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Sphingolipids with Inositolphosphate-Containing Head Groups

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I. Introduction

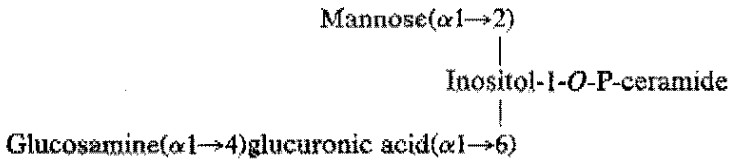
Since recent studies with yeast have shown the essentiality and possible function of one or more inositolphosphorylceramides, now appears to be an appropriate time for a review of this group of sphingolipids. The generalized structure of these lipids is ceramide-P-myoinositol-X, with X referring to polar substituents.

These compounds had been referred to as "phytoglycolipids" and "mycoglycolipids," indicating their origins. Since it is now clear that the distribution of these compounds is not limited to plants or fungi, we propose the neutral, generic designation, inositolphosphorylceramides, abbreviated here as InsPCers. Inositolphosphorylceramide itself will be abbreviated as IPC. We will refer to myoinositol as simply inositol.

II. Plant InsPCers

Studies on InsPCers derived from plants commenced over 50 years ago in a number of laboratories with the recognition of incompletely characterized inositol-containing lipid fractions from seed oils that yielded various sugars such as galactose and arabinose on acid hydrolysis. This material has been reviewed by Celmer and Carter (1952), Hawthorne (1960), and Allen and Good (1965). Much of this early work was hampered by inadequate purification methods applied to exceedingly complex mixtures of InsPCers.

H. Carter and associates at the University of Illinois carried out pioneering studies in the 1950s and 1960s on the so-named "phytoglycolipide" purified from seeds of corn, flax, and soybeans (Carter *et al.*, 1958b) and from bean leaves (Carter and Koob, 1969). Strong alkaline hydrolysis of these lipid mixtures yielded a series of (phospho)oligosaccharides of different sizes containing glucosaminido-glucuronido-inositol with variable amounts of galactose, mannose, arabinose, and fucose (Carter *et al.*, 1964) as well as (phospho)ceramide (Carter *et al.*, 1958b). Ceramides were mainly composed of phytosphingosine (D-*ribo*-1,3,4-trihydroxy-2-aminooctadecane) or dehydrophytosphingosine (D-*ribo*-1,3,4-trihydroxy-2-amino-8-*trans*-octadecene) (Carter and Hendrickson, 1963) and mainly a hydroxy C_{24} fatty acid (Carter and Koob, 1969). Detailed structural analysis was carried out only on the tetrasaccharide component derived from alkaline hydrolysis of corn seed InsPCers, resulting in the proposed structure (Carter *et al.*, 1969):



Wagner *et al.* (1969) purified a mixture of InsPCers from a commercial preparation of peanut lecithin which resembled the lipid and carbohydrate composition of the earlier described InsPCers from soy beans (Carter and Hendrickson, 1963).

Kaul and Lester (1975) devised a relatively mild extraction procedure that resulted in an InsPCer concentrate yielding about 100 μmol of P/kg fresh weight of tobacco leaves. This procedure avoided the potentially harsh treatments employed by Carter *et al.* (1958a), involving treatment with alkali to destroy acyl esters or refluxing with HCl-ethanol (Carter and Koob, 1969). From the tobacco InsPCer concentrate, pure lipids

were obtained which fell into two classes, the PSL-I group containing *N*-acetylglucosamine and the PSL-II group containing unacetylated glucosamine (Kaul and Lester, 1975, 1978). The structure of PSL-I was determined to be (Hsieh *et al.*, 1978):

Inositol-1-*O*-P-ceramide
|
N-Acetylglucosamine(α1→4)glucuronic acid(α1→2)

PSL-II is therefore:

Inositol-1-*O*-P-ceramide
|
Glucosamine(α1→4)glucuronic acid(α1→2)

The ceramides from both PSL-I and PSL-II comprised roughly equal amounts of phytosphingosine and dehydrophytosphingosine with OH-fatty acids ranging from C_{22} to C_{26}, with the OH-C_{24} homolog predominating. Thus, the hydrophobic components of the tobacco leaf InsPCers resemble those previously observed by Carter *et al.* from other plant sources. The other purified lipids (PSL-I/II-ABC) all have the trisaccharide components of PSL-I and PSL-II and are presumed to be derivatives of PSL-I and PSL-II; these are characterized as to composition but not as to sugar linkages and anomeric configuration (Kaul and Lester, 1978):

Compound	Structure
PSL-IA	PSL-I-[Arabinose$_2$Galactose$_2$]
PSL-IB	PSL-I-[Arabinose$_3$Galactose$_2$]
PSL-IC	PSL-I-[Arabinose$_4$Galactose$_2$]
PSL-IIA	PSL-II-[Arabinose$_3$Galactose]
PSL-IIB	PSL-II-[Arabinose$_{2\text{ or }3}$Galactose$_2$]
PSL-IIC	PSL-II-[Arabinose$_2$Galactose$_2$Mannose]

The InsPCers purified from tobacco leaves accounted for 50–60% of the InsPCer concentrate; many more additional components more complex than ceramide-P-nonasaccharides were not resolvable at that time (Kaul and Lester, 1978). Modern HPLC supports could probably resolve more of these components as intact lipids.

The complexity of tobacco leaf InsPCers was further probed by the work of Hsieh *et al.* (1981). These workers reduced the glucuronic acid

carboxyl group of the InsPCer concentrate, and liberated the oligosaccharides with strong alkali, followed by peracetylation and separation by reversed-phase high-performance liquid chromatography; at least 24 components were evident in the chromatographic profile. Alkaline hydrolysis and peracetylation completely eliminated the distinction between the PSL-I and the PSL-II series so that the complexity may be even greater than that observed. A major tetrasaccharide was identified:

Galactose(β1→4)*N*-acetylglucosamine(α1→4)glucuronic acid (α1→2)inositol

A minor tetrasaccharide was also partially characterized:

N-Acetylglucosamine(α1→4)glucuronic acid (α1→?)inositol(?←1)mannose

From black gram sprouts (*Vigna mungo*), Kondo and Nakano (1987) obtained a material from the upper phase of a Bligh–Dyer (1969) lipid extract that was further purified by gel filtration and ion-exchange chromatography, both with aqueous buffers. Analysis indicated the presence of the core plant InsPCer components, ceramide, P, inositol, glucuronic acid, and hexosamine as well as 5.3% protein. In addition, large amounts of galactose and arabinose were present, which their analysis suggests may consist of a β1,6-galactan substituted at some of the three positions with arabinose. No mannose was found. It is difficult to judge the purity of this material since the components, InsPCers and proteins, would be expected to exist together as mixed micelles under the conditions of isolation. No direct evidence for a covalent linkage between protein and lipid was given.

Thus far, only crop plants have been examined for the presence of InsPCers; however, they are probably widespread if not ubiquitous in plants. The complexity of the InsPCers in plants may be comparable to that of the glycosphingolipids in animals. The plant InsPCers await further studies of their structures, organ and intracellular distribution, biosynthesis, and function(s).

III. InsPCers of Fungi and Yeast

A. Analytical Methods

1. *Extraction of InsPCers*

A systematic and quantitative study of the extraction of InsPCers from *Saccharomyces cerevisiae* and from the mycelial phase of *Neurospora*

crassa was carried out by Hanson and Lester (1980). Cells uniformly labeled with [^{3}H]inositol were used to gauge the extraction efficiency of a variety of procedures, some previously employed by others to extract phospholipids. The best method involved treating the cells with 5% trichloroacetic acid at 0°C to destroy phospholipases, known to be activated by organic solvents during lipid extraction (Letters, 1968), followed by extraction with a slightly alkaline, warm, water-rich mixture of ethanol–diethylether–water–pyridine (Method IIIB, Hanson and Lester, 1980). This method approached 100% extraction of the labeled inositol; it is possible that inositol-containing lipid–protein anchors contribute to the minor amounts not extracted. Some water-poor solvents completely failed to extract the InsPCers. The adopted procedure has been used for InsPCer extraction from other fungi such as *Histoplasma capsulatum* (Barr and Lester, 1984) and from fresh tobacco leaves (Kaul and Lester, 1975) and has been used for the extraction of lipophosphoglycan from *Leishmania donovani* (Orlandi and Turco, 1987). In the absence of labeling of InsPCer components, efficacy of InsPCer extraction could be judged by monitoring total long-chain base and/or very-long-chain fatty acids (Dickson *et al.*, 1990).

2. *Purification of InsPCers*

Since the purification of InsPCers offers challenges no different from any very polar, acidic lipid, only details specific for InsPCers will be discussed. First, InsPCers precipitate almost quantitatively at low temperature after adjusting to pH 5 the initial lipid extracts from *S. cerevisiae* (Smith and Lester, 1974), *H. capsulatum* (Barr and Lester, 1984), *N. crassa* (Lester *et al.*, 1974), and tobacco leaves (Kaul and Lester, 1975). Second, mild alkaline methanolysis is a useful step to destroy the ester-containing lipids in crude or semipurified extracts (Barr and Lester, 1984); however, stronger alkaline conditions should be avoided owing to the lability of unsubstituted inositol attached by a phosphodiester bond (Smith and Lester, 1974). Finally, liquid chromatography on silica gel columns is a valuable technique for isolating InsPCers (Barr and Lester, 1984); low levels of salt in the eluting solvents are required to chromatograph macroscopic quantities of the very polar InsPCers. A solvent polar enough to dissolve a practical amount of the very polar InsPCers yields insufficient retention. Inclusion of salt increases the retention dramatically, presumably by providing a charged surface for the negatively charged InsPCers. In contrast, the salt had little influence on the retention of neutral lipids (R. L. Lester and G. B. Wells, unpublished).

B. Structures of InsPCers of Yeast and Fungi

1. Saccharomyces cerevisiae

The first report of an InsPCer in yeast was that of Wagner and Zofcsik (1966a,b,c), who characterized a so-called "mycoglycolipid" in *S. cerevisiae* and *Candida utilis* as

Ceramide-P-inositol-mannose

The ceramide was composed of C_{18} and C_{20} phytosphingosines, C_{18} dihydrosphingosine, and principally OH-C_{24}, OH-C_{26} (*C. utilis*), and C_{26} and OH-C_{24} (*S. cerevisiae*) fatty acids. Subsequent work with *S. cerevisiae* (Steiner *et al.*, 1969; Smith and Lester, 1974) indicated that the mycoglycolipid of Wagner and Zofcsik largely arose by alkaline hydrolysis (1 *N* KOH, 37°C, 24 hours) of a major sphingolipid with the composition mannose(inositol-P)$_2$ceramide, hereafter abbreviated as M(IP)$_2$C. These alkaline conditions were developed by Schmidt *et al.* (1946) and used widely in procedures for sphingomyelin isolation and analysis. The procedure of Schmidt *et al.* was based on the observation of Thudichum (1901) that sphingomyelin is base-stable relative to other phospholipids. In fact, the other major InsPCer in *S. cerevisiae*, IPC, was completely destroyed by strong alkaline treatment (Smith and Lester, 1974). The facile formation of a cyclic inositol phosphate accounts for the breakdown of these InsPCers; the stability of the mannose-inositol-P-ceramide (abbreviated MIPC) suggests that the mannose is vicinal to the phosphate and is thus unable to form a cyclic inositol-P:

$$\mathrm{M(IP)_2C + H_2O \xrightarrow{OH^-} cyc\text{-}1,2\text{-}Inositol\text{-}P + MIPC}$$

$$\mathrm{IPC + H_2O \xrightarrow{OH^-} cyc\text{-}1,2\text{-}Inositol\text{-}P + ceramide}$$

$$\mathrm{MIPC + H_2O \xrightarrow{OH^-} No\ reaction}$$

Small amounts of MIPC were found in *S. cerevisiae* untreated with strong alkali; however, M(IP)$_2$C and IPC were the major InsPCers. Collectively, the InsPCers comprised about 40% of the inositol in the lipid extract and about 15% of the total yeast phospholipid P (Smith and Lester, 1974).

Molecular species of IPC and MIPC with different levels of hydroxylation of the ceramide components were separated with difficulty on low-performance silica gel chromatographic supports by Smith and Lester (1974). Subsequent work with high-performance chromatographic sup-

ports (Wells and Lester, 1983; G. B. Wells and R. L. Lester, unpublished) showed that four major variously hydroxylated species of each of the InsPCers can be detected. In the order of elution from the column, these are as follows.

Species	Long-chain bases	Fatty acids
I	$C_{18/20}$ *erythro*-dihydrosphingosines	26 : 0
IIa	$C_{18/20}$ *erythro*-dihydrosphingosines	OH-26 : 0
IIb	$C_{18/20}$ phytosphingosines	26 : 0
III	$C_{18/20}$ phytosphingosines	OH-26 : 0
IV	$C_{18/20}$ phytosphingosines	$(OH)_2$-26 : 0

Molecular species IIa and IIb have the same total level of hydroxylation and are inseparable with standard methods; however, ordinarily they do not coexist. Species I is found only under anaerobic culture conditions (Wells and Lester, 1983). We propose this system of nomenclature to be superior to that used earlier by Smith and Lester (1974).

Preliminary nuclear magnetic resonance (NMR) analysis of the polar head group of $M(IP)_2C$ as well as other data (Shelling *et al.*, unpublished; R. L. Lester *et al.*, unpublished) suggest the following structure for $M(IP)_2C$.

Inositol-1-P-(6)mannose(α1,2)inositol-1-P-(1)ceramide

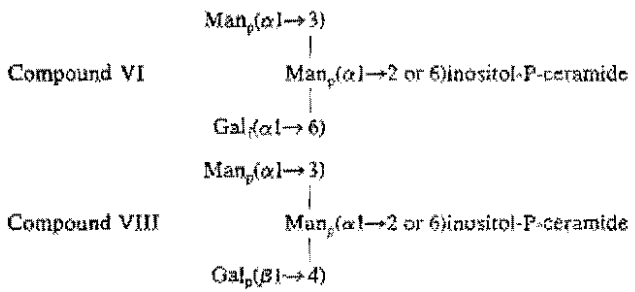
2. *Aspergillus niger*

Hackett and Brennan (1977) offered some evidence for a substance from *A. niger* that labels with [^{3}H]inositol and that has some of the chromatographic and chemical properties of inositol-P-ceramide. A brief communication by Brennan and Roe (1975) described the isolation of a glycosphingolipid that contained, in unspecified molar ratios, P, ceramide, inositol, galactose, mannose, and small amounts of glucosamine. This compound could be related to the InsPCers from *H. capsulatum* (see below).

3. *Histoplasma capsulatum*

From the yeast phase of the dimorphic, pathogenic fungus *H. capsulatum*, IPC as well as three other novel InsPCers were identified (Barr and Lester, 1984; Barr *et al.*, 1984):

Compound V $\quad$ $Man_p(\alpha 1 \rightarrow 3)$
$\qquad\qquad\qquad\qquad$ |
$\qquad\qquad\qquad\qquad$ $Man_p(\alpha 1 \rightarrow 2$ or $6)$inositol-P-ceramide



The ceramides were composed principally of C_{18} phytosphingosine and OH-C_{24} fatty acid. Compound VIII was the major InsPCer in both the yeast and the mycelial phase. Compounds V and VI were absent from the mycelial phase and the major *S. cerevisiae* InsPCer, $M(IP)_2C$, was absent from both phases. Antibodies reacting with these lipids were detected in sera from patients with histoplasmosis.

4. Neurospora crassa

Several strains of *N. crassa* exhibited an InsPCer with the composition

(Inositol-P)$_2$-ceramide

that accounted for 30–60% of the lipid-extractable inositol (Lester *et al.*, 1974). The compound was labile to alkali (1 *N* KOH, 37°C, 15 hours), yielding inositol monophosphates; the logical inference from the limited data was that the compound could be formulated as

Inositol-P-inositol-P-ceramide

with the details of the phosphate linkages undetermined. The ceramide was composed principally of C_{18} phytosphingosine and OH-C_{24} fatty acid.

5. Phytophtora capsici

A substance that elicits a defense reaction in peppers against *P. capsici* infection was isolated from this pathogenic fungus and was identified as an IPC (Lhomme *et al.*, 1990). The ceramide was unusual for fungal InsPCers: *N*(4-hydroxy-2-docosenoyl)C_{16}-sphingosine. The nature of the biological specificity of this sphingolipid is unclear, since these workers (Pivot *et al.*, 1991) have also elicited a defense reaction in peppers and wheat against *P. capsici* infection with a quite different lipid isolated from this fungus: dihexadecanoyl phosphatidylcholine.

IV. InsPCers in Protozoa

A. *Leishmania* sp.

Kaneshiro *et al.* (1986) showed that about 40% of the lipid-extractable inositol of *Leishmania donovani* promastigotes exhibited chromatographic and chemical properties of an IPC. The principal constituents of the ceramide were stearic acid and C_{16} and C_{18} sphingosines. *L. donovani* is the causative agent of the human disease visceral leishmaniasis.

Mass spectral evidence was offered in support of the occurrence in promastigotes of *Leishmania mexicana mexicana* of an InsPCer with a ceramide composed mainly of stearic acid and a C_{16} sphingosine (Singh *et al.*, 1988).

B. *Tritrichomonas foetus*

Singh *et al.* (1991) have studied the lipids resistant to mild alkali in *T. foetus*, a flagellated protozoan responsible for spontaneous abortion in cattle. IPC species as well as novel InsPCers were observed that both had ceramides consisting of palmitic acid and either C_{18} sphinganine or C_{18} sphingosine. The novel InsPCer was partially characterized as

Fucose-inositol-P-ceramide
|
Phosphoethanolamine (± *N*-acetyl)

C. *Acanthamoeba castellanii*

The soil amoeba *A. castellanii* was shown to contain complex related InsPCers, termed lipophosphoglycans, which constituted 29% of the plasma membrane mass. These were partially characterized (Dearborn *et al.*, 1976):

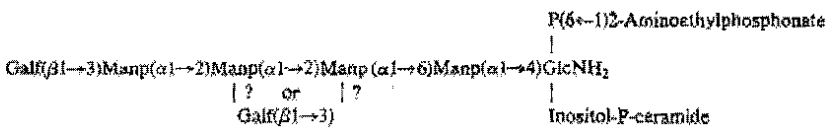
Galactosamine
|
Ceramide-P-inositol-[2-aminoethylphosphonate or 1-OH-2-aminoethylphosphonate]
|
[$Glucose_2 mannose_3 xylose_2$]
and
[$Glucose_{12} mannose_6 galactose_2$]

The major ceramide components were 2-OH normal fatty acids of chain lengths from 22 to 28 carbons and 2-OH branched fatty acids with chain

lengths of 21 to 25 carbons; the major long-chain bases were C_{24} and C_{25} phytosphingosines.

D. *Trypanosoma cruzi*

Beginning with the work of Lederkremer *et al.* (1976), a complex InsPCer in *T. cruzi* termed "lipopeptidophosphoglycan" has been the subject of numerous investigations; pertinent references can be found in Previato *et al.* (1990). *T. cruzi* is the causative agent of Chagas' disease and the surface glycoconjugates are actively studied because of their possible role in pathogenesis. The structure is thought to be (Previato *et al.*, 1990):



The ceramide is composed mainly of C_{18} sphinganine and C_{18} sphingosine with palmitic and lignoceric acids. The structure, -$(Man)_n$-$GlcNH_2$-inositol-P-, is found in phosphatidylinositol-linked membrane protein anchors (Ferguson and Williams, 1988) and in the surface glycoconjugate termed lipophosphoglycan from *L. donovani* (Turco *et al.*, 1989).

V. Metabolism of InsPCers

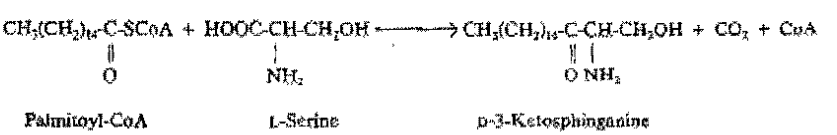
A. Synthesis of Long-Chain Bases

This review is limited to the organisms in which InsPCers have been identified; information is available only for *Hansenula ciferrii* and *S. cerevisiae*. For a general review of long-chain base synthesis, see Merrill and Jones (1990).

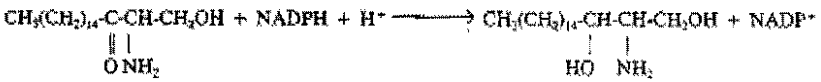
1. Hansenula ciferrii

At least three steps are required for the synthesis of phytosphingosine, the predominant long-chain base in InsPCers:

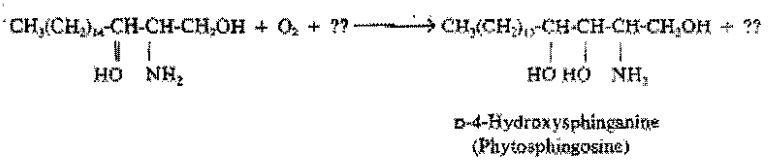
i. Serine Palmitoyltransferase (SPT):



ii. 3-Ketosphinganine (3-KDS) Reductase:



iii. Phytosphingosine Synthase:



Steps i and ii were first defined in the 1960s by E. E. Snell and coworkers with crude membranes of the fungus *H. ciferrii* (for summary, see Snell *et al.*, 1970). Subsequent work with other biological systems has confirmed these findings (reviewed in Merrill and Jones, 1990). The enzymes have never been purified.

Phytosphingosine is not limited to plant, fungal, and protozoan sphingolipids but has a very widespread distribution, including higher animals (Nishimura, 1987), fish (Li *et al.*, 1984), amphibians (Nohara-Uchida and Ohashi, 1987; Hidari *et al.*, 1991), and invertebrates such as sea urchins (Kochetkov *et al.*, 1976; Kubo *et al.*, 1990) and starfish (Sugita, 1979). Nevertheless, phytosphingosine synthesis has not yet been demonstrated *in vitro*. *In vivo* studies with *H. ciferrii* (Stoffel *et al.*, 1968a) and in the rat (Crossman and Hirschberg, 1977) showed conversion of labeled *erythro*-dihydrosphingosine to phytosphingosine; molecular oxygen appears to be the source of the 4-oxygen of phytosphingosine (Kulmacz and Schroepfer, 1978). One can speculate that a mixed-function oxidase system might catalyze step iii.

2. Saccharomyces cerevisiae

a. Serine Palmitoyltransferase. SPT activity was demonstrated in crude membranes from *S. cerevisiae* (Pinto *et al.*, 1992b). The conversion of labeled serine into 3-keto-dihydrosphingosine was completely dependent on a fatty acyl-CoA; C_{14}, C_{16}, and C_{18} fatty acyl-CoAs gave rise to C_{16}, C_{18}, and C_{20} products, respectively. The abundant C_{20} homolog of phytosphingosine found in yeast thus originates from the SPT-catalyzed condensation of stearyl-CoA with serine.

Two potent antifungal agents have been shown to inhibit SPT activity in membranes of *S. cerevisiae* at nanomolar levels (Zweerink *et al.*, 1992):

sphingofungin B (2S-amino-3R,4R,5S,14-tetrahydroxyeicos-6-enoic acid) and sphingofungin C (2S-amino-5S-acetoxy-3R,4R,14-trihydroxyeicos-6-enoic acid). These agents inhibited inositol incorporation into sphingolipids but not into phosphatidylinositol; this specificity suggests these compounds may prove to be useful for the study of sphingolipid metabolism/function.

Free sphingolipid long-chain bases did not inhibit SPT activity *in vitro*, nor did they repress enzyme activity levels when added to growing cultures (Pinto *et al.*, 1992b); this contrasts with the downregulation of SPT activity observed by Mandon *et al.* (1991) in cultured neurons with the addition of sphingolipid bases.

b. 3-Ketodihydrosphingosine Reductase. Crude membranes of *S. cerevisiae* were shown to catalyze the stereospecific reduction of 3-ketodihydrosphingosine with NADPH, forming the *erythro* isomer of dihydrosphingosine (Pinto *et al.*, 1992b), a reaction analogous to that shown earlier with animal microsomes (Stoffel *et al.*, 1968b). Further confirmation of the pathway for phytosphingosine synthesis was the observation that mutants defective in SPT could grow and make phytosphingosine-containing sphingolipids when supplemented with either 3-ketodihydrosphingosine or dihydrosphingosine (Pinto *et al.*, 1992a). The fact that both the *erythro* and the unnatural *threo* isomers of dihydrosphingosine could support growth of the SPT mutant (Pinto *et al.*, 1992a) suggests that these isomers are interconvertible.

c. Genes Responsible for SPT Activity. Molecular cloning of the *S. cerevisiae* gene(s) encoding SPT activity was greatly facilitated by the isolation of a mutant strain that required a sphingolipid *l*ong-chain *b*ase (*lcb*) (dihydrosphingosine or phytosphingosine) for growth (Wells and Lester, 1983). Isolation of an *lcb* mutant strain established that sphingolipids are essential for growth of *S. cerevisiae* which, in turn, suggested that one or more specific cellular functions required sphingolipids. The *lcb* strain also proved to be important because its behavior provided a simple procedure for enrichment of mutants defective in long-chain base biosynthesis. A buoyant density increase was noted when *lcb* mutant cells were starved for long-chain base. Consequently, mutant cells could be separated from normal cells by buoyant density centrifugation (Pinto *et al.*, 1992a). Using this enrichment procedure, numerous Lcb$^-$ mutants were isolated and placed into complementation groups, of which there turned out to be only two, *LCB1* and *LCB2* (Pinto *et al.*, 1992a). Both *lcb1* and *lcb2* mutant strains lack SPT activity (Pinto *et al.*, 1992b).

i. Characterization of the LCB1 gene. The *LCB1* gene was isolated from a *S. cerevisiae* recombinant genomic DNA library by complementation of the *Lcb*$^-$ phenotype of an *lcb1* mutant strain (Buede *et al.*, 1991). The cloned gene restored SPT activity as expected if the gene encoded SPT or a subunit of the enzyme. Southern blot analysis demonstrated that there was but a single copy of *LCB1* in the *S. cerevisiae* haploid genome. The gene is predicted to encode a protein of 558 amino acid residues. Since SPT activity is found in the membrane fractions of *S. cerevisiae* (Pinto *et al.*, 1992b) and mammalian cells (Merrill and Jones, 1990), we anticipated that the predicted LCB1 protein would have at least one membrane-spanning domain. The algorithms of Kyte and Doolittle (1982) and Eisenberg *et al.* (1984) predicted two membrane-associated helices spanning residues 12 to 32 (IPIPAFIVTTSSYLWYYFNLV) and residues 344 to 373 (ATAIDITVGSMATALGSTGGFVLG).

Three results suggest that *LCB1* encodes SPT or a subunit of the enzyme (Buede *et al.*, 1991). First, the cloned *LCB1* gene complements the Lcb$^-$ phenotype of an *lcb1* mutant and restores growth in media lacking long-chain base. Second, the cloned gene restores SPT activity in an *lcb1* deletion mutant. Finally, the predicted *LCB1* amino acid sequence shows amino acid similarity to the enzymes 5-aminolevulinic acid synthase (ALA synthase) and 2-amino-3-ketobutyrate-CoA ligase, enzymes that catalyze chemical reactions very similar to the one catalyzed by SPT. Moreover, all three enzymes use the cofactor pyridoxal phosphate.

ii. Characterization of the LCB2 gene. The *LCB2* gene of *S. cerevisiae* was isolated from a recombinant genomic DNA library by complementation of the Lcb$^-$ phenotype of an *lcb2*-defective strain (R. C. Dickson *et al.*, unpublished results). *LCB2*, like *LCB1*, is present as a single-copy gene in the yeast haploid genome. The predicted protein product of *LCB2* has 561 amino acids and is expected to contain two membrane-associated helices spanning residues 57 to 77 (PYYISLLTYLNYLILIILGHV) and residues 443 to 463 (LGFIVYGVADSPVIPLLLYCP). Comparison of the predicted LCB1 and LCB2 amino acid sequences showed 22.4% amino acid identity and 15.2% similarity over the entire sequences, which suggests that the proteins are related. The predicted LCB2 amino acid sequence shows about the same level of amino acid similarity to ALA synthase, as does the predicted LCB1 protein sequence. Taken together, the available data suggest that the LCB1 and LCB2 proteins are subunits of SPT. This possibility awaits biochemical confirmation. With the availability of both the *LCB* genes, it should be possible to overexpress the genes in yeast or bacteria, which ought to facilitate purification and characterization of SPT.

B. Synthesis of Very Long Chain (Hydroxy) Fatty Acids

Information is incomplete on the synthesis of the very long chain, often hydroxylated, fatty acids found in the InsPCers. In animals the 16:0 product of the fatty acid synthase can be elongated in mitochondria by a system that shares some of the β-oxidation pathway enzymes; elongation in the endoplasmic reticulum proceeds as acyl-CoA derivatives by condensation with malonyl-CoA and reduction with reduced pyridine nucleotides (Seubert and Podack, 1973; Bernert and Sprecher, 1977). In no case have individual enzymes been highly purified or the system reconstituted with solubilized enzymes.

Information on fatty acid elongation is even scantier in those organisms with InsPCers. Evidence for a malonyl-CoA-primed elongation system in plants has been presented (Harwood, 1979; Walker and Harwood, 1986). In yeast, evidence for fatty acid elongation comes from observing the conversion of labeled fatty acids to longer chain acids *in vivo* with wild-type *C. utilis* and *S. cerevisiae* (Fulco, 1967) and with a fatty acid synthetase mutant of *S. cerevisiae* (Orme *et al.*, 1972). *In vitro* evidence for fatty acid elongation in *S. cerevisiae* comes from the work of Blanchardie *et al.* (1977) showing that purified membrane fractions could convert labeled stearoyl-CoA in the presence of malonyl-CoA and NADPH to fatty acids with 20–30 carbons. However, Schweizer (1984) questions the relevance of malonyl-CoA-dependent chain elongation in yeast.

Fatty acid α-hydroxylation has been studied in animal systems; however, molecular mechanisms remain incompletely understood, although molecular oxygen and NADPH are required (Kishimoto, 1983; Shigematsu *et al.*, 1990). The nature of the substrate is unclear, i.e., whether it is a fatty acyl-CoA, a ceramide, or a more complex sphingolipid. The electron transport chain of events is also unclear.

In *Tetrahymena pyriformis*, Kaya *et al.* (1984) provided *in vivo* evidence for the direct hydroxylation of ceramide 2-aminoethylphosphonate, converting the bound palmitic acid to α-hydroxypalmitic acid. On raising the growth temperature from 15 to 39°C, a rapid increase in hydroxylated sphingolipid was observed. Hydroxylation of sphingolipid *in vitro* was not observed. Indirect evidence for direct sphingolipid hydroxylation comes from the observations of Dickson *et al.* (1990) with a mutant of *S. cerevisiae* that makes no detectable sphingolipid; nevertheless, this mutant contains normal levels of C_{26} fatty acid which is strikingly underhydroxylated. The origin of the 2-oxygen in hydroxy fatty acids in the InsPCers of *S. cerevisiae* appears to be molecular oxygen, since anaerobically cultured cells have no hydroxy fatty acids in the InsPCers (R. L. Lester *et al.*, unpublished).

C. Synthesis of Ceramide

Ceramide synthesis has been studied in higher animals; it occurs by the reaction of a fatty acyl-CoA with a free sphingolipid long-chain base (Kishimoto, 1983). No information is available concerning ceramide synthesis in the organisms with InsPCers.

D. Synthesis of InsPCers

In spite of the widespread distribution of these major membrane constituents, little is known about their assembly and metabolism; the only available information comes from studies in yeast. A pathway has been proposed as a working hypothesis (Becker and Lester, 1980) whereby phosphatidylinositol is the precursor of the inositol-P groups, reacting with ceramide and MIPC, and with GDP-mannose as a mannose donor:

i. Phosphatidylinositol + ceramide → Inositol-P-ceramide + diacylglycerol (DAG)
ii. Inositol-P-ceramide + GDP-mannose → Mannose-inositol-P-ceramide (MIPC) + GDP
iii. MIPC + phosphatidylinositol → $M(IP)_2C$ + DAG

In vivo evidence supporting this scheme was obtained by Angus and Lester (1972). Synthesis of $M(IP)_2C$ in *S. cerevisiae* was studied by uniformly labeling cells with $^{32}P_i$ and [^{3}H]inositol, chasing with unlabeled isotopes for several cell generations, and monitoring the changes in all the major phospholipids. Half of both the ^{32}P and [^{3}H]inositol that disappeared from the phosphatidylinositol pool accumulated in the $M(IP)_2C$ pool with the identical label ratio; the other half appeared in the culture medium as glycerophosphoinositol. These data are wholly compatible with phosphatidylinositol providing both inositol-P groups intact to form $M(IP)_2C$. Little turnover of $M(IP)_2C$ was observed; however, the IPC + MIPC fraction did exhibit turnover compatible with roles as precursors of $M(IP)_2C$. Previous work by Tanner (1968, 1969) is consistent with these conclusions. The synthesis of the InsPCers may occur in the Golgi apparatus because Puoti *et al.* (1991) have shown that synthesis of InsPCers from labeled inositol ceases in temperature-sensitive mutants that cannot carry out vesicular transport from the endoplasmic reticulum to the Golgi at the restrictive temperature.

In vitro evidence supporting reactions (i) and (ii) was obtained using crude membranes from *S. cerevisiae* which catalyze the incorporation of phosphoinositol into IPC and $M(IP)_2C$ from added labeled phosphatidylinositol (Becker and Lester, 1980). Presumably, endogenous ceramide and MIPC served as substrates. Work from this laboratory has shown

that exogenous labeled ceramide can serve as a precursor of IPC (W. J. Pinto *et al.*, unpublished) and that mannose-labeled GDP-mannose can serve as a mannose donor (G. W. Becker *et al.*, unpublished) in this *in vitro* system. The possibility that mannosylphosphoryldolichol serves as the ultimate mannose donor cannot be ruled out. We are undertaking purification of the synthetic enzymes away from endogenous lipids in order to establish definitively the stoichiometry of reactions i–iii. Evidence for reaction (i) in *A. niger* was obtained by Hackett and Brennan (1977). The postulated phosphoinositol transferase reaction for IPC synthesis is analogous to the phosphocholine transferase in the formation of sphingomyelin (Merrill and Jones, 1990).

VI. Functions of Sphingolipids in *Saccharomyces cerevisiae*

All eukaryotic cells, including animals (Hakomori, 1983), higher plants (Laine *et al.*, 1980), and fungi (Brennan and Losel, 1978), contain sphingolipids, particularly in their plasma membrane. In animals, sphingolipids are thought to play roles as modulators of membrane signal transducers, resulting in the regulation of cell growth and differentiation, and as mediators of cell-to-cell or cell-to-substratum recognition (Hannun and Bell, 1989; Hakomori, 1990). Additional biological roles for sphingolipid intermediates and breakdown products including ceramide and sphingosine have been reported by several workers (reviewed in Hannun and Bell, 1989; Merrill, 1991). Further effort is needed, however, to establish the physiological function of most sphingolipids. A particularly nagging problem in deciphering the function of a specific sphingolipid is the presence of dozens, perhaps hundreds, of sphingolipids in most animal cells (Hakomori, 1983).

To try to overcome the problems posed by the presence of many sphingolipids in a cell, we have begun to examine the function of sphingolipids in *S. cerevisiae*, since there is only one major sphingolipid ($M(IP)_2C$) and two minor sphingolipids (IPC and MIPC). The discovery of sphingolipid long-chain base auxotrophs of *S. cerevisiae* unable to make sphingolipids owing to defective SPT activity (Wells and Lester, 1983; Pinto *et al.*, 1992a,b) opened the possibility of using molecular genetic techniques to study sphingolipid functions. The inability to grow and the loss of viability of such mutant strains if not supplemented with a sphingolipid long-chain base strongly suggests that there are one or more vital functions for yeast sphingolipid. Besides contributing to general membrane properties such as charge and fluidity, sphingolipids may contribute unique functions such as specific binding to a protein and membrane anchors for proteins (see

below). Recent *in vitro* studies have demonstrated that the phospholipid requirement of the plasma membrane H^+-ATPase is effectively satisfied by certain InsPCers (Patton and Lester, 1992), suggesting that the physiological activity of the H^+-ATPase is dependent on InsPCers for normal function.

A. Sphingolipid Compensation Strains

One strategy for determining the physiological function(s) of sphingolipids was to compare the phenotype of a strain that has been manipulated so as to either contain or lack sphingolipids. Phenotypes that required sphingolipids, particularly ones dependent on the plasma membrane, could point the way to more detailed experiments which would reveal the function of a sphingolipid at the molecular level. To apply this strategy to *S. cerevisiae*, we isolated strains that can grow without making sphingolipids (Dickson *et al.*, 1990). Such strains have two essential mutations. First, the *lcb1* gene is deleted so that the strain cannot make the long-chain base component of sphingolipids. Second, the strains carry a semidominant mutation termed SLC (*s*phingo*l*ipid *c*ompensation) that enables the cell to suppress or bypass the *lcb1* defect and to grow without exogenous phytosphingosine. When exogenous phytosphingosine is supplied to the culture medium, the strains make a normal complement of sphingolipids.

Conditions that required sphingolipids for growth were searched for by comparing the behavior of SLC strains either containing or lacking sphingolipids. Sphingolipids were not required for growth under normal nonstressing culture conditions (Dickson *et al.*, 1990), but were required when the pH of the medium was reduced to 3.5, when the temperature of incubation was increased to 37°C, when the medium contained 0.75 *M* NaCl or KCl, and when the medium contained sodium acetate buffered at pH 6 (Patton *et al.*, 1992). These growth-inhibiting conditions seemed unrelated, but, when compared to previously published data (McCusker *et al.*, 1987), they showed a striking similarity to results obtained for some mutant strains defective in *pma1*, the gene coding for the plasma membrane H^+-ATPase, a major constituent of the plasma membrane of *S. cerevisiae*. This enzyme pumps protons out of the cell to create an electrochemical gradient that is utilized for transporting a variety of nutrients into the cell (for reviews, see Serrano *et al.*, 1989; Sigler and Hoefer, 1991). Furthermore, the InsPCers of *S. cerevisiae* are highly localized in the plasma membrane and represent a large fraction of its phospholipid (Patton and Lester, 1991). One interpretation of the behavior of SLC cells lacking sphingolipids is that the InsPCers are essential for H^+-ATPase activity when cells are subjected to environmental extremes.

This possibility was examined in more detail using whole cells to follow net extrusion of protons into the suspension medium (Patton *et al.*, 1992). The proton extrusion assay was initiated by adding glucose to the suspension medium. SLC cells containing sphingolipids behaved like wild-type cells and showed net proton extrusion even when the suspension medium was buffered at pH 4. In contrast, SLC cells lacking sphingolipids did not show net proton extrusion at pH 4 and, in addition, the suspension medium became alkaline. Alkalinization seemed to be due to increased permeability of sphingolipid-deficient cells to protons. Increased permeability was not due merely to formation of large holes or pores in the plasma membrane because cells did not leak proteins into the suspension medium and lactose did not freely enter cells. The effects of the low pH treatment occurred rapidly (within 1 minute) and were irreversible.

One explanation for these observations is that the SLC suppressor mutation is in *PMA1*. This is not the case, however, because the cloned *SLC1* suppressor gene is not *PMA1* (see below). At this time, it is not clear how the lack of sphingolipids affects membrane permeability and/or the H^+-ATPase under environmental extremes; there may or may not be one unifying explanation.

The predicted protein product of the *SLC1* suppressor gene (M. M. Nagiec, G. B. Wells, R. L. Lester, and R. C. Dickson, unpublished data) shows homology to the *PlsC* gene of *Escherichia coli* which encodes 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (Coleman, 1992). Such homology suggests that the wild-type allele of *SLC1* may have a similar function in *S. cerevisiae*. It is pertinent to note that SLC strains accumulate significant quantities of novel lipids when cultured without a long-chain base: mono- and di-fattyacyl versions of phosphatidylinositol (PI), mannosyl-PI, and inositol-P-(mannosyl-PI), each containing one mole of C_{26} fatty acid, ordinarily found in yeast sphingolipids but not abundantly found in glycerophospholipids (Lester, *et al.*, 1993). Thus the polar head groups and hydrophobic portions of these novel lipids are strikingly similar to *S. cerevisiae* sphingolipids found in wild-type cells, and it was speculated that the novel lipids that structurally mimic sphingolipids partially compensate for some sphingolipid function(s) necessary for growth. A reasonable hypothesis is that the suppressor *SLC1* allele could be responsible for a fattyacyl transferase that inserts a C_{26} fatty acid into a precursor of the novel lipids.

B. InsPCers May Act as Anchors for Proteins

It is now well established in several organisms that some proteins are anchored to a cellular membrane by covalent attachment to a glycophos-

phatidylinositol (GPI) (reviewed in Ferguson and Williams, 1988). The lipid moiety in these anchors is normally either a diacylglycerol or an alkylacylglycerol. Two reports indicate that some proteins may be anchored by attachment to a ceramide-containing glycolipid. The contact site A glycoprotein of *Dictyostelium discoideum* is an adhesion molecule expressed at the aggregation stage of development. The protein is anchored to the plasma membrane. The fatty acid in the anchor lipid is not released by mild alkaline hydrolysis but is released by strong alkaline hydrolysis, indicating amide linkage to the lipid. Incubation of the anchor with sphingomyelinase releases a lipid with the chromatographic behavior of a ceramide. Thus, the available evidence suggests a ceramide-containing lipid anchor (Stadler *et al.*, 1989).

Several proteins in *S. cerevisiae* appear to be anchored to the plasma membrane by two different types of phosphoinositol-containing lipid anchors (Conzelmann *et al.*, 1992). GPI is one type of anchor and the other type contains ceramide in place of diacylglycerol. Evidence supports the idea that the GPI anchor is attached first to proteins and is then remodeled by replacement of the diacylglycerol with ceramide. The route of synthesis of these novel lipid anchors remains to be determined as do their precise structures and function and the function of the proteins they anchor. How widely distributed in nature are the InsPCer protein anchors also remains to be determined.

VII. Summary

InsPCers have been characterized in many plants, fungi, and protozoans but not in animals. There are no well-documented reports of the absence of InsPCers in organisms of these categories and one might possibly consider these lipids to be ubiquitous in plants, fungi, and protozoans. The polar headgroups of these lipids display quite heterogeneous structures depending on the source, including attachment to proteins as possible membrane anchors. The ceramides are with some exceptions composed of phytosphingosine and a very long-chain, usually hydroxylated, fatty acid. The vital nature of such sphingolipids in the plasma membrane is indicated in *S. cerevisiae*. Clearly, much remains to be discovered about the structure, metabolism, and function of the InsPCers.

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