*ISOLATION AND PARTIAL CHARACTERIZATION OF A MAJOR INOSITOL-CONTAINING LIPID IN BAKER'S YEAST, MANNOSYL-DIINOSITOL, DIPHOSPHORYL-CERAMIDE**

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Abstract.—A polar lipid containing about 20 per cent of the lipid soluble inositol has been purified from cells of *Saccharomyces cerevisiae*. This compound labeled with ^{3}H-inositol, $^{32}P_i$, or ^{14}C glucose was isolated from aqueous pyridine extracts by means of paper chromatography. Unlabeled compound was obtained by a differential solubility scheme. Analytical and degradation studies suggest that this compound can be formulated as a mannosyl-diinositol, diphosphoryl-ceramide. It can be converted by treatment with base or acid into a mannosylmonoinositol phosphoryl-ceramide, a composition which relates it to the basic structure of the phytoglycolipids.

Previous work has led to the characterization of several inositol-containing phospholipids in yeast. Among these are the glycerol containing inositides,

(Fatty acyl)$_2$-glycerol-phosphate-inositol(phosphate)$_n$.

Monophosphoinositide (n = 0), diphosphoinositide (n = 1), and triphosphoinositide (n = 2) had been characterized earlier from higher plant and animal sources as well.[1, 2] Wagner and Zofcsik[3] recently isolated and characterized a phytosphingosine-containing lipid from yeast which they termed mycoglycolipid in analogy to the phytoglycolipid from plants studied by Carter and his associates.[4] They proposed the following structure for mycoglycolipid shown here schematically:

Mannose-inositol-phosphate-ceramide.

Phytoglycolipid has, in addition, a complex oligosaccharide attached to the inositol moiety.[4]

We have obtained evidence for the presence of a major inositol-containing phospholipid in *Saccharomyces cerevisiae* related to the phytoglycolipid family but unique in that it contains two inositol-phosphate moieties. Schematically, this compound appears to be:

mannose-(inositol phosphate)$_2$-ceramide.

One of these inositol-phosphate moieties can be released by treatment with either acid or alkali.

This compound, labeled with ^{14}C, ^{3}H, and ^{32}P, has been isolated by means of paper chromatography from cells grown in the presence of ^{3}H-inositol, ^{32}P-orthophosphate, and uniformly labeled ^{14}C-glucose. Unlabeled material was purified by a fractionation scheme based on differential solubility.

Experimental.—Isolation of labeled compound: Cells were grown for a minimum of eight generations on a synthetic medium (Difco, Yeast Nitrogen Base) supplemented with 4% (w/v) glucose and 0.048 *M* Na succinate buffer, pH 5.0. To obtain ^{14}C- and ^{32}P-labeled cells, 2 mCi, ^{32}P orthophosphate (carrier free, Tracerlab), and 10 mCi, uniformly

labeled glucose (220 mCi/mmole, International Chemical and Nuclear Corp.) were added to 10 ml of culture medium. For phosphorus and tritium labeling, 2 mCi ^{32}P orthophosphate (carrier free) and 0.2 mCi 2-^{3}H-myoinositol (1.27 Ci/mmole, Tracerlab) were added per 10 ml of medium. When the culture reached an absorbancy (650 nm) of 10 to 15, growth was stopped by adding Cl_3CCOOH to a final concentration of 5% (w/v). The cells were harvested by centrifugation and washed twice with 5% Cl_3CCOOH and twice with 0.5% (w/v) KH_2PO_4. To obtain a polar lipid extract, the pellet was suspended in approximately 10 volumes of pyridine: H_2O (1:1, v/v). After 12 hr, the suspension was centrifuged and the pellet re-extracted as before with 50% pyridine for an additional 12 hr. The combined extracts were streaked on silica gel paper (Whatman SG-81) impregnated with EDTA. Ascending chromatography was performed for $2^1/_2$ hr with solvent I, $CHCl_3$:CH_3OH: 4N NH_4OH (9:7:2, v/v). The radioactive zones were located by autoradiography. A major, well-separated zone at an R_F of approximately 0.4 was eluted with solvent II, $CHCl_3$:CH_3OH:H_2O: pyridine (40:56:12:2, v/v). The major glycerophosphatides are arrayed at R_F's of 0.75 to 0.90; thus, the low R_F of the zone eluted would indicate the presence of a quiet polar lipid. The exact R_F observed with this crude extract is to some extent dependent upon the amount loaded on the chromatogram. The material thus eluted was further purified by two-dimensional chromatography on SG-81 paper; it was developed in the first dimension with solvent I. The second dimension was developed with solvent III, $CHCl_3$:CH_3OH: gl. CH_3COOH: H_2O (15:6:4: 1.6, v/v). The resulting single radioactive spot could not be further resolved by chromatography on thin-layer cellulose (Eastman Chromagram) with the solvent systems: solvent IV, *n*-butanol:pyridine:H_2O (6:4:3, v/v) and isobutyric acid: conc. NH_4OH:H_2O (66:1:33, v/v). Thus, we have purified a substance labeled in one experiment with tritium from inositol and ^{32}P and in another experiment labeled with ^{14}C and ^{32}P. The yield of purified material for 10 ml of culture medium in several experiments was from 2 to 5 μCi of each of the isotopes. This compound represented about 5 to 8% of the lipid soluble ^{32}P counts and about 15 to 20% of the lipid soluble tritium counts.

Validity of labeling technique: Since our knowledge of the nature of this compound partly depends on data obtained with labeled material, it is necessary to consider certain features of the growth medium which could influence the labeling. The overwhelming carbon source is glucose (40 g/liter) although small amounts (50 mg/liter, total) of histidine, methionine, and tryptophan are added; thus, uniform labeling of carbon compounds should be anticipated when uniformly labeled glucose is used. The succinate buffer used does not serve as a carbon source for growth of *S. cerevisiae*, particularly in the presence of glucose. The sole phosphate source is orthophosphate, and, therefore, uniform labeling of phosphorus-containing compounds can be expected in the presence of ^{32}P orthophosphate. Myoinositol is present in the basal medium at a level of 2 mg/liter; one might expect that this high level of the vitamin would lead to minimal incorporation of glucose carbon into inositol.

The validity of the above reasoning was established in part by isolating the deacylated products of several glycerophosphatides[5] from a lipid extract of the labeled cells; the isotope ratios of these easily purified and well-characterized compounds could thus be compared. The reference compounds isolated were glycerophosphorylethanolamine, glycerophosphorylserine, di(glycerophosphoryl)glycerol, glycerophosphorylinositol, glycerophosphorylcholine, and glycerophosphorylglycerol. Since the absolute specific radioactivity was not measured, the count ratio for the ethanolamine derivative was used as an internal standard from which the carbon:phosphorus ratios of the other deacylated phospholipids could be calculated. The carbon:phosphorus ratios for the other derivatives were in the order given above: (6.3); (4.35); (3.25); (7.5); (5.7). It can be seen that the values obtained deviate less than 10% from the values expected for uniform labeling. In particular, note that the phosphoinositide data suggests that only slight labeling of the inositol occurred from glucose; complete labeling would, of course, give a C/P ratio of 9.0. Therefore, we can also conclude that the carbon labeling will largely reflect noninositol carbon.

When a lipid extract from cells labeled with ^{3}H-inositol and $^{32}P_i$ was deacylated, the only water-soluble products with significant tritium labeling were the glycerophosphoinositide and its monophospho- and diphospho- derivatives.[2] Assuming that the $^{32}P/^3H$ count ratio reflects the phosphorus:inositol ratio of one in the monophosphoinositide, then we obtained phosphorus:inositol ratios of 2.16 for the diphosphoinositide and 3.29 for the triphosphoinositide compound.

Isolation of unlabeled compound: Cells were grown on a complex medium as described by Lester and Steiner[2] to an absorbance (650 nm) of 20 to 25. Growth was stopped by adding Cl_3CCOOH to a final concentration of 5%. The cells were harvested by centrifugation and the pellet was washed twice with 20 vol of 0.5% KH_2PO_4 and resuspended in 2.5 vol of 0.5% KH_2PO_4. To 250 ml of this suspension was added 150 ml of H_2O and 100 ml of 0.02 *M* K citrate-phosphate and the mixture was adjusted to pH 7.4. This suspension was treated in the Ribi cell fractionator at 35,000 psi. Lipid was extracted by a modification of the method of Lester and Steiner.[2] To 100 ml of the broken cell extract adjusted to pH 10.0 with NH_4OH was added 500 ml of 95% ethanol-diethyl ether (3:1, v/v). After standing overnight, 300 ml of petroleum ether and 600 ml of 1 *M* KCl were added. The mixture was shaken vigorously and the upper phase removed. The lower phase was then re-extracted with 370 ml of the artificial upper phase of H_2O-ethanol-diethyl ether-petroleum ether-1 *M* KCl (1:3.75:1.25:3:6). The combined upper phases were taken to dryness *in vacuo* and redissolved with artificial upper phase equal to 1/40 of the original volume. Lipid was precipitated by the addition of one vol of petroleum ether and 2 vol of 95% ethanol. Neutral lipid and a major fraction of the glycerol phospholipids remained in the supernatant fraction. The precipitate was dried, redissolved in the artificial upper phase, and a second precipitation was carried out. The resulting precipitate was then dried and washed with CH_2Cl_2—CH_3OH—H_2O (12:6:1, v/v). Following removal of the wash, the precipitate was dried, redissolved in 30% pyridine in H_2O, and reprecipitated by adding 2 vol of 95% ethanol. An aqueous suspension of the precipitate was adjusted to pH 6.0 with dilute formic acid and mixed with an equal volume of isopropanol-diethyl ether (1:1, v/v). The upper phase was removed and the lower phase was re-extracted six times with a volume of artificial upper phase equal to the amount removed. The lower phase was reduced in volume to one-third, pyridine was added to a final concentration of 30%, and the final product was precipitated with 2 vol of 95% ethanol. About 100 mg of compound were obtained from 48 liters of culture medium.

Analytical Methods.—Long-chain base was measured by the method of Lauter and Trams[6] after hydrolysis and extraction according to the method of Sweeley and Moscatelli[7] except that hydrolysis was carried out in stoppered tubes for 12 hr at 75°. Hexose was estimated after sulfuric acid hydrolysis[8] by the phenol-sulfuric acid[9] method with mannose as standard. Hexuronic acid was measured by the carbazole[10] method with glucuronic acid as standard. Sugars were detected on chromatograms with the reagent of Schweiger.[11] Phosphorus was estimated by the Bartlett[12] method.

Results.—Studies with the labeled compound. The availability of compound labeled with ^{3}H, ^{14}C, and ^{32}P as described in *Experimental* allows us to obtain some useful compositional data. With the count ratio of GPE[17] (prepared from the same batch of cells) as internal standard, the carbon (noninositol) to phosphorus ratio of the ^{32}P, ^{14}C compound was found to be 24. Similarly, with glycerophosphophorylinositol used as internal standard, the inositol:phosphorus ratio was found to be 1.0 for the ^{3}H-, ^{32}P-labeled compound. Hydrolysis of the ^{32}P-, ^{14}C-labeled compound with 2 *N* HCl (aqueous) for 150 minutes at 100° gave rise to two principal water-soluble radioactive products; these were mannose and an inositol monophosphate. Chromatography of the water-soluble fraction on thin-layer cellulose with solvent IV revealed a spot (after

autoradiography) with only ^{14}C at an R_F of 0.46; this was chromatographed in the presence of carrier ^{12}C-mannose and the radioactive spot revealed by autoradiography and the mannose detected chemically[11] were superimposable. Similar hydrolysis of the ^{3}H, ^{32}P compound gave rise to one major water-soluble ^{32}P-containing product with a ^{3}H:^{32}P ratio expected for an inositol monophosphate.

Partial hydrolysis with acid or base provided evidence for the existence of two or more phosphorus and inositol moieties per molecule. After partial acid hydrolysis (Table 1) the organic soluble products were chromatographed on SG

TABLE 1. *Partial hydrolysis of the lipid.*

	Partial Acid Hydrolysis			Partial Base Hydrolysis		
	^{32}P (cpm)	^{14}C (cpm)	C/P	^{32}P (cpm)	^{14}C (cpm)	C/P
Starting material	20,206	58,844	24	7,260	45,590	24
H_2O soluble after hydrolysis	4,051	1,221		785	326	
Organic-soluble fraction after hydrolysis	13,901	49,624		4,970	36,210	
Eluates of organic-soluble fraction after chromatography						
At original R_F	10,214	32,239	25	4,318	27,094	24
High R_F product	1,244	7,089	47	651	8,356	49
Near front	0	2,462		0	760	

Acid hydrolysis: A sample of the ^{14}C-, ^{32}P-labeled purified compound (approx. 10 cpm ^{32}P/pmole) dissolved in solvent II was taken to dryness in a stream of N_2. Hydrolysis was carried out at 100° for 30 min after the addition of 1.5 ml of 9:1 CH_3OH:H_2O which was 0.05 N in HCl. After the successive additions of 1 ml H_2O and 2 ml $CHCl_3$, the tube was shaken vigorously and centrifuged. The aqueous layer was removed, and the $CHCl_3$ layer was washed once with 1 ml of H_2O. The aqueous extracts were combined and counted. The remaining contents were evaporated to dryness in a stream of N_2 and redissolved in 1 ml of solvent II; this constitutes the organic soluble fraction. Aliquots (*ca.* 1/3) of this fraction were chromatographed on SG 81 paper developed with solvent I. After autoradiography the spots were cut out and eluted with solvent II prior to counting. The same results were obtained after two-dimensional chromatography, solvent I followed by solvent III. The first and second dimension R_F's were, respectively, starting compound (0.4, .1), "high R_F product" (0.7, 0.6), "near front" (0.85–1.0, 1.0).

Alkaline hydrolysis: A 0.2-ml sample dissolved in solvent II was mixed with 0.8 ml CH_3OH followed with 3 ml 1.33 N KOH (aqueous). After 45 min at 37°, the mixture was neutralized with concentrated formic acid and extracted with 2 ml $CHCl_3$. The aqueous phase was removed from the tube and extracted twice more with 2 ml $CHCl_3$. This aqueous phase constitutes the H_2O-soluble fraction. The $CHCl_3$ extracts were combined in the original tube, washed once with 1 ml H_2O, evaporated, and redissolved in solvent II; this constitutes the organic soluble fraction which was analyzed as described above in the acid hydrolysis experiment.

81 paper, two dimensionally, with solvent I followed by solvent III. A significant fraction of the phosphorus and carbon counts that disappeared from the starting material appears, at a higher R_f ("high R_F product," Table 1) in both dimensions, as a single well-defined spot. The carbon:phosphorus ratio of this hydrolysis product, calculated from its count ratio as described above, was close to twice that of the starting compound. In addition, near the front there was considerable ^{14}C radioactivity. An entirely parallel experiment carried out with compound labeled with ^{32}P and ^{3}H gave a ^{32}P-labeled product at the identical high R_F's observed for the ^{14}C- and ^{32}P-labeled product. The ^{32}P:^{3}H ratio indicated that this compound had a mole ratio of one phosphorus:one inositol. This degradation product with the higher C:P ratio, labeled with ^{32}P and ^{14}C, was eluted from a chromatogram of a partial acid hydrolysate and subjected to 2

N HCl hydrolysis; mannose was identified as the principal ^{14}C-labeled water-soluble product.

The high R_F product can also be obtained in significant yield by a short treatment with 1 *N* KOH at 37° (Table 1). It should be noted that less than 2 per cent of the starting carbon appears at the front after chromatography of the organic soluble fraction. Since this is the region where fatty acids migrate, we can conclude that fatty acid esters are not a significant structural feature of this compound, nor is there adventitious contamination with such material. Treatment of the dry ^{14}C, ^{32}P-labeled compound with aqueous 1 *N* KOH for 17 hours converted 58 per cent of the phosphorus to a water-soluble form. Chromatography of the organic-soluble fraction revealed that only 8 per cent of the ^{14}C was in the original compound, 66 per cent of the ^{14}C was in the high R_F product, and the rest of the ^{14}C was at the front. Identical treatment (17 hr, 1 *N* KOH) of the ^{3}H-, ^{32}P-labeled compound gave precisely half the ^{3}H and ^{32}P in the water-soluble fraction. Ion-exchange chromatography[2] of the water-soluble fraction showed that virtually all the ^{3}H and ^{32}P counts migrated with unlabeled inositol-1-phosphate; treatment of this water-soluble fraction with alkaline phosphatase[2] showed complete conversion of the counts to ^{32}P-inorganic phosphate and ^{3}H-inositol.

The simplest interpretation of the above findings is that the original compound contains two inositol-phosphate moieties. Acid or base treatment results in the release of half of the inositol-phosphate moieties as inositol monophosphate plus a mannose-containing phospholipid with twice the carbon (noninositol):phosphorus ratio. The similarity of the partial hydrolysis lipid product to the compound isolated by Wagner and Zofcsik[3] was apparent; it should be noted that these workers isolated their compound after treatment of the crude lipid extract with aqueous alkali for 24 hours.[13] The ^{14}C-, ^{32}P-labeled compound was hydrolyzed and partitioned according to the method of Sweeley and Moscatelli[7] appropriate for sphingolipids. It can be seen from Table 2 that the distribution of carbon fits a compound composed of a long-chain fatty acid (average carbon length = 24),[3] a long-chain base (average carbon length = 19),[3] and one hexose.

TABLE 2. *Distribution of carbon and phosphorus after hydrolysis in 2 N HCl in methanol.*

Fraction	Per Cent ^{14}C		Per Cent ^{32}P	
	Expected*	Found	Expected*	Found
Water-soluble	12	14	100	100
Fatty acid ester	49	51	0	0
Long-chain base	39	35	0	0

A sample of ^{32}P-, ^{14}C-labeled compound was hydrolyzed as indicated in *Experimental* and fractionated according to Sweeley and Moscatelli,[7] fatty acid esters in the petroleum ether extract, and long-chain base(s) in the diethyl ether extract.

* The expected percentages are calculated assuming a C_{24} fatty acid, a C_{19} long-chain base, one hexose, and no ^{14}C in the inositol moieties.

Studies on the unlabeled compounds: Macroscopic amounts of this compound were prepared so that the above key stoichiometric findings could be confirmed by independent chemical methods. An isolation procedure was developed (*Experimental*) which avoided harsh conditions. The product was chromatographically indistinguishable from the radioactive material isolated by means of

TABLE 3. *Compositional analysis of the mannosyl-diinositol, diphosphoryl-ceramide.*

	(%) Dry Weight		
	Calculated*	Found	Mole ratio
Phosphorus	4.13	3.98	2.00
Hexose	11.99	12.2	1.05
Long-chain base	22.05	21.5	0.99

See *Experimental* for analytical methods.

* Based on an empirical formula of $C_{71}H_{129}O_{26}N_3P_2$ for the dipyridinium salt, assuming the *average* fatty acid to be a C_{24} hydroxy fatty acid[3] and the long-chain base to be on the *average* a "C_{19}" phytosphingosine.[3]

paper chromatography; only trace impurities were evident. The final product was analyzed for content of phosphorus, hexose, and long-chain base. These values (Table 3) confirm the expected content and the stoichiometry of two phosphorus to one long-chain base to one hexose. A test for glucuronic acid was negative. The exact nature of the fatty acids and the long chain base(s) remain to be described; however, it is anticipated that these would be quite similar to those reported by Wagner and Zofcsik[3] for the monoinositolphospholipid. It is hoped that the detailed degradation studies underway will delineate the precise structure, particularly the linkage of the base labile inositol-phosphate moiety.

Discussion.—The relative stability of sphingomyelin to base was recognized by Thudichum.[14] Treatment of lipid extracts with 1 *N* KOH at 37° for 24 hours to hydrolyze acyl ester containing lipids was introduced by Schmidt *et al.*[15] to serve as the basis of a widely used analytical method for sphingomyelin. It is clear that this procedure is not applicable to the complex sphingolipids discussed in this paper. Carter and Kisic[16] have recently pointed out the need for methods that obviate alkaline hydrolysis in the isolation of compounds of the phytoglycolipid family and report progress in developing countercurrent distribution methods to this end.

Thus, it would appear useful to examine higher plants for the possible presence of diinositolphosphorylceramide-containing compounds; likewise, it would seem worthwhile to examine yeast lipids carefully for the presence of oligosaccharide-containing sphingolipids.

The compound we have isolated is quite amphipathic; only half its mass is nonpolar. We hope that study of the metabolism and function of this compound proves to be of interest.

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[17] GPE = glycerophosphorylethanolamine.