THIS THESIS IS DEDICATED TO

MY PARENTS

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ABBREVIATIONS AND NOMENCLATURE

NL	Neutral Lipid
PA	Phosphatidic Acid
PC	Phosphatidyl Choline
PE	Phosphatidyl Ethanolamine
PG	Phosphatidyl Glycerol
PL	Phospholipid
PI	Phosphatidyl Inositol
PS	Phosphatidyl Serine
GLC	Gas-liquid Chromatography
TLC	Thin-layer Chromatography

FATTY ACIDS: In several sections of this text, fatty acids have been appreviated in the form n:x where n denotes the number of carbon atoms in the fatty acid chain and x the number of double bonds in the chain.

PHOSPHOGLYCERIDES: The IUPAC-IUB rules for stereospecific numbering of phosphoglycerides are followed where applicable. IUPAC-IUB rules for nomenclature of phosphoglycerides are followed with the exception that the prefix 3-sn-, where applicable, has been omitted.

ABSTRACT

Studies were performed on the fatty acids of a mesophilic yeast, Candida lipolytica, strain NRRLY 1094, grown at 10°C and 25°C. Gas-liquid chromatographic analyses of the fatty acids of the total lipids extracted from yeast cells harvested at different stages of growth at both temperatures confirmed the findings of Kates and Baxter (1962) that the degree of unsaturation of cellular lipids varies inversely with the temperature and age culture. Total lipids were separated by thin-layer chromatography into four fractions: phosphatidyl choline, phosphatidyl inositol+ phosphatidyl serine, phosphatidyl ethanolamine, and neutral lipids. Gas-liquid chromatographic analysis of the fatty acids obtained from each of these fractions showed that:

(i) Fatty acids of all components are more unsaturated at 10°C than at 25°C. Unsaturation of fatty acids is maintained at a higher level later into the linear phase of growth at 10°C than at 25°C.

(ii) A reciprocal relationship between levels of oleic acid and linoleic acid during growth at either temperature appears to exist in all of the phospholipids.

(iii) Of the phospholipids, phosphatidyl choline contributes most to the unsaturation effect mentioned above. The fraction containing phosphatidyl inositol + phosphatidyl serine also shows the relationship mentioned above but contains much higher levels of palmitic acid. Phosphatidyl ethanolamine shows changes in fatty acid composition

similar to but much less pronounced than those in phosphatidyl choline. A study of the positional distribution of fatty acids in phosphatidyl choline and phosphatidyl ethanolamine shows that desaturation during growth at both temperatures appears to occur non-specifically with respect to the position of esterified fatty acids in phosphatidyl choline, but specifically in the 2-position of phosphatidyl ethanolamine.

(iv) Silver nitrate chromatography of lecithin samples obtained from cells grown at either temperature indicates the existence of molecular species containing four, three and two or one double bonds. Calculations of the probable distribution of molecular species strongly suggest that desaturation of lecithin to a tetraenoic species occurs at both temperatures by stepwise desaturation of dienoic and trienoic molecular species during growth. A similar pathway, with some modifications, is envisioned for phosphatidyl ethanolamine.

INTRODUCTION

I. The Organism

The yeast Candida lipolytica is a member of the plant family Cryptococcaceae. Though some investigations concerning such anascogenous yeasts were carried out in the nineteenth century, the first direct studies on this family were performed by Will (1916). Will used the name Monilia (from the German "garungsmonilien", fermentation industries), to include the genus now recognized as Candida. The name Candida was coined by Berkhout (1923), and approved in 1954 by the 8th Botanical Congress (Paris).

The most outstanding feature of the genus Candida is the presence of pseudomycelia, as well as, in some species (including C. lipolytica), true mycelial structures. Members of this genus are widely distributed in nature, usually existing as saprophytes in association with both plants and animals. Some species are pathogenic, most notably C. albicans. In industry, a wide variety of Candida species are found as contaminants, in beverages, dairy products, stock yeast cultures and even wood pulp.

Candida lipolytica is most often found on fatty substances such as margarine, butter, and olives. Certain strains of C. lipolytica appear to be associated with meat spoilage and contamination of refrigerated grape juices, whereas other strains have been implicated in some infections of the skin and nails. Probably the most important biochemical characteristic of this yeast is its production of lipases, a factor which permits its growth on media containing

lipoidal materials. This property simplifies isolation of the organism and is not only responsible for its major adverse commercial effects, but is also the basis of its only planned industrial uses. Investigations have been carried out to study the feasibility of using C. lipolytica as a "starter" (Peters and Nelson, 1948), in the manufacture of cheese. More recent research has led to the possible use of this yeast, in a processed form, as a food source, since it may be grown on readily available, inexpensive, high-boiling petroleum fractions (Kosaric et al., 1969).

II. Growth of C. lipolytica

Interest in the growth of C. lipolytica on hydrocarbon-containing media has resulted in a dearth of information concerning its growth on various carbohydrates normally used for taxonomic purposes. However, Lawrence et al. (1959), in the original isolation of the particular strain used in the present studies, observed that growth of C. lipolytica occurred in media containing glucose, galactose, sucrose or maltose, but did not occur in media containing lactose or raffinose. Studies by Borukaeva et al. (1969), comparing the growth of C. lipolytica on hydrocarbon and glucose media, showed that rate of cell division, as well as size and number of mitochondria were all increased in hydrocarbon containing media. The authors suggest that an adaptation to a hydrocarbon environment may be indicated. In this respect the interest shown in growth in hydrocarbon media may be justifiable; a mathematical treatment of the growth of both C. lipolytica and C. tropicalis in hydrocarbon media has been given by Nikolaev and Sokolov (1968).

Shavlovskii and Ksheminskaya (1965), have shown in their studies of the vitamin requirements of a great variety of yeasts that C. lipolytica requires thiamine for growth but is capable of growth in the absence of all other vitamins investigated, including biotin, riboflavin, nicotinic acid, pantothenic acid, p-aminobenzoic acid and inositol. Limited data has been obtained concerning growth requirements for amino acids or trace minerals; Klug and Markovetz (1967) report growth on a mineral-salts medium containing salts of K, Na, Mg, Ca, Cu, Fe, Mn, Zn and Mo with ammonium sulfate as a nitrogen source.

Few specific studies have been performed on the effects of temperature and pH on yeast growth. Yeast are generally found to grow best at temperatures between 10°C and 30°C, though the optimal growth temperature of most mesophiles is usually in the upper half of this range. Similarly, acidic media are usually found to be preferable for the growth of yeasts, although the pH limits and optima vary substantially with the type of yeast grown. It is of interest however, in relation to the present studies, that Castelli et al. (1969) have shown pH to have essentially negligible effects on the lipid composition of Saccharomyces cerevisiae.

III. Lipid Metabolism

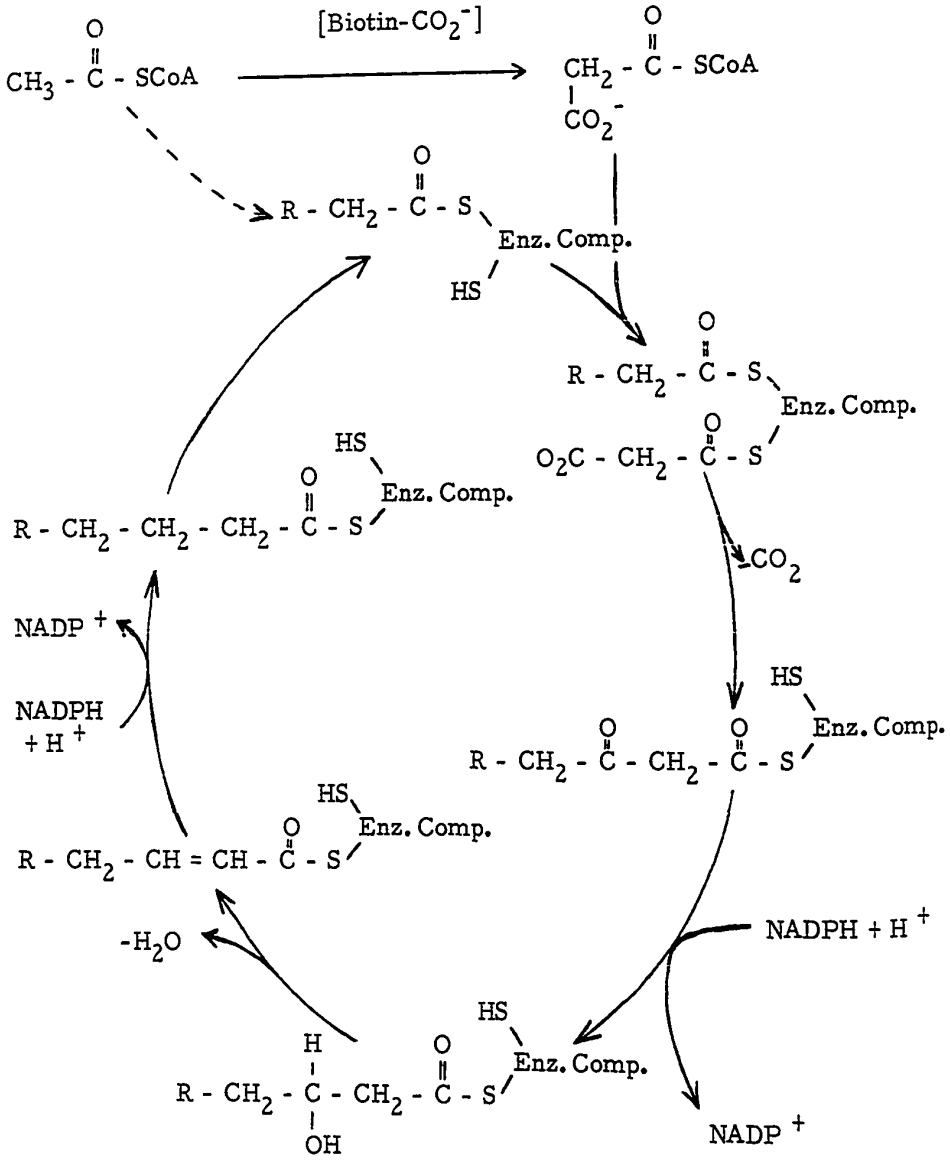
A. Fatty Acids

1. Saturated Fatty Acid Biosynthesis

The fatty acid synthethase system of yeast consists of a complex containing seven enzymes, currently visualized as being grouped around a 'central' sulfhydryl group (Lynen, 1968). A 'peripheral' sulfhydryl is also involved in functioning of the system.

SCHEME I

Biosynthesis of Saturated Fatty Acids (Karlson, 1968)



Biosynthesis appears to be initiated by binding of malonyl-S-CoA to the 'central' thiol and acetyl-S-CoA to the 'peripheral' thiol followed by a condensation reaction releasing CO_2 and forming an acetoacetyl residue bound to the 'central' thiol (Scheme I). This β-ketoacyl derivative is then reduced to the corresponding β-hydroxyacyl-S-enzyme which is dehydrated and reduced to the corresponding enoyl-S-enzyme and saturated acyl-S-enzyme. The saturated acyl derivative, still bound to the 'central' sulfhydryl group of the enzyme complex is then transferred to the 'peripheral' sulfhydryl permitting malonyl-S-CoA to be bound again to the 'central' thiol for repetition of the cycle. The synthetic process is terminated when a saturated fatty acyl residue of 14-18 carbon atoms bound to the 'central' thiol, is transferred to CoA-SH. The final product of fatty acid biosynthesis in yeast is the fatty acyl-S-CoA rather than the free fatty acid and all intermediates remain bound to the enzyme complex.

Comp.

In bacteria no similar tightly bound enzyme complex appears to exist. The fatty acid synthetase system is readily dissociable into individual enzymes. Alberts and Vagelos, (1961) and Wakil et al. (1964) have further shown that in bacterial systems, the acyl groups of acetyl-S-CoA, malonyl-S-CoA and longer chain acyl-S-CoA derivatives are rapidly transferred to a low molecular weight protein (acyl carrier protein, ACP) on which they are bound through the sulfhydryl group of the 4'-phosphopantetheine prosthetic group. Acyl-S-ACP rather than acyl-S-CoA appears to be the normal metabolite in bacterial fatty acid synthesis. It has also been possible to isolate intermediates such as β-ketoacyl-S-ACP (Toomey and Wakil, 1966) from bacterial systems and use

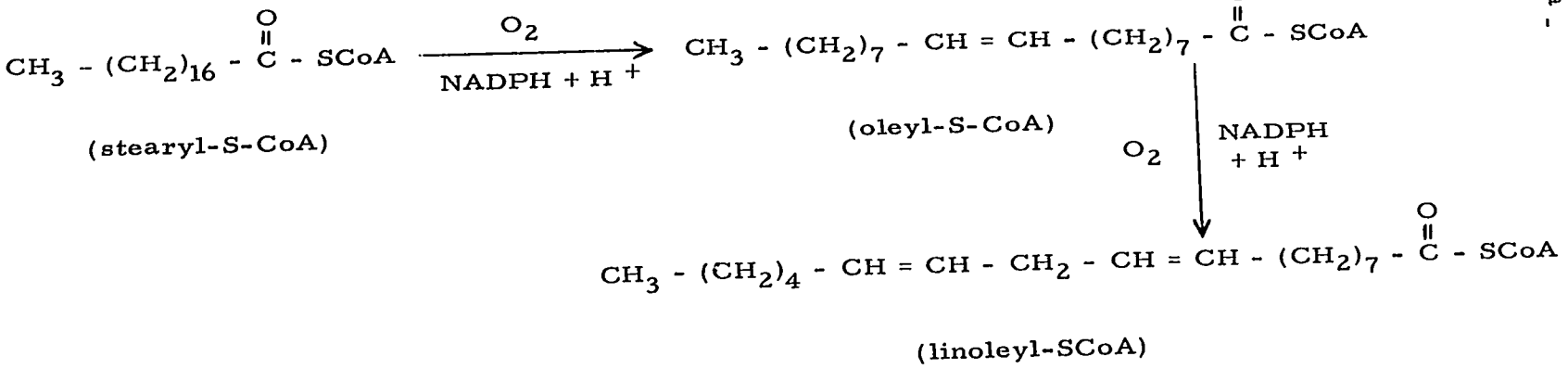
z. Comp.

[+ H^+

SCHEME II

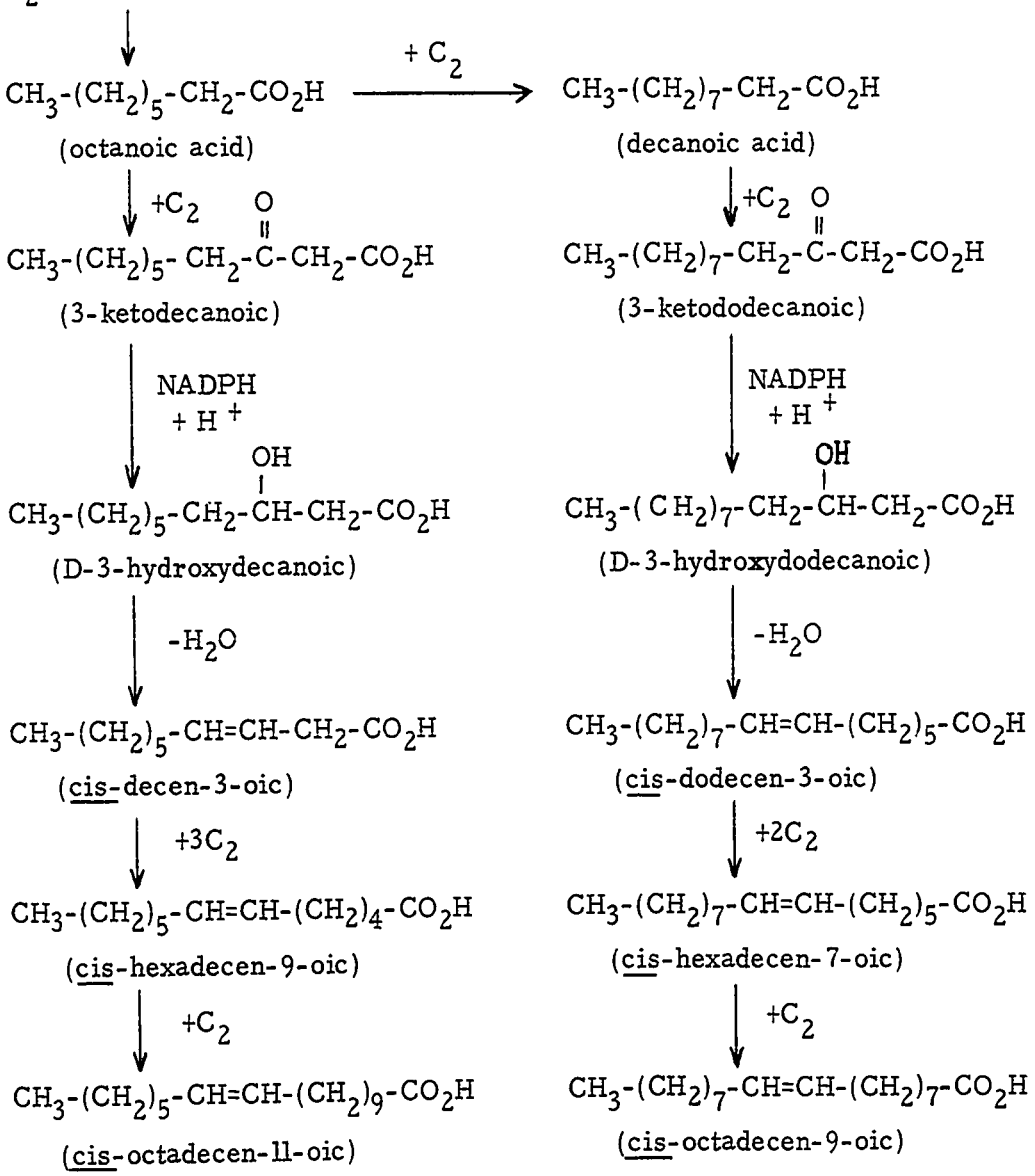
Biosynthesis of Unsaturated Fatty Acids

(a) Aerobic (Yuan and Bloch, 1961)



SCHEME II (ctd.)

(b) Anaerobic (Scheuerbrandt et al, 1961)



them to stimulate fatty acid synthesis, whereas similar intermediates have not been isolated from yeast, and added intermediates in the biosynthetic pathway have little stimulatory effect.

It should be noted that fatty acyl-S-CoA derivatives such as propionyl-S-CoA may substitute for acetyl-S-CoA in the initiation of biosynthesis as described above and that this scheme therefore also lends itself to the biosynthesis of odd-numbered fatty acids.

Though the reversal of the catabolic pathway of β-oxidation in mitochondria (Scheme IIIa) was originally believed to be of major importance in de novo biosynthesis of fatty acids, its significance in this respect may be limited. One possible role of this metabolic route may be in chain elongation of acyl-CoA (Harlan and Wakil, 1963).

2. Unsaturated Fatty Acid Biosynthesis

The biosynthesis of unsaturated fatty acids may be divided into two major metabolic pathways. The first is the 'aerobic' pathway (Scheme IIa) which, with some modifications, is utilized by most organisms other than Eubacteriales; the other is the 'anaerobic' pathway (Scheme IIb) which is found in most bacteria of the order Eubacteriales, but not in higher organisms.

The 'aerobic' pathway involves an enzyme system linked to molecular oxygen (Erwin and Bloch, 1964) which desaturates preformed, saturated fatty acids, usually at C-9 (Bloomfield and Bloch, 1960). The intermediate steps in aerobic desaturation are not known. Light et al. (1962) have shown that hydroxy-fatty acids do not appear

to act as precursors of the corresponding unsaturated acids. Desaturation of monoenoic to dienoic acids also requires oxygen but also does not appear to occur by formation of hydroxylated intermediates (Yuan and Bloch, 1961). The formation of more highly unsaturated fatty acids, though of less significance in yeasts than in animals and higher plants, appears to occur by a similar mechanism.

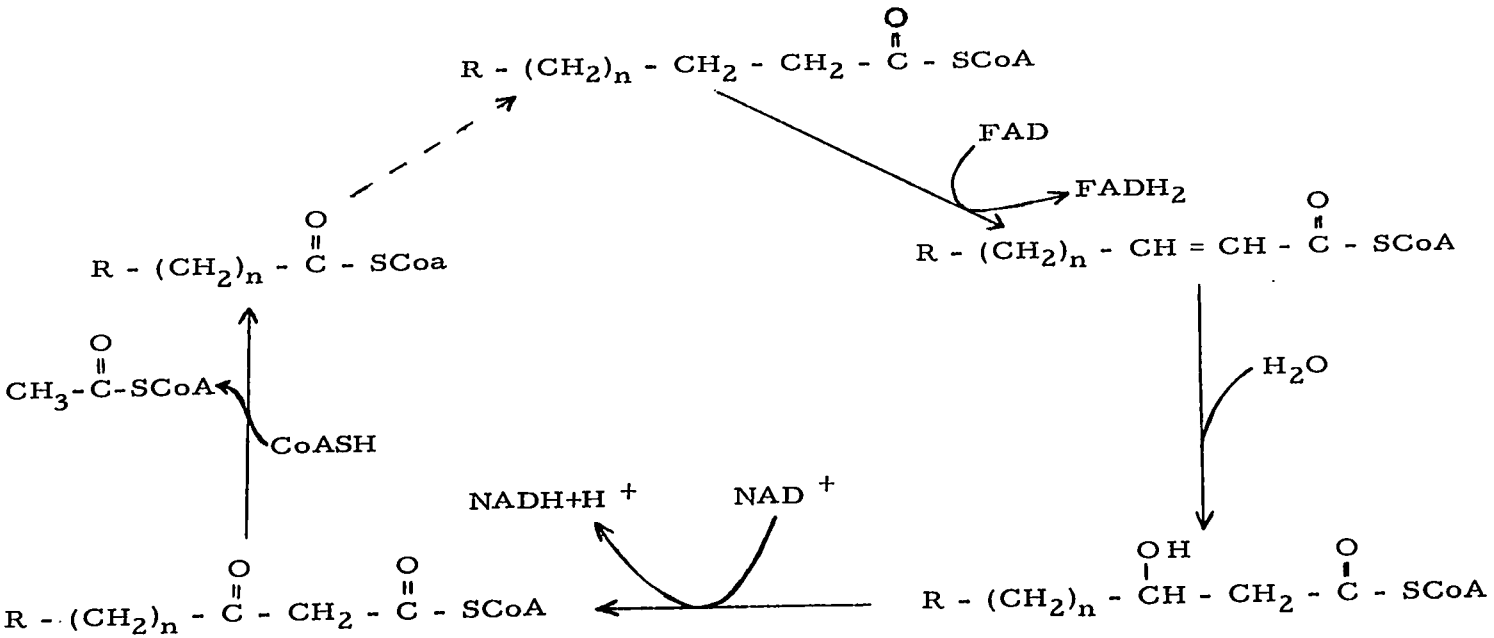
The normal substrate for desaturation in yeast is fatty acyl-S-CoA (Bloomfield and Bloch, 1960); however, Nagai and Bloch (1965, 1968) have shown that fatty acyl-ACP may also be a substrate for desaturation in Euglena gracilis and spinach chloroplasts. In the case of E. gracilis, Nagai and Bloch (1966) have shown that the complete desaturase system contains an NADPH-oxidase, ferredoxin, and a desaturase enzyme which is dependent on the former two components for its activity.

'Anaerobic' desaturation involves the formation of a cis-double bond which is not reduced during the course of biosynthesis. As shown in Scheme IIb, subsequent chain elongation can lead to the production of a homologous series of unsaturated fatty acids (Scheuerbrandt and Bloch, 1962). The existence of several isomeric unsaturated acids as well as relatively short-chain hydroxy acids (precursors of the unsaturated acids before chain elongation), predicted by this scheme is strong evidence in support of this pathway (Kates, 1964).

Nichols et al. (1967) and Gurr et al. (1969) have suggested that aerobic desaturation of oleic acid to linoleic acid in Chlorella vulgaris may occur while the acid is esterified to

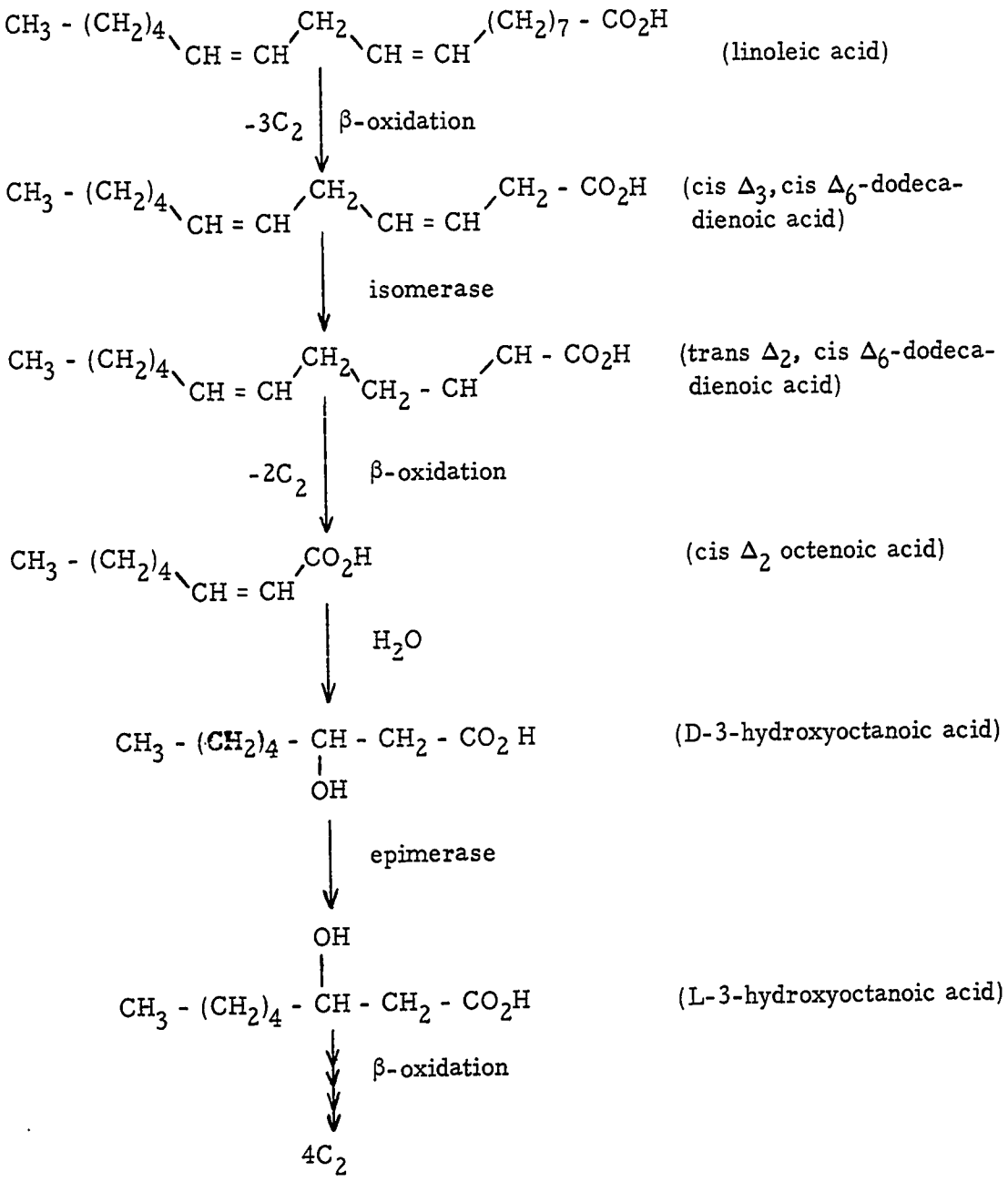
SCHEME IIIa

β-Oxidation of Saturated Fatty Acids



SCHEME IIIb

Oxidation of Linoleic Acid



phosphatidyl choline. An analogous mechanism was previously demonstrated by Zalkin et al. (1963) for the formation of cyclopropane fatty acids from the corresponding monoenoic acids esterified to phosphatidyl ethanolamine. Desaturation of fatty acids esterified to phospholipids will be discussed further in relation to the findings presented in this thesis.

3. Fatty Acid Oxidation

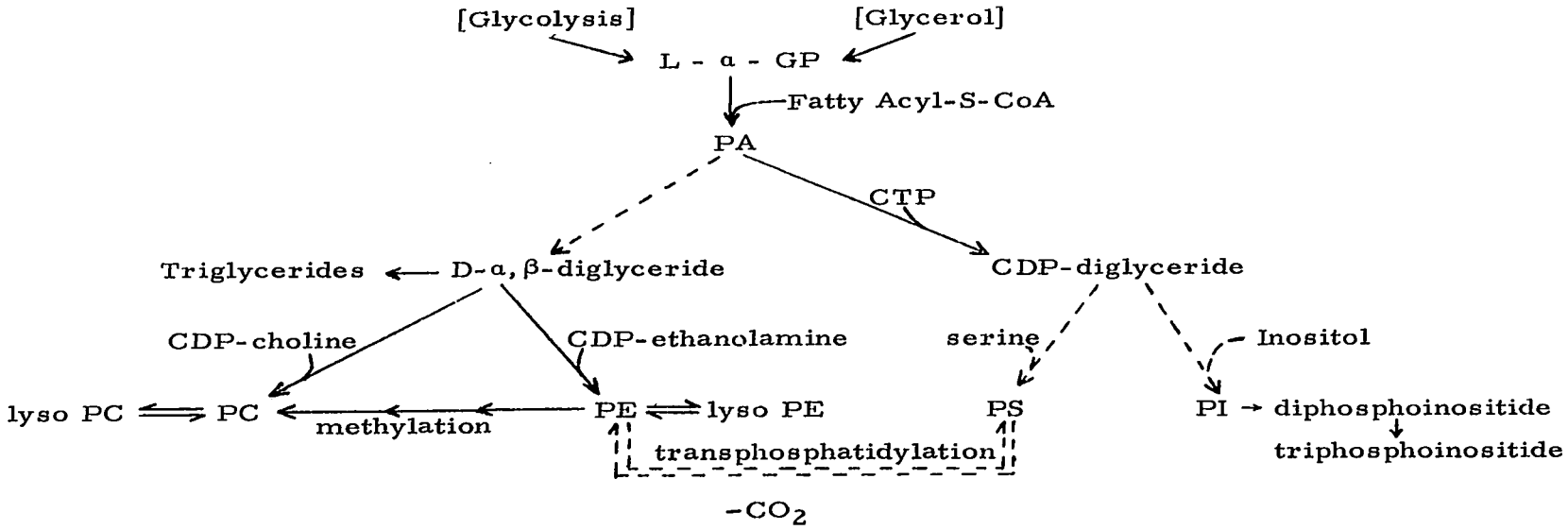
β-oxidation of fatty acids involves the initial desaturation of fatty acyl-S-CoA to the corresponding enoyl derivative, hydration of this compound to the β-hydroxy acyl-S-CoA then oxidation to the β-ketoacyl-S-CoA, followed by cleavage (as shown in Scheme IIIa) to yield acetyl-S-CoA and a two-carbon shorter chain fatty acyl-S-CoA. This oxidative mechanism may be repeated until the fatty acid has been completely oxidized.

The exact mechanism for catabolism of polyunsaturated and highly branched-chain fatty acids awaits clarification. Stoffel et al. (1965) have suggested that polyunsaturated acids may be oxidized by a process similar to β-oxidation but involving isomerization of the double bonds. Scheme IIIb indicates the probable sequence of reactions in the oxidation of linoleic acid.

Enzyme systems capable of oxidizing the ω-carbon of fatty acids to yield dicarboxylic acids have also been found in microorganisms (Kusunose et al., 1964). Fulco and Mead (1961) have shown that saturated fatty acids may be oxidized to the corresponding α-hydroxy acids, then further oxidized and decarboxylated to give fatty acids shorter by one carbon atom.

SCHEME IV

Biosynthetic Pathways for Phosphatides in Yeast*



* - - - - - metabolic routes not yet demonstrated in yeast.
For abbreviations see page ix.

B. Phospholipids

The principal phospholipids of C. lipolytica and of most yeasts are: phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), and phosphatidyl serine (PS). The discussion of phospholipid metabolism in this section will be limited to these phosphatides. It should be noted that most of the work elucidating the pathways of phospholipid metabolism has been performed with mammalian or bacterial systems; and where conclusive evidence is not available for particular pathways in yeast, the pathways demonstrated in other biological systems are presented as probable metabolic routes.

The initial steps of phospholipid biosynthesis (see Scheme IV) have been well established in yeast. sn-3-glycerophosphate (sn-3-GP), produced by glycolysis or by phosphorylation of endogenous glycerol, is acted upon by a fatty acyl-S-CoA transferase to give the corresponding diacylated derivative, phosphatidic acid (PA), (Kuhn and Lynen, 1965). Recent studies by Possmayer et al. (1969) suggest that asymmetric incorporation of fatty acids into PA of rat liver may occur, unsaturated fatty acids being preferentially incorporated into the 2-position. Yeast, unlike bacteria, demonstrate no capability for utilizing fatty acyl-ACP in the transferase reaction. At this point, as indicated in Scheme IV, PA may be dephosphorylated to give a D-α-β-diglyceride as in chicken liver (Smith et al., 1957), and/or it may be acted upon by a cytidyl transferase to give a CDP-diglyceride (Hutchison and Cronan, 1968).

D-α-β-diglycerides are the direct precursors of at least two of the four major phosphatides of interest, PC and PE. For these phosphatides to be produced it is necessary that the

diglyceride be condensed with an 'activated' form of the base. Activation is accomplished by phosphorylation of the base with ATP, then condensation of the phosphorylated base with CTP to yield a CDP-base. Phosphorylation of choline in yeast was demonstrated by Wittenberg and Kornberg (1953); subsequent activation by CTP was shown by Berger and Gimenez (1956). Kennedy and Weiss (1956) showed that CDP-choline and D-α-β-diglycerides could be condensed by a yeast enzyme preparation to produce PC. The analogous steps of phosphorylation and activation of ethanolamine have not been demonstrated in yeast, but Kennedy and Weiss also showed that CDP-ethanolamine and D-α-β-diglycerides could be utilized by yeast to synthesize PE.

Phosphatidyl ethanolamine in yeast may be methylated in a stepwise fashion by S-adenosyl methionine to produce phosphatidyl choline (Letters, 1966). Arvidson (1968) has suggested that such methylation in mammalian systems may occur preferentially for unsaturated species of PE. Recent studies by Ansell and Chojnacki (1966, 1967) on a mammalian brain preparation suggest that the enzyme system responsible for synthesis of CDP-ethanolamine is quite specific for ethanolamine, whereas the system responsible for incorporation of CDP-ethanolamine into PE is quite non-specific. A variety of ethanolamine analogues, including mono-and dimethyl ethanolamine, longer and branched-chain amines, may be incorporated into phosphatides by this latter system. It should be noted in discussing the 'diglyceride pathway' that D-α-β-diglycerides may also be interconverted with mono-and triglycerides, thus linking phosphatide biosynthesis with the metabolism of neutral lipids.

Biosynthesis of phospholipids through the 'CDP-diglyceride pathway' has not been extensively investigated in yeast. Paulus and Kennedy (1960) demonstrated that a chicken liver preparation could produce PI from CDP-diglyceride and inositol (Scheme IV). The operation of a similar pathway in mammalian brain tissue has been demonstrated (Thompson et al., 1959) although metabolic studies involving phosphoinositides of brain are hampered by simultaneous synthesis of polyphosphoinositides. However, the mechanism of biosynthesis of PI by yeast has yet to be clarified. Lester and Steiner (1968) have demonstrated the occurrence of both diphospho- and triphosphoinositide in S. cerevisiae suggesting a similarity between yeast and mammalian biosynthesis of PI. White and Hawthorne (1970) have recently shown that PI in the yeast Schizosaccharomyces pombe may be formed from inositol in the absence of CDP-diglyceride, possibly by exchange of the nitrogen base of PE with inositol.

The synthesis of PS from L-serine and CDP-diglyceride in E. coli was shown by Kanfer and Kennedy (1962). A 'transphosphatidylation' reaction in which L-serine is substituted for ethanolamine in the conversion of PE to PS by rat liver has also been demonstrated by Kennedy (Borkenhagen et al., 1961). Kennedy (1961) has suggested that a ready interconversion of PS and PE may occur in rat liver by decarboxylation of PS to PE accompanied by the transphosphatidylation noted above. No similar studies have been performed with yeast.

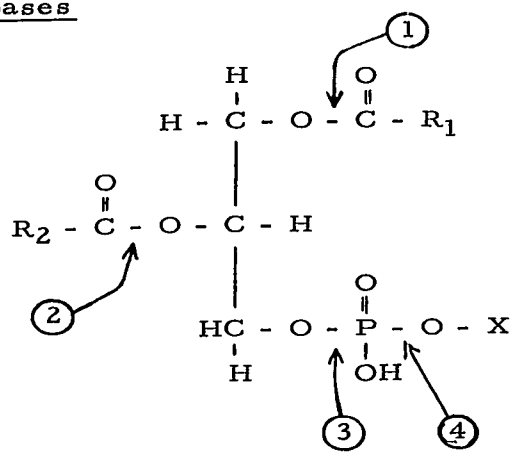
Smith and Law (1970) have shown that both the 'methylation pathway' and the 'CDP-choline pathway' may be utilized by the protozoan Tetrahymena pyriformis for the synthesis of PC. The

relative importance of each pathway has unfortunately proved difficult to determine. Waechter et al. (1969) and Steiner and Lester (1969) have intimated that a similar dual pathway for the synthesis of PC exists in S. cerevisiae, and that the presence of an exogenous source of choline during growth may depress the 'methylation pathway' while enhancing PC synthesis through the 'CDP-choline pathway'.

The fatty acid composition of phosphatides is also subject to control and modification. In situ modification of fatty acids to give cyclopropane and unsaturated fatty acids have been mentioned in the previous section. Control of the fatty acid composition of phosphatides may be exerted by preferential incorporation of specific fatty acids during biosynthetic steps involving acylation, or by the condensation of bases with specific types of diglycerides or CDP-diglycerides. Lands (1965) has suggested that the catabolism of phosphatides to their lyso derivatives and reacylation of the lyso compounds may represent an additional manner in which fatty acid composition of phosphatides may be regulated. Studies of mammalian lipids suggest that unsaturated fatty acids may be preferentially incorporated into the 2-position of many lysophosphatides (Lands, 1966). Metabolism of lysophosphatides may occur in a different manner in yeast. Kokke et al. (1963) showed that lyso PC in yeast could give rise to PC when no fatty-acyl-CoA was available. A transacylation mechanism was proposed whereby an acyl group was transferred from one molecule of lyso PC to another yielding a molecule of PC and a molecule of glycerol-phosphorylcholine. A similar reaction has been demonstrated for lyso PE.

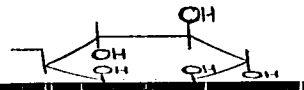
SCHEME V

Site of Action of Phospholipases



(1) Phospholipase A_1
(2) Phospholipase A_2
(3) Phospholipase D
(4) Phospholipase C

Lysophosphalipase: cleaves the acyl residues esterified in either the 1- or the 2-position in lysophosphatides.
Phospholipase B = 1 + 2 + lysophospholipase.

Phosphatide	X
PC	$-CH_2-CH_2-\overset{+}{N}(CH_3)_3$
PE	$-CH_2-CH_2-NH_2$
PS	$-CH(CO_2H)-CH_2-NH_2$
PI	

C. Enzymatic Hydrolysis of Lipids

The catabolism of phosphatides occurs by the intervention of several well characterized enzymes (Kates, 1960). These are phosphatidases A_1, A_2, B, C, and D and lysophospholipase. These enzymes catalyze the cleavage of acyl-ester and phosphate-ester bonds as shown in Scheme V. Lipases responsible for the hydrolysis of acyl-ester bonds in glycerides may also play a role in phosphatide metabolism by way of the 'diglyceride pathway'. In yeast the phospholipid-hydrolyzing enzymes are most likely phospholipase A_1, lysophospholipase and possibly phospholipase C (Letters, 1968).

D. Lipid Composition of C. lipolytica

1. Fatty Acids

As has been discussed in Section I b), a variety of media have been used for culturing C. lipolytica. The fatty acid composition of this yeast must be discussed with this fact in mind, since substantial differences in fatty acid composition have been shown between cells grown in the standard glucose medium and cells grown in specialized hydrocarbon media.

Kates and Baxter (1962) have shown that the major fatty acids of C. lipolytica when grown on a glucose-based medium are palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1) and linoleic (18:2) acids. Small amounts of shorter chain acids as well as odd-carbon numbered (15:0, 17:0) acids were found. Klug and Markovetz (1967) report a very similar fatty acid composition for C. lipolytica also grown on a glucose-containing medium. Ciarlini et al. (1966) have conducted a survey of the fatty acid composition

of seven other species (49 strains) of Candida yeasts. All species contained the same acids as C. lipolytica; in addition to these, linolenic acid (18:3) and substantially greater amounts of shorter-chain, even-numbered fatty acids were found in some species. Klug and Markovetz (1967) found that growth of C. lipolytica on n-alkanes and 1-alkenes did not change the type of fatty acids produced by the yeast but did radically alter the distribution of acids. Growth on alkanes or alkenes resulted in a net increase of fatty acids having the same number of carbon atoms. Growth on alkenes may also result in higher levels of unsaturated fatty acids in the yeast. Unsaturated alcohols and mono-carboxylic acids may also be produced by yeasts grown on alkanes.

Maurice (1967) has shown that C. lipolytica when grown on a high-boiling petroleum fraction as carbon source contained the 'normal' fatty acids, 18:2, 18:1, 16:1, 16:0 but also contained high levels of a fatty acid tentatively identified as phytanic acid. Alimova et al. (1968) have observed differences in the fatty acid composition of C. intermedia and C. tropicalis when these yeasts were grown on hydrocarbon or glucose-containing media. Growth in hydrocarbon media tended to result in the production of odd-carbon numbered fatty acids (up to 50% of total fatty acids), whereas growth in glucose was marked by production of even-numbered fatty acids. In both cases 18:1 and 18:2 were major fatty acid components. It would appear that growth in a glucose containing medium results in biosynthesis of fatty acids by the 'malonyl-CoA pathway' (Scheme I), yielding the expected even-numbered fatty acids. The small amounts of odd-numbered fatty acids may also be produced by starting with propionate as initiator of this pathway. Since

unsaturated fatty acids appear to be of the Δ^9-type, and the poly-unsaturated acid, linoleic ($\Delta^{9,12}$-octadecadienoic) acid, is found in this yeast, and no unusual hydroxy or cyclopropane fatty acids have yet been demonstrated in C. lipolytica, it seem reasonable to assume that desaturation occurs by an 'aerobic' mechanism. Growth on alkanes and alkenes does not appear to alter the fatty acid composition in a qualitative way. Klug and Markovetz have suggested that the quantitative changes observed may be due to direct oxidation and incorporation of hydrocarbons into cellular lipids. The evidence of Kates and Baxter (1962) that content of linoleic acid is related reciprocally to that of oleic acid, is further evidence in support of an 'aerobic' mechanism for fatty acid desaturation.

2. Phosphatides

Kates and Baxter (1962) have analyzed the lipids of C. lipolytica and have found the following components: phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, diglycerides, free fatty acids, and sterols. Phosphatides were by far the most common components, comprising 80% of the total lipids. Of the phosphatides, phosphatidyl choline was the most prevalent (35-40%), followed by phosphatidyl ethanolamine (20-30%). Traces of phosphatidic acid were found in the yeast lipids along with small amounts of unidentified components, (e.g. a slow-moving ninhydrin-positive spot on paper chromatography). These analyses are quite similar to the phosphatide compositions reported for other species of Candida including C. bogoriensis and C. reukaufii (Letters, 1968). Several points concerning lipid composition of

Candida species seem worthy of note. No significant amounts of lysophosphatides were found; free diglycerides were found in the neutral lipids; and combined levels of PE and PC were close to twice as high as combined levels of PS and PI. These experimental facts could be explained by the existence of a 'diglyceride pathway' for phosphatide metabolism in the yeast, as well as a 'CDP-diglyceride pathway' of lower activity. Lysophosphatide metabolism may not be of great significance.

E. The Effects of Temperature and Growth Phase on Microbial Lipid Composition

Since Henriques and Hansen demonstrated at the turn of the century that swine kept at low temperatures contained more highly unsaturated sub-cutaneous lipids than swine kept at elevated temperatures, researchers have been able to demonstrate similar temperature dependent effects in the lipids of almost all types of organisms.

Kates (1964) has reviewed temperature effects on bacterial lipids. The content of unsaturated fatty acids in bacteria is increased at temperatures below that required for optimal growth, whereas the content of cyclopropane and saturated acids is decreased. The converse effect of increased lipid saturation at temperature above the growth optimum has been shown in several bacteria, however some species, particularly the thermophilic bacteria, show either no change in the saturation of their lipids or a decrease in saturation at higher temperatures (Long and Williams, 1960). Kates and Baxter (1962) demonstrated that mesophilic and psychrophilic strains of C. lipolytica had higher levels of unsaturated fatty acids at $10^{o}C$

than at 25°C. Meyer and Bloch (1963) demonstrated that a cell free extract of C. utilis could convert fatty acyl-CoA to unsaturated derivatives to a greater extent at 19°C than at 30°C. They showed that this phenomenon could readily be explained by the stimulation of desaturating enzymes associated with the particulate phase of their cell-free system.

Fulco (1969) has shown that desaturation of palmitate by several species of bacilli is dependent on two distinct desaturating systems. The first, which introduces a double bond into the 8, 9 or 10 position is temperature independent; the second which produces desaturation in the 5 position is under strict temperature control, being active and inducible at 20°C but inactive at 30°C.

A second temperature-dependent effect in microorganisms is that involving changes in fatty acid chain length. Patterson (1970) has observed over a range of seven temperatures, from 14°C to 38°C, that the fatty acids of Chlorella sorokiniana vary not only in desaturation but also in the relative amounts of 16 carbon and 18 carbon fatty acids. The ratio of 16 to 18 carbon fatty acids varies from less than 0.9 to 14°C to almost 1.6 at 38°C. Brown and Rose (1969) have shown a different correlation in the fatty acids of Candida Utilis. They grew this yeast chemostically under glucose and ammonia limitation at different temperatures and dissolved oxygen tensions. At any particular temperature the degree of fatty acid unsaturation and the level of 18 carbon acids was directly proportional to the dissolved oxygen tension. Jollow, Kellerman and Linane (1968) found that the fatty acid composition of S. cerevisiae could be altered in a similar manner if cells were grown anaerobically. Under conditions of glucose limitation, above a critical level of

dissolved oxygen tension, Brown and Rose found that fatty acids obtained from cold-grown cells had a higher degree of unsaturation but a lower level of 18 carbon fatty acids. These results are somewhat surprising since high levels of unsaturation are generally associated with increased synthesis of unsaturated 18 carbon acids such as oleic, linoleic and linolenic acids. It seems possible that an inverse relationship may exist between the amount of 18 carbon acids produced and the amount of linolenic acid synthesized by this yeast. No similar effect of fatty-acid chain length variations was observed when cells were grown under ammonia limitation.

De Siervo (1969) has found that the phospholipid composition of E. Coli B changes with growth temperature. Cells grown at 27°C tend to accumulate phospholipids whereas cells grown at 37°C show an overall depletion of phospholipids per gram cell weight. Differences in the relative percent of individual phospholipids at the two temperatures were also observed. Kates and Baxter (1962), however, indicated that no significant changes occur in the phospholipid composition of a mesophilic strain of C. lipolytica grown at different temperatures.

The lipid composition of microorganisms has long been known to vary with the age of the culture being examined. Such variations may involve the content of total lipids or classes of lipids (e.g. phosphatides, neutral lipids) or may concern the fatty acid composition of the lipids. Asselineau (1951) found that the lipid content of Mycobacterium tuberculosis increased with age, a phenomenon explainable by an increase in neutral lipids. Kates et al. (1964) demonstrated substantial changes in the lipid content of Serratia marcescens during growth. The phosphatide composition of many microorganisms is also subject to growth-related changes.

De Siervo (1969) has noted the complex quantitative changes in the phospholipids of E. coli during growth at two temperatures; Urakami and Umetani (1968) have reported changes in the phosphatides of Bacillus natto during growth; and Randle et al. (1969) have examined the effect of growth phase on the phospholipid content of several gram negative bacteria. These results strongly indicate that phosphatides are actively metabolized in microorganisms, however McElhaney and Tourtelotte (1970) have indicated that the phospholipids of Mycoplasma laidlawii are metabolically stable.

Changes in the proportions of saturated and unsaturated fatty acids in the lipids of S. marcescens have been observed by Kates et al. (1964). Law (1963) has noted the relationship between growth phase and production of cyclopropane fatty acids in E. coli, S. marcescens and Agrobacterium tumefaciens. Both of the above-mentioned studies suggest that unsaturated fatty acids are produced in greatest amount during the early stages of exponential growth then decrease in amount as growth continues, whereas the saturated and cyclopropane acids show an inverse effect. Few similar studies have been carried out with yeast. Kates and Baxter (1962) showed that linoleic acid in C. lipolytica, grown at both $10^{\circ}C$ and $25^{\circ}C$, was produced in large amounts during early log phase and decreased in amount during late log phase, while oleic acid, its precursor, was present in minimal amounts early in growth and increased as growth continued.

It is thus clear that the lipid composition of yeast and of microorganisms in general is greatly dependent both on external, environmental factors and on internal controls.

IV. Purpose of the Research

The purpose of the work performed by the author and reported in this thesis was to examine in greater detail the temperature and growth effects on the fatty acids of C. lipolytica first reported by Kates and Baxter in 1962. Specifically it was our purpose to examine these effects with respect to component lipids of the yeast, especially during the earlier phase of growth in which changes in fatty acid composition were most marked, and during which the reciprocal relationship between oleic and linoleic acids was most in evidence. The ultimate aim of these detailed analyses was to shed light on the biosynthetic mechanisms responsible for the observed effects of temperature and growth on the degree of unsaturation of the lipids.

MATERIALS AND METHODS

I. MATERIALS

A. Yeast

The organism used was the mesophilic yeast, Candida lipolytica, strain NRRLY 1094. This is the same strain which was isolated from contaminated samples of refrigerated grape juice by Lawrence et al. (1959), and studied by Kates and Baxter (1962). Cultures were obtained in a lyophilized form from the National Research Council of Canada, Ottawa and maintained on agar slants at 8°C. The procedures for both agar and liquid culture of the organism is described in Section II A.

B. Chemicals

All solvents used were redistilled over glass. All other chemicals used were of 'Reagent Grade' quality unless otherwise specified. The enzyme preparation used for studies of the positional distribution of fatty acids in phosphatides was a lyophilized sample of Agkistrodon piscivorus venom, obtained from the Ross Allen Reptile Institute, Florida.

II. METHODS

A. Growth of the Organism

1. Purity of Cultures

The purity of stock cultures was checked by the homogeneity of colonies on agar slants and plates and by the appearance of cells under light microscopy. Colonies on agar are white in color

and produce mycelial strands which are visible using low power magnification. Under light microscopy cells may appear as ovoid single cells but are usually found in branching filamentous structures. Reproduction occurs by budding.

2. Growth Medium

The medium used for growth of the organism was a general yeast medium, the same as that used by Lawrence et al. (1959) in the initial isolation of the organism and by Kates and Baxter (1962) in their studies of the lipids of C. lipolytica. The composition of the liquid medium is as follows:

Tryptone, 10 g; Yeast extract 3 g; Glucose 20 g; KH_2PO_4 4 g; Tap water 1000 ml. The pH of the medium was adjusted to 6.0 with dilute NaOH or dilute HCl.

Agar slants were composed of the same medium with 15 g of nutrient agar per 1000 ml of water included.

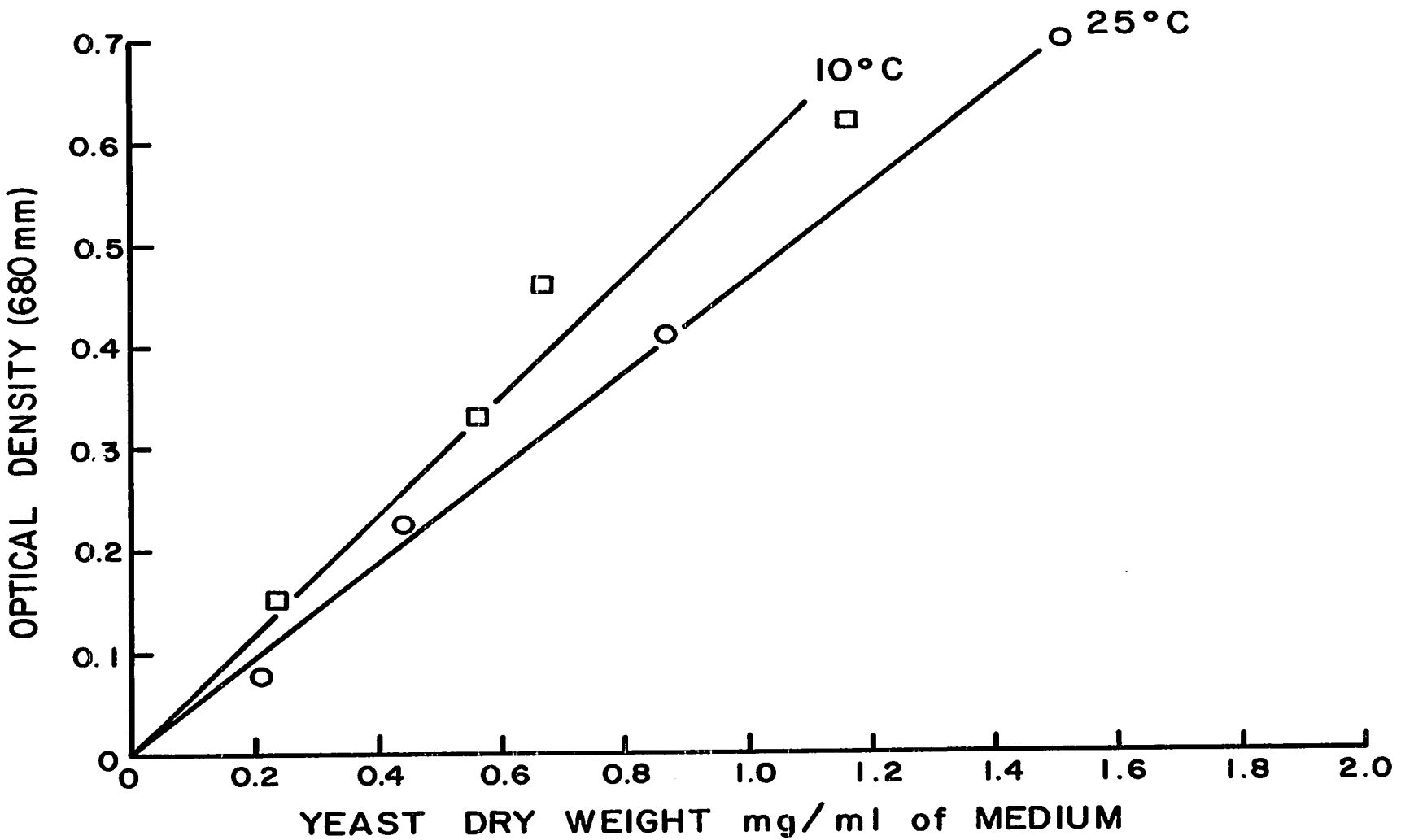
All samples were grown in liquid medium in Erlenmeyer flasks. A ratio of 1:5, v/v for medium volume:flask volume was used to maintain a similar degree of aeration in all cultures. When several samples were to be grown simultaneously, a large volume of medium was prepared, then subdivided, (before autoclaving), into smaller volumes for individual samples. In this way, initial differences in medium composition between cultures were minimized.

3. Inoculum

100 ml of autoclaved medium was loop inoculated and incubated at 25°C to an optical density of 1.4-1.5. This inoculum was then used to initiate growth in larger volumes of medium to be

FIGURE 1

Yeast cell yield at 10°C and 25°C. Values are plotted as mg. of yeast cell dry weight against the optical density of the cell culture



grown at both 10°C and 25°C. The ratio of inoculum volume to medium volume was maintained at 1:80, v/v.

4. Growth Curves

Growth curves of the yeast were determined at 10°C and 25°C for loop-inoculated and liquid-inoculated cultures, by optical density measurement, as follows:

i) 100 ml cultures were incubated in 500 ml Erlenmeyer flasks with sealed-on side-arm tube (18 x 150 mm). The optical density of the culture was measured at 680 nm in a Coleman Junior spectrophotometer by tipping the flask so as to fill the side-arm tube with the culture. The instrument was adjusted to 100% T using uninoculated culture medium as a blank contained in 500 ml Erlenmeyer flask with an optically matched sealed-on side-arm tube.

ii) For cultures of larger volume, 7 ml aliquots were removed periodically and their optical density measured in 18 x 100 mm cuvettes against a blank of uninoculated culture medium.

Incubation of cells was carried out in a Controlled Environment Incubator-Shaker (New Brunswick Co. Inc.), at either 10°C or 25°C at a shaking rate of 120 rpm.

iii) A calibration curve (see Figure I) relating the cell dry wt./ml of culture volume to the optical density of the culture harvested was determined as follows:

Cells were harvested (as described below), from cultures grown to a known optical density. An aliquot of an aqueous suspension of washed cells, usually 5% of the total volume, was used for determination of yeast cell yield. The suspension was concentrated at 40-50°C in a stream of nitrogen, then dried to constant weight in vacuo in a dessicator over KOH.

B. Harvesting of Cells

When cultures reached the appropriate optical density, samples were removed from the shaker-incubator. The pH of cultures was measured using a Radiometer, pH meter 28 (Copenhagen), and the volume of medium to be harvested was noted.

Large volumes of medium were centrifuged in 250 ml plastic buckets in a Sorvall RC2-B Automatic Refrigerated Centrifuge (Ivan Sorvall Inc.), for five minutes at 3,000 rpm. Samples of smaller volume (200 ml or 100 ml), were centrifuged in 90 ml Pyrex centrifuge tubes in a Sorvall GLC-1 centrifuge for five minutes at 2,600 rpm.

All samples gave cream-colored sediments and a clear supernatant solution. The supernatant was discarded and the cells washed onced with 40-50 ml of distilled water. The washed cells were centrifuged as described above then resuspended in a known volume of distilled water to give a dilute suspension (10-30 mg cell dry wt./ml of suspension, determined from a calibration curve of cell dry weight yield, Figure I).

The entire harvesting procedure required approximately twenty minutes.

C. Extraction of Yeast Lipids

Immediately after resuspension the washed cells were extracted by a modification (Kates et al., 1962) of the method of Bligh and Dyer (1959). To one volume of cell suspension 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 a 90 ml Pyrex centrifuge tube with vigorous mixing on a Vortex-Genie mixer (Scientific Industries). After mixing, the suspension was allowed to stand for 15-20 minutes, then centrifuged in a Sorvall GLC-1 centrifuge for five minutes at 2,600 rpm. The supernatant was decanted into an appropriate container, and the residue re-extracted in the manner previously described. The second supernatant was combined with the first and the residue discarded.

The pooled extracts were placed in a separatory funnel and appropriate volumes of chloroform and distilled water were added to give the ratio methanol:chloroform:water, 1:1:0.9, v/v. Although such a ratio of these solvents should give a biphasic system with chloroform as the lower layer and methanol/water as the upper layer, emulsions were often obtained at this stage. Emulsions usually cleared into two easily separable layers after standing for one to two hours; more rapid separation could be obtained by a brief, low-speed centrifugation of the emulsion.

The lower, chloroform layer was retained and the methanol-water layer discarded. The chloroform layer was evaporated to near dryness on a Rotavapor 'R' (Buchi, Switzerland) at 30°C. Traces of water were removed by re-evaporation in the

presence of benzene. The extracted lipids were then dissolved in a known volume of chloroform and stored at 8°C.

When required, an aliquot of this solution was removed for a determination of the total or percentage lipid yield of the sample. This was accomplished by placing the aliquot in a weighed vial then drying it under a stream of nitrogen and finally under vacuum in a dsssicator, over KOH pellets, to constant weight.

D. Analysis of Total Lipids

The term 'total lipids' will henceforth be used to refer to those lipids extracted from a sample of harvested cells, as described in the previous section.

1) Yield of Lipid

The yield of lipid (mg dry weight of lipid per 100 mg dry weight of cells) was determined by measuring the dry weight of lipid (as described in Section II C) obtained from a measured dry weight of cells (as described in Section II B).

2) Lipid Phosphorus

Phosphorus in the total lipids was determined by the method of Allen (1940), as follows:

> The sample solution, containing 30 - 60 µgP, was concentrated to dryness (under a stream of nitrogen), in a 25 ml digestion tube. Additional tubes containing 30 µgP and 60 µgP respectively of a standard phosphate solution, as well as an empty tube (to be used as a blank), were included in the determination.

To each tube was added 2.0 ml of 72% perchloric acid, along with a washed glass bead. Tubes containing lipid samples were heated in a digestion apparatus over a bunsen flame for several minutes until clear, then permitted to cool to room temperature. Each tube was then made up to the 12.5 ml mark with distilled water.

To each tube was added 2.0 ml of Amidol solution* followed by vigorous mixing, then 1.0 ml of ammonium molybdate solution** followed again by vigorous mixing. Color was allowed to develop over a period of twenty minutes, with intermittent mixing. Water was then added up to the 25.0 ml mark of each tube, and the contents of all tubes were vigorously mixed by inversion. The percentage transmission of the solutions was then measured at 680 nm in a Coleman Junior Spectrophotometer against the blank previously mentioned.

An alternative procedure, utilizing the methanol-water phase from the preparation of fatty acid methyl esters (see Analysis of Total Lipids, Section II D 5) was used when insufficient total lipid sample was available for a separate phosphorus analysis. This method differs from that described above only insofar as the methanol-water solution must be evaporated under nitrogen at elevated temperature (50-60°C) to remove solvent; and an additional blank is run to determine any phosphorus contamination from the methanolysis procedure.

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*Amidol solution: 1.0 g 2,4-diaminophenol dihydrochloride (Amidol) and 20.0 g sodium metabisulfite dissolved in 100 ml of distilled water and filtered.

**Ammonium Molybdate solution: 8.3 g ammonium molybdate dissolved in 100 ml distilled water.

Results by the two methods are reproducible and comparable within an error range of 2-3%.

3) Chromatography on Silicic Acid Impregnated Paper

Total lipid samples were spotted and run on silicic acid impregnated paper according to the method of Marinetti et al. (1957, 1958). The procedure for the preparation of chromatographic paper is as follows:

Whatman 3MM paper was cut into 11.5 cm x 45.0 cm strips, a pencil line being drawn 2 cm from one end of each strip to mark off a 'handling zone'. A solution of sodium silicate was prepared by slowly adding 115 g of silicic acid (100 mesh, Malinckrodt) with stirring, to 1 litre of 7.2 N sodium hydroxide solution. The solution was permitted to cool to room temperature and was then diluted to 1500 ml with water. The paper strips were dipped through the sodium silicate solution and hung to drain for three to four minutes. They were then immersed in 6N hydrochloric acid solution for thirty minutes with intermittent rocking of the acid tray. Papers were then washed in running tap water for two hours and finally in several changes of distilled water for one to two hours. Drippings from the paper were tested for the presence of chloride ions with acidified silver nitrate solution and washing continued till a negative test result was obtained. Papers were then hung to dry overnight. Dried papers were placed in a closed cylindrical jar (15 cm x 45 cm), with their lower ends immersed in a solution of chloroform:methanol, (1:1), until the solvent front had risen to within a few inches of the top (20-24 hours). Papers were again hung to dry, then pressed between glass plates for several hours.

Prior to use, papers, prepared and washed as above, were marked with a 'spotting line' two inches above the 'handling line' and with additional 'handling lines' across each of the upper corners. Spots for the application of samples were marked on the 'spotting line' at regular intervals (no more than six spots were placed on one sheet of paper). Samples were then applied to the spots by micropipet or capillary tube. Individual phospholipids were applied in an amount calculated to contain 1-2 μgP; total lipid samples were applied in an amount calculated to contain 5-6 μgP (these amounts having been determined on the basis of previous experiments). Once a paper was spotted, handling areas were cut off and the lower end of the paper immersed (in a cylindrical jar as for washing), in about 150 ml of developing solvent.

The developing solvent used to separate polar lipids was: 2, 6-dimethyl 4-heptanone:acetic acid:water (8:5:1, v/v). The developing solvent was permitted to rise to within a few inches of the top of the paper. The papers were then permitted to dry in fume hood for 15-20 minutes. Lipids chromatographed on silicic acid impregnated paper were located by staining with a 0.001% aqueous solution of Rhodamine-6G and viewing under U. V. light (388 nm). Components appear as spots fluorescing with a characteristic color: blue for phosphatidyl inositol and phosphatidyl serine; yellow for phosphatidyl choline and phosphatidyl ethanolamine; yellow to orange for the neutral lipids (Marinetti et al., 1957, 1958). Rhodamine-stained chromatograms were usually oversprayed with a 0.25% solution of Ninhydrin for detection of aminolipids. Rf's of component lipids and standard lipids were measured.

4) Thin-layer Chromatography of Total Lipids

Thin-layer chromatography of total lipids was used primarily to determine the ability of a variety of solvents to separate the total lipids into those components which were of most interest. Of primary importance is the solvent system chloroform: methanol:acetic acid:water (25:15:4:2, v/v) used in all later preparative separations of total lipids.

TLC plates were prepared in the following manner: 40 g of Silica gel H-Stahl (Merck, Darmstadt, West Germany) was uniformly suspended in 90 ml of 0.01% sodium carbonate solution. The suspension was applied to 20 cm x 20 cm and 10 cm x 20 cm glass plates or 2.5 cm x 7.5 cm glass microslides with a Desaga Stainless Steel Applicator (Heidelberg), to the desired thickness (0.5 mm - 1.0 mm). After the silica had set, plates were oven dried. Plates were washed by immersing their lower ends in about 120 ml of chloroform:methanol (1:1, v/v), solution, in a paper-lined, glass chromatography jar. The solvent was permitted to rise to the top of the plates. Washed plates were activated by oven drying.

Total lipids and standard lipids were applied as spots/ (0.1-0.5 mg/spot) in a mount sufficient to give good visualization. For preparative detection of lipid component bands, plates were permitted to air dry for approximately one minute and were then sprayed with a 0.1% aqueous solution of Rhodamine-6G and viewed under U.V. light (UVL-22, Fisher Scientific Corp.) at 388 nm.

Plates used for qualitative purposes only were sprayed with a solution of 30-50% H_2SO_4 in ethanol and charred over a hot-plate.

5) Methanolysis of Total Lipids

Methanolysis is defined as the conversion of both free and esterified fatty acids to their corresponding methyl esters, by treatment of the lipids with a solution of hydrogen chloride in methanol.

A 2.5% (w/v) solution of hydrogen chloride in methanol was obtained by bubbling hydrogen chloride gas from a cylinder into a known volume of methanol. A sample of total lipid (1-5 mg) in chloroform was blown to dryness under a stream of nitrogen in a 50 ml Erlenmeyer flask with a sealed-on 5 ml side-arm tube. To the dried sample was added 4.5 ml of 2.5% methanolic-HCl. The flask was attached to a condenser and drying tube and refluxed for approximately one hour. The solution was permitted to cool, diluted with 0.5 ml of distilled water and extracted three times with 5 ml of petroleum ether (b.p. 40-60°C), (the petroleum ether being easily decanted off while the aqueous methanol phase remained in the side-arm tube). The petroleum ether extracts containing the fatty acid methyl esters were collected and blown to dryness under a stream of nitrogen, methyl ester samples then being taken up into a small volume of chloroform for injection into the GLC.

6) Hydrogenation of Fatty Acid Methyl Esters

A solution of 5 mg of fatty acid methyl ester (obtained by hydrolysis of lipids from cells grown at 10°C and 25°C) in 10 ml methanol was hydrogenated for one hour at room temperature and atmospheric pressure with stirring over $PtO_2.H_2O$. The solution was then filtered to remove the catalyst and evaporated to near dryness under a stream of nitrogen. Methyl esters were then dissolved in chloroform for analysis by GLC.

7) Gas-Liquid Chromatography of Total Lipid Fatty Acid Methyl Esters

i) Preparation of GLC Columns

2 g of butanediol succinate (BDS) polyester was dissolved in 70 ml of warm chloroform and placed in a 1 litre flask. To this solution was added 18 g of Gas Chrom A. The solvent was removed by evaporation on a rotary evaporator at 35°C-40°C. The residue was dried in a dessicator (high vacuum), for two hours. A coiled 3 ft. glass column fitted to the inlet-outlet channels of a Carlo Erba Fractovap unit was plugged at one end with glass wool, and packed with the coated solid phase using a water pump for suction. The other end of the column was then also plugged with glass wool and the column fitted to the Fractovap unit.

ii) GLC Operating Conditions

Newly packed GLC columns were equilibrated in the Fractovap column oven at 180°C for 24 hours at a carrier gas pressure of approximately 1 kg/cm^2.

GLC operating conditions were as follows: Column oven temperature 172-173°C, injector port temperature 225°C, detector (flame-ionization unit) temperature 235°C, nitrogen carrier gas pressure 0.5 kg/cm^2. 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 320-1600. The system, once detector gases were lit, was allowed to equilibrate for one to two hours. The recorder pen was set at zero using the zero-adjustment knob of the electrometer. A steady base-line tracing of 15-20 minutes was taken as indicative of complete equilibration.

A solution of standard fatty acid methyl esters (14:0, 16:0, 16:1, 18:0, 18:1 or 16:0, 16:1, 18:0, 18:1, 18:2) in chloroform was prepared in a suitable concentration (1-10%, w/v). Samples (0.5-1.0 µl) were injected by a 10 µl Hamilton syringe through the rubber diaphragm of the injector port, (the syringe plunger being depressed and the syringe needle being kept inside the injection port for a standard period of five seconds). The syringe was then quickly removed and the polarization switch of the electrometer switched quickly to negative polarization then back to positive polarization as a zero time mark. Samples were injected repeatedly to check reproducibility of retention times and peak heights.

iii) Maintenance of GLC Apparatus

Several factors affecting the performance of the GLC system may be optimized by periodic maintenance. Gases used in the system pass through molecular filters which must be periodically replaced. Rubber diaphragms used in the injection port are subject to contamination and must also be replaced after sustained use. The detector was cleaned by sand-blasting after prolonged periods of use to remove accumulated combustion products and rust. Recorder sensitivity must be optimized for the particular attenuations being used.

E. Analysis of Component Lipids

1) Chromatographic Separation and Identification of Components

Total lipid samples in chloroform solution were applied (10-15 mg/plate) to TLC plates as streaks by the use of a TLC sample streaker (Applied Science Laboratories, Inc.). Plates were run in chloroform:

methanol:acetic acid:water (25:15:4:2, v/v), component bands being located by the techniques described in section Section II D iv), and scraped into 19 ml of methanol:chloroform:water (2:1:0.8), solution in 90 ml round bottomed centrifuged tubes. The mixtures were well shaken on a Vortex-Genie. The procedure for extraction of component lipids from the silica was the same as that described for extraction of lipids from yeast cells (Section II C). Each fraction obtained from TLC was extracted a total of four times with 19 ml of extracting solvent. Separation of the chloroform phase from the extracts and preparation of stock component lipid solutions in chloroform is the same as that described for total lipids in Section II C.

Component lipids were chromatographically identified on silicic acid impregnated paper and TLC plates by observation of their Rf's and staining behaviour relative to that of standard lipids. Procedures followed for the application and development as well as staining or charring of chromatograms are described in Sections II D 3 and II D 4, with the exception of the component lipid identified as lecithin (PC). PC and Rhodamine-6G (present in lipid components eluted from Rhodamine-stained TLC plates), were shown to have the same Rf on silicic acid impregnated paper. Before application to chromatographic paper, the PC was separated from Rhodamine-6G by TLC on a microslide, using chloroform: methanol 3:1 as the developing solvent. PC (which was left near the origin), was then scraped off the slide, extracted from the silica as described above, and obtained as a colorless chloroform solution for application to silicic acid impregnated paper.

An attempt was made to separate the two phospholipid components PS and PI, obtained in the same TLC fraction, by preparative chromatography on silicic acid impregnated paper. Twenty spots of the TLC fraction were spotted on a paper, developed and stained with Rhodamine-6G as described in Section II D 3. Three component bands were observed rather than two (see Results section). Each band was marked off under U. V. light and cut from the paper. The paper strips obtained were then subjected to descending chromatography with distilled methanol (overnight). Eluates were rechromatographed on silicic acid impregnated paper for identification as described above.

2) Phosphorus Content of Components

The percentage P in each of the four TLC fractions obtained from the total lipids of samples of yeast grown at 10°C and 25°C was determined using the procedure described in Section II D 2. Phosphorus was determined in the methanolic phase remaining after extraction of fatty acid methyl esters from the methanolysis of component lipids. The silica of an unused, Rhodamine-6G stained TLC plate was extracted, the extracts subjected to methanolysis and the methanolic phase from this procedure analysed for P, to determine any contamination which might affect the accuracy of component lipid percentage P determinations.

3) Methanolysis and GLC Analysis of Component Lipids

The procedures for methanolysis and GLC analysis of component lipids are the same as those described for total lipids in Section II D 5 and II D 6. The petroleum ether extracts of the

sample used to check P contamination in Section II E ii were run on GLC to determine any fatty acid contaminants which might affect the accuracy of fatty acid determinations in the lipid components. The eluates of two of the three bands isolated in the attempted separation of PS and PI by paper chromatography, shown to contain PS and PI, were also subjected to methanolysis and GLC analysis as described in Sections II D 5 and II D 6 . The eluate from a blank strip of silicic acid impregnated paper treated in the same manner as those from which the PS and PI components were obtained, was analysed on GLC after methanolysis to check the reliability of fatty acid analyses for these two samples.

F. Studies of the Positional Distribution of Fatty Acids in PC and PE

1) Enzymatic Cleavage with Snake-Venom Preparation

Procedures for enzymatic cleavage of esterified fatty acids were first checked using a Rhodamine-6G containing preparation of standard PC as follows:

1.2 mg of standard PC was dissolved in 1.0 ml of a 6% methanolic solution of diethyl ether saturated with Rhodamine-6G. To this solution was added 0.01 ml of an ammoniacal (0.05 N), solution of diethyl ether containing 3% methanol, and 0.01 ml of snake venom solution (1 mg snake venom/ml of 0.005 M $CaCl_2$). The reaction was carried out in a 2 ml tapered centrifuge tube. Progress of the reaction was followed by TLC on microslides of samples of reaction mixture withdrawn at intervals up to ninety minutes. Over this period of time substantial evaporation of solvent occurred and reaction volume was maintained at, or near,

its original level by dropwise addition of diethyl ether. The reaction solution was periodically swirled on a Vortex-Genie, and became cloudy 15-20 minutes after addition of the snake venom solution. TLC indicated that reaction was complete with respect to disappearance of PC after approximately 60 minutes of incubation with the venom. At this time the precipitate formed during the reaction was collected by centrifugation and separated from the supernatant. Both phases were subjected to TLC. Samples of PC and PE obtained from yeast grown at 10°C and 25°C were processed in the same manner. PE samples, however, required a longer incubation period, 3-4 hours.

2) Preparation of Fatty Acid Methyl Esters

A solution of diazomethane in ether was prepared as follows:

2 g of N-nitrosomethylurea was slowly added, with stirring, to an ice-cooled biphasic system containing 6 ml of 40% KOH in water and 20 ml of dry ethyl ether in a 100 ml round bottom flask. Diazomethane was distilled from this system at 30-40°C through a water-cooled condenser and collected in an appropriate volume of diethyl ether (Fieser and Fieser, 1967). After an appropriate period of incubation the total reaction mixture was evaporated under a stream of nitrogen to a small volume (0.1-0.2 ml), to which was added approximately 1 ml of the diazomethane solution. This solution was allowed to stand for one hour at room temperature, and was then evaporated to dryness under a stream of nitrogen 0.5-0.7 ml of methanol:water (8:1, v/v), solution was immediately added to the residue. Addition of 0.5-0.7 ml of

petroleum ether, (b.p. 40-60°C), produced a biphasic system which was well mixed on a Vortex-Genie. The petroleum ether phase was removed from the methanolic phase by capillary pipet, and the methanolic phase re-extracted several times with petroleum ether till a volume of approximately 4-5 ml of extract had been collected. The extracts were evaporated to dryness under a stream of nitrogen and the residue redissolved in a small volume of chloroform for injection into the GLC (Section II D 6). The aqueous methanol phase containing the lysolecithin was subjected to methanolysis and the fatty acid methyl esters analysed by GLC as described in Sections II D 5 and II D 6 . Two controls were subjected to the methanolysis as described in this section after snake-venom treatment as described in Section II F 1. The first contained all reagents with standard lyso PC instead of PC, the second contained all reagents but the phosphatide.

3) Argentation Chromatography of PC Obtained from Yeast grown at 10°C and 25°C

TLC plates containing silver-nitrate were prepared according to the method of Arvidson (1965), in the following manner:

40 g of binder-free Silica gel H (Merck, Darmstaadt), was mixed with 110-115 ml of water containing 12 g of $AgNO_3$. The thin slurry was spread on 20 cm x 20 cm and 5 cm x 20 cm glass plates, by means of a stainless steel applicator, to a thickness of 0.35 mm. Plates were dried at room temperature, then heated at 175°C for 4 hours and stored in a dessicator over P_2O_5. Plates were spotted or streaked and molecular species of PC located as described in Section II D 4 . The solvent used was chloroform: methanol:water (65:25:4, v/v). In preparative separation of

molecular species of PC, fractions located on TLC plates, (which had been left to air dry for approximately five minutes) were scraped directly into 50 ml Erlenmeyer flasks (with sealed-on side-arm tubes), for methanolysis and consequent GLC analysis of methyl esters as described in Sections II D 5 and II D 6 . A band of blank $AgNO_3$-silica was also processed in this manner for eventual GLC analysis.

RESULTS

I. Growth of the Organism

The yield of yeast cells (mg. dry weight) at both 10°C and 25°C (Fig. 1) was essentially linear with optical density of the culture (up to O.D. 0.7). The yield of cells per unit O.D. was, however, somewhat lower at 10°C than at 25°C. No marked changes in the morphology of the yeast during growth at either temperature was observed, and at both temperatures cultures appeared to be free from contamination.

II. Analysis of Total Lipids

Cells grown at 10°C had a lipid content more than 20% greater than cells grown at 25°C (Table I). Lipids from the 10°C cells also had a higher phosphorus content and consequently a relatively higher content of phosphatides and smaller amounts of neutral lipids. In terms of the absolute amounts of phosphatides and neutral lipids, however, it would appear that the increase in lipid content of 10°C cells is due almost entirely to an increase in phosphatides at that temperature.

Chromatography of lipids obtained from cells grown at both temperatures showed essentially no qualitative differences in the neutral or polar lipids throughout growth (Figs. 2 and 3).

The major phospholipids were identified as PC, PE, PS and PI (Table II). A few minor unidentified phosphatide components were detected on silicic acid impregnated paper chromatography(Fig. 3). The major neutral lipids were free fatty acids and sterols. Traces of diglycerides and triglycerides were also found in all samples but monoglycerides were not detected (Fig. 2B).

TABLE I

Content and Composition of Lipids from C. lipolytica*

Analysis	Growth Temperature	
	25°C	10°C
Total lipid content		
g/100 g dry cells	5.7	7.3
P, %	2.85	3.07
Phosphatides**		
% of total lipids	71	76
g/100 g dry cells	4.0	5.5
Neutral lipids‡		
% of total lipids	29	24
g/100 g dry cells	1.7	1.8

* Cells were grown to an O.D. of 1.0 - 1.45.

** Calculated as follows: % phosphatides = % P x 25. This calculation is based on the assumption that the average molecular weight of phosphatides is approximately 800.

‡ Calculated by difference.

FIGURE 2

A. TLC of phosphatide components of the lipids of C. lipolytica.

Solvent: Chloroform-Methanol-Acetic acid-Water (25:15:4:2)

Material applied:

a. Total lipids, Inoculum
b. Total lipids, 25°C, 4.5 hr.
c. Total lipids, 10°C, 33 hr.
d. Standard PC
e. Standard PI
f. Standard PS
g. Standard PE

B. TLC of neutral lipids of C. lipolytica.

Solvent: Diethyl ether-Benzene-Ethanol-Acetic acid (40:50:2:0.2).

Material applied:

a. Total lipids, Inoculum
b. Total lipids, 25°C, 4.5 hr
c. Total lipids, 10°C, 33 hr.
d. Monoglycerides (MG)
e. Diglycerides (DG)
f. Triglycerides (CH)
g. Fatty acids (TG)
h. Cholesterol (FA)

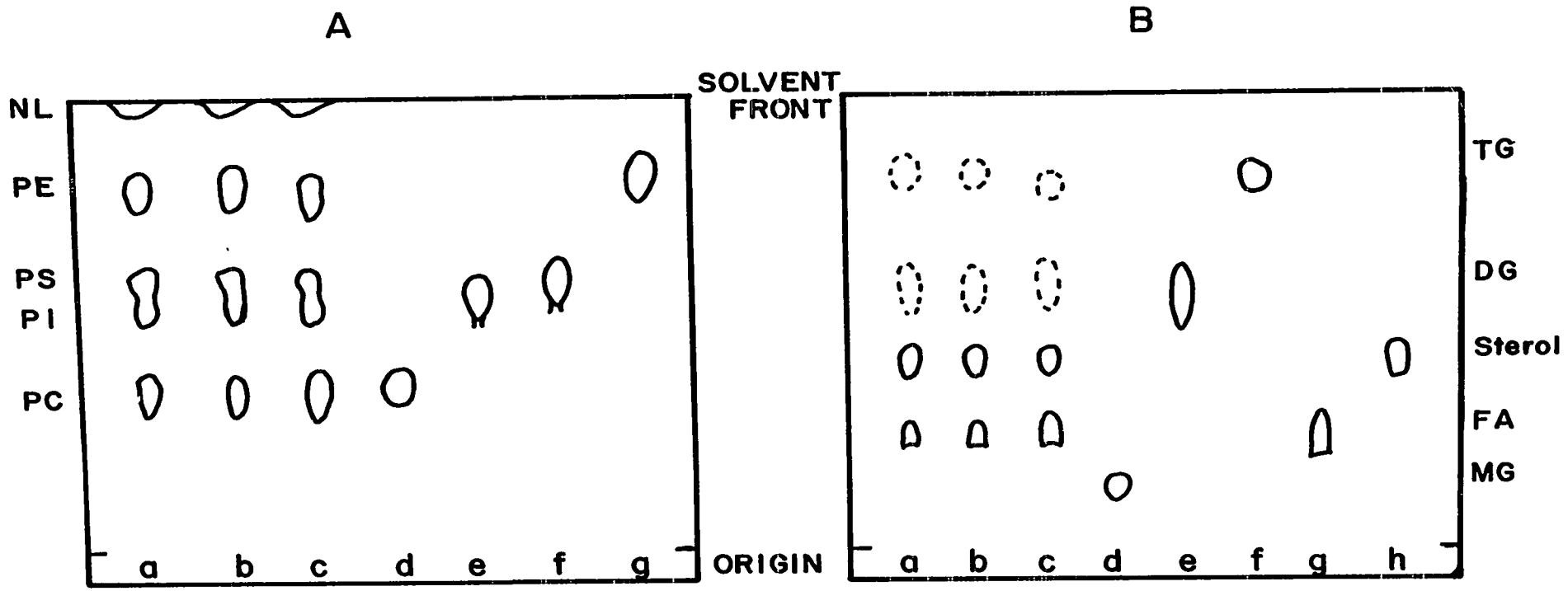


FIGURE 3

Chromatography of the phosphatides of C. lipolytica on silicic acid impregnated paper.

Solvent: 2, 6-dimethyl-4-heptanone-acetic acid-water (8:5:1)

Material applied:

a. Total lipids 10°C, 33 hr.

b. Total lipids 25°C, 4.5 hr.

c. PC fraction from TLC

d. PI + PS fraction from TLC

e. PE fraction from TLC

Mixture of standard phosphatides (PI, PC, PS, PE)

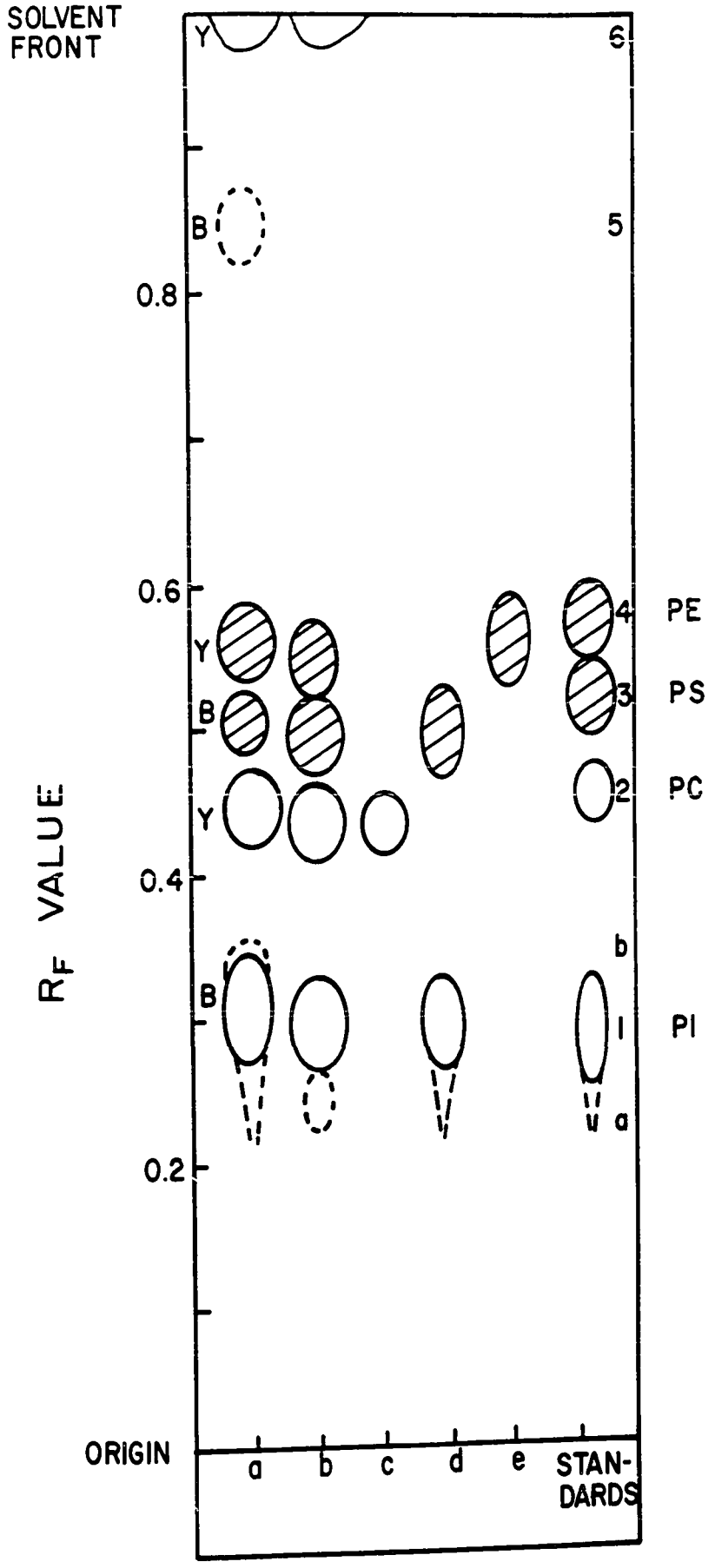

SOLVENT FRONT
Y
6
B
5
0.8
0.6
Y
4
PE
B
3
PS
Y
2
PC
0.4
b
B
1
PI
a
0.2
ORIGIN
a
b
c
d
e
STAN-DARDS
R_F VALUE

TABLE II

Phosphatide Components of C. lipolytica*

Spot No.	Rf value (av.)	Rhodamine 6G stain	Ninhydrin stain	P, %**		Tentative Identity of Component
				25°C	10°C	
'a'	0.26	B_F	-	ND	ND	Unknown
1	0.32	B_S	-	+	+	PI
'b'	0.35	B_F	F	ND	ND	Unknown
2	0.47	Y_S	-	40	36	PC
3	0.53	B_S	M	+	+	PS
4	0.59	Y_S	S	24	29	PE
5	0.85	B_F	-	ND	ND	Unknown
6	0.98	O	-	ND	ND	Neutral lipids

*See Figure 2; abbreviations: B, blue; Y, yellow; O, orange; F, faint; M, medium; S, strong; -, negative. Rf's and staining behaviour from chromatography on silicic impregnated paper using Marinetti's solvent.

**Phosphorus content of components was determined after separation by TLC (Materials and Methods). Components 'a', 'b', 5 and 6 were not determined (ND); values are given as % of total P applied to plates.
+Sum of PI and PS; 25°C, 32% P; 10°C, 28% P.

Quantitative differences in the major phospholipids at the two temperatures do exist (Table II). Small decreases in the relative amounts of PC and PS + PI and a small increase in PE at 10°C compared to those at 25°C were observed. It should be noted that these values for phosphatide composition were obtained from lipids extracted from cultures grown to a high optical density and may not be representative of the phosphatide composition during other phases of growth.

TLC of lipids in neutral lipid solvent (Fig. 2B) indicated that substantially more free fatty acids are produced at 10°C than at 25°C and that some qualitative differences may exist between the triglyceride components of 10°C and 25°C lipids.

III. Changes in Fatty Acid Composition

Fatty acids were tentatively identified by comparison of their retention times with those of standard fatty acid methyl esters (Table III), and quantitized by the retention time x peak height method of Carroll (1961) as shown in Table IV. Hydrogenation of a mixed sample of 25°C and 10°C fatty acid methyl esters supports the assignment of identities of the unsaturated acids, insofar as the latter disappeared on hydrogenation and corresponding saturated peaks increased by an equivalent amount. At both temperatures the major acids identified were linoleic (18:2), oleic (18:1), palmitoleic (16:1) and palmitic (16:0) acids; small amounts of stearic (18:0), heptadecanoic (17:0) and heptadecenoic (17:1) acids as well as traces of myristic (14:0) and pentadecanoic (15:0) acids were present in all samples. The GLC chromatograms (Figs. 4A and 4B) indicate the type of quantitative changes which occur between samples containing maximum (Fig. 4A) and minimum (Fig. 4B) proportions of 18:2.

TABLE III

GLC Identification of Fatty Acid Methyl Esters of C. lipolytica

Fatty Acid	Retention* time (mm)	Retention Relative to 16:0	% Composition** Original Sample	% Composition** Hydrogenated Sample
16:0	57.0	1.00	27.3	32.9
16:1	64.9	1.14	7.4	0
17:0	83.4	1.46	0.9	2.5
17:1	91.0	1.59	1.1	0
18:0	113.8	2.00	4.9	64.5
18:1	125.8	2.21	27.3	0
18:2	153.1	2.69	31.1	0
Standard Fatty Acids				
Palmitic	57.2	1.00		
Palmitoleic	65.0	1.14		
Stearic	113.3	1.98		
Oleic	125.3	2.19		
Linoleic	152.7	2.67		

*On butanediol succinate polyester at 172°C.

**The sample analyzed before and after catalytic (Pt) hydrogenation was a pooled fatty acid methyl ester preparation obtained from methanolysis of lipids from both 10°C and 25°C cells. Both the original and the hydrogenated samples contained < 1% of 14:0 and 15:0.

TABLE IV

Calculation of Fatty Acid Peak Areas as Percentages of Total Peak Areas by Method of Carroll (1961)*

Fatty Acid Methyl Ester	Peak height (mm) h	Retention time (mm) R	Product hR	% to Total
16:0	70.0	58.0	4060.0	17.1
16:1	34.0	65.7	2233.8	9.4
17:0	2.2	83.8	184.4	0.8
17:1	3.8	91.0	346.6	1.5
18:0	6.1	113.9	694.8	2.9
18:1	63.0	125.2	7887.6	33.1
18:2	55.0	152.3	8376.5	35.2
		Total	23,783.7	100.0

*Calculation given for chromatogram B, Figure IV; 14:0 and 15:0 peaks (< 1%) are not included.

FIGURE 4

Chromatogram A

Fatty acid methyl esters from total lipids of cells grown at 10°C for 33 hr.

Chromatogram B

Fatty acid methyl esters from total lipids of cells grown at 25°C for 36 hr. (inoculum)

Column: Butanediol-succinate polyester at 173°C.

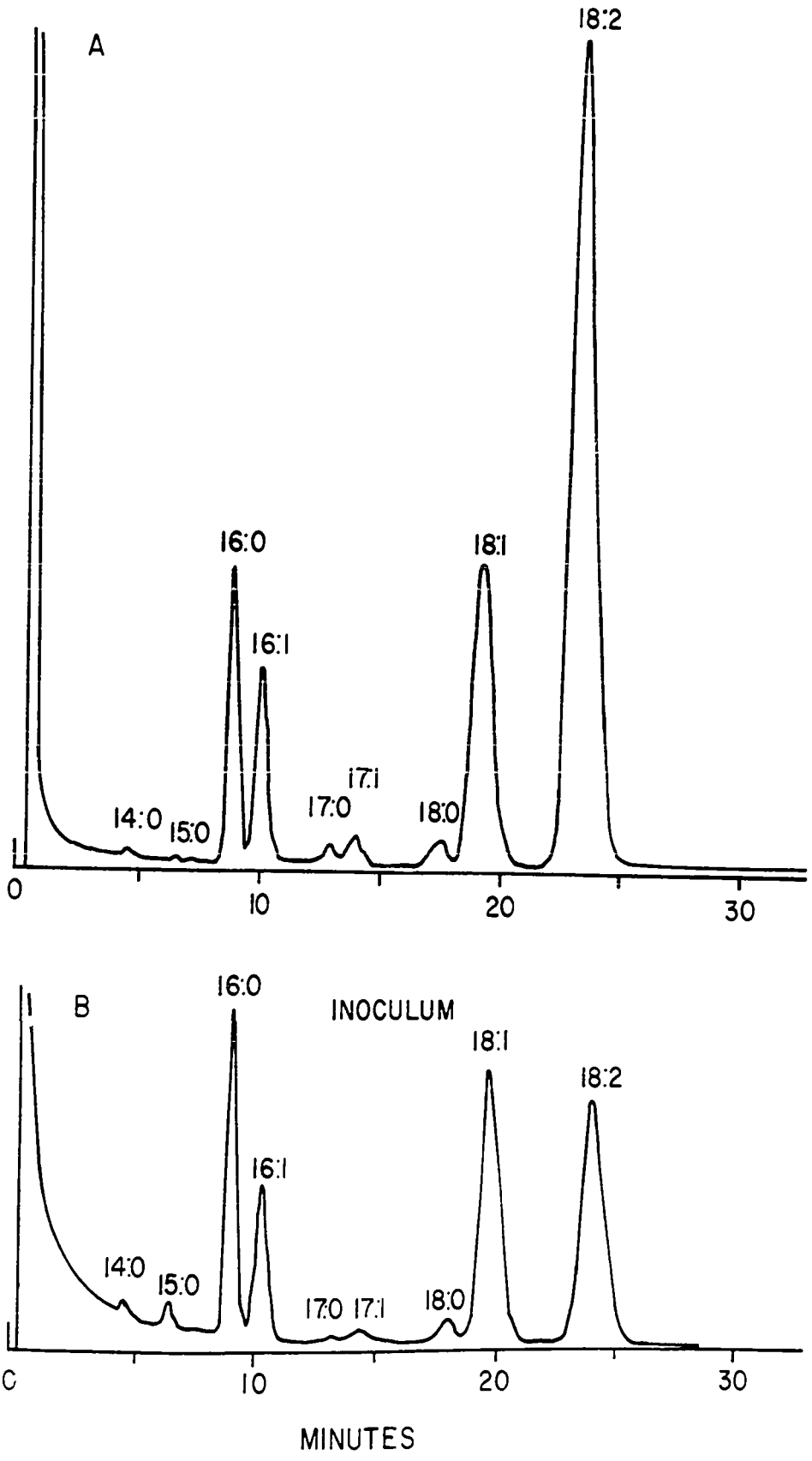
Nitrogen carrier gas pressure: 0.5 kg/cm^2.

Attenuation: Chromatogram A, 640

Chromatogram B, 320

grown

grown


A
18:2
16:0
18:1
16:1
17:1
17:0
18:0
14:0
15:0
0
10
20
30
B
INOCULUM
16:0
18:1
18:2
16:1
14:0
15:0
17:0
17:1
18:0
0
10
20
30
MINUTES

For the purpose of the following discussion only significant changes ($\geq$ 4%) in fatty acid composition will be considered. With rare exceptions, no such changes are found in the 17:0, 17:1 and 18:0 components.

A. Total Lipids

At 25°C the level of 18:2 acid increased from the inoculum value of 35% to a maximum of 55% in early cultures (4.5 hr) while a concurrent decrease of 18:1 acid from 33% to a minimum of 17% took place (Fig. 5; Table V). After 4.5 hr. linoleic acid decreased slowly to 42% while oleic acid increased slowly to 34%. No other significant changes in fatty acid content occurred other than a relatively small decrease of 16:0 to a minimum value during early growth.

At 10°C the same general type of changes were noted, except that the maximum level of linoleic acid was almost 10% higher at 10°C than at 25°C and high levels of 18:2 acid were maintained later in growth than at 25°C (Fig. 5; Table VI). The level of oleic acid though decreasing to the same minimum value as at 25°C, did not increase again to as high a final level as at 25°C. The level of 16:0 acid decreased to a somewhat lower level at 10°C than at 25°C.

At both temperatures, maximum levels of linoleic acid and minimum levels of oleic acid are reached during the early stages of growth.

TABLE V

Changes in Fatty Acid Composition of Lipids During Cell Growth at 25°C

Sample	Time (hr)	Fatty Acid Compostion, %*						
		16:0	16:1	17:0	17:1	18:0	18:1	18:2
Total lipids	0	17.1	9.4	0.8	1.5	2.9	33.1	35.2
	4.5	14.3	8.0	1.2	2.1	2.1	17.3	54.5
	7	10.9	7.2	1.1	2.8	2.7	20.7	54.0
	8	10.3	6.7	0.9	2.2	2.7	25.5	51.3
	11.5	12.1	6.9	0.7	1.6	2.5	33.8	42.1
PC	0	5.7	12.9	0.9	1.5	2.3	31.2	45.5
	4.5	5.3	9.4	1.8	2.0	1.9	11.7	67.9
	7	5.2	8.3	1.5	2.5	2.8	13.4	65.7
	8	5.1	7.6	1.1	2.2	2.6	18.4	63.0
	11.5	4.2	8.1	1.0	1.8	2.2	26.8	55.3
PE	0	14.0	10.4	0.4	1.3	1.4	36.3	36.2
	4.5	18.6	9.2	0.4	2.0	1.2	21.8	46.7
	7	17.0	7.7	0.4	2.7	3.3	25.3	43.6
	8	13.4	7.2	0.4	1.6	1.7	30.6	45.1
	11.5	14.2	7.7	0.2	1.5	1.3	40.4	34.6
PS and PI	0	38.5	6.0	0.6	0.6	5.3	33.8	15.2
	4.5	33.4	5.6	0.5	1.7	1.7	22.4	34.8
	7	24.2	6.2	0.6	2.6	2.2	29.3	34.8
	8	24.8	5.3	0.4	1.6	2.9	33.4	31.6
	11.5	28.6	5.1	0.2	0.8	2.6	40.3	22.2
Neutral lipids	0	13.8	5.9	0.4	0.7	11.0	27.3	37.1
	4.5	14.3	4.9	0.5	1.3	5.2	26.8	47.0
	7	8.0	6.2	0.6	1.8	4.3	27.8	51.8
	8	8.2	5.3	0.7	1.7	6.5	30.8	46.8
	11.5	12.7	5.3	0.5	1.0	8.6	33.8	38.1

*All samples contained < 1% of 14:0 and 15:0 fatty acid methyl esters.

TABLE VI

Changes in Fatty Acid Composition of Lipids During Cell Growth at 10°C

		Fatty Acid Composition*						
Sample	Time(hr.)	16:0	16:1	17:0	17:1	18:0	18:1	18:2
Total lipids	0	17.1	9.4	0.8	1.5	2.9	33.1	35.2
	13	14.4	10.1	1.1	1.2	2.5	21.3	49.4
	24.5	8.2	6.9	1.0	1.4	1.3	17.1	63.4
	33	8.4	6.5	0.8	1.4	1.5	18.8	62.2
	41.5	8.2	6.7	0.8	1.0	1.5	24.5	56.6
	48.5	11.0	6.9	0.8	0.9	1.6	29.2	48.8
PC	0	5.7	12.9	0.9	1.5	2.3	31.2	45.5
	13	3.7	11.8	1.8	1.2	1.4	12.8	67.3
	24.5	2.0	8.9	1.6	1.0	1.7	13.4	71.4
	33	3.1	6.3	1.1	0.9	1.1	11.4	76.1
	41.5	2.8	7.3	1.2	0.8	1.3	18.3	68.2
	48.5	2.1	7.5	1.0	0.8	1.3	20.1	67.1
PE	0	14.0	10.4	0.4	1.3	1.4	36.3	36.2
	13	16.6	11.7	0.6	1.3	1.2	24.9	43.7
	24.5	12.8	9.2	0.6	1.8	1.0	25.2	49.4
	33	15.7	9.8	0.4	1.9	1.4	22.2	48.7
	41.5	13.2	8.5	0.4	1.4	1.0	33.1	42.3
	48.5	13.0	8.4	0.4	1.1	2.5	33.3	41.3
PS and PI	0	38.5	6.0	0.6	0.6	5.3	33.8	15.2
	13	34.4	7.0	0.2	0.8	2.2	26.2	29.2
	24.5	22.6	5.3	0.5	1.3	2.2	28.0	40.0
	33	22.8	6.2	0.2	1.4	1.9	27.6	39.8
	41.5	21.4	4.8	0.3	0.7	1.9	33.6	37.3
	48.5	26.0	5.5	0.3	0.7	2.3	39.5	25.7
Neutral lipids	0	13.8	5.9	0.4	0.7	11.0	27.3	37.1
	13	4.1	5.8	1.0	1.3	1.5	15.7	69.8
	24.5	12.0	6.7	0.7	1.4	2.5	27.5	49.2
	33	4.8	5.4	0.8	0.9	1.7	17.0	69.4
	41.5	3.2	4.8	0.4	0.7	1.2	23.5	64.6
	48.5	5.1	5.6	0.4	0.7	2.0	26.6	59.0

*All samples contained < 1% of 14:0 and 15:0 fatty acid methyl esters.

B. Individual Lipid Components

1. Phosphatidyl Choline

At 25°C, 18:2 acid in the PC fraction increased to a maximum level of almost 68% and 18:1 acid decreased reciprocally to a minimum of 12% in early cultures (Fig. 5; Table V). Thereafter 18:2 acid decreased and 18:1 acid increased gradually to values approaching those of the inoculum; 16:0 acid was present in low, constant amounts during growth, whereas 16:1 acid decreased to a minimum halfway through the period of linear growth. As with the total lipids, similar changes occurred in the fatty acid composition of PC at 10°C, except that the maximum level of 18:2 was again more than 10% higher at 10°C than at 25°C and was maintained at a higher level later into the linear growth phase along with lower levels of 18:1. The decrease in 16:1 is more marked at 10°C than at 25°C, and 16:0 is somewhat lower throughout growth at the lower temperature (Fig. 5, Table VI). Maxima and minima of 18:2 and 18:1 at both temperatures occur during early stages of growth.

2. Phosphatidyl Ethanolamine

Changes in the fatty acid composition of PE obtained from cells grown at either temperature were not as great as those occurring in PC (Fig. 5; Tables V and VI). At both temperatures 18:2 increased to a maximum level of 45 - 50% during early growth, while 18:1 reached a minimum level of approximately 22%. PE samples consistently contained higher levels of 16:0 acid and lower levels of 18:2 acid than the PC samples. During later stages of linear growth, a higher level of 18:2 was maintained in PE at 10°C than at 25°C.

3. Phosphatidyl Serine and Phosphatidyl Inositol

The most notable characteristic of the PS + PI fraction was its high proportion of 16:0 acid and comparatively small proportion of 18:2 acid, both at 10° and at 25°C (Tables V and VI; Fig. 6). At both temperatures, the level of 16:0 acid was five to ten times higher than in corresponding PC fractions, and twice as high as in corresponding PE fractions throughout growth. The levels of 18:2 acid in PS + PI, however, were only half as high as in PC during growth at either temperature.

At both temperatures, large increases in 18:2 acid occurred, again during early stages of growth, followed by decreases in the level of 18:1 2 as growth continued; reciprocal changes in the level of 18:2 acid were also observed (Fig. 6, Tables V and VI).

Analysis of PS and PI separated by paper chromatography of a PS + PI TLC fraction suggests that the composition of these two lipids may be quite similar (Table VII). However, it should be noted that the analyses were performed on a PS + PI fraction obtained from lipids of cells grown at 25°C for 36 hr. and may not necessarily be representative of the fatty acid composition of these two phosphatides at 10°C or at other stages of growth.

4. Neutral Lipids

At 25°C the level of 18:2 acid in neutral lipids increased to a maximum of 52%, from 37% in the inoculum, halfway through the linear phase of growth; then decreased as growth continued. The level of 18:1 acid, however, remained near 27% throughout the early linear phase of growth, increasing slightly at the end of linear growth (Fig. 6, Table V). The level of 16:0 showed only a small decrease during mid-

TABLE VII

Fatty Acid Composition of Phosphatidyl Serine and Phosphatidyl Inositol separated by Chromatography on Silcic Acid Impregnated Paper*

	Fatty Acids Composition‡						
Sample	16:0	16:1	17:0	17:1	18:0	18:1	18:2
Total lipids	15.9	11.8	0.6	1.3	1.4	34.9	34.2
PS and PI	31.1	6.5	1.9	0.9	8.0	34.2	18.2
PS	35.3	6.0	1.2	-	2.2	35.8	19.6
PI	29.3	10.6	1.2	-	1.7	40.9	16.3

*Total lipids, obtained from a 36 hr., 25°C culture, were subjected to TLC (Materials and Methods). The PS and PI fraction was obtained from TLC and was then separated into PS and PI by chromatography on silicic acid impregnated paper in Marinetti's solvent. Some breakdown of the PS component during the separation was observed.

‡All samples contained < 1% of 14:0 and 15:0 fatty acid methyl esters.

FIGURE 5

Changes in fatty acid composition of total lipids, lecithin and phosphatidyl ethanolamine at 25°C and 10°C.

Growth curves

Linoleic acid

Oleic acid

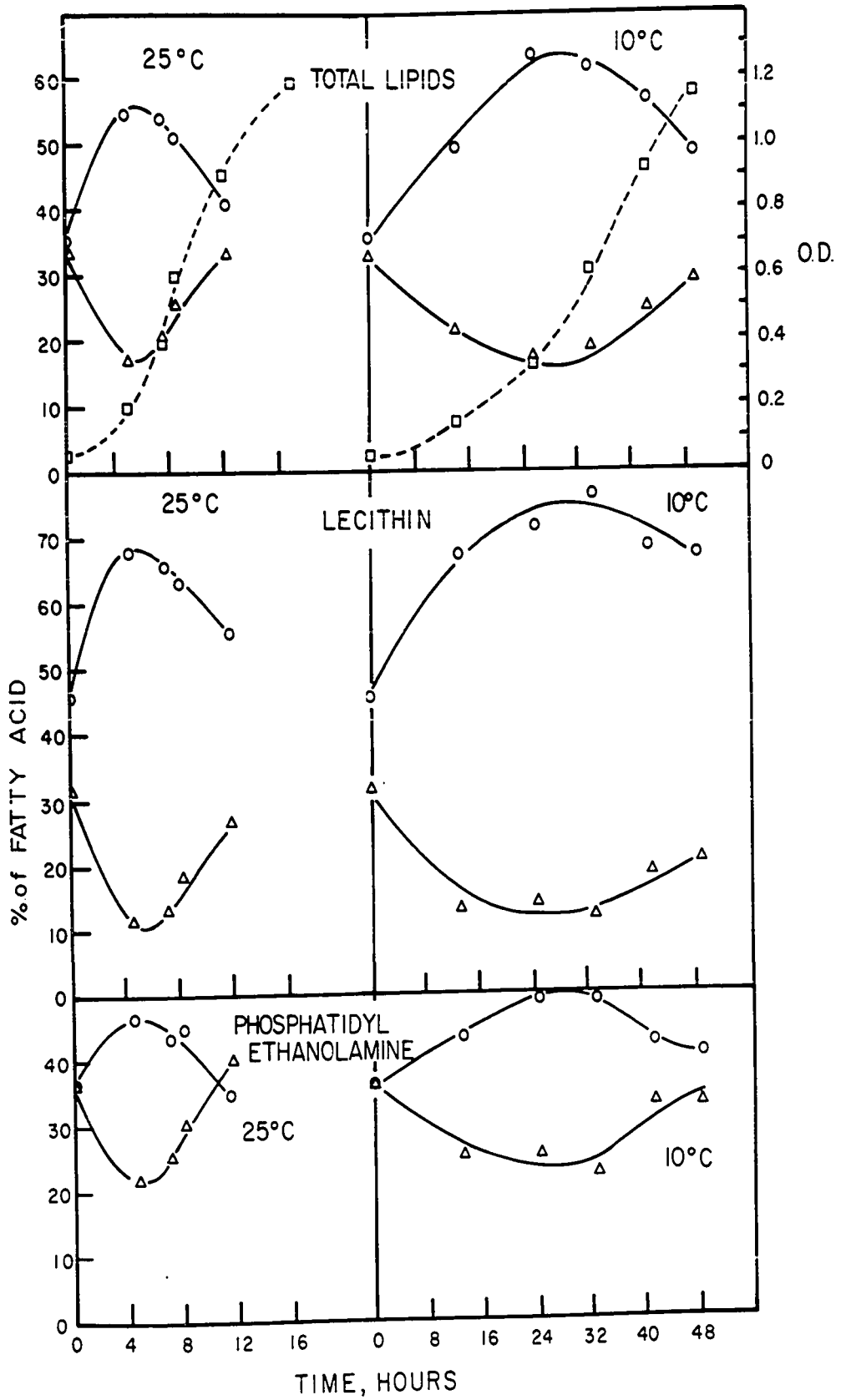

25°C
TOTAL LIPIDS
10°C
O.D.
25°C
LECITHIN
10°C
%of FATTY ACID
PHOSPHATIDYL
ETHANOLAMINE
25°C
10°C
TIME, HOURS

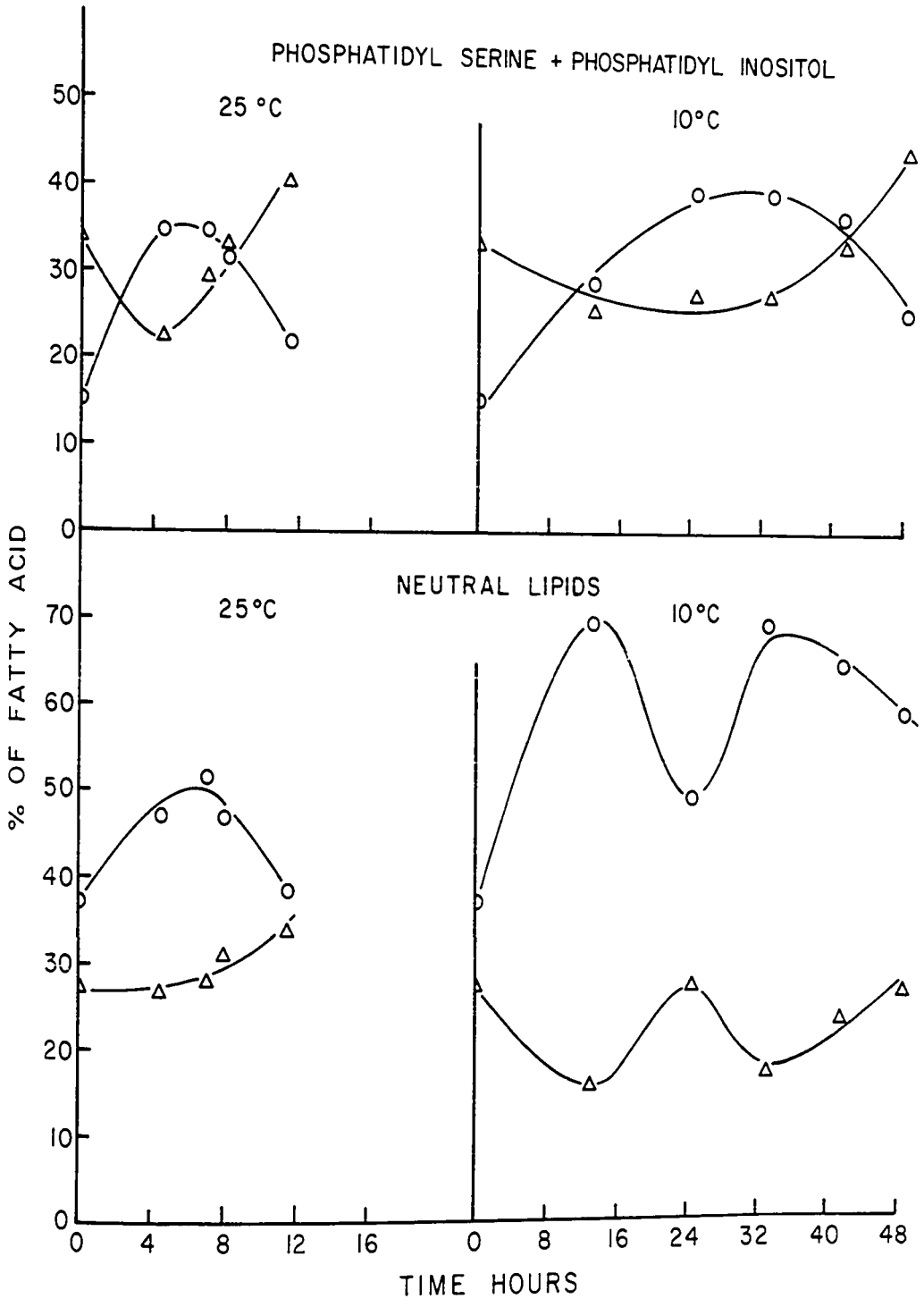
FIGURE 6

Changes in fatty acid composition of phosphatidyl serine + phosphatidyl inositol and neutral lipids at 25°C and 10°C.

Linoleic acid O–O–O

Oleic acid Δ–Δ–Δ

hosphatidyl



linear growth phase, increasing to the original level of 13% in late linear growth phase. It may be worthy of note that appreciably high levels of 18:0 acid (6 - 11%) were found only in the neutral lipids from cells grown at 25°C.

At 10°C, levels of 18:2 in neutral lipids were generally much higher than at 25°C, but in contrast, showed two maximum levels of almost 70% first at the early linear growth phase and again mid-way through the linear phase (Fig. 6), this high level of 18:2 being maintained into the late growth stage. Both 18:1 and 16:0 showed significant changes which appeared to be reciprocally related to the above changes in levels of 18:2 acid. Unlike neutral lipids from 25°C cells, 10°C neutral lipids contained only traces of stearic acid.

C. Summary of Overall Changes

Table VIII indicates that on the average the fatty acids of all lipid components at 10°C were more unsaturated than at 25°C. At both temperatures, PC was the most unsaturated component, followed by neutral lipids, PE and PS + PI. At both temperatures, also, the ratio of 18-carbon atom fatty acids to 16-carbon atom fatty acids was greatest in PC, followed by neutral lipids, PE and PS + PI. Each lipid component tended to contain higher levels of 18-carbon fatty acids at 10°C than at 25°C, except for PE which was not affected in this respect by temperature. During growth at 25°, only PC showed a maximum degree of unsaturation in the very early growth stage (4.5 hr), the other components showing maxima at the mid-linear phase (7 - 8 hr). In sharp contrast, at 10° all components showed maximal degrees of unsaturation in the early linear phase of growth.

TABLE VIII

Changes in 18-Carbon/16-Carbon Fatty Acid Ratios and Degrees of Unsaturation for Lipids of C. Lipolytica

Growth Temp(°C)	Age of Culture(hr.)	18-C/16-C(molar ratio)* Total Lipids	PC	PE	PS and PI	Neutral Lipids	Δ-Values, (double-bonds/mole fatty acid)** Total Lipids	PC	PE	PS and PI	Neutral Lipids
25°	0	2.7	4.3	3.0	1.2	3.8	1.14	1.37	1.10	0.71	1.08
	4.5	3.3	5.5	2.5	1.5	4.1	1.36	1.59	1.26	0.99	1.27
	7	4.3	6.1	3.3	2.2	5.9	1.39	1.55	1.23	1.08	1.39
	8	4.7	6.6	3.8	2.3	6.2	1.37	1.54	1.30	1.04	1.31
	11.5	4.1	6.9	3.5	1.9	4.9	1.27	1.47	1.19	0.91	1.16
	Average	3.8	5.9	3.2	1.8	5.0	1.31	1.50	1.22	0.95	1.24
10°	0	2.7	4.3	3.0	1.2	3.8	1.14	1.37	1.10	0.71	1.08
	13	3.0	5.3	2.5	1.4	8.8	1.31	1.60	1.25	0.92	1.62
	24.5	5.4	7.9	3.4	2.5	4.2	1.52	1.66	1.35	1.15	1.34
	33	5.5	9.4	2.8	2.4	8.6	1.51	1.71	1.31	1.15	1.62
	41.5	4.9	8.7	3.5	2.8	11.2	1.45	1.63	1.28	1.14	1.58
	48.5	4.5	9.2	3.6	2.1	7.3	1.27	1.63	1.25	0.97	1.51
	Average	4.3	7.5	3.1	2.1	7.3	1.37	1.60	1.26	1.01	1.46

*Calculated as follows: (% 18:0 + 18:1 + % 18:2 / % 16:0 + % 16:1).

**Calculated as follows: Δ = 1.0 x (% monoene/100) + 2.0 x (% diene/100).

IV. Changes in the Positional Distribution of Fatty Acids in PC and PE

A. PC

Several quantitative differences are found between the acids esterified in the α and β positions (Table IX). At both temperatures the β position contains 20 - 25% more 18:2. The α-position at both 10°C and 25°C contains slightly more 18:1 and significantly higher levels of 16-carbon acids (approximately 2 - 3 times more in the α position than in the β position). Consequently, the β position appears to be a locus of high levels of 18-carbon fatty acids and unsaturation, whereas the α position is less unsaturated and contains higher levels of the shorter chain acids (Table XI).

At both temperatures changes in the fatty acid composition of PC in both the α and β positions (Fig. 7) are similar to those observed in the total fatty acids of PC (cf. Tables V and VI). Thus, 18:2 in all cases shows an increase to a maximum level during the early exponential phase of growth, then decreases somewhat as growth continues, while 18:1 decreases to a minimum during early growth and increases as growth continues.

B. PE

At both temperatures, levels of 16-carbon atoms in the α position of PE are very much higher than in the β position (5 - 7 times more 16-carbon acids in the α position than in the β position), while levels of 18:2 and 18:1 are substantially higher in the β position (Table X).

TABLE IX

Positional Changes in Fatty Acid Composition of Phosphatidyl Choline Samples Obtained From Cells Grown at 25°C and 10°C

		% of Fatty Acids in each Position*						
Temp.	Time(hr.)	16:0	16:1	17:0	17:1	18:0	18:1	18:2
					α-position			
25°C	0	13.4	15.9	0.8	1.1	2.8	32.6	33.2
	4.5	11.9	13.7	2.2	2.8	3.4	13.3	52.7
	7	10.8	10.7	1.6	3.4	4.0	15.6	53.9
	8	12.8	10.5	1.0	2.9	4.2	22.1	46.6
	11.5	8.7	10.2	0.8	2.3	2.8	32.8	42.5
					β-position			
	0	2.3	11.9	0.8	1.2	1.2	28.3	51.6
	4.5	1.9	7.2	1.3	1.3	1.2	10.1	76.6
	7	1.8	7.0	1.2	1.8	1.9	12.1	73.9
	8	1.7	6.5	1.1	1.6	1.8	16.6	70.9
	11.5	1.3	6.5	0.9	1.1	1.1	21.4	66.9
					α-position			
10°C	0	13.4	15.9	0.8	1.1	2.8	32.6	33.2
	13	10.3	17.1	1.9	1.6	3.0	16.6	49.0
	24.5	5.6	7.2	2.3	2.7	3.0	20.2	59.0
	33	7.7	12.6	0.5	0.8	1.1	16.5	61.0
	41.5	6.5	12.7	1.4	1.5	1.8	17.0	59.1
	48.5	4.6	8.0	0.7	1.0	1.5	28.5	55.7
					β-position			
	0	2.3	11.9	0.8	1.2	1.2	28.3	51.6
	13	2.3	10.1	1.4	0.4	0.9	11.3	72.6
	24.5	0.9	4.5	1.1	1.4	1.2	9.7	78.1
	33	1.7	4.9	1.1	0.8	0.9	9.7	78.8
	41.5	1.3	4.9	0.9	0.5	0.9	14.8	73.2
	48.5	1.0	4.9	0.7	0.4	0.7	15.0	74.8

*All samples contained < 1% of 14:0 and 15:0 fatty acid methyl esters.

TABLE X

Positional Changes in Fatty Acid Composition of Phosphatidyl Ethanolamine Samples obtained from Cells grown at 10°C and 25°C

Sample	Time(hr.)	% of Total Fatty Acids in each Position*						
		16:0	16:1	17:0	17:1	18:0	18:1	18:2
				α-position				
25°C	0	27.2	15.2	0.4	2.0	2.4	26.6	26.3
	4.5	28.2	12.8	0.4	2.1	2.5	24.5	29.4
	7	28.0	11.5	0.4	2.7	2.5	24.2	30.0
	8	27.0	10.7	0.5	3.8	2.6	26.7	28.6
	11.5	27.8	12.5	0.3	3.1	2.7	22.7	30.8
				β-position				
	0	2.5	7.3	0.3	0.8	0.6	45.8	42.4
	4.5	3.6	4.8	0.2	0.2	0.9	30.8	59.4
	7	3.9	4.0	-	1.0	-	32.2	57.8
	8	3.3	4.1	-	0.6	0.5	37.3	54.1
	11.5	2.9	4.3	0.2	0.9	0.8	50.3	41.4
				α-position				
10°C	0	27.2	15.2	0.4	2.0	2.4	26.6	26.3
	13	33.7	14.8	0.7	3.2	1.8	20.6	25.3
	24.5	32.0	12.2	0.8	5.0	3.0	21.2	25.8
	33	33.2	13.3	0.4	2.3	1.6	20.7	28.5
	41.5	26.0	14.9	0.6	2.3	2.2	27.6	26.4
	48.5	27.8	14.8	0.6	2.0	2.2	27.9	24.6
				β-position				
	0	2.5	7.3	0.3	0.8	0.6	45.8	42.4
	13	2.9	4.9	-	1.3	0.6	29.2	61.1
	24.5	2.5	4.7	-	0.8	-	25.9	66.5
	33	4.9	5.6	-	0.3	0.6	29.8	59.7
	41.5	1.6	4.6	0.3	0.4	0.5	35.2	57.2
	48.5	2.8	4.7	0.7	-	-	39.3	52.3

* All samples contained 1% of 14:0 and 15:0 fatty acid methyl esters

TABLE XI

Positional Changes in Degree of Unsaturation and 18-Carbon/16-Carbon Ratios for Lecithin and Phosphatidyl Ethanolamine

Δ-values (double bonds/mole fatty acid)*

			25°C					10°C				
		Time(hr.)	0	4.5	7	8	11.5	0	13	24.5	33	41.5	48.5
PC	α		1.16	1.35	1.38	1.29	1.30	1.16	1.33	1.48	1.52	1.49	1.49
	β		1.45	1.72	1.69	1.67	1.63	1.45	1.67	1.72	1.73	1.67	1.70
PE	α		0.96	0.98	0.98	0.98	1.00	0.96	0.89	0.90	0.93	0.98	0.94
	β		1.39	1.55	1.53	1.50	1.38	1.39	1.58	1.64	1.55	1.55	1.49

18-C/16-C (molar ratio)**

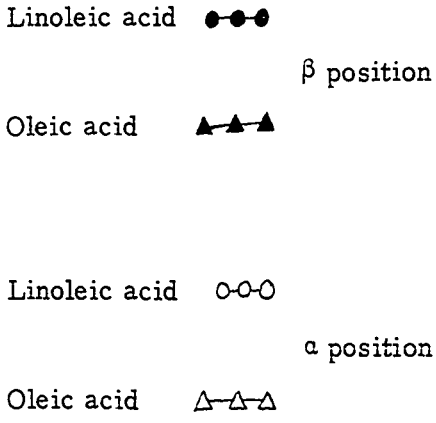
			25°C					10°C					
		Time(hr.)	0	4.5	7	8	11.5	0	13	24.5	33	41.5	48.5
PC	α		2.34	2.71	3.42	3.13	4.13	2.34	2.50	6.42	3.87	4.06	6.80
	β		5.71	9.66	9.99	10.90	11.46	5.71	6.84	16.48	13.55	14.34	15.34
PE	α		1.30	1.38	1.44	1.54	1.39	1.30	0.98	1.13	1.09	1.37	1.28
	β		9.06	10.85	11.39	12.42	12.50	9.06	11.65	12.83	8.58	14.98	12.21

*Calculated as follows: Δ = 1.0 x (% monoene/100) + 2.0 x (% diene/100).

**Calculated as follows: (% 18:0 + % 18:1 + % 18:2/% 16:0 + % 16:1)

FIGURE 7

Changes in positional distribution of fatty acids in lecithin at 25°C and 10°C



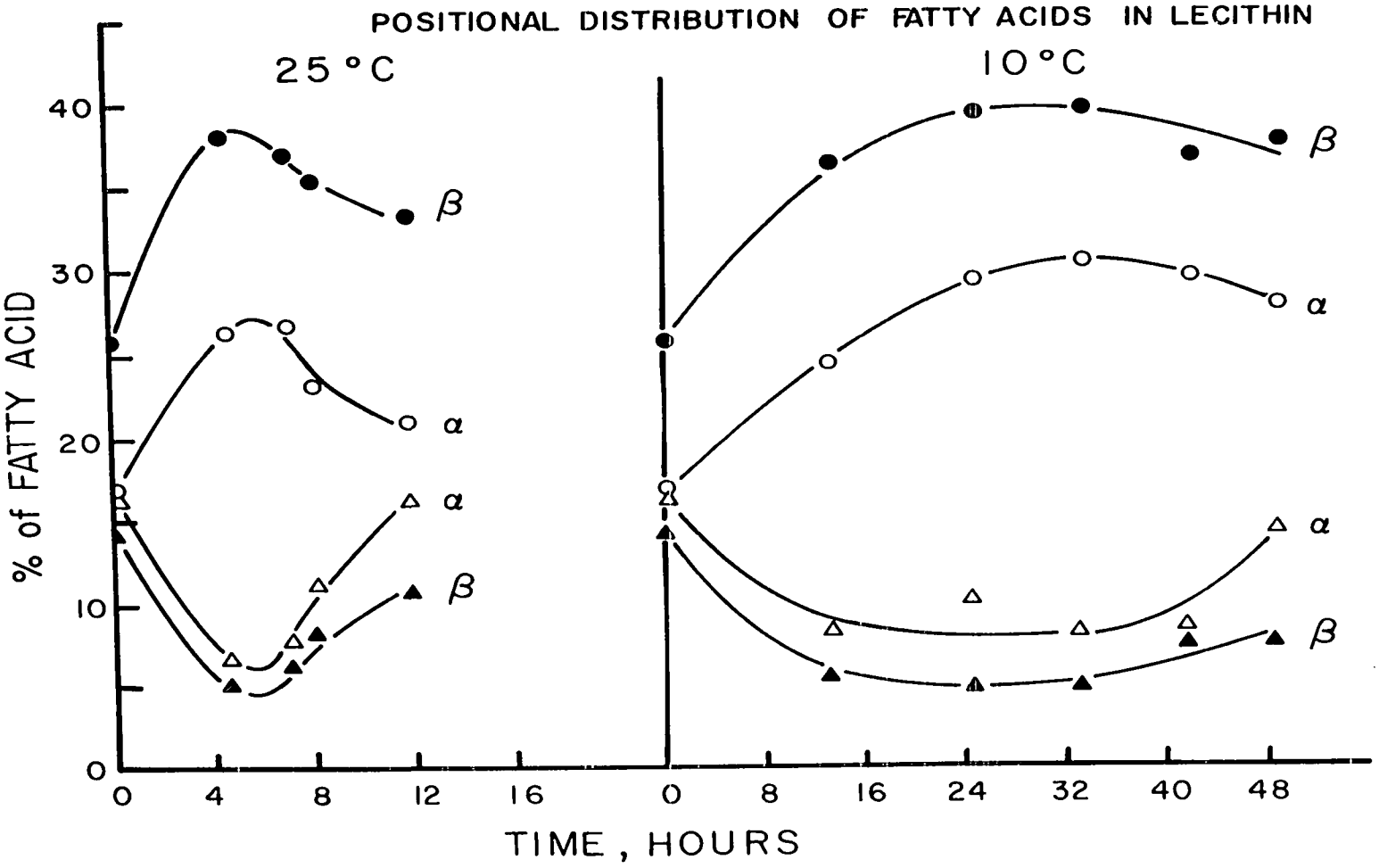

POSITIONAL DISTRIBUTION OF FATTY ACIDS IN LECITHIN
25 °C
10 °C
% of FATTY ACID
TIME, HOURS
β
α
α
β
β
α
α
β
0
10
20
30
40
0
4
8
12
16
0
8
16
24
32
40
48

FIGURE 8

Changes in positional distribution of fatty acids in phosphatidyl ethanolamine at 25°C and 10°C

Linoleic acid ●–●–●

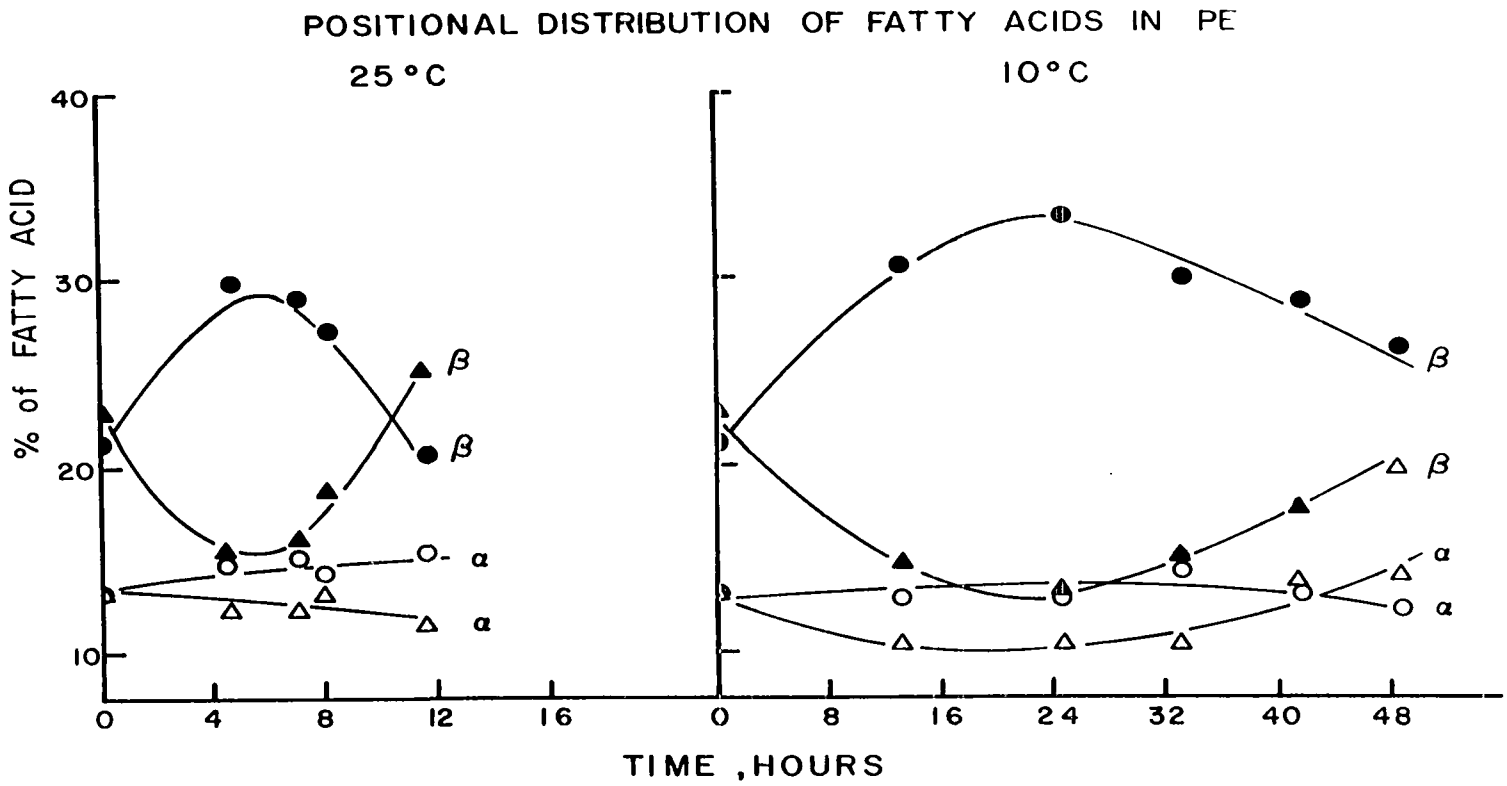
β position

Oleic acid ▲–▲–▲

Linoleic acid o–o–o

α position

Oleic acid Δ–Δ–Δ


POSITIONAL DISTRIBUTION OF FATTY ACIDS IN PE
25 °C
10 °C
% of FATTY ACID
40
30
20
10
β
β
α
α
β
β
α
α
0
4
8
12
16
0
8
16
24
32
40
48
TIME , HOURS

Changes which occur in the total fatty acids of PE at both temperatures are mirrored only in the fatty acid composition of β esterified fatty acids (Fig. 8). In this position at both 10°C and 25°C, 18:2 increases to a maximum early in growth, then decreases, while 18:1 reaches an early minimum and increases towards late exponential phase. All other acids in the β position are present in small amounts and remain relatively constant throughout growth.

In the α position of PE at 10°C only very minor changes in the relative amounts of 18:1 and 18:2 occur during growth and 16:0 shows a slight increase during early exponential phase (Fig. 8, Table X). At 25°C there appears to be a slight tendency for 18:2 to increase and 18:1 to decrease throughout growth, all other acids remaining relatively constant.

Thus the α position of PE at both temperatures shows negligible changes in fatty acid composition throughout growth, along with very low levels of total 18-carbon acids and unsaturation (Table XI). The β position shows large changes in fatty acid composition with high levels of unsaturation and 18-carbon fatty acids.

V. Molecular Species of PC and PE

A. $AgNO_3$-TLC of Lecithin

As shown in Figure 9, PC was separated by $AgNO_3$-TLC into three classes of molecular species. These were identified by GLC analysis of their fatty acids (Table XII) as monoenes and dienes (band I and II), trienes (band III), and tetraenes (band IV). No fully saturated lecithin species was detected. TLC (Figure 9) of the PC sample from cells grown at 10°C for 33 hr showed that the concentrations of the classes of molecular species were in the order tetraene > triene > mono + diene. In contrast, the classes of PC from the inoculum were in the order triene > mono + diene > tetraene.

TABLE XII

TLC- $AgNO_3$ Fractionation of the Lecithin Components*

		Fatty Acid Composition, % of Total							
Band No.	Rf	16:0	16:1	17:0	17:1	18:0	18:1	18:2	Unsaturated Species
I and II	0.57	24.0	8.4	3.0	2.7	9.9	16.4	36.0	monoenes and dienes
III	0.36	3.6	14.5	0.5	1.4	1.0	32.8	46.2	trienes
IV	0.21	t	9.9	t	t	t	3.2	86.8	tetraenes

*Analysis was performed on a pooled sample of PC obtained from cells grown at 10°C and 25°C (see Results). Abbreviation: t, trace amounts.

FIGURE 9

Silver nitrate-TLC of lecithin components

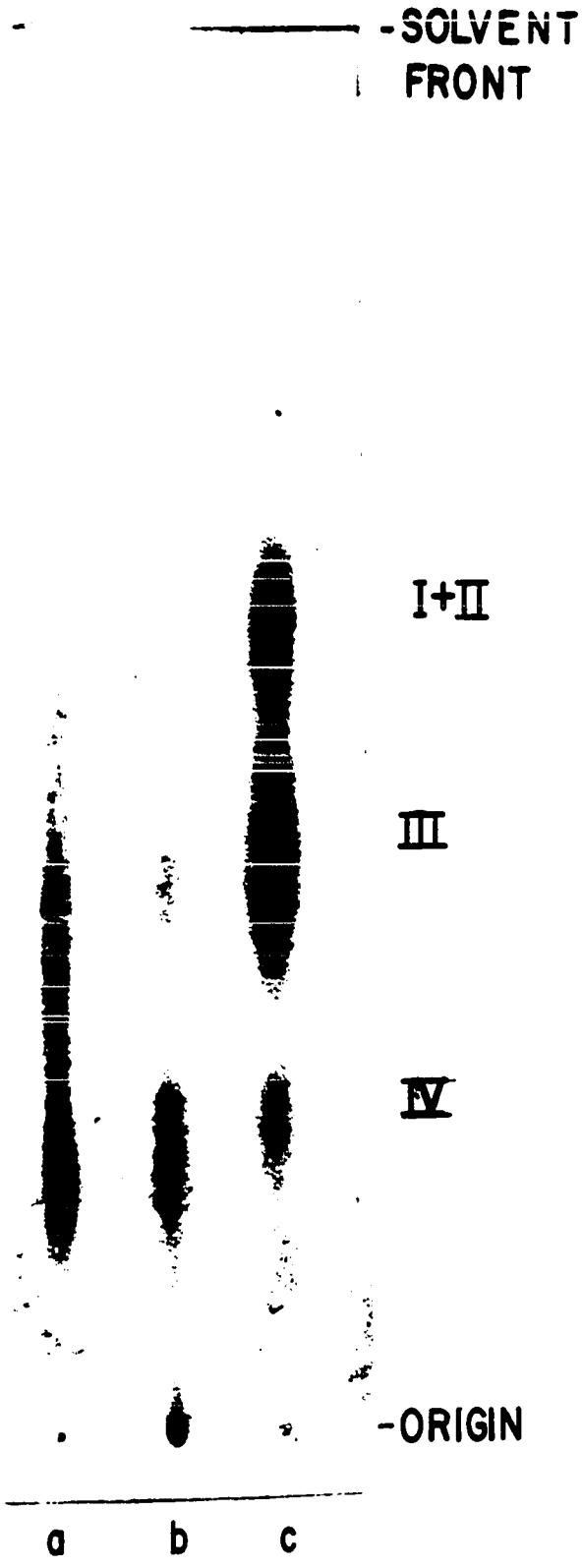
Solvent: Chloroform-methanol-water (65:25:4)

Material applied:

a. Pooled PC fraction ($10^{\circ}C$ and $25^{\circ}C$)

b. PC from cells grown at $10^{\circ}C$ for 33 hr.

c. PC from cells grown at $25^{\circ}C$ for 36 hr (Inoculum)


SOLVENT FRONT
I+II
III
IV
ORIGIN
a
b
c

B. Calculated Molecular Species of Lecithin

The most probable molecular species of lecithin at different times during growth at 10°C and 25°C may be calculated from the data in Table IX on the basis of a "restricted random" hypothesis of fatty acid distribution in lecithin (Coleman, 1963). This theory stipulates that the distribution of fatty acids is restricted with respect to positional specificity but random with respect to the nature of the fatty acid in the adjacent position of the molecule. This theory has been successfully applied in predicting the distribution of molecular species of glycerides (Coleman, 1963). The major molecular species of PC were thus calculated to be as follows (Table XIII): monoenes, 16:0 - 18:1 ; dienes, 16:0 - 18:2 ; 18:1 - 18:1 ; trienes, 18:1 - 18:2 and/or 18:2 - 18:1 ; 16:1 - 18:2 ; and tetraenes, 18:2 - 18:2.

Table XIII also indicates the predicted changes in distribution of the molecular species at 10°Cand 25°C. At both temperatures tetraenoic PC rises to a maximum then decreases. 18-Carbon trienoic species of PC show a reciprocal decrease during growth followed by an increase as growth continues, as does the dienoic 18-carbon molecular species 18:1 - 18:1. The 'mixed' 16-carbon and 18-carbon trienes do not show the same changes as the 18-carbon trienes; rather 16:1 - 18:2, like the tetraene, reaches a maximum level during early growth then decreases. All of the remaining dienes (except 16:0 - 18:2) which are of the mixed variety, show early decreases followed by later increases.

The major differences between the changes in molecular species at 10°C and at 25°C are as follows: at 10°C the maximum level of the tetraenoic PC is somewhat higher than the maximum at 25°C, and high levels of tetraene are maintained for a longer period of time; dienes at the lower temperature decrease and remain at a lower level than at 25°C. However, little difference is apparent in changes in the trienoic species during growth at the two temperatures.

TABLE XIII

Calculated Changes in Proportions of Major Molecular Species of Phosphatidyl Choline During Growth

Molecular Species		25°C			10°C			
		0 hr.	4.5 hr	11.5 hr.	0 hr.	13 hr.	33 hr.	48.5 hr.
	α β	moles %						
Monoenes								
	16:0-18:1	4.4	1.4	2.1	4.4	1.3	0.8	0.8
Dienes								
	16:0-18:2	8.1	10.7	6.6	8.1	8.7	6.7	3.8
	16:1-16:1	2.2	1.2	0.7	2.2	2.0	0.7	0.4
	16:1-18:1	5.3	1.6	2.5	5.3	2.2	1.3	1.3
	18:1-16:1	4.5	1.1	2.4	4.5	1.9	0.9	1.5
	18:1-18:1	10.8	1.6	7.9	10.8	2.2	1.8	4.7
	Total	30.9	16.2	20.1	30.9	17.0	11.4	11.7
Trienes								
	16:1-18:2	9.6	12.3	7.7	9.6	14.4	10.9	6.5
	18:2-16:1	4.6	4.5	3.1	4.6	5.7	3.3	3.0
	18:1-18:2	19.6	12.0	24.7	19.6	14.0	14.3	23.3
	18:2-18:1	11.0	6.2	10.3	11.0	6.4	6.5	9.1
	Total	44.8	35.0	45.8	44.8	40.5	35.0	42.9
Tetraene								
	18:2-18:2	20.0	47.4	32.0	20.0	41.2	52.8	45.6

C. Calculated Molecular Species of Phosphatidyl Ethanolamine

The major molecular species of PE calculated from the data in Table X on the basis of the "restricted random" hypothesis are as follows (Table XIV): monoenes, 18:0 - 18:1 ; dienes, 16:0 - 18:2, 16:1 - 18:1, 18:1 - 18:1 ; trienes, 18:1 - 18:2, 18:2 - 18:1, 16:1 - 18:2 ; and tetraenes, 18:2 - 18:2. These are the same species as are found in lecithin, and the changes in their proportions during growth - although on a much lower level - were similar with two major exceptions: (a) the triene 18:1 - 18:2 did not show a reciprocal relationship with 18:2 - 18:2 as was the case for lecithin; (b) very little change in 16:1 - 18:2 occurred during growth.

Temperature effects were similar to those occurring with lecithin, except that again the 18:1 - 18:2 species showed anomalous behaviour.

Table XIV

Calculated Changes in Proportions of Major Molecular Species of Phosphatidyl Ethanolamine During Growth

Molecular Species *		25°C			10°C			
		0 hr	4.5	11.5	0 hr	13	33	48.5
	α β	moles %						
Monoenes	16:0 - 18:1	14.8	10.2	16.3	14.8	11.5	11.6	12.5
Dienes	16:0 - 18:2	13.7	19.6	13.4	13.7	24.2	23.1	16.7
	16:1 - 18:1	8.3	4.6	7.3	8.3	5.1	4.6	6.7
	18:1 - 18:1	14.5	8.8	13.3	14.5	7.1	7.2	12.6
	Total	40.5	33.0	34.0	40.5	37.4	34.9	36.0
Trienes	16:1 - 18:2	7.7	8.9	6.0	7.7	10.6	9.3	8.9
	18:1 - 18:2	13.4	17.0	10.9	13.4	14.8	14.4	16.7
	18:2 - 18:1	14.3	10.6	18.0	14.3	8.7	9.9	11.1
	Total	35.4	36.5	34.9	35.4	34.1	33.6	36.7
Tetraene	18:2 - 18:2	13.3	20.4	14.8	13.3	18.1	19.9	14.8

* Species 16:1 - 16:1, 18:1 - 16:1 and 18:2 - 16:1 were neglected for these calculations.

DISCUSSION

I. Errors

A. Measurement of Fatty Acid Composition

The method of Carroll (1961), used to measure the peak areas of fatty acid methyl esters on GLC analysis, is based on the assumption that peaks obtained closely approximate Gaussian distribution curves. Dijkstra (1961) has pointed out the shortcomings of this method of calculation, especially in determinations of fatty acid composition with a probable error $\leq \pm 1\%$. Carroll's method however, was used here since it is rapid, and convenient, allowing the analysis of large numbers of samples within a reasonably acceptable 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 through errors in measurement of peak height retention time, giving an overall error range of ± 2.0 - 3.0% (absolute). Consequently, only fatty acid changes of $\geq 4\%$ were considered significant in the present studies. Absolute values and changes reported for the minor fatty acids (17:0, 17:1, 18:0) cannot be regarded as significant, and therefore will not be considered in the Discussion.

B. Variations in Culturing Cells

Precautions were taken to minimize the possibilities of contamination by other microorganisms and to ensure uniformity of growth conditions. It should be noted, however, that slight differences may be introduced as a result of procedures such as autoclaving, low

temperature incubation, and aeration by shaking. In addition non-uniform sampling of inocula used to initiate growth of cultures may lead to variability and non-reproducibility of the growth curves, particularly in samples incubated over a long period of time. Non-uniform sampling of aliquots taken for O. D. measurement, as well as sedimentation of cells during such measurements may also produce additional errors in the determination of the growth phase of cultures examined. However, such errors were minimal in the present studies, since the growth curves were reproducible within ± 0. 02 O. D. units; furthermore growth-related changes in lipid composition were completely reproducible with growth phase.

II. Growth-related Changes in Fatty Acid Composition

Analyses of the fatty acid composition of the total lipids of C. lipolytica grown at both 10°C and 25°C confirm the previous finding by Kates and Baxter (1962) of a large increase in 18:2 and a reciprocal decrease in 18:1 during early stages of growth, followed by a decrease and increase in 18:2 and 18:1, respectively (Fig. 5, Tables V and VI) as growth continues. Results presented here (Table VIII) further show that at those stages of growth during which unsaturation of fatty acids is greatest the relative amount of 18-carbon fatty acids is also at a maximum.

Growth-related changes in fatty acid composition occur to a greater or lesser extent in all of the components investigated, but the most pronounced changes occur in the lecithin component (Tables V and VI, Figs. 5 and 6).

It should be noted that PS + PI fractions also show large changes in relative content of 18:2. However, these are less significant to the overall desaturation since they occur at a much lower concentration level of 18:2 and furthermore this fraction is present in much smaller amounts than PC. PE shows the least pronounced changes in fatty acid composition throughout growth.

Considerable changes in content of 18:2 acid were also observed at both temperatures in the neutral lipid fraction (Fig. 6), but these are most likely confined to free fatty acids, the only major neutral lipid component which could be involved (Fig. 2B). Furthermore, the changes were reciprocal with those in 18:1 acid (Fig. 6).

Changes in the positional distribution of fatty acids in PC and PE during cell growth at 10°C and 25°C show a remarkable difference in positional specificity. The reciprocal changes in 18:1 and 18:2 acids occur both in the α and the β positions of PC (Fig. 7) but only in the β position of PE.

III. Temperature-related Changes in Fatty Acid Composition

In general, the total lipids of cells grown at 10°C contained at all times during growth higher levels of 18:2 acid than lipids of cells grown at 25°C (Fig. 5), as was found previously by Kates and Baxter (1962). Furthermore, it was also shown that the overall degree of unsaturation and high levels of linoleic acid persisted to a later stage of growth at 10°C than at 25°C. In the present study

these effects of temperature were also found to hold for the individual phospholipid components. Different temperature effects were observed for the neutral lipids (essentially free fatty acids): an unexplainable decrease in 18:2 acid during early linear growth phase was observed at 10° but not at 25°C. This decrease at 10°C was repeatedly observed and may reflect changes in free fatty acid pools at the lower temperature.

The temperature effects observed for changes in the total fatty acids of PC during growth were essentially the same as for fatty acid changes in the α and β positions (compare Fig. 5 with Fig. 7). This also holds true for the β position of PE but fatty acid in the α position, as mentioned earlier, showed negligible changes due to growth or temperature (compare Fig. 5 with Fig. 8).

IV. Changes in Molecular Species

To follow up the data on α,β-positional distribution of fatty acids in PC and PE, analysis of molecular species of PC and PE and their changes with time and temperature was required. These analyses would involve $AgNO_3$-TLC and GLC of the fatty acids of the separated species for several time periods at the two temperatures. Such a detailed study was not possible since insufficient material was available for these analyses. However, sufficient material was available for $AgNO_3$-TLC of two representative samples, the inoculum and the 10°C 33 hr (maximum 18:2) sample; the results (Fig. 9) showed that the major classes of species in the inoculum were trienes, mono- + dienes and tetraenes in decreasing order and in the 10°C sample tetraenes, trienes and mono- + dienes in decreasing order.

Changes in overall desaturation of the fatty acids appear to be both temperature and growth-phase dependent, and to occur generally in all the components. Furthermore, these changes occur to different extents in each of the components, and in the case of PE, the changes are confined to the β-position. To explain these complex changes the following general mechanisms for control of fatty acid desaturation may be considered;

(a) One fundamental type of control mechanism would be that occurring on the level of fatty acyl-CoA desaturation, as shown in Scheme IIA. Desaturation at this level would result in a common fatty acid composition for the phosphatidic acid and diglyceride precursors of phospholipids (Scheme IV). Provided no selective utilization of PA or diglyceride intermediates for phospholipid synthesis occurs, control of the rate of fatty acid desaturation by temperature or growth-rate dependent factors would produce the same general changes in the fatty acid composition of all components. Examples of control factors would be differences at high and low temperatures in the availability of oxygen necessary for desaturation and changes in oxidative metabolism during growth.

(b) Another mechanism must now be invoked to account for the fact that each phospholipid shows a characteristic fatty acid composition during growth. Such a mechanism would require specific selection by the corresponding diglyceride transferases of the appropriate diglyceride moieties of the phospholipids. For example, selectivity would be required in the utilization of PA for the synthesis of PS and PI to explain the unusually high levels of palmitic acid found in the PS + PI fraction; similarly, selectivity would be required in diglyceride utilization

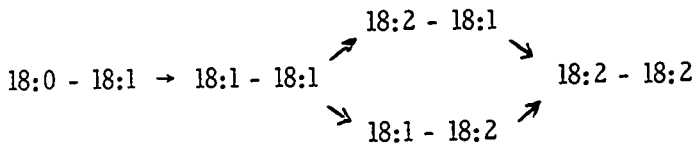
to ensure the incorporation of more highly unsaturated species of diglyceride into PC rather than into PE. Furthermore, diglyceride selectivity would have to change during growth of cells in such a way as to explain specificity of β-positional changes in the fatty acids of only PE and the non-specific positional changes in the fatty acids of PC. Such changes in selectivity would presumably require synthesis of a new set of transferases with the appropriate specificities during the growth cycle. This control mechanism appears unlikely since it would require an unnecessarily complex and cumbersome genetic control system.

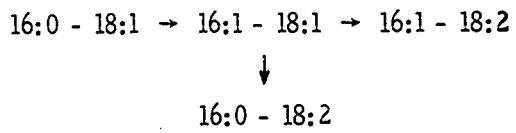
(c) An alternative mechanism to explain the specific changes in fatty acid composition would involve the action of transacylases which would specifically exchange oleic acid for linoleic acid in the β-position of PE or either position of PC. On this basis one would expect the free fatty acid pool to show changes opposite to those observed in PE and PC, namely a decrease in linoleic acid and an increase in oleic acid during early growth phase. The fatty acid changes observed in the neutral lipids may be attributed largely to changes in free fatty acids (Figs. 2B and 6). It may therefore be significant that the changes at 10°C (at which temperature the transacylation would be expected to be slower than the fatty acid desaturation step) show a pronounced reciprocal decrease in 18:2 and increase in 18:1 acids precisely at the time when 18:2 is at a maximum in the phospholipids (cf. Figs. 5 and 6). This observation would therefore support the participation of transacylases as described above. It should be noted that the transacylases would be required to have different positional specificities for PC and PE.

SCHEME VI

Proposed Pathways for Desaturation of Molecular Species of:

A. Lecithin





B. Phosphatidyl Ethanolamine

18:1 - 18:1 → 18:1 - 18:2

18:2 - 18:1 → 18:2 - 18:2

16:0 - 18:1 → 16:0 - 18:2

(d) Another alternative mechanism would require changes in fatty acid composition to occur on the phosphatide level. This would involve in situ desaturation of fatty acids esterified to phospholipids in membranes. Such a mechanism would require a non-positionally specific desaturase for PC and a β-positionally specific desaturase for PE. The desaturase systems for all phosphatides would have to be temperature sensitive in the same way (so as to increase the proportion of unsaturated acids in all phosphatides at lower temperatures). The growth-phase dependence of all desaturase systems would also have to be similar (to cause changes in unsaturation of phosphatides during the same growth phase). In fact the desaturase systems would be similar in all aspects other than positional specificity for desaturating activity. Non-specificity in the case of PC would explain the higher levels of unsaturation in this phosphatide, whereas increasing degrees of positional specificity for desaturating activity, as in PE, would necessarily result in lower overall levels of fatty acid unsaturation. This mechanism would be consistent with the calculated changes in molecular species of PC and PE during growth at 10°C and 25°C (Tables XIII and XIV). The individual steps (proposed for desaturation of PC and PE) are shown in Scheme VI.

V. The Physiological Role of Fatty Acid Desaturation

Phospholipids, being structural components of biological membranes, are probably involved in determining the permeability properties of the membrane, thereby playing an important role in cellular metabolism and physiology. It is reasonable to assume that close controls would be exerted by a cell on phospholipid composition at different stages

of growth and with respect to different environmental conditions. For example, total phospholipid content of C. lipolytica, and hence total membrane material, appears to be greater at 10°C than at 25°C. Similarly, changes in composition of phospholipids and their fatty acid components appear to be related to different phases of microbial growth.

Since degree of unsaturation is known to have a large effect on physical properties of fatty acids (such as melting or boiling point) it has long been intimated (see Chapman, 1968) that the degree of unsaturation of fatty acids esterified to phospholipids in membranes should have a similar effect on fluidity of the membrane and thus on its permeability (i.e. the greater the degree of unsaturation, the greater the fluidity and permeability). Studies on lipid micelles formed from phospholipids differing in degree of unsaturation tend to support the hypothesis that membrane permeability increases with increasing unsaturation (Haest et al, 1969). On this basis the large increases in unsaturation in lecithin, the major membrane phospholipid, in the early growth phase would indicate increased membrane permeability during this period. This is reasonable in view of greater requirements for exogenous nutrients during the phase of active growth. Decreases in unsaturation during later stages of growth would correlate with decreased membrane permeability expected in older cells.

In order to maintain high permeability of membranes an even higher degree of fatty acid unsaturation would be required to counteract the effect of decreased membrane fluidity at low temperatures. This is consistent with the findings that at all times during growth at 10°C higher levels of unsaturation are maintained in the lipids of C. lipolytica.

SUMMARY

1. Fatty acids of all component lipids of C. lipolytica were shown to have a degree of unsaturation which varied inversely with the temperature and age of cell cultures.

2. Of the phospholipids, phosphatidyl choline contributed most to overall unsaturation of lipids. The phosphatide fraction containing phosphatidyl serine + phosphatidyl inositol contained high levels of palmitic acid. Phosphatidyl ethanolamine showed the least pronounced changes in fatty acid unsaturation during growth at $10^{o}C$ or $25^{o}C$.

3. Changes in the fatty acid composition of phosphatidyl ethanolamine during growth at both temperatures occurred specifically in the β-position, whereas changes in the fatty acid composition of phosphatidyl choline occurred without positional specificity.

4. Calculations of the changes in molecular species of PC and PE strongly suggest that desaturation of these phosphatides at both temperatures occurs in a stepwise manner from diene to triene to tetraene (with the restriction that only β-desaturation occurs in PE).

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