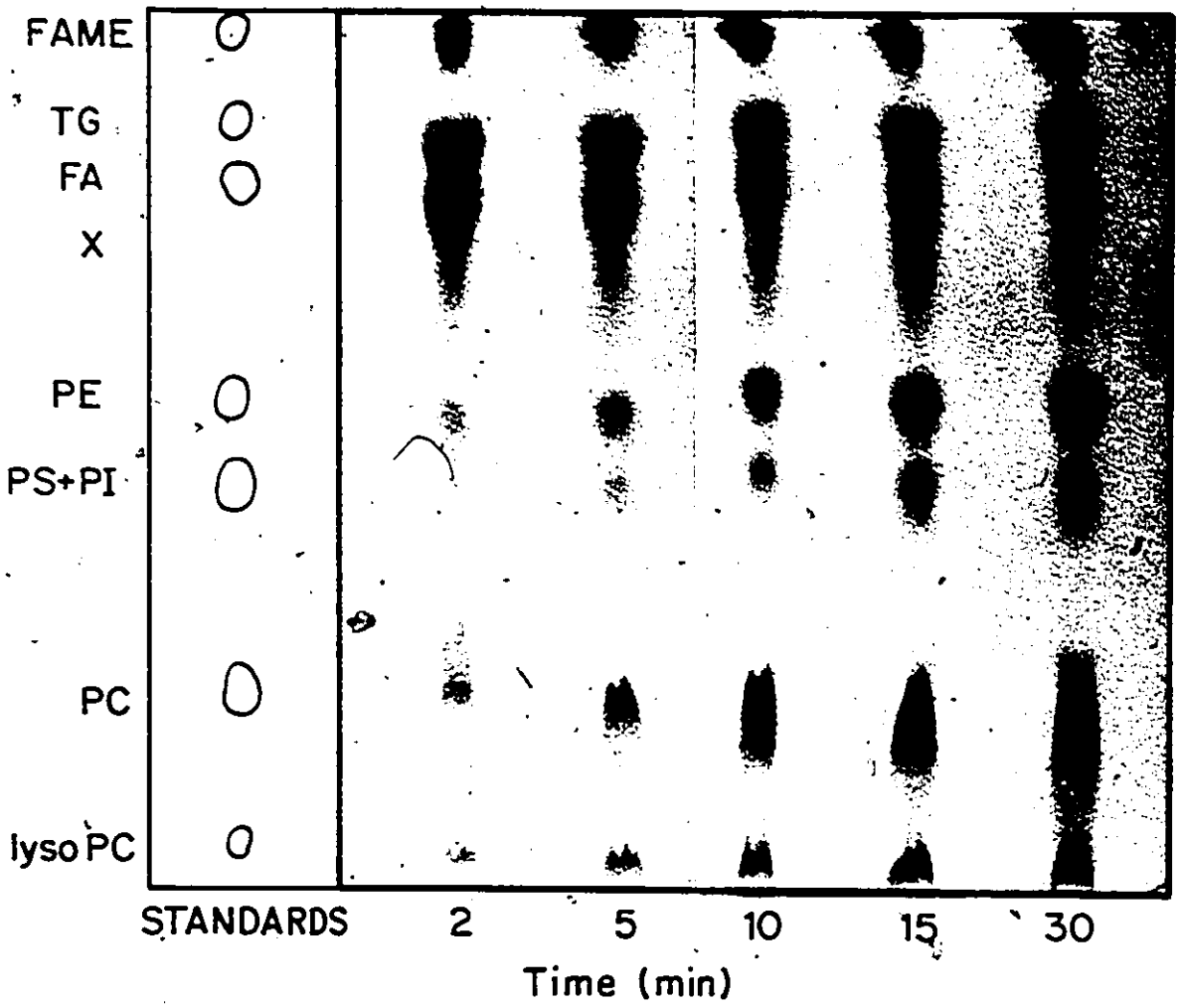
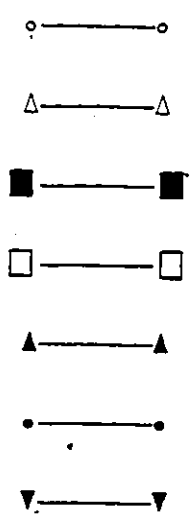
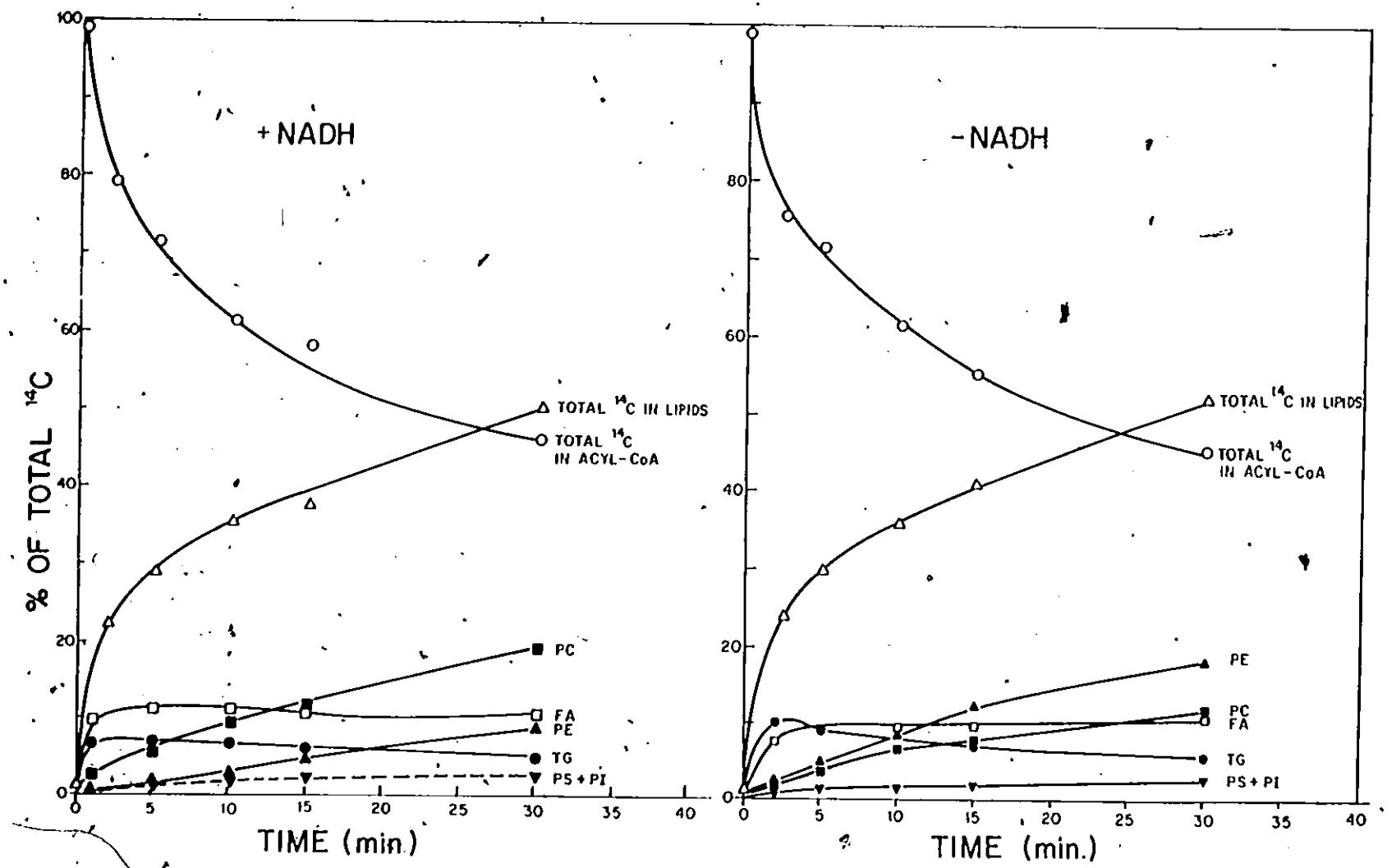

FAME
TG
FA
X
PE
PS+PI
PC
lyso PC
STANDARDS
2
5
10
15
30
Time (min)

FIGURE 15. Distribution of [^{14}C] in acyl-CoA and acyl lipids during incubation of [1-^{14}C]oleoyl-CoA with microsomes of Candida lipolytica grown in the mid-log phase at 25°C. Incubations were performed in presence of ATP and $MgCl_2$ as described in Section IID, A, in the presence and B, in absence of NADH; lipids were separated by TLC (see Section IIK) and counted for ^{14}C.




+ NADH
- NADH
% OF TOTAL ^{14}C
TIME (min.)
TOTAL ^{14}C IN LIPIDS
TOTAL ^{14}C IN ACYL-CoA
PC
FA
PE
TG
PS + PI

presence of NADH, desaturation of 18:1 → 18:2 occurred and analysis of phospholipids, neutral lipids, and acyl-CoA fraction (see Fig. 14 and Section II. K.) revealed that after 30 min. 11% desaturation occurred in the phospholipids (summarized in Fig. 16), trace amounts were found in the neutral lipids, while about 4% was recovered in the acyl-CoA fraction. Tables IX and X summarize the extent of desaturation in each of the lipid classes and in individual lipid components. Phosphatidylcholine was always more rapidly labelled than the other phosphatides, and its extent of desaturation always exceeded that of phosphatidylethanolamine or phosphatidylserine plus phosphatidylinositol.

FIGURE 16. Time course of disappearance of [^{14}C]oleoyl-CoA (o---o) and appearance of [^{14}C]oleate (X——X) and [^{14}C]-linoleate in phospholipids (o——o) and [^{14}C]linoleate in acyl-CoA (•——•). [^{14}C]oleoyl-CoA was incubated with microsomes of C. lipolytica prepared from cells grown to mid-log phase at 25°C. Incubations were performed as described in Section II K and radioactivity expressed as percentage of total radioactivity.

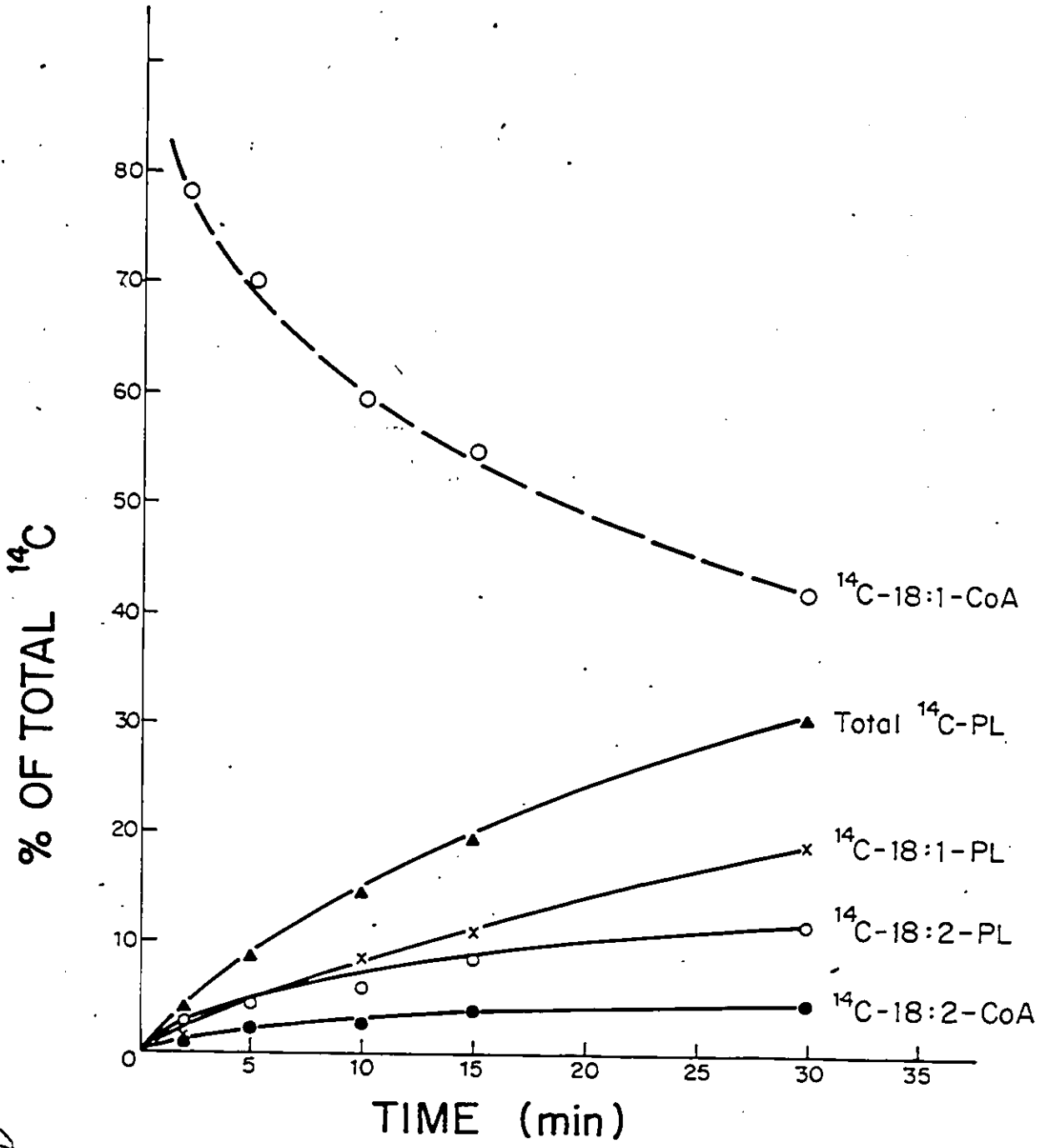

% OF TOTAL ^{14}C
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
TIME (min)
^{14}C-18:1-CoA
Total ^{14}C-PL
^{14}C-18:1-PL
^{14}C-18:2-PL
^{14}C-18:2-CoA

TABLE IX

% Distribution of [^{14}C] with Time[a]

Time	Acyl Lipids ($CHCl_3$-soluble)			Acyl-CoA (CH_3OH/H_2O-soluble)		
min.	18:1	18:2	Total	18:1	18:2	Total
0	1.13	0	1.13	98.2	0	98.2
2	19.3	2.8	22.1	79.0	0.7	79.7
5	24.2	4.7	28.9	69.7	1.8	71.5
10	28.7	6.5	35.2	59.4	1.8	61.2
15	28.5	9.0	37.5	54.8	3.4	58.2
30	38.9	11.3	50.2	42.0	4.4	46.4

[a]In the presence of NADH.

TABLE X

% Distribution of [^{14}C] Among Phospholipid and Netural Lipid Classes[a]

Time	PC[b]		PE		PS + PI		Total [^{14}C] in PL	TG		FFA[c]		Total [^{14}C] in NL
min.	18:1	18:2	18:1	18:2	18:1	18:2		18:1	18:2	18:1	18:2	
0	0	0	0	0	0	0	0	0	0	1.13	0	1.13
2	0.8	1.7	0.4	0.4	0.1	0.5	3.9	6.6	0.1	9.8	0.1	16.6
5	3.0	2.6	1.0	0.8	0.2	1.0	8.6	6.9	0.1	10.8	0.2	18.0
10	5.9	3.3	1.7	1.2	0.8	1.2	14.1	6.2	0.3	10.7	0.5	17.7
15	6.9	4.9	2.9	2.0	0.9	1.6	19.2	6.0	0.2	10.5	0.3	17.0
30	14.0	5.1	5.4	3.7	0.6	2.1	30.9	5.2	0.2	10.7	0.2	16.3

[a] In presence of NADH.

[b] Including traces of Lyso-PC.

[c] Including fatty acid methyl esters (FAME).

Discussion

I. Errors

a) Measurement of Fatty Acid Composition

The method of Carroll (1961) was used to measure the peak areas of fatty acid methyl esters on the GLC analysis. The method is based on the assumption that peaks obtained closely approximate the Gaussian distribution curve. However, Dijkstra (1961) pointed out some of the shortcomings of this method especially with respect to fatty acid analysis determination. Carroll's method, however was used because of its rapidity and convenience which allow analysis of large numbers of samples within a short time and range of error. The probable error inherent in Carroll's method is ± 1.5-2.0% (Carroll, 1961); in addition errors of 0.5-1.0% may be introduced as result of measurements of peak height and retention time, giving a total error of ± 2.0 - 3.0%. Therefore, only fatty acid changes >4% were considered significant and are commented upon in the discussion.

b) Variation in Culture of Cells

Precautions were taken to minimize contamination of culture flasks by other microorganisms and to ensure the reproducibility of growth conditions throughout the study. Provided that a uniform and constant sample of the inoculum was taken each time, it was possible to reproduce the growth curves and activity measurements very accurately. Thus, the

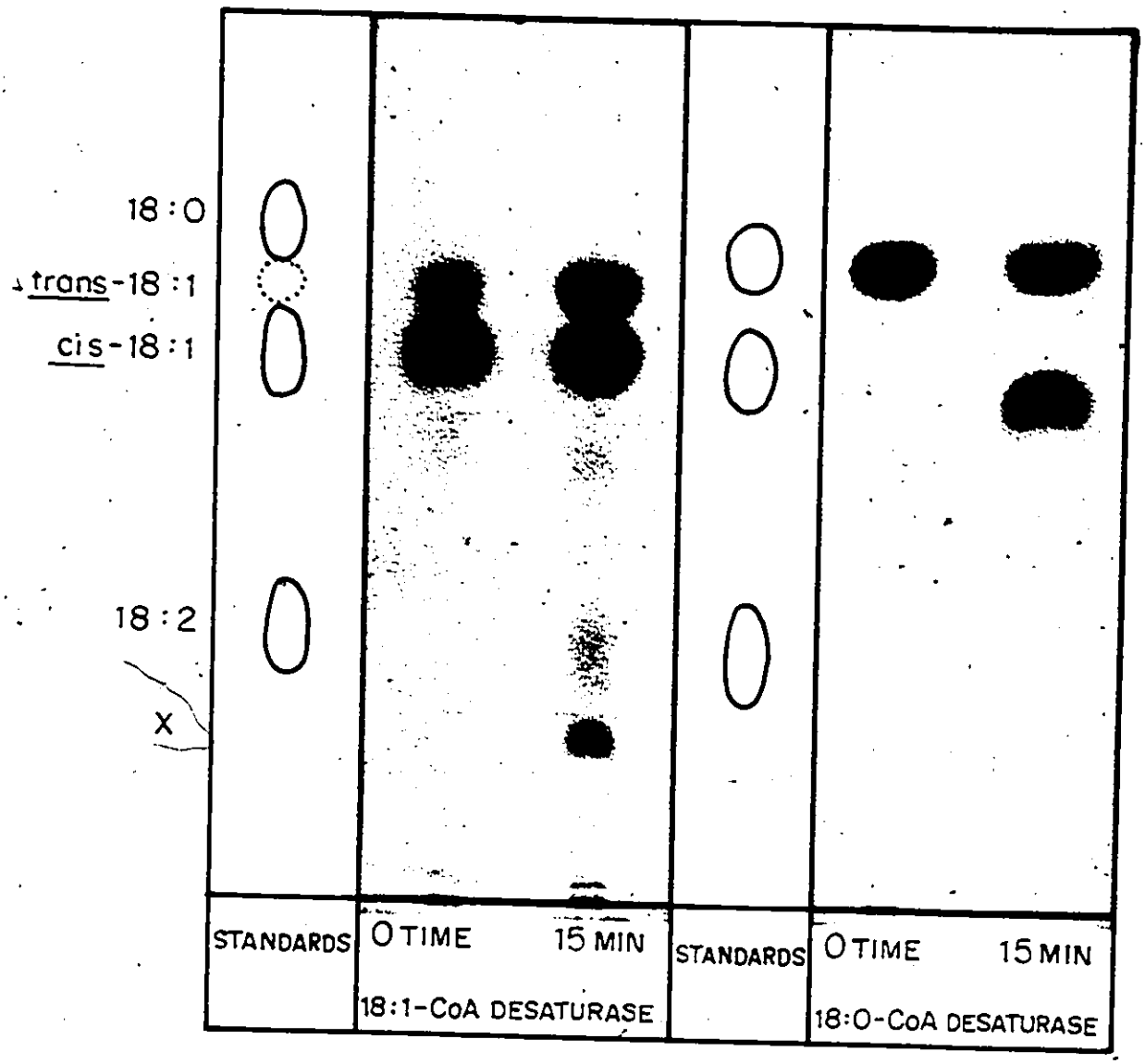
growth points were reproducible within ± 0.01 - 0.02 O.D. units and enzyme activities were within ± 5% (see Table VII and VIII). Variations in fatty acid composition in different microsomal preparation were within ± <1% (Table V, VI).

II. General Properties of 18:1-CoA Desaturase

Cell fractionation studies revealed that the 18:1-CoA desaturase activity was present both in the 105,000 x g or 160,000 x g pellets and their respective supernatants (Table IIA, B); the bulk of the activity was still present in the 160,000 x g supernatant. The high speed supernatant may represent either a solubilized form of the desaturase enzymes or may be a finely dispersed form of the membrane-bound enzyme system. Further investigation of the supernatant activity is required to settle this point. The specific activities recorded in Table II are much higher than those found in subsequent experiments; this may be due to the fact that only in this experiment was the 18:2 band counted together with a radioactive band (X, Fig. 17) formed during the incubation, which migrated close to it and could only be detected on autoradiograms. In the experiments recorded in Table II, the spots were revealed by 2',7'-dichlorofluorescein which did not discriminate between the 18:2 band and the X. Band X may be an oxidized form of 18:1 or 18:2 acids.

Lineweaver-Burk plot indicated an apparent Km of 250 μM, a value similar to that previously reported by Pugh and Kates

FIGURE 17. Autoradiogram of a thin-layer chromatogram on 10% $AgNO_3$-Silica Gel G showing the formation of 18:2 and 18:1 acids from 18:1-CoA and 18:0-CoA, respectively. The band marked X appeared only with 18:1-CoA as a substrate, and may be an oxidized form of 18:1 or 18:2 acids produced during the incubation in air.


18:0
trans-18:1
cis-18:1
18:2
X
STANDARDS
0 TIME
15 MIN
18:1-CoA DESATURASE
STANDARDS
0 TIME
15 MIN
18:0-CoA DESATURASE

(1975) for 1-acyl-2-oleoyl-phosphatidylcholine as a substrate This information, together with the subsequent work described in this thesis, confirmed the presence of a phospholipid desaturase. Assuming the presence of lysophospholipids in membranes, these could be acylated with 18:1-CoA to form 1-acyl-2-oleoyl-phospholipid which then could undergo desaturation to 1-acyl-2-linoleoyl-phospholipid.

III. Changes Due to Aeration Rate

An almost doubling in aeration rate from 70 to 130 rpm resulted in a corresponding doubling in the fatty acid mole ratio from 0.81 to 1.62. Since the fatty acid mole ratio is usually taken as an index of membrane unsaturation, it appears than an increase in aeration rate results in an increase in membrane unsaturation. The increase in linoleic acid with increasing shaking speed may be explained by the increased supply of oxygen for the desaturase. This conclusion, however, is contrary to the observation of Thompson et al (1980) while studying the palmitoyl-CoA desaturase in the Tetrahymena pyriformis whose membrane fatty acids did not change with a change in O_2 tension at either 15°C or 39.5°C. In our experiments, both 18:0-CoA and PC desaturase activities remained independent of aeration rate while 18:1-CoA desaturase activity showed a large increase in activity. However, it should be kept in mind that 18:1-CoA activity shows a sharp maximum strongly dependent on the age of the culture (Fig. 11B) while the other desaturases show

broader ranges of activities. Thus the decrease in 18:1-CoA desaturase activities in cells grown at decreased aeration rates may be due to the fact that these cells are actually older than those grown at the higher aeration rate. However, more detailed studies are required to explain this aeration effect unambiguously.

In contrast 18:0-CoA desaturase is almost independent of age after 12 hours (Fig. 11B) and so would not be expected to change with aeration rate.

IV. Growth and Temperature Related Changes in Fatty Acid Composition and Desaturase Activity

The changes in fatty acid composition of microsomes of C. lipolytica with growth at either 10°C or 25°C found in our studies, were similar to those reported previously by Kates and Baxter (1962) and Kates and Paradis (1973) for total cellular lipids: a large increase in proportions 18:2 acid and a reciprocal decrease in proportions of 18:1 acid occurring very early in the log-phase, followed by a decrease and increase in proportions of 18:2 and 18:1 acids respectively as growth continued (Fig. 10A & B). In membranes of 25°C grown cells the proportions of 18:2 and 18:1 acids returned to the original inoculum value late in the growth phase, but in membranes of 10°C grown cells a higher level of 18:2 acid was maintained throughout the growth phase. A shift in temperature from 25° to 10°C during the growth period, could increase the rigidity of the membranes, or create a more

viscous lipid milieu in the lipid bilayer, especially in the early stages of growth, and such a stimulus could result in an increase in desaturase activity (see further discussion in Section VII) leading to higher levels of 18:2 in 10°C membranes than in 25°C membranes.

In absolute values, the total fatty acid content expressed as nmoles per mg microsomal protein was higher at 10°C than 25°C and increased with age of the cells at both temperatures (Tables V and VI). Activities of 18:0-CoA, 18:1-CoA and PC-desaturases in 25°C membranes were all maximal in mid log-phase (12 hours) of growth (Table VII, Fig. 11B), coinciding with the maximum absolute contents of 18:2 acid (Fig. 11A). However, the maximum and minimum proportions of 18:2 and 18:1 acids, respectively, occurred in early log-phase (4.5 hours, Fig. 10A). In contrast, microsomal membranes from 10°C cells, had maximum 18:0-CoA, 18:1-CoA and 18:1-PC desaturase activities in the early stages of growth (42-47 hours), coinciding with maximum and minimum proportions of 18:2 and 18:1 acids, respectively (Fig. 10B) but not precisely with the maximum absolute content of 18:2 reached at 72 hours of growth. Actually, the maximum rate of conversion of 18:1 → 18:2 acids (Fig. 12A, Table VIII) occurred early in the log-phase (37 hours), just preceeding the maximum 18:1-CoA activity at 42 hours of growth. After 42 hours, the absolute amounts of 18:2 acid still increased but less rapidly to a maximum level in the stationary phase (72 hours); the low levels of 18:1-CoA activity during this

period probably suffice to account for the slow increase in 18:2 acid. Furthermore, it should be noted that the maximum 18:1-CoA activity in 10°C membranes was nearly double that in 25°C membranes. PC-desaturase activity was lower in 10°C membranes than in 25°C membranes, but in both cases the general pattern of activity throughout the growth cycle was similar to that of 18:1-CoA desaturase. The 18:0-CoA desaturase activity was also lower in 10°C membranes than in 25°C membranes, but the overall pattern of activity was the same in both membranes. In the 10°C membranes the high activity of 18:0-CoA together with the lower 18:1-CoA activity after the mid-log phase would explain why the rate of accumulation of 18:1 acid in membranes proceeds faster than the 18:2 acid during the late period of growth.

It should be mentioned that the high activity of 18:0-CoA desaturase (per mg microsomal protein) throughout the growth phase at both temperature is probably responsible for maintaining the low content of 18:0 acid in the membranes at both temperatures. The question now arises as to which enzyme, 18:1-CoA or PC-desaturase, is responsible for the high 18:2 content in 10°C membranes. The high 18:1-CoA desaturase activity observed in 10°C membranes would suggest that this enzyme is responsible for the higher 18:2 content in this membrane. However, the kinetic studies described above (Section V of Results) raise the possibility that the observed 18:1-CoA activity might be accounted for by a phospholipid desaturase and this will be discussed further in

Section V of Discussion.

Another point that arises, concerns the question whether the increase in desaturase activity per mg protein in early log phase at 10°C and mid-log phase at 25°C is due to de novo synthesis of the enzymes or to activation of the desaturase enzyme system. Previous studies by Pugh and Kates (1975) have shown that the Km of the PC-desaturase from 25°C and 10°C grown cells at maximum activities remains constant while Vmax was higher for the desaturase 25°C grown cells. These results would suggest that the changes in activity may be due to changes in conformation of the enzyme system (see Fig. 18) but further studies are necessary to resolve this point. These studies would involve the use of protein inhibitors such as cycloheximide to see whether de novo enzyme synthesis is required and activity measurement of the membrane dissociated and reconstituted desaturase system.

V. Kinetic Studies on the Desaturase Activity

The rapid hydrolysis of acyl-CoA catalyzed by a thiolase, could be greatly reduced by addition of ATP, $MgCl_2$ and CoA to stimulate resynthesis of acyl-CoA by membrane bound acyl-CoA ligase (thiokinase). In the presence or absence of reduced nucleotides, the 18:1-CoA was rapidly incorporated into phospholipids and neutral lipids, indicating that acyl transfer was independent of reduced nucleotides. The only puzzling observation was the reversal of labelling of PC and PE in the presence or absence of reduced nucleotides: while

PC predominated in the presence of reduced nucleotide, PE was the major phospholipid in the absence of reduced nucleotides (Fig. 15)..

In the presence of NADH, the newly formed 18:2 acid appeared in the phospholipids at a faster rate than in the acyl-CoA fraction (Fig. 16, Table X). After 2 min. of incubation, 67% of the [^{14}C]acyl groups present in phospholipids was found to be 18:2 acid, whereas only 0.9% of [^{14}C] 18:1-CoA had been converted to [^{14}C] 18:2-CoA (Table X). After 30 min. of incubation, the newly formed 18:2 acid in phospholipids decreased to 35% while the 18:2 in acyl-CoA increased to 10%. These findings indicate that 18:1-CoA, apart from being hydrolysed by a thiolase to the free acid and CoA, was also rapidly transacylated into phospholipids and that the 18:1-phospholipid was desaturated to 18:2-phospholipid at a greater rate than desaturation of 18:1-CoA (Figs. 15, 16).

The phospholipids involved in the desaturation of oleate, were found to be PC, PE, and PS + PI. Only trace amounts of 18:2 were found in the neutral lipids, TG and free fatty acids (Table X). In early stages of incubation (2 min.), 65% of the 18:2 in phospholipids was found in PC, 15% in PE and 19% in PS + PI, while at the end of 30 min., 18:2 in PC decreased to 47%, increased to 34% in PE and remained constant (19%) in PS + PI..

The above results indicate that linoleic acid appears to be formed largely by action of a phospholipid desaturase and

to some extent also by a 18:1-CoA desaturase. However, recent studies by Stymne and Glad (1981) on microsomes of developing soya bean catyledons demonstrated conclusively that oleate from oleoyl-CoA was rapidly transacylated into phospholipids primarily PC and desaturated to linoleate while part of the phospholipid molecule. This transacylation was followed by a reverse exchange of the PC acyl groups (labelled and unlabelled) with free CoA, resulting in the appearance of low amounts of [^{14}C]18:2 in acyl-CoA. If a similar reverse exchange reaction occurred in the Candida lipolytica microsomal system, then the small amounts of [^{14}C]18:2-CoA formed at early stages (Table IX) might have resulted, at least partly, by back exchange with [^{14}C]18:2-PC, which at all times is present in higher concentrations than [^{14}C]18:2-CoA. It may therefore be concluded that a considerable portion of linoleic acid present in the Candida lipolytica microsomes may have been formed by the action of the phospholipid desaturase, acting predominantly on PC and PE, and that this enzyme may play an important role in control of membrane fluidity.

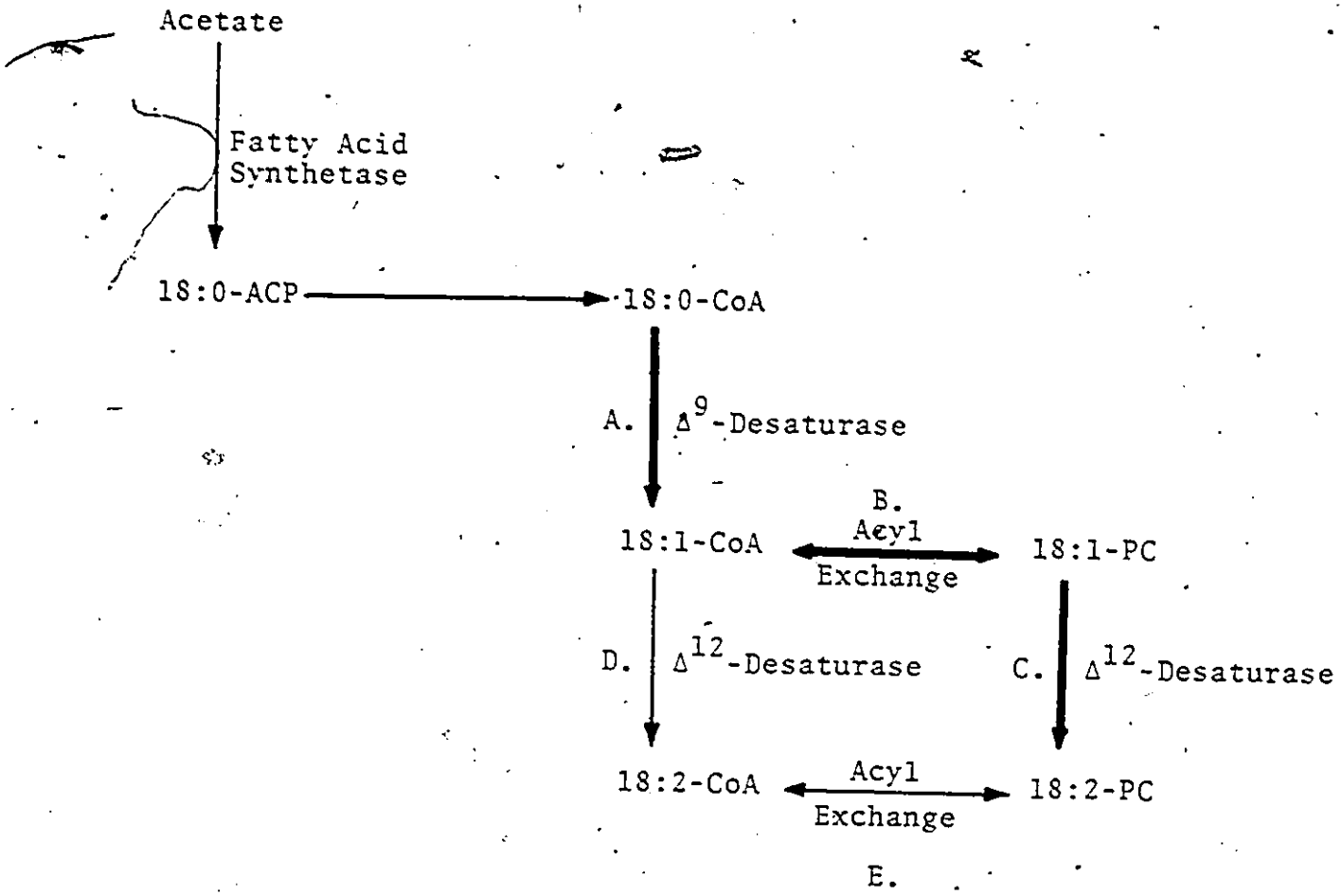
VI. Proposed Pathway of 18:1 and 18:2 Synthesis

On the basis of the foregoing results and previous work done in our laboratory (Pugh and Kates, 1973 and 1975) it is possible to postulate the following pathways for the synthesis of oleic and linoleic acids in the yeast Candida lipolytica (Scheme V).

Stearoyl-CoA, synthesized by the FAS complex or formed

SCHEME VI

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.


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

from free stearic acid and CoA by the microsomal ligase, is desaturated to 18:1-CoA by a Δ9-desaturase (pathway A). (Bloomfield and Bloch, 1960). The 18:1-CoA then undergoes acyl exchange with phospholipids (mainly PC, pathway B), which undergo further desaturation (Δ12-desaturase, pathway C) to 18:2-phospholipids (mainly 18:2-PC). Alternatively, 18:1-CoA could be desaturated directly to 18:2-CoA which could then undergo acyl exchange to form 18:2-PC (pathway D and E).

As shown in Fig. 16 the rate of appearance of [^{14}C]18:2 in PC is at all times more than twice that in acyl-CoA. Such kinetics are consistent with the pathways B, C and not with pathways D, E as major route to 18:2-formation. If pathway D, E was predominant the rate of [^{14}C]18:2-CoA formation would initially be much higher than that of [^{14}C]18:2-PC, which is clearly not the case. This latter route is therefore presumed to be a minor one, relative to the pathway involving direct desaturation of 18:1-PC (pathway B, C).

VII. The Physiological Role of Fatty Acid Desaturation

Phospholipids are structural components of biological membranes and because of this, they may play important roles in cellular metabolism and physiology. As demonstrated by Kates and Paradis (1973) in the yeast Candida lipolytica, changes in phospholipid composition do occur at different growth temperatures. For example, total lipid and total

phospholipid content of Candida lipolytica (Kates and Paradis, 1973) and total microsomal lipid content (This thesis Tables V, VI), appear to be greater at 10°C than 25°C grown cells. Similarly, the fatty acid composition of phospholipids also changes with respect to growth and temperature (Kates and Paradis, 1973). Thus it is reasonable to assume that a close control is exerted by the cell on fatty acid and phospholipid synthesis as a function of growth stage and temperature. Such control mechanisms, will determine how well the cell will adapt to and survive under changed environmental conditions.

The degree of fatty acid unsaturation has a large effect on its physical properties, such as melting or boiling points; 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 to which the fatty acids are esterified. Thus the greater the degree of unsaturation in phospholipid fatty acids the greater the fluidity and hence the permeability of the membrane. On this basis, the large increase in unsaturation in the major membrane phospholipids, PC and PE, in the early log phase would indicate an increased membrane permeability during this period. This is reasonable in view of the greater requirements for exogenous nutrients during the active growth cycle. Decreases in unsaturation during later stages of growth would correlate with decreased membrane permeability expected for older cells (Chapman, 1968). At low

temperatures, an even higher degree of membrane fluidity would be required and this in turn would necessitate a higher degree of fatty acid unsaturation than at normal temperatures, as was observed in the present studies (Table VI).

A model for control of fatty acid unsaturation that would provide increased lipid unsaturation required for increased membrane fluidity would involve the membrane-bound desaturase enzyme system itself. It would be recalled that this enzyme system consists of two electron transporting proteins, cytochrome b_5 reductase and cytochrome b_5, and the terminal desaturase, which fit into the lipid bilayer of the microsomal membrane as shown in Fig. 18. The model proposes that the three proteins are optimally oriented only in the membrane of low fluidity (Fig. 18A), i.e., in the case of Candida lipolytica, when 18:2/18:1 molar ratio is low. The desaturase system will therefore have optimal activity when the membrane has low degree of unsaturation. As more linoleic acid is formed the lipid chains become more fluid allowing the enzymes to move apart, thus decreasing their interaction and hence reducing the desaturase activity (Fig. 18B). The system therefore consitutes a self-regulating mechanism, shutting itself off when the membrane becomes too unsaturated or fluid and turning itself on again when the unsaturation or fluidity decreases due to insertion of less unsaturated fatty acids into the membrane phospholipids. In this way, the optimum physical state within the lipid bilayer of the cellular membranes would be maintained at all times.

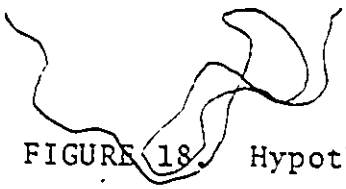
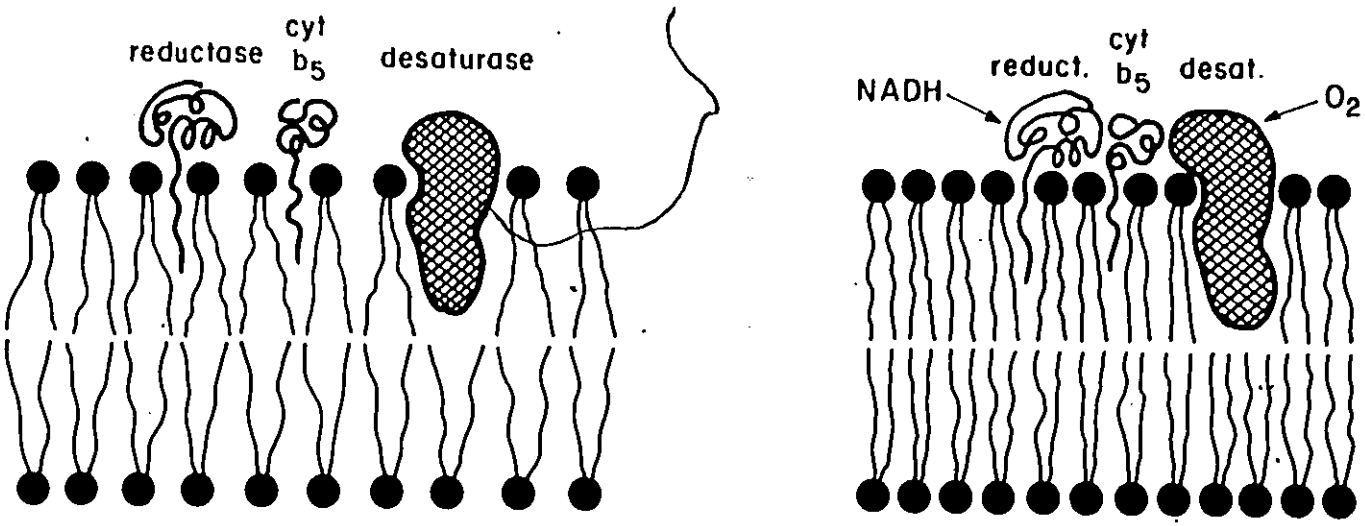


FIGURE 18. Hypothetical model for self-regulation of phospholipid desaturase activity by membrane fluidity.



INACTIVE

ACTIVE

Summary

1. The proportions of 18:1 and 18:2 fatty acids of Candida lipolytica microsomes were shown to have a reciprocal relationship during the early log phase of growth at both 10° and 25°C.
2. Microsomal membranes prepared from 10°C grown cells had a higher lipid content and degree of unsaturation than membranes prepared from 25°C grown cells.
3. The absolute amounts of 18:1 and 18:2 fatty acids in microsomal membranes from 25°C grown cells increased simultaneously, with the 18:2 acid predominating over 18:1 acid up to 12 hours of growth while 18:1 acid predominated over 18:2 acid in the later stages of growth.
4. The absolute amount of 18:2 acid in microsomal membranes from 10°C grown cells was much higher than that of 18:1 acid early in the log-phase; thereafter, 18:2 increased slowly while 18:1 increased very rapidly.
5. The specific activities of 18:0-CoA, 18:1-PC and 18:1-CoA desaturases showed maximum values corresponding to maximum absolute contents of 18:1 and 18:2 acids in 25°C microsomal membranes.
6. In 10°C membranes 18:0-CoA and 18:1-PC desaturase specific activities decreased, while that of the 18:1-CoA desaturase nearly doubled indicating that the latter enzyme may be responsible for the increased content of 18:2 in 10°C microsomal membranes.

7. Lineweaver-Burk plot of oleoyl-CoA as substrate for desaturase activity gave a Km value of 250 μM similar to that of 1-acyl-2-oleoyl-phosphatidylcholine as a substrate (Pugh and Kates, 1975).

8. An increase in aeration rate resulted in an increase in 18:2/18:1 mole ratio from 0.81 at 70 rpm to 1.62 at 130 rpm. The 50% increase in 18:2/18:1 ratio was reflected in a corresponding increase in the 18:1-CoA activity.

9. Kinetic studies of 18:1-CoA desaturase performed in the presence of ATP, $MgCl_2$ and CoA showed a low level of hydrolysis of the substrate (10% in 30 min.) and rapid transacylation [^{14}C]acyl groups into phospholipids (31% in 30 min) plus triglycerides (10% in 30 min.). In the presence of NADH, 74% of the newly formed [^{14}C]18:2 acid appeared in the phospholipids while about 20% was associated with acyl-CoA after 2 min. PC was more rapidly labelled and contained more 18:2 than any other phospholipid during the incubation period. These studies suggest that the observed 18:1 → 18:2 conversion occurs largely by desaturation at the level of phospholipids primarily PC and to some extent PE. However these studies do not entirely rule out the operation of an acyl-CoA desaturase.

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