JOURNAL OF BACTERIOLOGY, May 1981, p. 775-783
0021-9193/81/050775-09$02.00/0

Vol. 146, No. 2

Metabolism of *myo*-Inositol During Sporulation of *myo*-Inositol-Requiring *Saccharomyces cerevisiae*

RENÉE SCHROEDER AND MICHAEL BREITENBACH*

Institut für Allgemeine Biochemie and Ludwig Boltzmann-Forschungsstelle für Biochemie, A-1090 Vienna, Austria

Received 25 August 1980/Accepted 6 February 1981

We investigated the sporulation properties of a series of diploid *Saccharomyces cerevisiae* strains homozygous for inositol auxotrophic markers. The strains required different amounts of inositol for the completion of sporulation. Shift experiments revealed two phases of inositol requirement during sporulation which coincided with the two phases of lipid synthesis found by earlier workers. Phase I was at the beginning and during premeiotic deoxyribonucleic acid synthesis; phase II immediately preceded the appearance of mature asci. Of the inositol taken up by sporulating cells, 90% was incorporated into inositol phospholipids. By two-dimensional thin-layer chromatography, eight compounds were resolved, one of which was sporulation specific. The majority of the inositol phospholipids were, however, identical to those found in vegetatively growing cells. In the absence of inositol, the cells did not sporulate but, after a certain time, were unable to return to vegetative growth. These nonsporulating cells did, however, incorporate acetate into lipids and double their deoxyribonucleic acid content in the premeiotic phase. We believe that it is this lack of coordination of biosynthetic events which causes inositol-less death on sporulation media without inositol.

The purpose of the present investigation was to study the metabolism of *myo*-inositol (referred to as "inositol" hereafter) during sporulation of *Saccharomyces cerevisiae* and to compare it with the vegetative phase. Previous authors (13, 14) have studied lipid synthesis during sporulation by labeling with [^{14}C]acetate and $^{32}PO_4^{3-}$. A general lipid accumulation during sporulation, but no sporulation-specific lipid, has been found in this way. Two phases of phospholipid synthesis occur.

Lester and co-workers (20, 21) separated up to seven inositol-containing lipids from vegetative cells of *S. cerevisiae*. This was done by incorporation of [2-^{3}H]inositol into wild-type yeast cells. We have used diploid strains of *S. cerevisiae*, which are homozygous for inositol auxotrophy, for labeling inositol-containing lipids during sporulation. Only those mutants which are also inositol requiring in the sporulation phase provide us with the possibility of labeling putative sporulation-specific inositol lipids.

Generally, very few biochemical events or compounds are known which are truly sporulation specific in yeast cells. One good example is the catabolism of glycogen (7). Therefore, we thought that it was worthwhile to look for additional biochemical markers of sporulation. Quantitative and qualitative changes in the inositol-containing lipids could be useful as such markers.

In addition to this, we wanted to determine the timing of the inositol requirement during sporulation by means of shift experiments and to find out which stage of sporulation could be reached in the absence of inositol.

MATERIALS AND METHODS

Yeast strains. All of the haploid inositol-requiring strains used in this study were kindly provided by M. C. Culbertson and S. A. Henry (5). We crossed the corresponding **a**- and α-strains carrying identical inositol markers by standard genetic techniques. In this way, the diploids described in Table 1 were obtained.

Chemicals and radiochemicals. Glucose, inositol, and potassium acetate were of the highest available purity (E. Merck AG, Darmstadt, Germany). Agar, yeast nitrogen base without amino acids, vitamin-free yeast base, peptone, and yeast extract were all from Difco Laboratories, Detroit, Mich. Glusulase was from ENDO Laboratories, Garden City, New York, N.Y.; calf thymus DNA (type I, sodium salt) was from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade.

myo-[*U*-^{14}C]inositol (specific activity, 278 mCi/mmol) and [*U*-^{14}C]acetic acid (sodium salt; specific activity, 40 to 60 mCi/mmol) were from the Radiochemical Centre, Amersham, England.

Culture media, growth, and sporulation conditions. Yeast stocks were maintained on YPD (2%

TABLE 1. *Diploids used*

Strain	Genotype				
259	**a**	*ino1-13*	*ade1*	+	
	α	*ino1-13*	+	*lys2*	
308	**a**	*ino1-14*	*ade1*	+	
	α	*ino1-14*	+	*lys2*	
310	**a**	*ino2-2*	*ade1*	+	
	α	*ino2-2*	+	*lys2*	
311	**a**	*ino2-21*	*ade1*	+	
	α	*ino2-21*	+	*lys2*	
312	**a**	*ino4-8*	*ade5*	+	
	α	*ino4-8*	+	*lys2*	
318	**a**	*ino1-13*	*ino4-8*	*ade1*	+
	α	*ino1-13*	*ino4-8*	+	*lys2*

agar, 2% glucose, 2% peptone, and 1% yeast extract). All other experiments were performed with liquid media. For vegetative growth and presporulation, 0.3% yeast extract–0.5% peptone–1% glucose was used. Inositol starvation medium was a synthetic medium containing: glucose, 1%; vitamin-free yeast nitrogen base, 16.75 g/liter; biotin, 2 μg/liter; calcium pantothenate, 400 μg/liter; folic acid, 2 μg/liter; niacin, 400 μg/liter; *p*-aminobenzoic acid, 200 μg/liter; pyridoxine hydrochloride, 400 μg/liter; riboflavin, 200 μg/liter; and thiamine hydrochloride, 400 μg/liter.

Sporulation medium consisted of 1% potassium acetate with the addition of inositol (0 to 200 μM, as required).

All experiments were performed with synchronized cells obtained by separating small and large stationary-phase cells by differential centrifugation in 15% mannitol (8).

Presporulation and sporulation conditions have been described elsewhere (9). The cell density in sporulation medium was 2×10^7 cells per ml. The percentage of asci was counted in a modified Neubauer hemacytometer. Cells containing buds were counted as single cells.

Electron microscopy. Cells were fixed in 4% glutaraldehyde–0.05 M phosphate buffer (pH 7) for 4 h. They were then treated with 0.1 M mercaptoethanol–0.02 M EDTA–0.2 M Tris (pH 8.5) for 10 min at 30°C. Vegetative cell walls were then digested with glusulase for 1 to 3 h at 30°C. Glusulase had been diluted 1:40 with 0.3 M KCl–0.01 M $MgCl_2$–0.01 M sodium citrate-phosphate buffer (pH 6) (D. Zickler, personal communication).

The sample was then washed three times and incubated in 1% OsO_4 (18) overnight at 4°C, followed by treatment with 2.5% aqueous uranyl acetate at room temperature for 2 h. After dehydration, the cells were embedded in Epon 812. Ultrathin sections (60 nm) were stained with lead citrate and uranyl acetate and examined with a Phillips EM 400 electron microscope.

DNA determination. DNA determination was performed by the modification of Kissane and Robbins (16). Cells (5×10^7) were washed three times and hydrolyzed in 1 ml of 1 N NaOH for 24 h at room temperature. After addition of 3 ml of 5% trichloroacetic acid, the mixture was centrifuged at 3,000 rpm for 10 min (GLC2; Ivan Sorvall, Inc., Norwalk, Conn.), and the supernatant was discarded. The pellet was washed with 1 ml of 5% trichloroacetic acid, then with 1 ml of 0.1 M potassium acetate in 95% ethanol, and then with 1 ml of absolute ethanol and dried at 60°C for 1 h.

Subsequently, 0.25 ml of a solution of 0.3 g of 3,5-diaminobenzoic acid in 1 ml of 4 N HCl (treated with Darco-activated carbon to remove impurities) was added, and the mixture was kept at 60°C for 30 min. $HClO_4$ (3 ml, 0.6 N) was added, the sample was centrifuged at 3,000 rpm for 10 min, and the supernatant was transferred to a fluorescence cuvette and measured in a Perkin-Elmer MPF 44A fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) (excitation wavelength, 408 nm; emission wavelength, 508 nm; band width, 10 nm in emission and excitation). A calibration curve was obtained by weighing a sample of the purest available calf thymus DNA (Sigma), dissolving it in 1 N NH_3, and treating serial dilutions as described above. The instrument response was calibrated with a solid-state fluorescence standard.

Oxygen uptake. Respiration curves were obtained with samples of 2×10^7 cells in 3 ml of sporulation medium at 30°C with a Yellow Springs Instruments model 53 oxygen monitor as described previously (10).

Isotope incorporation. Cells (2×10^8) were labeled for 30 h with [^{14}C]acetate (5 μCi) in a total volume of 5 ml during sporulation in the absence and presence of 200 μM inositol. Unlabeled potassium acetate (0.4%) had been added to each experiment. During vegetative growth, cells were labeled for 4 h in 5 ml of inositol starvation medium containing 2 μCi of [*U*-^{14}C]inositol and 200 μM "cold" inositol. During sporulation, cells were labeled in 1% potassium acetate containing 2 μCi of [*U*-^{14}C]inositol and 200 μM "cold" inositol.

The incorporation of radioactivity into whole cells was measured after a sample of 10^8 cells was solubilized for 5 min at 90°C in 100 μl of a medium containing 10 mM phosphate buffer (pH 6.8), 2.5% sodium dodecyl sulfate, and 10 mM dithiothreitol. The suspension was then directly transferred to a scintillation vial containing 1 ml of distilled water and 10 ml of scintillation mixture [666 ml of toluene, 5.35 g of 2,5-diphenyloxazole (PPO; Sigma), 0.1 g of 1,4-bis-(5-phenyloxazolyl)benzene (POPOP; Sigma), and 333 ml of Triton X-100] and counted in a Beckman LS-230 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Extraction and identification of lipids. Lipids were extracted from a sample containing 2×10^8 labeled and 2×10^9 unlabeled cells, which served as a carrier. The combined cells were treated with 5 ml of 6% trichloroacetic acid for 15 min at 0°C and then washed twice with 5 ml of 0.5% aqueous KH_2PO_4. Lipids were then extracted as described by Angus and Lester (1). Neutral lipids were separated by one-dimensional thin-layer chromatography, using plates (20 by 20 cm) coated with a layer (0.25 mm) of silica without fluorescence indicator, in *n*-hexane–diethyl ether–acetic acid (40:10:1, by volume). Phospholipids were separated by two-dimensional thin-layer chromatography, using chloroform–methanol–4 N ammonia (9:7:2, by volume) in the first direction and chloroform-acetone-methanol-acetic acid-water (10:4:2:2:

1, by volume) in the second direction.

Lipids were located with a UV lamp in an iodine atmosphere. Phosphatidylserine and phosphatidylethanolamine were detected with ninhydrin spray; glycolipids were detected with a spray containing 20 mg of orcinol in 12 M H_2SO_4 and by heating the plates for 30 min to 110°C. When labeled spots were to be detected, 0.1% PPO was added to the chromatographic medium in the second step of the two-dimensional chromatography, and the spots were visualized by autoradiography, using Kodak X-R5 film. The exposure period was 3 weeks. Spots containing 10 to 20 cpm could be detected. For quantitative detection, the spots were removed from the plates and directly counted in 10 ml of scintillation mixture.

Extraction and identification of trichloroacetic acid-soluble compounds containing inositol. [*U*-^{14}C]inositol-labeled cells and asci (2×10^8) and unlabeled carrier cells (2×10^9) were quickly frozen in liquid nitrogen and ground with sea sand in a mortar until no intact cells could be detected with a light microscope. Trichloroacetic acid (1 ml, 6%) was then added, and the pellet was homogenized in an ice-cooled Potter homogenizer. The suspension was centrifuged for 15 min at 3,000 rpm (Sorvall GL-C2). The pellet was extracted twice more with 6% trichloroacetic acid, and the extracts were combined. The trichloroacetic acid was extracted with ether, and the aqueous phase was transferred to a Dowex 1×4 (100 to 200 mesh) column in the OH^- form. The neutral fraction was eluted with 200 ml of distilled water, and the acid fraction was eluted with 150 ml of 2 N HCl. Both fractions were evaporated to dryness in a Rotavapor at 30°C and suspended in 1 ml of water. The neutral fraction was submitted to paper chromatography with acetone-water (85:15, by volume). *myo*-Inositol, glucose, galactinol, glycerol, mannose, and lactose were used as standards. The acid fraction was separated by paper electrophoresis in 0.2 M ammonium acetate (pH 3.6) at 2,000 V and 30 mA. Inositol, inositol 1-phosphate, inositol 1,2-bis-phosphate, glucuronic acid, and P_i were used as standards. 1-*myo*-Inositol α-D-*manno*-pyranoside (mannosylinositol) was identified by treatment with α-mannosidase from jack beans (Sigma).

RESULTS

Sporulation of inositol-requiring mutants. The strains were analyzed for growth and sporulation in inositol-free media. The results of these experiments were as follows. All of the diploid strains constructed as described above were unable to grow on vegetative media lacking inositol. However, strains 310, 311, and 312 sporulated in the absence of inositol, but the yield of asci was considerably less than in the presence of inositol. Strains 259, 308, and 318 showed an absolute requirement for inositol during sporulation. Strains 259 and 318 were used for all of the remaining experiments. They were checked for sporulation in media containing different amounts of inositol. Strain 259 needed less inositol than strain 318, which had an optimal yield of asci with 200 μM inositol (Fig. 1). The sample at the start of presporulation consisted of single, large synchronized cells. During presporulation, over 90% of the cells developed a bud. They were transferred to the sporulation medium before commitment to a second mitoic cycle (after 90 min). In the sporulation medium, asci formed only in the mother cell and never in the bud.

During sporulation in the absence of inositol, no four-spored asci were detectable. The cells vacuolized (Fig. 2A), and after 30 h they were not able to bud when returned to the growth medium, nor were they able to sporulate when inositol was subsequently added. A very small number (<1%) of defective one-spored asci were found. By means of micromanipulation, the spores were found to be unable to form haploid colonies. These single spores were investigated by electron microscopy. The spore wall was found to be much thicker than usual, and no membrane systems were visible in these aberrant spores (Fig. 2B).

Normal asci obtained on 1% potassium acetate–200 μM inositol are shown for comparison in Fig. 2C. In these normal spores, the membrane systems are clearly visible (nuclear membrane, plasma membrane).

Two phases of inositol requirement. Strains 259 and 318 were submitted to shift experiments. Strain 259 was shifted from an inositol-free sporulation medium to a sporulation medium containing 100 μM inositol and, in another experiment, from sporulation medium containing 100 μM inositol to an inositol-free sporulation medium. The shift from the first to the second medium was carried out after various lengths of time (Fig. 3A). A similar experiment was performed with strain 318 but with 200 μM

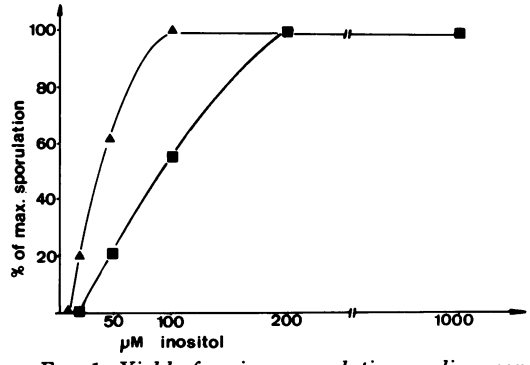
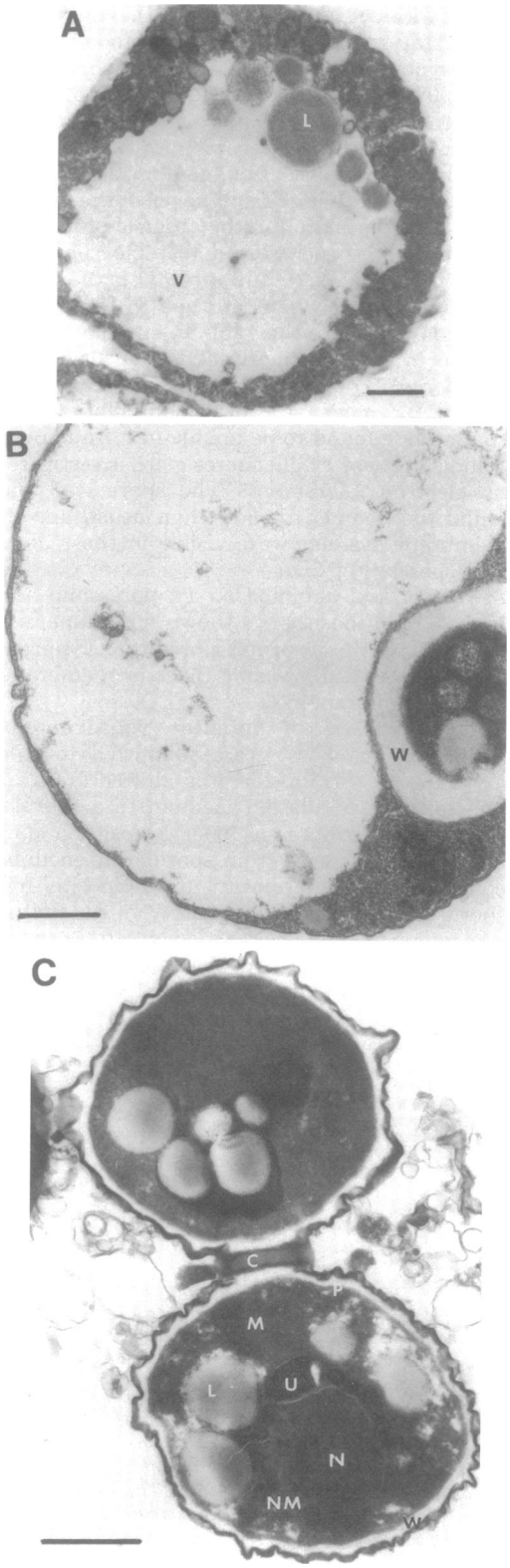


FIG. 1. *Yield of asci on sporulation medium containing various amounts of inositol. Asci were counted after 3 days, and the yield of asci is expressed as a percentage of the maximum (max.) yield. Symbols: ▲, strain 259; ■, strain 318. The actual maximum counts of asci were 60% for strain 259 and 70% for strain 318.*



inositol (Fig. 3B). Strains 259 and 318 were sensitive to inositol at T_{20} to T_{23} and T_{10}, respectively. (T_x means x hours after transfer to sporulation medium.) When strain 318 was shifted from 10 to 200 μM inositol and vice versa, the sensitive phase could be retarded to T_{18} to T_{20} (Fig. 3C).

Oxygen uptake and DNA synthesis during sporulation. Strain 318 was tested for oxygen uptake and DNA synthesis during sporulation in the absence and presence of 200 μM inositol (Fig. 4). Up to 35 h after transfer to the sporulation medium there was no difference between sporulating cells with inositol and nonsporulating cells in the absence of inositol. Both sporulating and nonsporulating cells showed a typical respiration curve, and both doubled their DNA contents in the premeiotic phase.

Uptake of acetate during sporulation. Strain 318 was checked for uptake of [U-^{14}C]-acetate during sporulation in 0.4% potassium acetate in the presence and absence of 200 μM inositol (Fig. 5). There was no difference between sporulating and nonsporulating cells. In the absence of inositol, no lipid accumulation exceeding the normal level for sporulation took place (11). The total acetate uptake and incorporation into lipids did not differ significantly (Table 2 and 3). Only the amount of phosphatidylinositol decreased from 34% of the total amount of phospholipid to 13%, as was expected.

Inositol-containing lipids of sporulating and vegetative cells. To search for sporulation-specific, inositol-containing lipids, both vegetative and sporulating cells were labeled with [U-^{14}C]inositol as described in Materials and Methods. The lipids were extracted and subjected to two-dimensional thin-layer chromatography (Fig. 6). Ninety percent of the incorporated inositol was extracted as lipid. The two-dimensional patterns differed both quantitatively and qualitatively. The lipid extract of the asci showed eight spots containing inositol. Such spots have been identified by Smith and Lester (20) and Steiner and Lester (21), using a system

FIG. 2. *(A) Cells of strain 318 after 3 days on sporulation medium without inositol. L, lipid globules; V, vacuole. The cells are no longer viable. (B) Defective one-spored ascus of strain 318 formed in low yield after 3 days on sporulation medium without inositol. W, Enlarged spore wall. (C) Part of a normal ascus of strain 318 formed on sporulation medium with 200 μM inositol. Cytoplasmic structures of the mother cell are still connected to the spores. The ascus wall has been digested with glusulase. N, Nucleus; NM, nuclear membrane; L, lipid globules; M, mitochondria; P, plasma membrane; W, spore wall; C, cement connecting the two spores; U, unidentified structures. Scale bars represent 0.5 μm.*

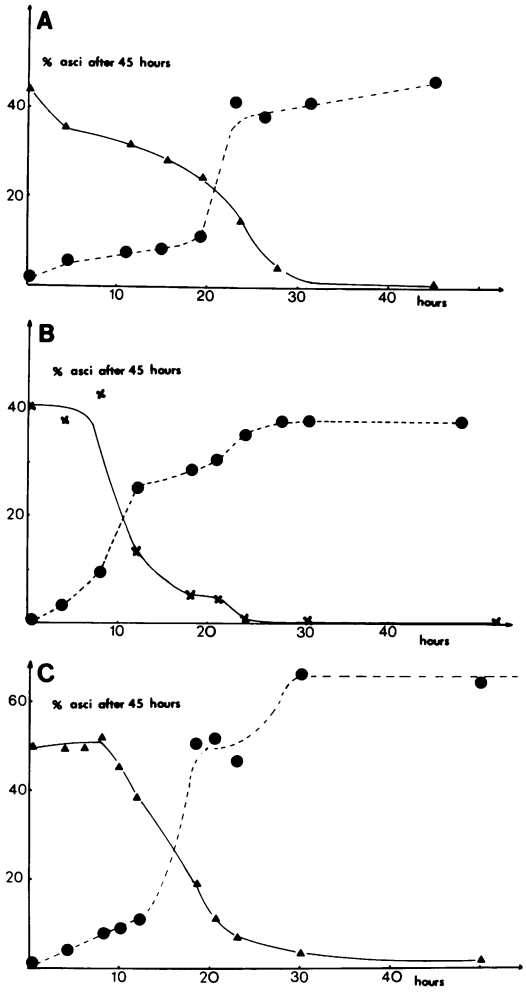


FIG. 3. *(A) Shift experiments with strain 259. Symbols: ▲, shift from potassium acetate to potassium acetate plus inositol; ●, shift from potassium acetate plus inositol to potassium acetate. The cells were shifted at the times indicated on the abscissa, and asci counted after 45 h. (B) Shift experiments with strain 318. Symbols: ×, shift from potassium acetate to potassium acetate plus inositol; ●, shift from potassium acetate plus inositol to potassium acetate. (C) Shift experiments with strain 318. Symbols: ▲, shift from potassium acetate plus 10 μM inositol to potassium acetate plus 200 μM inositol; ●, shift from potassium acetate plus 200 μM to potassium acetate plus 10 μM inositol.*

that was essentially identical to the one used for the present investigation. For some of the substances, the exact structures are unknown, but the stoichiometry of the components (inositol, phosphate, sphingosine, long-chain fatty acids) is known. The similarity of the two-dimensional patterns found by Steiner and Lester (21) and by us for vegetative cells is very striking and was used for the identification of inositol lipids in the present work. Spot 1 was identified as phosphatidylinositol; 3, as phosphatidylinositol 4-phosphate; and 2, as mannosyl(inositol phosphate)$_2$-ceramide. Spots 5 through 9 could not be identified, but Smith and Lester (20) and Steiner and Lester (21) described three of these spots as consisting of five components: namely, inositol phosphate-ceramide-I, -II, and -III, and mannosylinositol phosphate-ceramide-I and -II. The two-dimensional pattern for the vegetative cells showed only seven spots. Spot 8 was missing. Phosphatidylinositol 4,5-bisphosphate (spot 4 of Steiner and Lester [21]) could not be detected in vegetative nor sporulating cells.

Inositol metabolism (nonlipid material). The trichloroacetic acid-soluble compounds were checked for differences between vegetative and sporulating cells. The [*U*-^{14}C]inositol-labeled asci and vegetative cells were disrupted, and the radioactivity was extracted with 6% trichloroacetic acid. The extracts were passed through an anion-exchange column and divided into a neutral fraction and an acid fraction. The neutral fraction was subjected to paper electrophoresis. The same procedure was performed with the media of both sporulating and vegetative cells. Inositol 1-phosphate and glucuronic

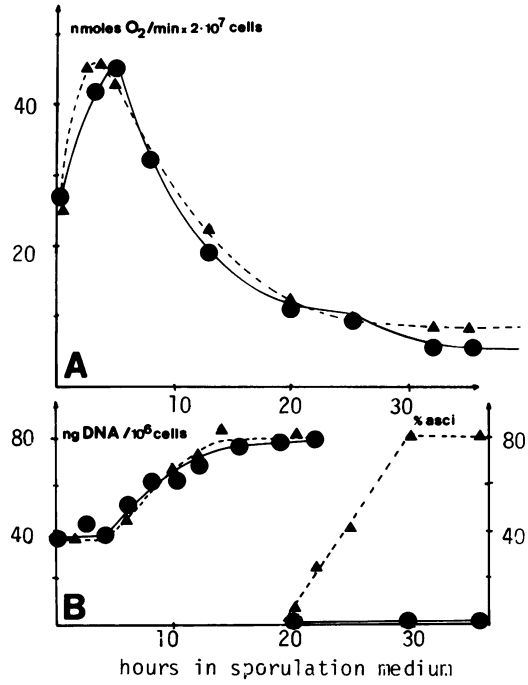


FIG. 4. *(A) Oxygen uptake; (B) DNA synthesis and the percentage of asci during sporulation of strain 318. Symbols: ▲, sporulation medium plus 200 μM inositol; ●, sporulation medium without inositol.*

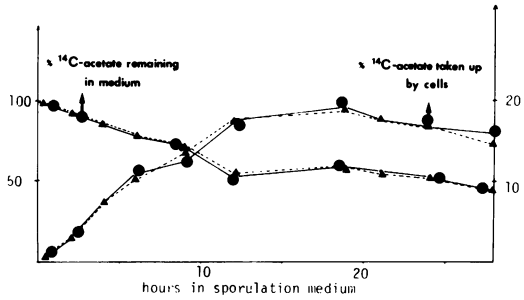


FIG. 5. *[U-^{14}C]acetate metabolism during sporulation of strain 318. Symbols: ▲, sporulation medium plus 200 μM inositol; ●, sporulation medium without inositol. The figures for [U-^{14}C]acetate remaining in the medium and [U-^{14}C]acetate taken up by the cells do not add up to 100% because $^{14}CO_2$ in the gas phase was not measured.*

TABLE 2. *Distribution of [^{14}C]acetate-labeled lipids of strain 318 in sporulation medium containing and lacking inositol*

Lipid class	% of total lipids[a]	
	Sporulation medium without inositol	Sporulation medium with inositol
Sterol ester	11	8
Triglycerides	18	21
Fatty acids	9.2	7.4
Sterols	5.3	7.3
Diglycerides	10	7.4
Phospholipids	45	48

[a] The absolute amount of incorporation of [^{14}C]acetate into total lipid (= total counts extractable by the method of Lester) was 340,000 cpm or 60 μmol of acetate in 2×10^8 cells.

acid were found in the acid fraction of both vegetative cells and spores. The two compounds were also found in the media.

Inositol, glucose, and mannosylinositol were found in the neutral fractions of both vegetative cells and spores. After treatment of mannosylinositol with α-mannosidase from jack beans, all of the radioactivity was found as inositol. These three compounds were also found in the neutral fractions of the media.

DISCUSSION

Our results show that inositol is an absolute requirement during sporulation. Some of the inositol-auxotrophic strains (259, 318, and 308) sporulated only on media containing inositol. Others (310, 311, and 312) sporulated without inositol, but to a lesser degree than in the presence of inositol. In principle, three explanations for this phenomenon are possible.

(i) Of the five homozygous diplod strains

TABLE 3. *Distribution of [^{14}C]acetate-labeled phospholipids of strain 318 in sporulation medium containing and lacking inositol*

Phospholipid	% of total phospholipids	
	Sporulation medium without inositol	Sporulation medium with inositol
Phosphatidylcholine	46	34
Phosphatidylethanolamine	23	29
Phosphatidylserine	7.1	6.5
Phosphatidylinositol	13	34
Phophatidic acid	7.1	3.2
CDP-diglyceride	2.5	2.9
Mannosyl(inositol phosphate)$_2$-ceramide	1	1

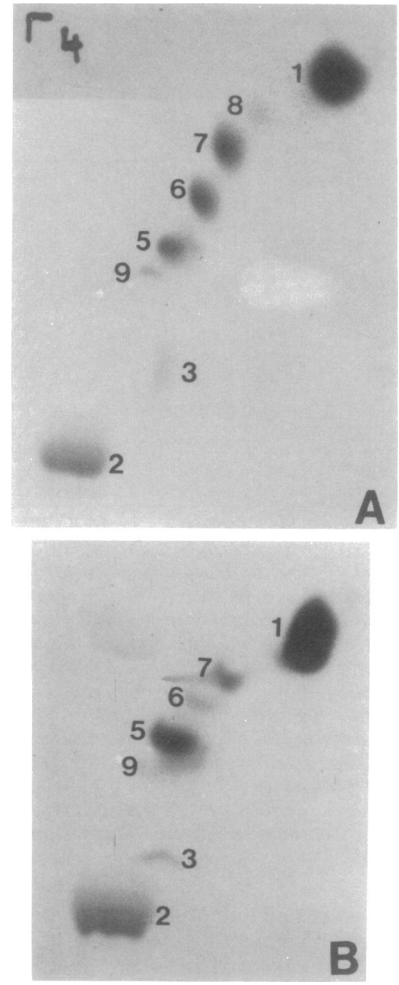


FIG. 6. *Autoradiograms of [U-^{14}C]inositol-labeled lipids after two-dimensional thin-layer chromatography. (A) sporulating cells; (B) vegetatively growing cells. The identity of the spots is discussed in the text.*

which carry only one inositol marker, only those in complementation group 1 are inositol dependent during sporulation. The others, belonging to complementation groups 2 and 4 (Table 1), are not inositol dependent during sporulation. This could mean that the ability to sporulate in the absence of inositol depends on the specific complementation group involved, i.e., on the specific genes and gene products that are correlated with complementation groups 1, 2, 4.

(ii) In those strains in which the inositol auxotrophy is not manifest during sporulation, there may be a large accumulation of intracellular inositol at the beginning of the sporulation phase, i.e., a large intracellular pool of inositol.

(iii) These strains may be "leaky" to a degree which allows sporulation but not growth.

Several lines of evidence speak against explanations ii and iii.

If the fact that the diploid strains are nearly isogenic is taken into consideration, large differences in pool size seem improbable. The second explanation is therefore unlikely.

For a discussion of "leakiness" we refer to data recently obtained by T. F. Donahue and S. A. Henry (personal communication). These authors have shown, by measurements of *myo*-inositol 1-phosphate synthase (EC 5.5.1.5) (4) in crude extracts, that strains ino-1-13, ino-4-8, and ino-2-21 produce about 1 to 2% of the wild-type enzyme activity. There is no significant difference in terms of "leakiness" (on vegetative growth media) among the three strains, all of which do not grow on inositol-free media. Another strain (ino-1-16; which was not available to us) possesses about 10% of the wild-type enzyme activity and, consequently, grows slowly on inositol-free media. Therefore, we think that differences in "leakiness" are not likely to be the cause of the different sporulation requirements of the strains used by us.

Explanation i has recently gained substantial support by the identification of the structural gene for *myo*-inositol 1-phosphate synthase (Donahue and Henry, personal communication). Most of the mutants in complementation group 1 (for instance, mutant ino-1-13) contain a protein which is precipitated by an antibody against *myo*-inositol 1-phosphate synthase but has a very low synthase activity. Complementation group 1 represents, therefore, the structural gene for *myo*-inositol 1-phosphate synthase.

Complementation groups 2 and 4 can be shown to contain regulatory genetic loci (S. A. Henry et al., personal communication). *ino*-4 is probably the gene for a lipid-methylating enzyme (acting on phosphatidyl ethanolamine). The defect in lipid methylation by some unknown mechanism "down-regulates" the expression of the *ino-1* gene by a factor of 50 on vegetative growth media. As a tentative explanation for the sporulation properties of strains *ino-2* and *ino-4*, we suggest that the above-mentioned regulatory interaction is not acting to the same degree under sporulation conditions.

Strain 318 is homozygous for two different inositol loci belonging to two different complementation groups. The effects of the two mutations are multiplicative, one of them leading to a *myo*-inositol 1-phosphate synthase with a low enzyme activity and the other leading to down-regulation of gene expression of the synthase. Therefore, strain 318 is ideally suited for investigations of the inositol requirement during sporulation.

The ultimate biological fate of the compounds synthesized from inositol is not known, but there is some evidence, both from the literature and from the results presented here, that the inositol lipids are needed for the synthesis of membranes. Matile (17) has shown that the inositol phospholipids of *Neurospora crassa* are incorporated into the membranes of lysosomes, mitochondria, and a third unidentified membrane fraction. Illingworth et al. (14) have also presented some evidence that the phospholipids and sterols synthesized during sporulation are needed as building blocks for the plasma membrane, the endoplasmic reticulum, and the mitochondrial membranes of the spores. However, the specific role of inositol phospholipids was not investigated in their work. The apparent absence of membranes in defective spores (Fig. 2b) indicates that the absence of inositol inhibits membrane formation, presumably because inositol lipids are essential membrane components.

Conclusive evidence for the fate of incorporated inositol phospholipids can be obtained by electron microscopy-autoradiography. Experiments are at present being carried out in collaboration with A. Ellinger of the Institute of Micromorphology and Electron Microscopy, Vienna University.

Shift experiments define the time of inositol requirement during sporulation. After a certain time (T_{10} for strain 318; T_{20} to T_{23} for strain 259) in potassium acetate plus inositol, the shift to potassium acetate without inositol can no longer stop sporulation. The reverse shift experiments showed that after the same time in potassium acetate, the addition of inositol no longer restored sporulation. In conjunction with this, the two series of experiments showed that only around T_{10} (strain 318) and T_{20} to T_{23} (strain 259) was inositol needed for sporulation. T_{10} is the time of premeiotic DNA synthesis and of the commitment to sporulation (6). T_{20} is shortly before the appearance of mature asci.

When strain 318 was shifted from potassium acetate plus 10 μM inositol to potassium acetate plus 200 μM inositol and vice versa, the time of inositol requirement then appeared at T_{18} to T_{20}, i.e., similar to that of strain 259. In our opinion, the surprising difference between the two strains is explained by their different "leakiness." Strain 318, which is nearly ideally "nonleaky" (see above), already requires inositol during the first of the two phases of inositol phospholipid synthesis (phase I). When this strain was given just enough inositol (10 μM) to complete the syntheses necessary during phase I, the time of inositol requirement of this strain was delayed to T_{18-20}. Strain 259 seems to be "leaky" to a minor degree, its capacity to synthesize inositol being sufficient for phase I but not for phase II (T_{20-23}). The fact that strain 318 needed more inositol during sporulation than strain 259 supports this theory. Strains 310, 311, and 312 synthesized enough inositol for phase I and phase II.

The two phases of inositol requirement revealed by our shift experiments coincide with the phases of lipid synthesis found by Henry and Halvorson (13). These authors used incorporation of [^{14}C]acetate and [^{32}P]phosphate into total lipids as a measure of lipid synthesis during the two phases. Acetate is incorporated during both phases, whereas very little phosphate is incorporated during phase II. As all the known inositol lipids contain phosphorus and we showed a requirement for inositol during phase II, there appears to be a contradiction. One has to consider, however, that inositol phospholipids are only a fraction (1/3) of the total phospholipids and that this might well be accommodated in the small increase in phosphate incorporation during phase II, as observed by Henry and Halvorson. From electron microscope investigations, it can be concluded that extensive membrane formation takes place at the time immediately preceding the appearance of mature asci (T_{20-23}).

It was the main aim of the present investigation to compare the inositol phospholipids formed in vegetative and sporulating cells and to look for sporulation-specific compounds belonging to this class of lipids. Of the labeled inositol 90% is incorporated into lipids in both the vegetative phase and the sporulative phase. This therefore seems to be the most important pathway of inositol metabolism in yeast cells. We did not obtain enough radioactive material to investigate the exact chemical structure of these complex phospholipids. The identification of individual lipids is based on comparison with the two-dimensional thin-layer chromatograms of Steiner and Lester (21). As can be seen from the autoradiograms (Fig. 6), the major inositol phospholipids (spots 1 through 3) are approximately identical in their relative amounts during sporulation and vegetative growth. Spot 5, which is a major product in vegetative cells, is less abundant in sporulating cells. Spot 8 seems to be sporulation specific but is only found in very small amounts. It is difficult at present to assess the importance of this minor product for the sporulation process. Most of the inositol phospholipid material synthesized in vegetative and sporulating cells seems to be identical. In our view, this reflects the fact that the biomembranes formed during both phases are of similar compositions.

The metabolism of inositol in spores and vegetative cells does not differ with regard to trichloroacetic acid-soluble compounds. It was found that inositol is degraded to glucuronic acid and further, as described by Sivak and Hoffmann-Ostenhof (19), which explains why glucose was found after labeling with inositol. Inositol 1-phosphate and mannosylinositol were found in the medium. Mannosylinositol is thought to be a degradation product of mannosylinositol phosphate and mannosyl(inositol phosphate)$_2$-ceramide.

A last point of interest is that several biochemical "markers" of sporulation (premeiotic DNA synthesis and incorporation of [^{14}C]acetate into cellular lipids), which occur after the first inositol-requiring and lipid-synthesizing phase (T_{10}), can be observed even in the absence of inositol. One of the series of shift experiments with strain 318 showed that after T_{10} the cells could not return to sporulation when inositol was added. This means that DNA synthesis and acetate incorporation occur to the same extent as in sporulating cells even after the cells are irreversibly prevented from sporulation. These cells are also unable to return to the vegetative cell cycle when they are resuspended in growth medium. In other words, inositol-less death occurs also on sporulation media lacking inositol in the appropriate inositol-auxotrophic strains.

All of the above-mentioned facts can be understood if we assume that the cells lack a regulatory mechanism preventing other metabolic pathways and stopping the mitotic cycle in the absence of inositol. Such mechanisms do exist, for instance, in the case of an amino acid auxotrophic strain kept on a medium without the required amino acid (22). When such mechanisms are absent, uncoordinated synthesis of cellular components takes place, leading eventually to cell death, as pointed out in the current theory of inositol-less death (2, 3, 12, 15).

ACKNOWLEDGMENTS

We thank S. A. Henry and M. R. Culbertson for supplying the haploid strains used in this study, D. Zickler and A. Ellinger for help with the electron microscopy of the yeast cells, F. Paltauf for advice about the separation of yeast lipids, and O. Hoffmann-Ostenhof for critically reviewing the manuscript.

LITERATURE CITED

1. **Angus, W. W., and R. L. Lester.** 1972. Turnover of inositol- and phosphorus-containing lipids in *Saccharomyces cerevisiae*; extracellular accumulation of glycerophosphorylinositol derived from phosphatidylinositol. Arch. Biochem. Biophys. **151**:483–495.
2. **Atkinson, K. D., A. I. Kolat, and S. A. Henry.** 1977. Osmotic imbalance in inositol-starved spheroplasts of *Saccharomyces cerevisiae*. J. Bacteriol. **132**:806–817.
3. **Becker, G. W., and R. L. Lester.** 1977. Changes in phospholipids of *Saccharomyces cerevisiae* associated with inositolless death. J. Biol. Chem. **252**:8684–8691.
4. **Culbertson, M. R., T. F. Donahue, and S. A. Henry.** 1976. Control of inositol biosynthesis in *Saccharomyces cerevisiae*: inositol-phosphate synthetase mutants. J. Bacteriol. **126**:243–250.
5. **Culbertson, M. R., and S. A. Henry.** 1975. Inositol-requiring mutants of *Saccharomyces cerevisiae*. Genetics **80**:23–40.
6. **Esposito, R. E., and M. S. Esposito.** Genetic recombination and commitment to meiosis in *Saccharomyces*. Proc. Natl. Acad. Sci. U.S.A. **71**:3172–3176.
7. **Fonzi, W. A., M. Shanley, and D. J. Opheim.** 1979. Relationship of glycolytic intermediates, glycolytic enzymes, and ammonia to glycogen metabolism during sporulation in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. **137**:285–294.
8. **Golombek, J., and E. Wintersberger.** 1974. Glucose uptake in the cell cycle of *Saccharomyces cerevisiae*. Exp. Cell Res. **86**:199–202.
9. **Hartig, A., and M. Breitenbach.** 1977. Intracellular cAMP in synchronous sporulation of *Saccharomyces cerevisiae*. FEMS Lett. **1**:79–82.
10. **Hartig, A., and M. Breitenbach.** 1980. Sporulation in mitochondrial oxi3 mutants of *Saccharomyces cerevisiae*. Curr. Genet. **1**:97–102.
11. **Hayashi, E., R. Hasegawa, and T. Tomita.** 1976. Accumulation of neural lipids in *Saccharomyces carlsbergensis* by *myo*-inositol deficiency and its mechanism. J. Biol. Chem. **251**:5759–5769.
12. **Henry, S. A., K. D. Atkinson, A. I. Kolat, and M. R. Culbertson.** 1977. Growth and metabolism of inositol-starved *Saccharomyces cerevisiae*. J. Bacteriol. **130**: 472–484.
13. **Henry, S. A., and H. O. Halvorson.** 1973. Lipid synthesis during sporulation of *Saccharomyces cerevisiae*. J. Bacteriol. **113**:1158–1163.
14. **Illingworth, R. F., A. H. Rose, and A. Beckett.** Changes in the lipid of composition and fine structure of *Saccharomyces cerevisiae* during ascus formation. J. Bacteriol. **113**:373–386.
15. **Keith, A. D., E. C. Pollard, W. Snipes, S. A. Henry, and M. R. Culbertson.** 1977. Inositol-less death in yeast results in a simultaneous increase in intracellular viscosity. Biophys. J. **17**:205–212.
16. **Kissane, J. M., and E. Robbins** 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. J. Biol. Chem **233**:184–188.
17. **Matile, P.** 1966. Inositol deficiency resulting in death: an explanation of its occurrence in *Neurospora crassa*. Science **151**:86–88.
18. **Palade, G. E.** 1952. A study of fixation for electron microscopy. J. Exp. Med. **95**:285–297.
19. **Sivak, A., and O. Hoffmann-Ostenhof.** 1962. Enzyme des meso-Inositabbaus in *Schwanniomyces occidentalis*. Biochem. Z. **336**:229–240.
20. **Smith, S. W., and R. L. Lester.** 1974. Inositol phosphorylceramide, a novel substance and the chief member of a major group of yeast sphingolipids containing a single inositol phosphate. J. Biol. Chem. **249**:3395–3405.
21. **Steiner, S., and R. L. Lester.** 1972. Studies on the diversity of inositol-containing yeast phospholipids: incorporation of 2-deoxyglucose into lipid. J. Bacteriol. **109**:81–88.
22. **Unger, M. W., and L. H. Hartwell.** 1976. Control of cell division by methionyl-tRNA. Proc. Natl. Acad. Sci. U.S.A. **73**:1664–1668.