The extraction of inositol-containing phospholipids and phosphatidylcholine from *Saccharomyces cerevisiae* and *Neurospora crassa*

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Abstract By use of fungi grown in the presence of [^{3}H]-inositol and [^{14}C]choline, we have explored methods for the quantitative extraction of inositol-containing phospholipids and phosphatidylcholine. Slightly alkaline mixtures of both ethanol–water and ethanol–diethylether–water at elevated temperatures were shown to effectively extract these lipids from intact *Saccharomyces cerevisiae* and *Neurospora crassa.* Some previously published procedures fail to completely extract the very polar phosphoinositol-containing sphingolipids of these organisms. Trichloroacetic acid can be used with caution in killing cells prior to extraction; lipid destruction can occur at elevated concentrations and temperatures. Complete extraction of these very polar lipids with polar solvents also results in an extract containing significant amounts of non-lipids.—**Hanson, B. A., and R. L. Lester.** The extraction of inositol-containing phospholipids and phosphatidylcholine from *Saccharomyces cerevisiae* and *Neurospora crassa. J. Lipid Res.* 1980. **21:** 309–315.

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Phospholipid extraction procedures in yeast, based on those used originally for animal tissue, have employed mixtures of ethanol–diethylether (1) and chloroform–methanol (2). While these methods appeared to be effective for extraction of glycerophospholipids from broken cells, in general, poor extraction was obtained from whole cells (3–5). Another potential problem was the activation of potent phospholipases during the extraction procedure (3–5).

Previous workers have not attempted to evaluate the effectiveness of these various extraction procedures and have generally been satisfied with the method giving the highest amount extracted. Instead, we have labeled specifically certain phospholipids with radioactive isotopes in vivo enabling us to monitor the effectiveness of the extraction procedure. As a result we have found that some published procedures are not very effective in extracting certain phospholipids (1–5).

We have been especially interested in the study of the inositol-containing lipids of *S. cerevisiae* (6–8) and *Neurospora crassa* (9): phosphatidylinositol (PI); inositol-P-ceramide (IPC); mannosylinositol-P-ceramide (MIPC); mannosyl-(inositol-P)$_2$-ceramide (M(IP)$_2$C); (inositol-P)$_2$ceramide ((IP)$_2$C). Most of these compounds are very poorly extracted from whole cells by the procedures others have employed for extraction of glycerophospholipids.

The objective of this study was to develop procedures for phospholipid extraction that can be applied directly to intact cells that have been denatured to avoid action of degradative enzymes. For the extraction of certain phospholipids we have evaluated and identified the critical variables in procedures that have been successfully used in this laboratory with various modifications for some time (8, 10–13), and as well, have described a new method.

MATERIALS AND METHODS

Strains

An inositol-requiring double mutant of *Saccharomyces cerevisiae* MC6A *ino* 1–13, *ino* 4–8 was obtained from Dr. Susan Henry, Department of Genetics, Albert Einstein College of Medicine, New York. An inositol-requiring strain of *Neurospora crassa* (*inl* 89601) was obtained from the Fungal Genetics Stock Center, California State University, Humboldt, CA (14).

Culture conditions

The basic synthetic culture medium used for the growth of *S. cerevisiae* was Vitamin-Free Yeast Base [Difco Laboratories], each liter supplemented with 40 g of glucose, 0.05 g of myoinositol, 2 μg of biotin, 400

Abbreviations: PI, phosphatidylinositol; IPC, inositolphosphoceramide; MIPC, mannosylinositolphosphoceramide; M(IP)$_2$C, mannosyldiinositolphosphoceramide.

μg of calcium pantothenate, 2 μg of folic acid, 400 μg of p-aminobenzoic acid, 400 μg of pyridoxine hydrochloride, 20 μg of riboflavin, and 400 μg of thiamine hydrochloride. A complex medium employed contained 1% peptone, 1% yeast extract, 4% glucose, 0.5% KH_2PO_4, and 0.05 M sodium succinate, pH 5.

Yeast were maintained on 1.5% agar slants of complex medium. The cells from these slants were resuspended in water and used to inoculate the basic synthetic medium supplemented with either [2-^{3}H]*myo*-inositol, $^{32}P_i$, [1,2-^{14}C]choline hydrochloride (all from New England Nuclear) and 1 mM choline chloride (Eastman Chemicals). The cultures were incubated at 25°C in a rotary shaker (180 cycles/min). Cell turbidity was monitored by measurement of absorbance at 650 nm with a Zeiss PMQ-2 spectrophotometer.

Cells were harvested by centrifugation at 1610 $g \times 5$ min and washed twice with water at room temperature. Where indicated, some cultures were treated with trichloroacetic acid prior to harvesting.

Neurospora crassa was grown on 500 ml Vogel's medium (15) supplemented with 0.28 mM inositol, 2% glucose, and about 0.4 mCi of [2-^{3}H]*myo*-inositol. Cultures were grown in 1 liter flasks in a rotary shaker (180 cycles/min) at 25°C for 18–25 hr. These early stationary phase cultures were harvested by filtration through Whatman #1 paper and washed three times with water at room temperature. A weighed portion of the wet mycelia was used for lipid extraction and the remainder was weighed before and after drying at 100°C for 3 hr.

Lipid Extraction

The pellet of washed cells or mycelia labeled with $^{32}P_i$, [^{3}H]inositol, or [^{14}C]choline was extracted in screw-capped tubes with Teflon-lined caps by a variety of procedures listed below. The pooled lipid extracts and the extracted, hydrolyzed residues were assayed for ^{14}C or ^{3}H with a scintillation spectrometer. The unextracted cells as well as the lipid extracted cell residues were hydrolyzed with 6N HCl for 40 hr at 105°C.

Procedure IA. After Folch, Lees, and Sloane Stanley (2). The cell pellet was extracted with 5 ml of chloroform–methanol 2:1 (v/v) for 24 hr. The mixture was centrifuged and the supernatant was removed. The extraction of the pellet was repeated once more and the supernatants from the extractions were combined.

Procedure IB. After Pedersen (16). The washed cell pellet (original procedure: lyophilized cells were used) was extracted with 5 ml of chloroform–methanol 1:1 for 3 hr at room temperature. The mixture was centrifuged and the supernatant was removed. The pellet was extracted twice more in the same manner and the three supernatants were combined.

Procedure IC. After Hubbard and Brody (17). The washed cell pellet was treated with 5 ml of methanol for 24 hr and centrifuged. The pellet was re-extracted, 60 min and then 30 min with 5-ml volumes of chloroform–methanol 2:1 (v/v). The three extracts were combined.

Procedure ID. After Letters (4). The washed cell pellet was treated with 5 ml 95% ethanol at room temperature for 20 hr. The mixture was centrifuged and the supernatant was removed. The pellet was further extracted two times with 5-ml portions of chloroform–methanol 2:1 (v/v) at room temperature for 2 hr each. The pellet was extracted finally with 5 ml of chloroform–methanol–concentrated hydrochloric acid 124:65:1 (v/v/v) for another 2 hr at room temperature. The supernatants from the four extractions were combined.

Procedure II. After Hanahan and Jayko (18). The washed cell pellet was extracted with 5 ml of 95% ethanol for 24 hr at room temperature. Sufficient peroxide-free diethyl ether was added to make a 3:1 alcohol–ether mixture. After 24 hr the mixture was centrifuged and the supernatant was removed.

Procedure IIIA. After Angus and Lester (10). The washed pellet was extracted with 5 ml of 95% ethanol–water–diethylether–pyridine–conc. NH_4OH 15:15:5:1:0.018 (by vol) for 15 min at 60°C. The extract was removed after centrifugation and the pellet extracted twice more in the same manner.

Procedure IIIB. The culture was treated for 1 hr at 0°C with 5% trichloroacetic acid prior to washing the cells. The extraction was carried out as described for Procedure IIIA.

Procedure IIIC. This was the same as IIIB except that the trichloroacetic acid was neutralized with 6N NaOH after 1 hr at 0°C and was allowed to stand at 5°C for 18 hr prior to washing the cells.

Procedure IVA. The washed pellet was extracted with 5 ml of ethanol–water 4:1 at 100°C for 15 min and centrifuged. The pellet was extracted twice more in identical fashion.

Procedure IVB. After Letters (5). This was identical to Procedure IVA except that the extraction was carried out at 75°C.

Procedure IVC. This was identical to procedure IVA except extraction was carried out at 50°C.

Procedure IVD. This was identical to procedure IVA except that the culture medium was treated with 5% trichloroacetic acid for 1 hr at 0°C prior to washing the cells.

Procedure IVE. The culture was treated with 5% trichloroacetic acid for 1 hr at 0°C prior to washing the cells. The pellet was extracted with 5 ml of ethanol–water–pyridine–conc. NH_4OH 80:20:2.85:0.05 for 15 min at 100°C. The residue was centrifuged and extracted twice more in the same manner.

TABLE 1. Comparison of procedures for the extraction of inositol- and choline-containing lipids from *S. cerevisiae* grown with [^{3}H]inositol and [^{14}C]choline

Extraction Procedures	% Total ^{3}H- or ^{14}C-labeled Lipid Extracted	
	Logarithmic	Stationary
A. [^{3}H]inositol[a]		
IA. $CHCl_3$–CH_3OH 2:1	21.3	14.3
IB. $CHCl_3$–CH_3OH 1:1	27.9	5.2
IC. CH_3OH; $CHCl_3$–CH_3OH 2:1	44.6	28.9
ID. $CHCl_3$–CH_3OH–HCl 124:65:1	56.6	17.9
II. CH_3CH_2OH–$(C_2H_5)_2O$ 3:1	31.4	6.4
IIIA. CH_3CH_2OH–H_2O–$(C_2H_5)_2O$–pyridine–conc. NH_4OH 15:15:5:1:0.018	98.8	94.4
IIIB. CH_3CH_2OH–H_2O–$(C_2H_5)_2O$–pyridine–conc. NH_4OH 15:15:5:1:0.018[c]	99.2	95.5
IIIC. as IIIB except trichloroacetic acid neutralized[c]	98.0	92.3
IVA. CH_3CH_2OH–H_2O 4:1 100°C	97.3	94.8
IVB. CH_3CH_2OH–H_2O 4:1 75°C	88.7	47.5
IVC. CH_3CH_2OH–H_2O 4:1 50°C	30.9	8.0
IVD. CH_3CH_2OH–H_2O 4:1 100°C[c]	84.0	42.9
IVE. CH_3CH_2OH–H_2O–pyridine–conc. NH_4OH 80:20:2.85:0.05 100°C[c]	98.0	98.8
B. [^{14}C]choline[b]		
ID. $CHCl_3$–CH_3OH–HCl 124:65:1	21.4	8.7
IIIB. CH_3CH_2OH–H_2O–$(C_2H_5)_2O$–pyridine–conc. NH_4OH 15:15:5:1:0.018[c]	97.4	97.6
IVA. CH_3CH_2OH–H_2O 4:1 100°C	96.3	96.2
IVE. CH_3CH_2OH–H_2O–pyridine–conc. NH_4OH 80:20:2.85:0.05 100°C[c]	97.0	95.6

[a] Yeast were grown as described in Materials and Methods in 125 ml of the synthetic medium supplemented with 0.08 mCi of [2-^{3}H]myoinositol and sampled at an absorbance (650 nm) of 2.7 (16.5 hr) and 10 (40.5 hr). Extraction procedures are described in Materials and Methods. Dry weight per sample extracted: 6 mg (16.5 hr); 18 mg (40.5 hr).
[b] Yeast grown in 250 ml of synthetic medium supplemented with 0.05 mCi [1,2-^{14}C]choline and 1 mM choline–HCl were sampled at an absorbance (650 nm) of 4.2 (17 hr) and 13.2 (42 hr). Extraction of 6–8 mg dry weight samples and chromatography of 50 μl of the pooled extracts were carried out as described in Materials and Methods.
[c] Culture treated with 5% trichloroacetic acid: 1 hr at 0°C.

Chromatography of lipid extracts

The [^{3}H]inositol-labeled lipid extracts were chromatographed on 15 × 19 cm pieces of EDTA-treated silica gel impregnated paper (7) in one dimension with chloroform–methanol–4.2N NH_4OH 9:7:2 (by vol). The [^{14}C]choline-labeled lipid extracts were resolved on the same paper with chloroform–methanol–conc. NH_4OH 66:17:3 (by vol) (19). In both cases each sample lane was cut into 0.5 cm sections that were added to vials and counted in a previously described scintillation counting fluid (10). The phospholipid regions were compared to known standards. Inositolphosphorylceramide and mannosylinositolphosphorylceramide isomers were not well separated and were therefore reported as the sum.

^{32}P-Labeled lipid extracts were chromatographed in two dimensions on EDTA-treated silica gel-impregnated paper as previously described (12).

RESULTS

We have adopted labeling of yeast with [^{3}H]inositol as a reliable monitor for inositol-containing lipids since it had been shown that 99% of the lipid-extractable ^{3}H was in inositol (10). *S. cerevisiae* labeled with [^{3}H]inositol was extracted by procedures most of which previously have been employed for the extraction of phospholipids from fungi (**Table 1**). Extraction with water-free solvents, and mixtures of chloroform–methanol (procedures IA–ID) and of ethanol–diethyl ether (procedure II) failed to yield complete extraction of ^{3}H, with the poorest results obtained with stationary phase cells. On the other hand, water-rich solvents (procedures IIIA, IIIB, IVA and IVE) gave virtually complete extraction; the small amount left unextracted did not appear to be an unusual class of lipids. Repeated extractions of both yeast and *Neurospora* removed all but 0.3–0.5% of the total cell inositol and 97–99% of this additional extract chromatographed as the inositol-containing lipids.

Procedure IIIB, a modification of procedure IIIA, was previously shown to be effective (10) with cells treated with trichloroacetic acid, washed and heated with an 0.5% KH_2PO_4 solution. We subsequently found (12) that extraction of trichloroacetic acid-killed cells was successful after washing the cells with water if the extraction solvent was mildly alkaline (1–7 mM NH_4OH).

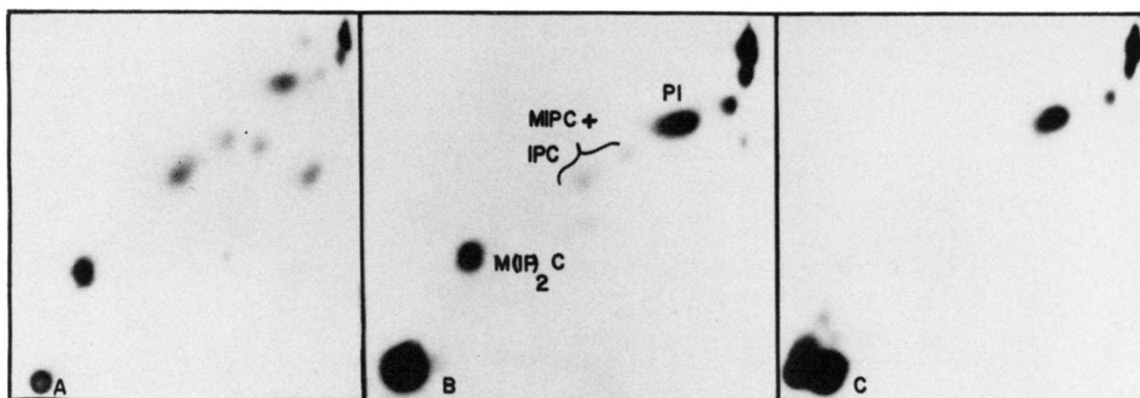


Fig. 1. Comparison of phospholipids extracted by three procedures. Yeast were grown in a 250 ml culture supplemented with 4.4 mCi $^{32}P_i$ to an absorbance (650 nm) of 1.8, and 10-mg dry weight samples were extracted as indicated. Samples (50 μl) of the pooled lipid extract were chromatographed in two dimensions and subjected to radioautography as described in Materials and Methods. A. Cells were suspended in 10% trichloroacetic acid for 24 hr at 22°C prior to extraction by procedure IIIA. B. Cells were processed directly by procedure IIIB. C. Extraction was carried out by procedure IVC.

Procedure IVA (20) is similar to that of Letters (5) who showed that extraction of whole cells with ethanol–water 4:1 at 75°C (procedure IVC) yielded a glycerophospholipid complement similar to that obtained from a broken cell suspension. Letters (5) did not assess the completeness of extraction, and in addition the very polar phosphoinositol-containing sphingolipids were not measured. Carrying out this extraction at 100°C (procedure IVA) is clearly more effective than at lower temperatures (Table 1). This procedure was not effective with trichloroacetic acid-treated cells as shown in procedure IVD (Table 1). Making the extraction solvent alkaline (procedure IVE, Table 1) permitted extraction of trichloroacetic acid-killed cells.

We have used yeast cells grown in the presence of [^{14}C]choline to monitor the extraction of phosphatidylcholine (19, 21). Fortunately, procedures IIIB, IVA, and IVE that extract inositol-containing phospholipids are also effective in the extraction of phosphatidylcholine from both stationary and logarithmic phase yeast cells (Table 1). Extraction with acidified chloroform–methanol (procedure ID) was found to be incomplete.

As shown by Letters (5), it is relatively easy to activate phospholipases in yeast, yielding massive degradation. We have made similar observations and have found that even storage of unkilled, washed cells at −20°C results in major degradation. It is imperative to either extract cells immediately after harvest or expose the culture medium to 5% trichloroacetic acid for 1–2 hr at 0°C followed by washing the cells several times with distilled water. Such cells have been stored at −20°C without significant lipid degradation. Excessive exposure of the cells to trichloroacetic acid can result in massive degradation of phospholipids. A two-dimensional chromatogram of ^{32}P-labeled lipids extracted from cells excessively exposed to trichloroacetic acid (**Fig. 1A**) shows the glycerophospholipids (upper right) considerably diminished as compared to a sample identical except for minimal exposure to trichloroacetic acid (Fig. 1B). For example, 75% of the phosphatidylinositol disappears with 10% trichloroacetic acid treatment for 18 hr at room temperature. In addition new ^{32}P-labeled spots are evident in Fig. 1A which presumably arise from lipid destruction; some of these are in the IPC + MIPC region and could lead to significantly erroneous values for these components. We have found that treatment of cells for 1 hr in 5% trichloroacetic acid at 0°C gives lipid values indistinguishable from cells extracted directly; overnight storage in 5% trichloroacetic acid at 0°C results in detectable destruction.

The use of water-rich solvents required to extract the very polar inositol-containing phosphosphingolipids predictably extracts significant amounts of materials that probably are largely non-lipids and are more polar than the most polar yeast phospholipid so far characterized (Fig. 1ABC, origins). Direct chemical analysis of such lipid extracts would be misleading because of the presence of non-lipid components.

Fig. 1 shows the selective extractability of certain inositol-containing phospholipids. A 17 hr (logarithmic) culture of yeast grown in the presence of $^{32}P_i$ was extracted by procedure IIIB (Fig. 1B) and by procedure IVC (Fig. 1C), and the lipid extracts were resolved into lipid classes by chromatography on silica gel-impregnated paper. It can be seen that the poor extraction by procedure IVC resulted in drastically low values for the inositol-containing sphingolipids. This poor extractability was found to be true for other procedures. For example, acid chloroform–methanol (procedure

ID) and ethanol–water treatment at 50°C (procedure IVC) extracted substantial amounts of phosphatidylinositol, about 53% and 35%, respectively, whereas only 10% and 0%, respectively, of the $M(IP)_2C$ and 22% and 22%, respectively, of the IPC + MIPC was extracted as compared to procedure IIIB as 100%.

Table 2 shows the amount and distribution of inositol-containing lipids extracted by the preferred extraction procedures from *S. cerevisiae* and *N. crassa*. There is little difference in these values for the preferred methods.

Using the most successful procedures (IIIA, IIIB, IVA, and IVE), as much as 38 mg dry weight of cells have been extracted with 5-ml volumes of solvent with undiminished effectiveness. These four procedures include three successive extractions with 5 ml; less than 5% of the extractable counts are present in the last extract. The volume of extracting solvent used for lipid extraction may be reduced. For example, in procedure IIIB, a 3-ml extraction followed by a 1-ml extraction gives virtually the same recoveries as the larger volumes. A more concentrated lipid extract reduces the amount necessary to spot on a chromatogram.

We have compared the extractability of [^{3}H]inositol-labeled lipids in *Neurospora crassa* by methods previously employed and by methods shown to be effective with yeast cells. Greater than 90% of the ^{3}H can be extracted with water-rich solvents (procedures IIIA, IIIB, IVA and IVE) (Table 2) whereas poor extraction, 44.6% and 58.6%, respectively, was obtained by methods (procedures IC and ID) similar to those previously published (4, 18). As with yeast, these latter procedures extract a large proportion of phosphatidylinositol (3.8 nmol/mg dry weight) but are especially ineffective in extracting the most polar sphingolipid, $(IP)_2C$ (0.24 and 0.83 nmol/mg dry weight, respectively).

Because of the difficulties in removing trichloroacetic acid from filamentous fungi such as *Neurospora*, procedure IVA is probably the preferred method for this organism if the cells can be extracted without delay.

DISCUSSION

For not entirely clear reasons, the complete extraction of phospholipids from intact fungi has been difficult. Quantitative data in the literature (22–24) obtained by extraction of intact cells should be suspect unless objective criteria were used to evaluate the efficiency of extraction.

The optimal lipid extraction method for yeast and other fungi should be effective under a variety of growth and species conditions, produce minimal extraction artifacts, and be rapid, with a minimal manipulation of the cells to be extracted. In addition, there

TABLE 2. Extraction of individual [^{3}H]inositol-containing lipids from *S. cerevisiae* and *N. crassa* by different extraction procedures

Extraction Procedure	$M(IP)_2C$	IPC + MIPC	PI	^{3}H-labeled Lipid Extracted[c]	n
		nmoles per mg dry wt			
S. cerevisiae[a]					
IIIA	2.2	1.8	8.4	98.0 ± 2.2	6
IIIB	2.3	2.0	7.7	95.4 ± 2.7	8
IVA	2.1	1.8	8.6	98.6 ± 2.8	12
IVE	1.9	1.9	8.4	98.0 ± 0.9	4
Mean	2.1 ± 0.15	1.9 ± 0.08	8.3 ± 0.30		
N. crassa[b]					
IIIB	2.5	0.6	4.7	92.0 ± 2.8	4
IVA	2.5	0.5	5.0	90.0 ± 1.8	8
IVE	2.5	0.4	4.8	92.0 ± 8.1	3
Mean	2.5 ± 0	0.5 ± 0.08	4.8 ± 0.12		

[a] Yeast were grown in 500 ml of synthetic medium supplemented with 1.0 mCi [2-^{3}H]inositol and sampled at an absorbance (650 nm) of 1.1 (17 hr). Extraction of 12 mg dry weight samples was carried out and 25 μl aliquots of the pooled extracts were chromatographed in one dimension as described in Materials and Methods.

[b] Procedures for growth, extraction, and estimation of lipids of *N. crassa* are described in Materials and Methods. Each sample extracted was from 150–250 mg dry weight and 50-μl aliquots of the pooled extracts were chromatographed.

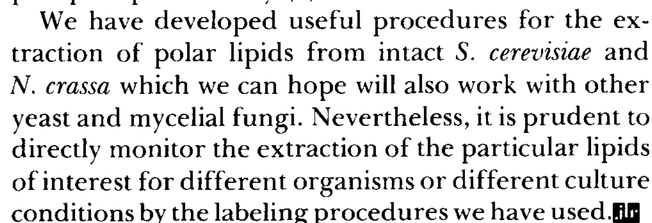
[c] Percent lipid extracted = (^{3}H-labeled lipid in lipid extract) ÷ (^{3}H-labeled lipid in lipid extract + total ^{3}H in residue) × 100 ± standard deviation and number (n) of experiments.

must be a method to verify that the extraction procedure used extracts all or most of the lipid from the cell. The preferred procedures IIIA, IIIB, IVA, and IVE are rapid, effective methods that give nearly complete extraction with minimum creation of artifacts, such as lysolipids, and do not require cell breakage, which is difficult to carry out reproducibly and conveniently on a micro scale. Procedure IIIB also has been employed for the extraction of glycophosphoceramides from plant leaves (25).

Water-rich, slightly alkaline solvents appear to be required for extraction of inositol-containing phospholipids. Letters (5) was the first to show that ethanol–water 4:1 at 75°C for 15 min was useful in directly extracting glycerophospholipids from unbroken yeast cells; the completeness of extraction, however, was not assessed. We were prompted to develop a water-rich extraction procedure (10) to extract and analyze the very polar sphingolipid: $M(IP)_2C$ and related sphingolipids (6–9, 25).

These lipids were found to be poorly soluble in anhydrous solvents, whereas they were soluble in a variety of mixtures such as chloroform–methanol–water, ethanol–diethylether–water, and pyridine–water. Therefore, at least for these lipids, the requirement for water is self-evident. Why water-rich solvents are required for extraction of phospholipids such as phosphatidylcholine that are soluble in relatively anhydrous solvents is not clear.

We have investigated trichloroacetic acid as a safe means to instantly kill cells in a culture and prevent action of phospholipases. Trichloroacetic acid must be used with caution since the samples cannot be stored in its presence indefinitely without degradation; this is especially true of very acid-labile lipids, e.g., dolichol pyrophosphoryl derivatives. Neutral solvents, such as acetone, methanol, etc., cannot be used to kill cells because they lead to phospholipid degradation via phospholipase activity (5).



We have developed useful procedures for the extraction of polar lipids from intact *S. cerevisiae* and *N. crassa* which we can hope will also work with other yeast and mycelial fungi. Nevertheless, it is prudent to directly monitor the extraction of the particular lipids of interest for different organisms or different culture conditions by the labeling procedures we have used.

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