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Studies on the Diversity of Inositol-Containing Yeast Phospholipids: Incorporation of 2-Deoxyglucose into Lipid

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Seven inositol-containing phospholipids in *Saccharomyces cerevisiae* can be resolved by two-dimensional chromatography on silicic acid-impregnated paper. Four of these lipids are stable to mild alkaline methanolysis; one of these has already been characterized as a mannosyl di(inositolphosphoryl) ceramide. Addition of labeled 2-deoxy-D-glucose to a growing culture results in label appearing in five lipids, some of which are alkali-stable and have the same R_F values as the alkali-stable inositol-containing phospholipids. These lipids are labeled rapidly. The deoxyglucose is incorporated intact, probably in a glycosidic link, since the deoxyglucose is released by mild acid treatment.

Yeasts have been convenient organisms in which to study the biological role of inositol. The effects of inositol deprivation (11, 20) as well as the effects of inositol antagonists (7) have been examined with inositol-requiring yeast strains. This work suggested a role for inositol in the metabolism of cell wall polysaccharides.

Delineation of the biological role of inositol at the molecular level requires some knowledge of the nature of the inositol-containing compounds of yeast. Most of the inositol found in yeast is in the form of lipid. For many years, phosphatidylinositol was the only well characterized inositol-containing yeast lipid. More recently, evidence was presented for the occurrence in yeast of diphosphoinositide and triphosphoinositide (17). In addition, inositol-containing sphingolipids have been shown to occur in yeast. Wagner and Zofcsik (26) isolated a lipid, termed "mycoglycolipid," to which they assigned the structure: ceramide-P-inositol-mannose. Steiner et al. (23) subsequently reported that the major inositol containing sphingolipid in baker's yeast had the composition: ceramide-(P-inositol)$_2$-mannose. In this report we show that from lipid extracts of yeast we can resolve chromatographically, in addition to the three glycerol-containing inositides, at least four other inositol-containing phospholipids which, because of their stability to alkaline methanolysis, are tentatively thought to be sphingolipids. Some aspects of yeast lipids have been recently reviewed (15).

The molecular diversity of the inositol-containing lipids suggests that a single metabolic role for inositol in yeast is improbable. Each inositol-containing lipid may serve a unique biological function. It has been noted that 2-deoxyglucose serves as a growth inhibitor of yeast by preventing proper cell wall formation, possibly by blocking some steps in the synthesis of wall polysaccharide or glycoprotein (9, 10, 13, 16, 18). These observations coupled with the above noted effects on inositol deprivation in yeast prompted us to examine the incorporation of labeled deoxyglucose into yeast lipid. Deoxyglucose incorporated into a glycolipid in place of the natural sugar of course might interfere with the function of the lipid. In this paper we show that 2-deoxyglucose is readily incorporated into several lipids, some of which have the chemical stability and chromatographic behavior of the alkali-stable inositol-containing phospholipids.

MATERIALS AND METHODS

Radioactive compounds. Purchased from New England Nuclear Corp. were myo-inositol-2-^3H (3.47 Ci/mmole), 2-deoxy-D-glucose-1-^{14}C (58 mCi/mmole), and 2-deoxy-D-glucose-*UL*-^3H (6.8 Ci/mmole). ^{32}P-orthophosphate, carrier-free, was obtained from Tracerlab and ICN Corp. Myo-inositol-*UL*-^{14}C (312

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mCi/mmole) was purchased from Amersham-Searle, and myo-inositol-2-^{14}C (9.8 mCi/mmole) was from Cal Atomic. D-Glucose-*UL*-^{14}C (180 mCi/mmole) was a product of ICN Corp.

Culture conditions. A strain of *Saccharomyces cerevisiae* isolated from commercial bakers' yeast and used in other experiments (17) was grown on a rotary shaker at 30 C, in air, with a medium consisting of 1% (w/v) peptone (Difco), 1% (w/v) yeast extract (Difco), and 0.5% (w/v) KH_2PO_4. The KH_2PO_4 was omitted in the experiment with ^{32}P. Either 2% (w/v) ethanol or 4% (w/v) glucose served as carbon source. Growth in the presence of radioactive compound was terminated by addition of CCl_3COOH to a final concentration of 5% (w/v). The cells were centrifuged, washed once with 5% CCl_3COOH and twice with 0.5% (w/v) KH_2PO_4 before lipid extraction.

Lipid extraction and methanolysis. Lipid was extracted from the cells as previously described (17). The total lipid extract was chromatographed directly, without concentration. Lipids were deacylated by a previously described methanolysis procedure (17) except that the reaction was carried out at 22 C for 30 min.

Paper chromatography of the total lipid extract. Silicic acid-impregnated paper (Whatman SG81) was treated with ethylenediaminetetraacetic acid (EDTA) as follows. The papers (46 by 57 cm) were dipped in a solution made by adding 100 g of EDTA to approximately 4 liters of water, adjusting the *p*H to 7.2 to 8.0 with NH_4OH, and adding water to 5 liters. The papers were air dried at 22 C and then cut into pieces 15 by 19 cm. Before use, the sheets were heated for 20 min at 110 C. The chromatograms were run in two dimensions in 2-quart Mason jars. The first dimension solvent mixture was $CHCl_3$:CH_3OH:4 N NH_4OH (9:7:2, v/v). After a drying period of 30 min at 22 C and 10 min at 45 C in a forced draft oven, the second dimension was run with the solvent mixture, $CHCl_3$:CH_3OH:CH_3COOH:H_2O (15:6:4:1.6, v/v). After chromatography, the spots were detected by autoradiography (Kodak No Screen X-ray film), cut out, and eluted with $CHCl_3$:CH_3OH:H_2O:pyridine (40:56:12:2, v/v).

Thin-layer chromatography of eluted spots. Compounds labeled with ^{14}C-inositol and ^{14}C-deoxyglucose were chromatographed in one dimension on 1-mm thin-layer silica gel plates (Linear-Q, Quantum Industries, Fairfield, N.J.). The compounds were spotted on the cellulose strip and developed into the silica gel with the solvent, $CHCl_3$:CH_3OH:4 N NH_4OH (9:7:2, v/v).

Hydrolysis of deoxyglucose-labeled lipid. The eluates of the spots labeled with deoxyglucose (either ^{14}C or ^{3}H) were dried in a stream of N_2 after addition of 2 μmoles of ^{12}C-2-deoxy-D-glucose. Hydrolysis was carried out for 4 min at 100 C after the addition of 1.5 ml of 0.05 N H_2SO_4. The acid was neutralized with excess $BaCO_3$. After centrifugation the supernatant fraction was extracted with an equal volume of $CHCl_3$. The aqueous phase was concentrated in a stream of N_2 before chromatography.

Chromatography and detection of the water-soluble hydrolysis products. Ascending chromatography was carried out with sheets of 589 Orange Ribbon paper 7.5 inches long (Carl Schleicher and Schuell, Inc.) with three separate one-dimensional systems: *n*-butanol:pyridine:H_2O (6:4:3, v/v); *n*-butanol:propionic acid:H_2O (6:3:4, v/v); and isopropanol:H_2O (4:1, v/v). ^{14}C was detected by autoradiography. Adjacent lanes contained ^{12}C- and ^{14}C-deoxyglucose as additional R_F markers. The carrier ^{12}C-deoxyglucose was detected by being sprayed with either a nonspecific sugar spray, *p*-anisidine-phthalate (22), or with a more specific reagent: $HClO_4$-acetone (27). In the experiment involving ^{3}H-deoxyglucose, the material was spotted in a band; half of the lane was sprayed to detect the carrier ^{12}C-deoxyglucose, and the other half was subdivided into small strips and the ^{3}H was located by scintillation counting.

Detection of radioactivity. For liquid scintillation counting, lipid samples and samples of the acid hydrolysates were added to ethanol:toluene:H_2O (50:50:4, v/v) containing 0.38% (w/v) 2,5-bis-2-(5-tertbutylbenzoxazolyl)-thiophene (BBOT). Regions of the chromatograms of the hydrolysate were counted directly in 0.4% (w/v) BBOT in toluene.

RESULTS

We have found that inositol-containing phospholipids of yeast can be separated effectively on silicic acid-impregnated paper. Crude lipid extracts were prepared from cells grown for many generations with either $^{32}P_i$ or ^{14}C-inositol. Figures 1 and 3 show the results of chromatography of these extracts according to the two-dimensional system described above. Inositol-labeled spots correspond in every case to ^{32}P-labeled spots. Both of these extracts were subjected to mild alkaline methanolysis, and the resulting alkali-stable, lipid-soluble fraction was chromatographed in the same system (Fig. 2 and 4).

Mild alkali-labile lipids. Three ^{14}C-inositol-containing lipids are labile to mild alkaline methanolysis. The major inositol-containing lipid is phosphatidylinositol (MPI), long known to occur in yeast; only a small fraction of the radioactivity remains in this region of the chromatogram after methanolysis; in most experiments, none remains. The other two inositol-containing, alkali-labile lipids have been identified as diphosphoinositide (DPI) and triphosphoinositide (TPI). The ^{32}P-labeled spots were eluted, and it was found that with each lipid ^{32}P was rendered completely water-soluble by mild alkaline methanolysis. These water-soluble fractions were chromatographed on an anion exchange column eluted with an ammonium formate-borate gradient (17); the methanolysate of each

FIG. 1. *Autoradiogram of total lipid extract from ^{32}P-labeled cells. Cells were grown overnight from a small inoculum in 20 ml of medium supplemented with 3.0 mCi of $^{32}P_i$ to an absorbance (650 nm) of 4.5. Glucose was the carbon source. Conditions of harvest, lipid extraction, and two-dimensional chromatography were as described in the text. The origin is in the lower left corner, and the first dimension was developed from bottom to top.*

lipid gave rise to a single peak at the elution volumes already noted (17) for glycerophosphorylinositol phosphate (from DPI) and glycerophosphorylinositol diphosphate (from TPI).

Lipids stable to mild alkaline methanolysis. Four lipid spots (numbered 2, 5, 6, and 7) remained after the methanolysis procedure and were labeled with both $^{32}P_i$ and ^{14}C-inositol (Fig. 2, 4). The major one of these (spot 2) has been characterized previously as a mannosyl di(inositolphosphoryl) ceramide (23), related to the previously described "mycoglycolipid" of Wagner and Zofscik (26). Although the other three substances are as yet incompletely characterized, one important point emerges from use of a double labeling approach described previously (23), which involves labeling cells uniformly with ^{3}H-inositol and $^{32}P_i$. The results thus obtained indicate that these three compounds have an inositol to phosphorus ratio of 1.0.

Incorporation of ^{14}C-deoxyglucose into alkali-stable lipids. The presence in yeast of inositol-containing glycosphingolipids in substantial amounts raises questions concerning their role in cellular metabolism. It has been shown that inositol deficiency during growth of yeast leads to altered morphology and cell wall composition (11, 20). Another possibly related finding was that growth inhibition of yeast by 2-deoxy-D-glucose somehow involved interference with polysaccharide biosynthesis (9, 10, 13, 16, 18). If deoxyglucose were incorporated into certain glycolipids in place of the normal sugar, this might interfere with the function of these compounds. We therefore added ^{14}C-deoxyglucose to a growing yeast culture, and after 4 hr we extracted the lipid. Radioactivity corresponding to approximately 2 nmoles of deoxyglucose per μmole of phospholipid P was found in the crude lipid extract. This extract was chromatographed two-dimensionally on silicic acid-impregnated paper, and the resulting autoradiogram is shown in Fig. 5. Five spots are evident, and three of these, A, B, and C, have R_F values similar to those of the alkali-stable, inositol-containing lipids shown in Fig.

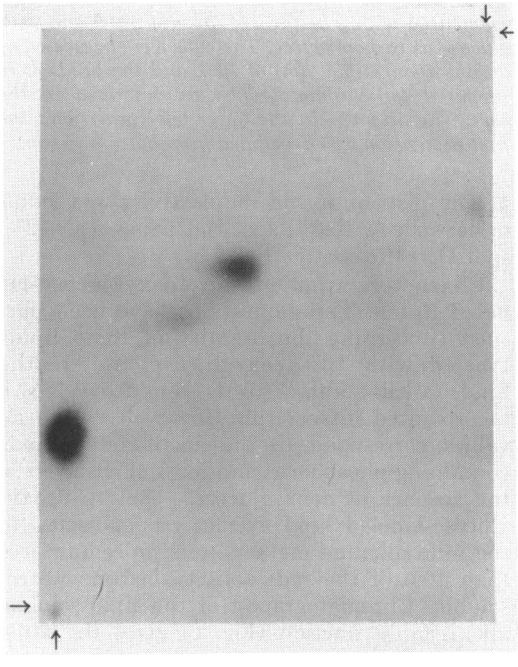

FIG. 2. *Autoradiogram of the lipid-soluble, alkali-stable fraction of ^{32}P-labeled lipids. Conditions of harvest, lipid extraction, and two-dimensional chromatography were as described in the text; growth conditions were as described in Fig. 1. The origin is in the lower left corner, and the first dimension was developed from bottom to top.*

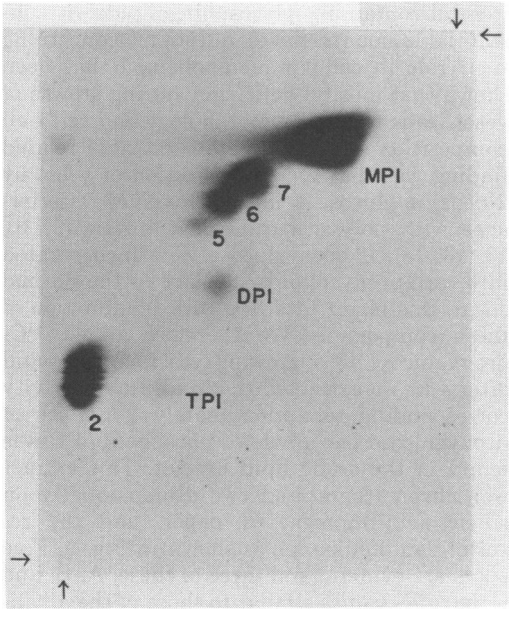

FIG. 3. *Autoradiogram of total lipid extract from cells labeled with ^{14}C-inositol. Cells were grown from a small inoculum in 20 ml of culture medium supplemented with 0.01 mCi of inositol-2-^{14}C and with glucose as carbon source. The cells were harvested at an absorbance (650 nm) of 13.0, and the lipids were prepared for chromatography as described in the text. The origin is in the lower left corner, and the first dimension was developed from bottom to top.*

4. The distribution of radioactivity was found to be: spot A, 10.4%; spot B, 32%; spot C, 42%; spot D, 5.4%; spot E, 10.2%.

Characterization of the deoxyglucose-labeled lipids. Three questions have been pursued concerning the identity of these lipids labeled with ^{14}C-deoxyglucose. (i) Are the lipids alkali-stable? (ii) Is the deoxyglucose incorporated intact? (iii) How well do the R_F values correspond to the alkali-stable lipids containing phosphorus and inositol observed in the absence of deoxyglucose? The ^{14}C-deoxyglucose-labeled lipid extract was subjected to the mild alkaline methanolysis procedure; less than 10% of the radioactivity became water-soluble. Chromatography of the lipid-soluble, alkali-stable fraction (Fig. 6) gives the same pattern of spots as before the methanolysis treatment except that spot D is absent, indicating that this lipid could contain acyl ester groups or is otherwise alkali-labile. Compound A is unambiguously present but has decreased about one-third relative to B and C after methanolysis of the extract. Compound 2 is poorly soluble in most lipid solvents, and quantitative difficulties have been encountered in its handling. We therefore think it likely that if A behaves like compound 2, then the low relative recovery of A is probably due to solubility difficulties rather than to alkali lability. Compounds A, B, C, and E were eluted from the chromatogram (Fig. 5) and subjected to a 4-min hydrolysis with 0.05 N H_2SO_4 at 100 C in the presence of carrier ^{12}C-deoxyglucose (*see above*). It was found (Table 1) that after this treatment the bulk of the radioactivity was water-soluble. Virtually all of this water-soluble radioactivity migrated with the R_F of authentic 2-deoxyglucose in three chromatographic systems (*see above*). The presence of glycosides of a deoxy sugar is implied, as these are well known to be labile to mild acid. Longer hydrolysis with stronger acid led to extensive destruction of the labeled material. To establish whether the alkali-stable deoxyglucose-labeled areas on the chromatogram in fact coincided with the alkali-stable phosphoinositol-containing lipids, compounds 2, 5, 6, and 7, labeled in independent experiments with ^{14}C-inositol or ^{14}C-deoxyglucose, were eluted from two-dimensional chromatograms

FIG. 4. *Autoradiogram of the lipid-soluble, alkali-stable fraction of ^{14}C-inositol-labeled lipids. Cells were grown and harvested as described in Fig. 3; lipids were prepared for chromatography as described in the text. The origin is in the lower left corner, and the first dimension was developed from bottom to top.*

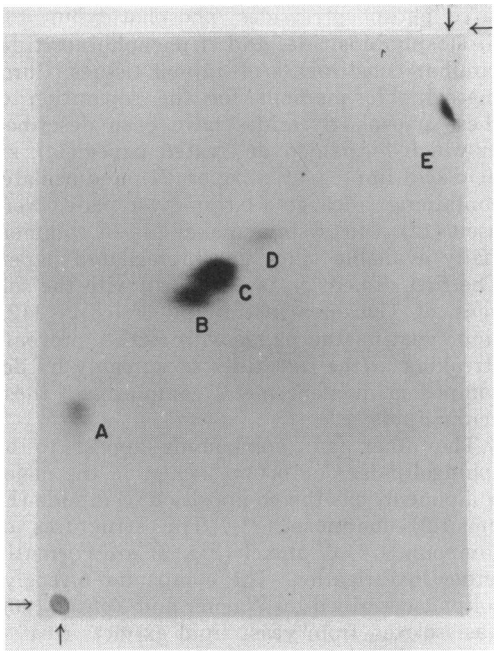

FIG. 5. *Autoradiogram of the total lipid extract from ^{14}C-deoxyglucose-labeled cells. Cells were grown in 35 ml of medium with ethanol as carbon source to an absorbance (650 nm) of 6.1. ^{14}C-deoxyglucose (0.1 mCi) was added, and the incubation was continued for 4 hr, at which time the cells were harvested and the lipid was prepared for chromatography as described in the text. The origin is in the lower left corner, and the first dimension was developed from bottom to top.*

as in Fig. 3 and 5. These were then subjected to thin-layer chromatography in parallel lanes and mixed in various combinations. This system gives somewhat higher resolution than was obtained with silicic acid paper. In this system compound B could not be resolved from compound 6, and compound C could not be resolved from compound 7. However, compound A had a slightly higher R_F than compound 2. We conclude, therefore, that the compounds labeled with deoxyglucose contain deoxyglucose per se and the two major spots (B and C) are at the same R_F values as alkali-stable phosphoinositol-containing lipids. If deoxyglucose took the place of a natural hexose, e.g., mannose or glucose, then one might expect a difference of one hydroxyl group to yield a somewhat less polar lipid with a higher R_F; evidently, these particular chromatographic systems do not have enough resolving power to achieve this resolution. On the other hand, it is quite possible that compound B is "deoxy-compound 5" and that compound C is "deoxy-compound 6" and compound A is "deoxy-compound 2"; only further chemical characterization could decide this point.

Kinetics of incorporation of glucose and deoxyglucose into lipid. The deoxyglucose labeling experiments cited above were carried out over fairly long periods. It seemed of interest to do a short-term experiment. Figure 7

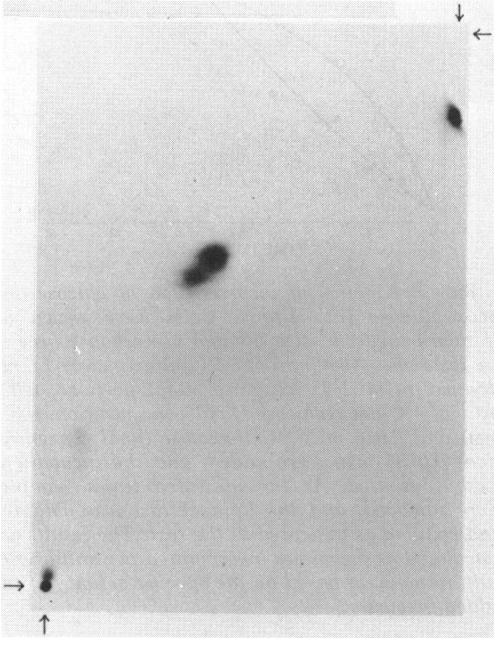

FIG. 6. *Autoradiogram of the lipid-soluble, alkali-stable fraction of ^{14}C-deoxyglucose-labeled lipids. Cells were grown and harvested as described for Fig. 5. The origin is in the lower left corner, and the first dimension was developed from bottom to top.*

TABLE 1. *Release of free deoxyglucose from labeled lipids by treatment with 0.05 N H_2SO_4*[a]

Compound	Percentage of ratioactivity			
	A	B	C	E
Starting material	100	100	100	100
$CHCl_3$-soluble after hydrolysis	14	4	1	8
H_2O-soluble after hydrolysis	94	82	77	70
Deoxyglucose in H_2O-soluble fraction after hydrolysis	97	96	98	92

[a] Compounds A, B, C, and E labeled with ^{14}C-deoxyglucose were isolated and hydrolyzed according to the procedure described in the text.

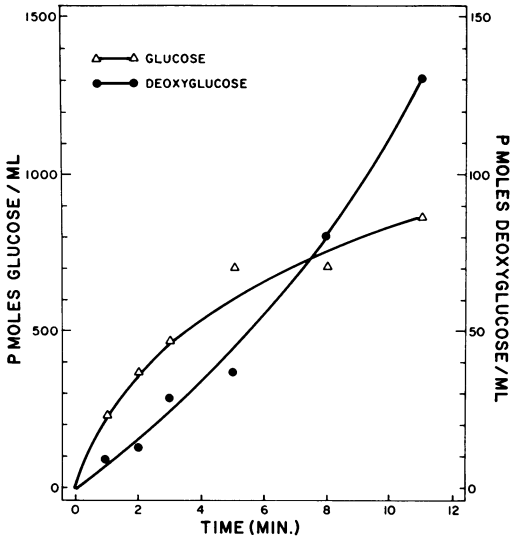

FIG. 7. *Kinetics of incorporation of glucose and deoxyglucose into lipids. Cells were grown on standard medium with ethanol as carbon source to an absorbance (650 nm) of 6.5 (approximately 1.6 mg dry weight/ml). To separate 30-ml portions, 0.025 mCi of ^{14}C-deoxyglucose (final concentration, 0.014 mM) and 0.076 mCi ^{14}C-D-glucose (final concentration, 0.0135 mM) were added, and the incubations were continued. At the indicated times, samples were removed, and the lipid extract was obtained and counted as indicated in the text. The results are calculated as picomoles incorporated per milliliter of culture medium based on the specific activity of the added precursor.*

shows the results of measuring the rate of incorporation of glucose and deoxyglucose into total lipid. In parallel, either ^{14}C-glucose or ^{14}C-deoxyglucose was added to a growing culture, and at the intervals noted samples were removed for lipid extraction. It can be seen that counts appear promptly and that over the time period measured deoxyglucose was incorporated at approximately one-tenth the rate of glucose. Thus, one might expect biological effects of deoxyglucose to appear early if these effects depended on the incorporation of deoxyglucose into lipid.

DISCUSSION

At least seven inositol-containing phospholipids can be resolved from a lipid extract of *S. cerevisiae* by use of chromatography on EDTA-treated, silicic acid-impregnated paper. These compounds account for no less than 98% of the total cellular inositol-containing lipid (W. A. Angus and R. L. Lester, *unpublished data*). Three of these compounds are the related phosphoglycerides, phosphatidylinositol, diphosphoinositide, and triphosphoinositide, familiar constituents of animal tissues. Chromatographic methods for the separation of these phosphoglycerides have been described in which formaldehyde-treated paper (14), silicic acid-impregnated paper (21), and oxalate-containing silica gel H thin-layer plates were used (12). Our system makes use of commercially available silica gel-impregnated paper. The first dimension is developed with the solvent of Gonzalez-Sastre and Folch-Pi (12). Impregnating the paper with EDTA prevents streaking of the inositides presumably by decomposing divalent metal complexes of these acidic lipids.

The other four compounds appear to be sphingolipids. Compound 2 (Fig. 3), the major component, has the composition, ceramide-(P-inositol)$_2$-mannose (23). The structures of compounds 5, 6, and 7 (Fig. 3) are currently under investigation. The compound mycoglycolipid, described by Wagner and Zofcsik (26), was isolated from yeast lipid extracts treated with 1 N KOH for 24 hr. It was shown that this treatment will cleave an inositol phosphate moiety from compound 2 resulting in a less polar lipid with a higher R_F (23). This less polar lipid might be similar to one of the three unidentified substances (compound 5, 6, or 7, Fig. 3). Although mycoglycolipid could have been artifactually produced from compound 2, it is entirely possible that it also occurs as such and may be identical to one of the three unidentified substances. Inositol-containing sphingolipids have not been shown to occur in animal tissues. As first pointed out by Wagner and Zofcsik (26), these yeast lipids are clearly related in structure to the phytoglycolipids of plants. Phytoglycolipids characterized by Carter et al. (6) share with the yeast compounds the structural feature, ceramide-P-inositol-mannose; they differ in that an additional trisaccharide moiety or more complex oligosaccharide is attached to the inositol. It is perhaps noteworthy that thus far these inositol-containing phosphosphingolipids have only been found in organisms with cell walls. Previous work (25), apparently with a chromatographic system of lower resolving power, disclosed only two inositol-containing lipid fractions from yeast. The chromatographic method described in this report should facilitate a broader survey of species for the occurrence of this class of compounds.

Early work on 2-deoxyglucose metabolism in yeast showed phosphorylation of this sugar and conversion to uridine diphosphodeoxyglucose

(2, 13), guanosine diphosphodeoxyglucose (3), and deoxygluconic acid (1). It was suggested that growth inhibition resulted from depletion of uridine nucleotide pools leading to decreased polysaccharide synthesis (13). Conversion of deoxyglucose to dideoxytrehalose (8) and the in vitro incorporation of uridine diphosphodeoxyglucose into glycogen (4) show that further transglycosylation reactions occur. Reports on the in vivo incorporation of deoxyglucose into wall material have been both negative (1, 9) and positive (5). Work with yeast protoplasts, recently reviewed by Nečas (19) indicates that a low ratio of 2-deoxyglucose to glucose gives an inhibition of the formation of the amorphous mannan-protein-containing component of the cell wall without affecting the formation of the fibrillar, glucan-containing component. At higher levels of 2-deoxyglucose, both components of the cell wall are affected. Effects at high 2-deoxyglucose concentrations may only reflect inhibition of glucose uptake and adenosine triphosphate production rather than a specific inhibition of some subsequent process. It should be noted that our experiments with deoxyglucose were carried out with cells growing in the absence of added glucose, ethanol serving as carbon source. Further evidence that deoxyglucose interferes with wall formation comes from reports that it not only inhibits growth but with several species of yeast gives cell lysis as well (13, 18) at loci concluded by Johnson (16) to be the sites of glucan synthesis.

Because it seemed reasonable that the role of the yeast glycosphingolipids might be related to cell wall structure or function, some experiments with 2-deoxyglucose were carried out. These experiments showed that deoxyglucose is readily incorporated intact into several lipids, some of which are stable to mild alkaline methanolysis and have chromatographic properties similar to some of the inositol-containing lipids. The deoxyglucose is apparently glycosidically linked, as it is released as such by mild acid treatment. Although the reasoning is somewhat circular, these data suggest that at least two of the three unidentified natural compounds (5, 6 or 7) contain sugar. Tanner (24) has presented evidence for a glycolipid intermediate in yeast mannan synthesis that is rapidly labeled by guanosine diphosphate (GDP)-mannose. We find (*unpublished data*) that the lipid labeled most rapidly in vitro with ^{14}C-GDP-mannose or in vivo with ^{14}C-mannose does not correspond in chromatographic behavior to any of the inositol-containing lipids described in this report.

It seems clear that the possibility should be examined further that some aspect of the inhibitory effects of 2-deoxyglucose on yeast stems from its incorporation into glycolipids. Future work will have to correlate in a temporal fashion the incorporation of deoxyglucose into specific lipids and the inhibition of specific biochemical reactions. Hopefully, these results with deoxyglucose will also lead to some insight concerning the role of these inositol-containing glycolipids.

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