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Biosynthesis of Phosphoinositol-Containing Sphingolipids from Phosphatidylinositol by a Membrane Preparation from *Saccharomyces cerevisiae*

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Incubation of membranes prepared from *Saccharomyces cerevisiae* with [^{32}P]phosphatidyl[^{3}H]inositol resulted in the transfer of both labels to two products which were characterized as two species of inositolphosphoceramide, differing in the ceramide portion of the molecule. The products were characterized on the basis of stability in mild alkali, mobility on silica gel-impregnated paper, chromatography on silicic acid columns, and release of inositol phosphate upon base hydrolysis. The reaction did not require the addition of metals, nor was it inhibited by ethylenediaminetetraacetic acid. The detergents Triton X-100 and Tween 20 provided little, if any, stimulation. At relatively high concentrations of phosphatidylinositol (1 to 4 mM), the in vitro rate was about 20% of the in vivo rate. Although ceramide was a logical substrate, the reaction could not be greatly stimulated by the addition of ceramides containing mono- and dihydroxy fatty acids. In addition, incubation of yeast membranes with [^{32}P]phosphatidylinositol gave rise to a product that was chromatographically indistinguishable from the major yeast phosphosphingolipid, mannose-(inositol-P)$_2$ ceramide.

Phosphosphingolipids containing inositol (in this paper, "inositol" will refer to the isomer "*myo*-inositol") have been reported in a number of plants (6, 9, 10) and in several fungi, including *Saccharomyces cerevisiae* (16, 19), *Neurospora crassa* (12), *Candida utilis* (21), *Aspergillus niger* (5, 7), and *Agaricus bisporus* (4).

In *S. cerevisiae*, three classes of these sphingolipids are known. The inositolphosphoceramides (IPCs) contain a single inositol phosphate (16); the mannosylinositolphosphoceramides (MIPCs) also contain a single inositol phosphate, but with a mannose unit attached to the inositol (16). The major sphingolipid, with composition, mannose (inositol-P)$_2$-ceramide [M-(IP)$_2$C], contains two inositol phosphates, with a mannose unit attached to one of the inositols (19). Diversity in the type of long-chain base and in the degree of hydroxylation and chain length of the fatty acid gives rise to several members of these three classes (16).

It was suggested by Angus and Lester (1), based on in vivo labeling experiments with *S. cerevisiae*, that phosphatidylinositol might be the source of the phosphoinositol portion of the phosphoinositol sphingolipids. This idea was supported by Hackett and Brennan (7), who showed that a soluble fraction prepared from *A. niger* catalyzed the transfer of phosphoinositol from phosphatidylinositol to IPC. This product was characterized by its stability in mild alkali and its mobility on thin-layer chromatography.

This report describes the in vitro biosynthesis of two types of IPCs by a membrane fraction of *S. cerevisiae* and provides a rigorous product characterization by high-performance column chromatography and degradative methods. The reaction conditions are optimized so that the rates of the in vitro reactions approach the in vivo rates.

In vitro synthesis of M(IP)$_2$C is also reported for the first time.

MATERIALS AND METHODS

Materials. The strain of yeast used to prepare membranes, *S. cerevisiae* MC13, was provided by Susan Henry (Albert Einstein College of Medicine). ^{32}P$_i$ and [2-^{3}H]inositol were purchased from New England Nuclear Corp. Chemical sources were as follows: Celite 545, Fisher Scientific Co.; yeast phosphatidylinositol, Koch-Light; Triton X-100 and soybean phosphatidylinositol, Sigma Chemical Co.; Porasil A60 (37 to 75 μm), Waters Associates; silica gel-impregnated paper SG-81, Whatman; and yeast extract and peptone, Difco Laboratories. Glycerophospho[^{14}C]inositol was prepared by deacylation of phosphatidylinositol obtained from a yeast culture grown with [*U*-^{14}C]inositol.

Preparation of yeast membranes. Yeast membranes were prepared from cells grown on a peptone-yeast extract medium (3) supplemented with inositol (20 mg/liter). A 1.0-liter culture was harvested at an absorbancy at 650 nm (1 cm) of 18 to 20 (ca. 4 mg/ml)

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by centrifugation for 10 min at 1,500 × *g* and washed three times with cold distilled water. The resulting pellet was suspended in water so that the total volume of the cell suspension was 50 ml (80 to 100 mg [dry weight] per ml). After 12.5 ml of cell suspension was combined with 12.5 ml of 0.4 M sucrose–0.1 M potassium phosphate (pH 7.2)–25 ml (dry volume) of acid-washed glass beads (0.45- to 0.50-mm diameter), the cells were broken by shaking in a Braun homogenizer cooled with CO_2. The suspension was shaken four times for 15 s, with 15-s intervals for cooling. The supernatant was decanted, and the beads were washed three times with 20-ml portions of 0.2 M sucrose–0.05 M potassium phosphate, pH 7.2. All of the supernatants and washes were combined and centrifuged at 2,000 × *g* for 10 min to remove unbroken cells and other debris. The membranes were then collected by centrifugation at 70,000 × *g* for 1 h. The membrane pellet was washed two times by homogenization in a Teflon pestle homogenizer with 60 ml of 0.2 M sucrose–0.05 M potassium phosphate, pH 7.2. The final pellet was homogenized in a small volume of the same buffer to give a suspension of about 30 to 50 mg of protein per ml, as measured by the modified biuret procedure (11). A typical preparation yielded 9 to 12 ml of membrane suspension, which was stable for several months at −20°C.

Preparation of [^{32}P]phosphatidylinositol. [^{32}P]phosphatidylinositol was prepared in vivo by growing yeast with $^{32}P_i$ and then isolating the labeled product by preparative thin-layer chromatography on thick silica gel plates (PLQ-1000, Quantum Industries). A 20-ml culture of yeast was grown on low-phosphate (2.2 mM) medium containing 20 to 25 mCi of carrier-free $^{32}P_i$, 1% peptone (Difco Laboratories), 1% yeast extract, 4% glucose, and 0.048 M sodium succinate, pH 5. After growth for 2 days at 30°C in a rotary shaker (the cells exhibited a sharply reduced growth rate), 1.0 ml of 100% trichloroacetic acid was added, and the culture was placed in an ice bath for 30 min. After centrifugation, the pellet was washed twice with 20-ml portions of distilled water. Lipids were extracted from the washed pellet with ethanol-water-diethyl ether-pyridine (15:15:5:1, vol/vol) (3). The lipid extract was applied to the loading zone of the preparative plate and developed with the solvent $CHCl_3$–CH_3OH–4.2 N NH_4OH (9:7:2, vol/vol). The region of the plate containing the [^{32}P]phosphatidylinositol (localized by autoradiography) was removed and eluted with $CHCl_3$–CH_3OH-water (16:16:5, vol/vol). The partially purified product was usually rechromatographed with the same system. By this procedure, a preparation was obtained that was at least 90 to 95% [^{32}P]phosphatidylinositol. The impurities present were other ^{32}P-labeled phospholipids, including variable amounts (<0.2%) of ^{32}P-labeled alkali-stable phospholipid with chromatographic properties similar to those of the monophosphoinositol-containing sphingolipids.

Preparation of phosphatidyl[^{3}H]inositol. A 10-ml culture of yeast was grown on yeast nitrogen base supplemented with 4% glucose, 0.048 M sodium succinate, 240 mg of lysine dihydrochloride per liter, and 1.0 mCi of [^{3}H]inositol (final inositol concentration, 4.9 mg/liter). The culture was grown at 30°C with shaking. The harvest of the cells and isolation of the phosphatidyl[^{3}H]inositol was carried out exactly as for [^{32}P]phosphatidylinositol.

Assay for the transfer of label from [^{32}P]phosphatidyl[^{3}H]inositol to alkali-stable phospholipids. Incubations were terminated by the addition of 6.4 volumes of $CHCl_3$-CH_3OH (1:1, vol/vol), and the insoluble pellet was removed by centrifugation. To 0.10 ml of the supernatant was added 0.02 ml of 0.6 N NaOH in methanol, and the mixture was placed in a 30°C bath for 30 min. After neutralization with 0.020 ml of 1.0 N acetic acid, the entire mixture was applied to EDTA-treated SG-81 paper (18) and chromatographed in $CHCl_3$–CH_3OH–4.2 N NH_4OH (9:7:2, vol/vol). ^{32}P-labeled components of this mixture were localized by autoradiography with Kodak No Screen X-ray film. The chromatograms were cut up, and the radioactivity was measured by scintillation counting with the fluid previously described (2). An example of such an assay is shown in Fig. 1.

Isolation of a sphingolipid fraction. The procedure described here was developed to produce a sphingolipid fraction, free of nonlipids, appropriate for liquid chromatography after a sample had been treated with mild alkaline methanol. The sample was taken to dryness under a stream of N_2 and dissolved in 1 ml of ethanol-water-diethyl ether-pyridine (15:15:5:1, vol/vol). After 1 ml of 0.2 N KOH in CH_3OH was added, the mixture was allowed to stand at room temperature for 1 h, and then 0.2 ml of 1.0 N acetic acid and 1.16 ml of water were added. This mixture was then applied to a column containing 0.2 g of Celite 545 and eluted three times with 2-ml portions of solvent with the same final composition as described above. This had been found to wash polar constituents from the column. To the column was added 1 ml of CH_3OH and 4 ml of $CHCl_3$-CH_3OH-water (16:16:5 [vol/vol], adjusted to pH 9.5 with NH_4OH), which eluted the sphingolipids from the column; the eluates were combined and constituted the sphingolipid fraction.

Preparation of unlabeled sphingolipid standard. The mixture of unlabeled sphingolipids used as standards was prepared from commercial bakers' yeast as described previously (16).

Column chromatography of sphingolipids. Chromatography was carried out on a column (0.32 by 600 cm) packed with base-treated (16) silicic acid (Porasil A60), equilibrated with the initial solvent, and maintained at 55°C. Two elution procedures were employed.

In procedure 1, a nonlinear gradient was provided by a two-pump solvent delivery system (model 6000A, Waters Associates) controlled by a model 600 programmer (Waters Associates) set to gradient no. 7. Solvent A was $CHCl_3$-CH_3OH-water (65:40:3, vol/vol), and solvent B was $CHCl_3$-CH_3OH-water (16:16:5, vol/vol). The initial solvent composition was 10% solvent B, and the final solvent composition was 90% solvent B. The total time for the gradient was 60 min. The percentage of solvent B at time t is given by $\%B = 10 + (90 - 10)\,(t/tf)^2$, where tf is the total gradient time. The flow rate was 2.0 ml/min.

In procedure 2, the conditions were as above except for the following differences. The injected sample was followed by pumping solvent A for 5 min. A nonlinear gradient (program no. 4) was then begun with 0%

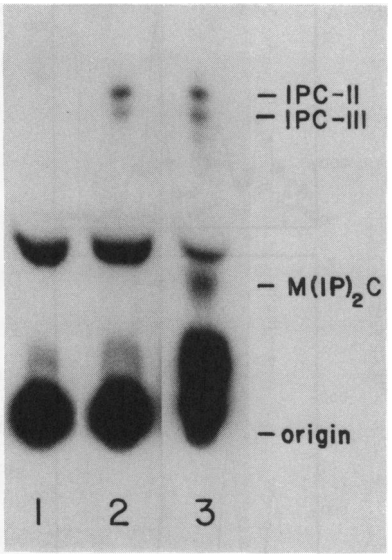


FIG. 1. *Autoradiograms prepared from chromatograms of incubation products and yeast lipids. Mixtures containing 0.44 mM [^{32}P]phosphatidyl[^{3}H]inositol (2.9 × 10^{6} cpm of ^{32}P and 4.0 × 10^{4} cpm of ^{3}H), 50 mM Tris-hydrochloride, pH 7, 5 mM $MnCl_2$, 7.5 mM $MgCl_2$, 0.2% Triton X-100, and yeast membranes (6.6 mg of protein) in a total volume of 0.5 ml were incubated at 30°C for 2 h. After the addition of 3.2 ml of $CHCl_3$-CH_3OH (1:1, vol/vol) and centrifugation, 0.05 ml of the supernatant was deacylated by the addition of 0.01 ml of 0.6 N NaOH in CH_3OH and heating at 30°C for 30 min. The mixture was neutralized with 0.01 ml of acetic acid, and the entire mixture was applied to silica gel-impregnated paper and chromatographed with the solvent $CHCl_3$–CH_3OH–4.2 N NH_4OH (9:7:2, vol/vol). (1) Result obtained when the yeast membranes were heat-inactivated before addition to the incubation mixture; (2) result obtained with active membranes; and (3) pattern obtained when a ^{32}P-labeled lipid extract (3) was treated with mild methanolic alkali. The identities of some of the ^{32}P-labeled spots appearing in (3) are indicated.*

solvent B. The percentage of solvent B at time t after the start of the gradient is given by $\%B = 75(t/60)^{1/3}$. After 50 min of the gradient, 100% solvent B was pumped for another 50 min. All solvents were pumped at 2.0 ml/min. Solvent A was $CHCl_3$-CH_3OH-concentrated NH_4OH (65:29:6, vol/vol); solvent B was $CHCl_3$-CH_3OH-concentrated NH_4OH-water (43:43:6:8, vol/vol).

RESULTS

Incorporation of ^{32}P and ^{3}H from [^{32}P]-phosphatidyl[^{3}H]inositol into alkali-stable products. Incubation of [^{32}P]phosphatidyl-[^{3}H]inositol with yeast membranes resulted in the transfer of both ^{32}P and ^{3}H to products which were stable to mild alkaline methanolysis and which had the same chromatographic mobilities on silica gel-impregnated paper as did the alkali-stable lipids prepared from a yeast lipid extract labeled in vivo. Figure 1 shows an autoradiogram of mild alkali-stable products obtained from such an incubation. Lane 2 shows two major alkali-stable spots labeled with ^{32}P that were not present when the membranes were heat inactivated before addition to the incubation mixture (lane 1). Lipids with the mobility of these two products were found in the chromatographic pattern obtained when a lipid extract of cells grown with $^{32}P_i$ were treated in a manner identical to that of the incubation mixtures (lane 3). The identities of the alkali-stable yeast lipids have been determined previously (16) and are shown in Fig. 1. The mobility of phosphatidylinositol in this system was just above the IPCs; this lipid was present only in trace amounts in samples subjected to alkaline methanolysis.

Both the ^{32}P and the ^{3}H of [^{32}P]phosphatidyl[^{3}H]inositol were transferred to both of the alkali-stable products (Table 1) in the same ratio as that in the precursor. This is consistent with the notion of the transfer of the intact phosphoinositol group from phosphatidylinositol to a lipid acceptor.

Further characterization of reaction products. In addition to their stability to mild alkali and their mobilities on silica gel-impregnated paper (Fig. 1), further evidence indicates that the two major products of the enzymatic transfer of the phosphoinositol moiety to endogenous lipid acceptors are two species of IPC, designated IPC-II and IPC-III. IPC-II contains phytosphingosine and a monohydroxy C_{26} fatty acid; IPC-III contains phytosphingosine and a dihydroxy C_{26} fatty acid (16). It was felt that

TABLE 1. *Conversion of [^{32}P]phosphatidyl[^{3}H]-inositol to alkali-stable products*[a]

Precursor/product	cpm		^{32}P/^{3}H
	^{32}P	^{3}H	
[^{32}P]phosphatidyl[^{3}H]-inositol	228,986	136,777	1.67
Alkali-stable products cut from paper chromatogram			
Upper spot	1,076	708	1.52
Lower spot	756	455	1.66

[a] Yeast membranes (0.59 mg of protein and 19 nmol of phosphatidylinositol) were incubated with [^{32}P]-phosphatidyl[^{3}H]inositol (0.98 nmol) in 50 mM Tris-hydrochloride, pH 7, containing 5 mM $MnCl_2$ and 0.05% Tween 20 (total volume, 0.05 ml). After 2 h, the reactions were terminated, and the incorporation of both labels into alkali-stable products was measured as described in the text.

comparison of the R_f's with one system, as done here and in the earlier work by Hackett and Brennan (7), ought to be substantiated further in light of the work of Smith and Lester (16), which showed the occurrence of closely related (and, hence, difficult-to-separate) inositol-containing phosphosphingolipids in yeast.

(i) Chromatography on silica gel columns. The ^3H-labeled products from an incubation of yeast membranes with phosphatidyl[^3H]inositol comigrated with authentic yeast IPC-II and IPC-III on a silica gel column. Figure 2 shows the sphingolipid region of the elution profile from a representative column. The incubation products (Fig. 2B), detected by measuring the ^3H, corresponded exactly to the authentic IPC-II and IPC-III which were added as internal standards and which were detected by a moving wire-flame ionization detector that detects all nonvolatile carbon compounds. An incubation mixture containing inactivated membranes showed only a trace of radioactivity in the sphingolipid region (Fig. 2A).

Mild alkali-stable products resulting from the incubation of [^{32}P]phosphatidylinositol with yeast membranes were also chromatographed on a silica gel column with internally added sphingolipid standards, employing elution conditions of somewhat greater resolving power (Fig. 3). Figure 3A shows the internal standards observed with the moving wire detector, and Fig. 3B shows the ^{32}P-labeled products from the control and the experimental incubation mixtures. Both control and experimental samples showed radioactivity in the region of phosphatidylinositol; the control also showed ^{32}P in the region just slightly more retarded than IPC-III. These represented about 0.01% of the starting radioactivity. All of the radioactivity in these peaks became water soluble after deacylation, suggesting that the first peak was indeed phosphatidylinositol and that the second was probably lysophosphatidylinositol. Evidently, despite two rounds of deacylation, these small amounts persisted. In the experimental sample, the second radioactive peak was perfectly superimposable on the IPC-II standard, and the third peak, after deacylation, was perfectly superimposable on the IPC-III standard. In the latter case, deacylation removed about the same amount of radioactivity as that observed in the control in this region.

Thus, both ^3H- and ^{32}P-labeled phosphatylinositol yielded two mild alkali-stable compounds with chromatographic behavior identical to those of IPC-II and IPC-III.

(ii) Base hydrolysis of reaction products. Hydrolysis by aqueous 1.0 N KOH at 37°C for

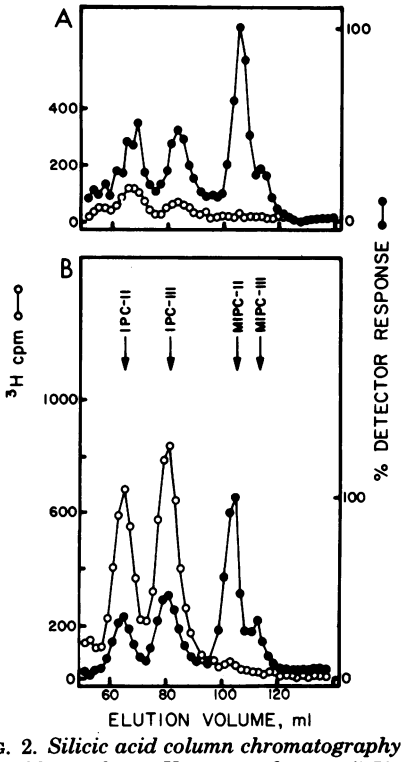


FIG. 2. *Silicic acid column chromatography of alkali-stable products. Yeast membranes (0.78 mg of protein) were incubated at 30°C for 2 h with 0.26 mM [^3H]phosphatidylinositol (1.5 × 10^6 cpm), 50 mM Tris-hydrochloride (pH 7), and 0.05% Tween 20 in a total volume of 0.10 ml. After termination of the reaction by the addition of 0.64 ml of $CHCl_3$-CH_3OH (1:1, vol/vol) and centrifugation, a sphingolipid fraction was isolated by a modification of the procedure given in the text. The deacylation was carried out in the same solvent, but for only 30 min. The reaction was not neutralized, but 2.0 ml of absolute ethanol was added to dilute the base. This mixture was then applied to a column containing 0.4 ml (dry volume) of Na^+ Chelex 100 (100 to 200 mesh, Bio-Rad Laboratories) and 1.0 ml (dry volume) of HiFlosil (60 to 200 mesh, Applied Science Laboratories). The column was eluted with absolute ethanol (3.0 ml), followed by 4 ml of $CHCl_3$-CH_3OH-water (16:16:5, vol/vol) adjusted to pH 9.5 with NH_4OH. The last milliliter of eluate contained the sphingolipid, free of the bulk of nonlipids. This fraction was taken to dryness under a stream of N_2 and redissolved in 0.25 ml of $CHCl_3$-CH_3OH-water (16:16:5, vol/vol) containing a mixture of unlabeled sphingolipids prepared from commercial bakers' yeast (3.0 mg/ml) (16). A sample (0.1 ml) of this mixture of ^3H-labeled reaction products and unlabeled standard sphingolipids was chromatographed according to procedure 1 in the text. Fractions were collected and taken to dryness under a stream of hot air, and the ^3H was measured in a scintillation counter. The unlabeled yeast sphingolipid standards were detected with a moving wire carbon detector (LCM2, Pye Unicam).*

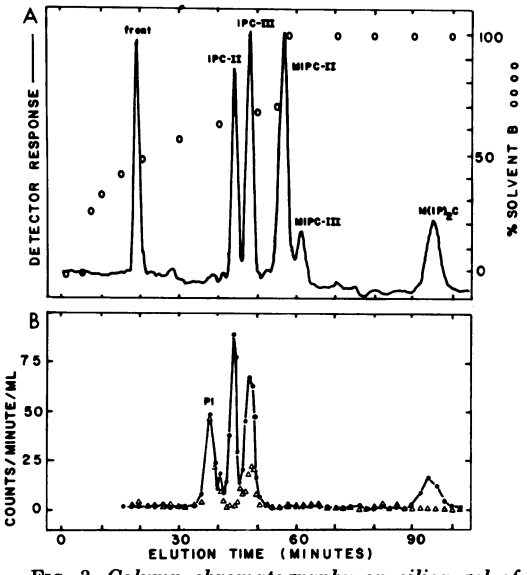


FIG. 3. *Column chromatography on silica gel of alkali-stable phospholipids resulting from incubation of yeast membranes with [^{32}P]phosphatidylinositol. The following components were incubated for 60 min at 30°C in a final volume of 0.1 ml: 50 mM Tris-hydrochloride (pH 7), 5 mM $MnCl_2$, 0.1% Triton X-100, 0.644 mg of membrane protein, and 1.22 mM [^{32}P]phosphatidylinositol (5.7 cpm/pmol). The reaction was terminated by addition of 0.64 ml of $CHCl_3$-CH_3OH (1:1, vol/vol). After centrifugation, the pellet was extracted three times with 0.50-ml volumes of $CHCl_3$-CH_3OH-water (16:16:5, vol/vol), and the extracts were pooled and combined with the original supernatant. The sphingolipid fraction was deacylated as described in the text, and the resulting lipid fraction was again put through the same deacylation procedure. The resulting alkali-stable lipid mixture was taken to dryness and dissolved in 0.50 ml of $CHCl_3$-CH_3OH-concentrated NH_4OH-water (16:16:1.03:3.97, vol/vol) containing 1.5 mg of the standard sphingolipid mixture. A 0.45-ml sample was chromatographed according to procedure 2 in the text. (A) Moving wire-flame ionization detection of nonradioactive standards. (B) Symbols: ●, experimental; △, control consisting of reaction terminated with $CHCl_3$-CH_3OH (1:1, vol/vol) before addition of membranes. ^{32}P was detected by Cerenkov counting.*

15 h is known to convert the phosphorus of the yeast sphingolipid IPC to a water-soluble organic form which appears to be an inositol monophosphate (16). Base-catalyzed hydrolysis of glycerophosphoinositol also results in the formation of inositol monophosphate and glycerol, as well as glyceromonophosphates and free inositol (1, 8).

To ^{32}P-labeled alkali-stable products isolated by preparative thin-layer chromatography was added glycerophospho[^{14}C]inositol as a standard, and the mixture was hydrolyzed with base. After neutralization, the products of the base hydrolysis were chromatographed in a two-dimensional system that separates the inositol phosphates from the isomeric glyceromonophosphates (1). Autoradiography revealed two spots which accounted for 100% of the ^{14}C and 79% of the ^{32}P. The upper spot, with the R_f expected for inositol, contained 56% of the ^{14}C and negligible amounts of ^{32}P (Table 2). The lower spot, with the R_f expected for inositol phosphate, contained both ^{32}P and ^{14}C in a constant ratio across the spot. From this result, it can be concluded that the ^{32}P-labeled alkali-stable products contain phosphate linked to inositol, since base hydrolysis yielded a product that moved with an R_f identical to that of the [^{14}C]inositol phosphate generated by hydrolysis of glycerophosphoinositol.

Properties of the sphingolipid-synthesizing system. Crude yeast membranes were found to be capable of synthesizing sphingolipids when incubated with only [^{32}P]phosphatidylinositol and Tris buffer. There does not appear to be a requirements for the three divalent metals tested, $MnCl_2$, $MgCl_2$, and $CaCl_2$. In fact, each of these metals at 5 mM actually inhibited the reaction slightly. The inclusion of 5 mM EDTA gave the same amount of product as the basal system, providing additional evidence for the lack of a metal requirement. The two detergents tested, 0.02% Triton X-100 and 0.05% Tween 20, gave a slight stimulation. Two varieties of ceramide isolated from yeast, one con-

TABLE 2. *Identification of the product of base hydrolysis of putative [^{32}P]IPC as inositol [^{32}P]-phosphate*[a]

Product	cpm	
	^{32}P	^{14}C
Free inositol zone	85	4,714
Inositol phosphate zone	1,084	3,636
Recovery (%)	79	103

[a] To a sample of the alkali-stable products (1,480 ^{32}P cpm) prepared as indicated in the legend to Fig. 1 and isolated by preparative thin-layer chromatography on a PLQ-1000 silica gel plate, using the solvent $CHCl_3$-CH_3OH-4.2 N NH_4OH (9:7:2, vol/vol), was added authentic glycerophospho[^{14}C]inositol (8,075 ^{14}C cpm). The mixture was hydrolyzed in aqueous 1.0 N KOH at 37°C for 15 h as described previously (16). After neutralization with Bio-Rex 70 (H^+; Bio-Rad Laboratories), the mixture was concentrated in vacuo, applied to a cellulose thin-layer plate (Eastman chromagram sheet 6064), and chromatographed in two dimensions with the solvent systems described previously (20). After localization by autoradiography, the radioactive zones were cut out and counted for ^{14}C and ^{32}P.

taining a monohydroxy fatty acid and the other containing a dihydroxy fatty acid, were added to reaction mixtures as suspensions in Tween 20. Neither variety was able to stimulate the synthesis of sphingolipid.

The incorporation of label from [^{32}P]phosphatidyl[^{3}H]inositol into alkali-stable products assayed by paper chromatography was linear only for the first 60 min but continued for at least 4 h.

The amount of product formed in a 2-h incubation was linear with the amount of membrane protein added, up to the maximum amount tested, 1.4 mg (27.4 mg of protein per ml).

The amount of product formed was increased by the addition of exogenous phosphatidylinositol (Fig. 4). Both soybean and yeast phosphatidylinositol stimulated product formation. This assay for products was carried out by column chromatography identical to that shown in Fig. 3: the IPC-III peak, contaminated with small amounts of what was possibly lysophosphatidylinositol (see above), was subjected to further deacylation to produce the data shown. The apparent K_m for phosphatidylinositol was about 0.5 mM. When we calculated the substrate concentration and the specific activity at each concentration, the contributions from the labeled precursor, the membranes (33 nmol of phosphatidylinositol per mg of protein), and exogenous material were all taken into account. Although the amount of product formed was much higher at higher substrate concentrations, the resulting reduction in specific activity of the substrate offset this advantage by reducing the sensitivity of the assay. It is for this reason that most of the experiments were performed at lower levels of phosphatidylinositol. We were concerned that the [^{32}P]phosphatidylinositol was always contaminated with a few percent of extraneous ^{32}P-labeled lipid, which might have accounted for the incorporation into ^{32}P-labeled sphingolipid. Addition of unlabeled phosphatidylinositol from several sources gave the profound decrease in net counts incorporated expected from such a decrease in specific activity, making it unlikely that the label found in the product was derived from sources other than phosphatidylinositol.

The maximum rates obtained (Fig. 4) were about 0.4 (IPC-II), 0.3 (IPC-II), and 0.1 [M(IP)$_2$C] nmol per mg of protein per h. Since the cells contained about 5 nmol of total phosphoinositol-containing sphingolipid per mg (dry weight) and had a generation time of about 2.5 h, one can calculate that the minimum in vivo rate was about 4 nmol of protein per mg per h. Thus, the maximal in vitro rates were about 20% of the minimum in vivo rates.

Synthesis of M(IP)$_2$C. Yeast membranes

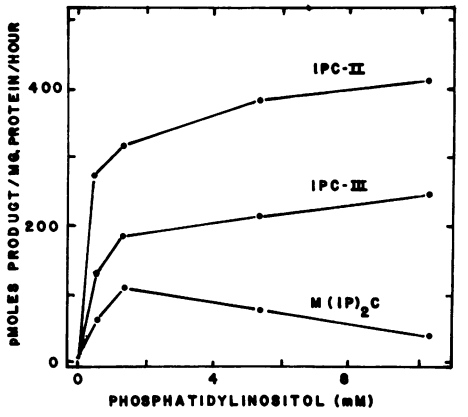


FIG. 4. *Sphingolipid synthesis as a function of phosphatidylinositol concentration. Yeast membranes were incubated with various concentrations of [^{32}P]phosphatidylinositol (3 to 7 cpm/pmol) under conditions otherwise identical to those indicated in the legend to Fig. 3. Lipids were extracted, deacylated, subjected to liquid chromatography, and counted as indicated in the legend to Fig. 3. The IPC-III peaks from each sample were subjected to the deacylation procedure, and the remaining alkali-stable lipid counts were used to calculate the results shown.*

catalyzed the conversion of [^{32}P]phosphatidylinositol to [^{32}P]M(IP)$_2$C. Figure 3B shows a radioactive peak that coincided with the mobility of the standard M(IP)$_2$C (Fig. 3A) which occurred in the experimental sample and was absent in the control. This putative [^{32}P]M(IP)$_2$C from the M(IP)$_2$C region of the silica gel column (Fig. 3) was spotted on a 100-μm-thick silica gel plate (PLQ 1000, Quantum Industries) and developed with $CHCl_3$–CH_3OH–4.2 N NH_4OH (9:7:2, vol/vol). Autoradiography disclosed one radioactive zone that coincided exactly with the unlabeled internal M(IP)$_2$C standard detected by spraying with 0.003% rhodamine 6G and with orcinol-H_2SO_4 (15). Presumably, the M(IP)$_2$C was generated by the reaction of phosphatidylinositol with an endogenous lipid, such as MIPC.

Because of this rather laborious column assay, not much is known about the optimum conditions for the formation of M(IP)$_2$C, except that the amount of product formed was sensitive to changes in the concentration of phosphatidylinositol (Fig.4).

DISCUSSION

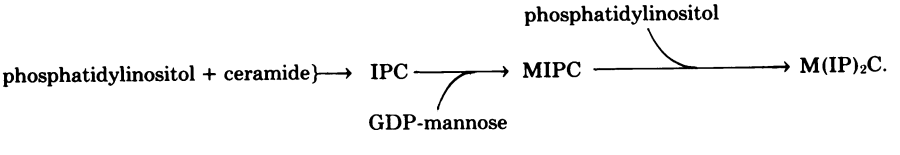
The results show that phosphatidylinositol is a precursor of IPC and M(IP)$_2$C. Under optimum conditions, the intact phosphoinositol group is transferred to endogenous acceptors in the cell-free system at about 20% of the synthesis rates during cell growth.

The simplest reaction one might imagine for the synthesis of IPC is a direct transfer: phosphatidylinositol + ceramide → IPC + diglyceride. We were unable to demonstrate a significant stimulation of this reaction by the addition of ceramides. It is unclear whether this failure was due to the existence of saturating levels of endogenous ceramides, the inability to properly present the exogenous ceramide, or the possibility that ceramide is not the substrate. Hackett and Brennan, however, did report a significant stimulation of IPC synthesis with ceramide in a soluble system from *A. niger* (7). It is possible that such a stimulation could be a misleading effect of adding lipid or detergent, and in view of our negative findings, it would seem important to carry out experiments with labeled ceramide to settle this important point.

More complicated reaction mechanisms which involve activated forms of phosphoinositol or ceramide or both are possible. For example, nucleoside diphosphoceramides might function in a manner analogous to that of CDP-diacylglycerol in the synthesis of phosphatidylinositol in animals (13) and yeast (17): nucleoside diphosphate-ceramide + inositol → IPC + nucleoside monophosphate. In fact, Schneider and Kennedy (14) searched without success for the existence of CDP-ceramide in yeast. The direct transfer of the intact phosphoinositol moiety is also supported by the in vitro data of Hackett and Brennan (7) and the earlier in vivo experiments of Angus and Lester (1); therefore, we need to consider only mechanisms such as the following: (i) phosphatidylinositol + X → inositol-P ~ X + diglyceride and inositol-P ~ X + ceramide → IPC + X and (ii) phosphatidylinositol + X ~ ceramide → IPC + X. Activated forms of either inositolphosphate or ceramide have yet to be found.

For the first time we have demonstrated the in vitro synthesis of $M(IP)_2C$, the major phosphosphingolipid of yeast. Again, the simplest mechanism of synthesis to consider is a direct transfer: phosphatidylinositol + MIPC → diglyceride + $M(IP)_2C$. Experiments with a labeled acceptor would also be required to test this hypothesis.

We have recently obtained evidence that GDP-mannose provides the mannose moieties of MIPC and $M(IP)_2C$ (unpublished data). An outline of a likely pathway for the synthesis of the yeast phosphosphingolipids is therefore:



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