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Inositol-Limited Growth, Repair, and Translocation in an Inositol-Requiring Mutant of *Neurospora crassa*

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The biochemical consequences of inositol limitation in an inositol auxotroph of *Neurospora crassa* have been examined as a means of disclosing the cellular role of inositol. The cellular levels of inositol in the *inl* mutant were proportional to the concentration of inositol in the growth medium whereas inositol phosphate levels remained relatively constant at about 0.1 μmol/g (dry weight). After 72 h of growth, about 57-fold more protein per milligram (dry weight) was released by the mutant grown on limiting inositol than by the inositol-supplemented control. When the inositol-limited growth medium was osmotically buffered with 1% NaCl, 3% NaCl, or 6% sorbitol, there was about 33, 74, or 54%, respectively, less protein released by the mutant. These results are consistent with cell lysis occurring in the mutant grown on limiting inositol because of a structurally weakened cell wall and membrane deterioration. When sufficient inositol for normal mycelial growth was supplied to an inositol-deficient mycelium, there was within 2 h a rapid incorporation of inositol to 85% of control levels. This incorporation occurred without significant growth by any area of the mycelium. About 10 to 15% of the total cell inositol was translocated forward from the older mycelial areas to the growing tips; only 2 to 5% of the total cell inositol was translocated backward toward the older mycelial areas. Possible mechanisms of translocation are discussed.

In *Neurospora crassa*, there is a unique group of mutations which specifically affect phospholipid synthesis and result in aberrant hyphal formation. These mutants are the choline (*chol*) and inositol (*inl*) auxotrophs (3, 6, 9, 10, 12, 15, 16, 22, 26, 29-32, 34, 38). Of the two mutant classes, the *inl* strain has been more extensively studied. When the *inl* mutant is grown on less than 5.6 μM inositol, changes occur in the intracellular levels of three inositol-containing lipid classes: phosphatidylinositol (PI), di(inositolphosphoryl) ceramide [$(IP)_2C$], and monoinositolphosphorylceramide (IPC) (11, 12, 17, 26, 36). These changes in the inositol metabolism of the *inl* mutant are the result of a defective inositol synthetase system (28, 38).

The syntheses of important cell wall components, specifically the amino sugars glucosamine and galactosamine (12), are perturbed in the *inl* mutant grown on limiting inositol. Glucosamine in the form of cell wall chitin (17, 27) is reduced by 50%, whereas there is no compensatory increase in the other structural polymer, β-1,3-glucan (12). This loss of chitin may greatly weaken the cell wall, as suggested by the work of Katz and Rosenberger with temperature-sensitive chitin sythetase mutants of *Aspergillus* (20, 21). They found that at nonpermissive temperatures their mutants produce little chitin and release cellular material into the growth medium. This leakage is reduced by osmotically buffering the growth medium. The *inl* mutant grown on limiting inositol (31) also releases significant amounts of cellular material into the medium, suggesting that the reduction in chitin in the *inl* mutant may be linked to the leakage of cellular material. Membrane deterioration and lipid peroxidation may also be causes of this leakage (25, 26, 30, 31, 34). No attempt has been made to determine whether the growth of the *inl* mutant in osmotically buffered medium would prevent cellular leakage and reverse the effects of inositol limitation on cell growth and morphology as in the case of the temperature-sensitive chitin synthetase mutants (20, 21).

These earlier studies also did not address the specific question of whether the cellular and chemical changes resulting from inositol-limited growth in the *inl* mutant are reversible when inositol is restored to the growth medium. Antioxidants such as nordihydroguaiaretic acid reduce but do not reverse the deleterious effects of inositol-limited growth (26, 30).

The purpose of this study was to determine which cellular changes occur in the *inl* mutant grown on limiting inositol and whether the severity of these changes could be reversed by increasing the osmolality of the growth medium.

In addition, the reversibility of inositol-limited growth was determined by supplying sufficient inositol for normal growth to the *inl* mutant grown originally on limiting inositol. Inositol incorporation was measured in the hyphal tips and older mycelial areas to determine whether the entire *inl* mycelium or these specific areas were capable of incorporating inositol for inositol-containing lipid synthesis with the resumption of normal growth. The intracellular movement or translocation (11, 14, 24, 37, 39, 40) of inositol also was measured to determine whether inositol from the growing tips could reverse the effects of limiting inositol in the older mycelial regions.

MATERIALS AND METHODS

Organisms. The Rockefeller-Lindegren wild-type (RL3-8A) and inositol-requiring (89601 *inl*) strains of *N. crassa* were obtained from the Fungal Genetics Stock Center, California State University, Humboldt. The *inl* strain has an RL3-8A background (3).

Media. In all experiments, the *inl* and wild-type strains were grown at 22°C on Vogel medium (VM) supplemented with 2% glucose and various concentrations of inositol (3.3 to 111.1 μM) (36). For experiments with solid medium, VM was solidified with 2% agar (Difco Laboratories, Detroit, Mich.).

Chemicals. The sugar and lipid standards for chromatography were obtained from Sigma Chemical Co., St. Louis, Mo. PI was obtained from Pierce Chemical Co., Rockford, Ill. The $(IP)_2C$ and IPC chromatographic standards were a gift of R. L. Lester, University of Kentucky, Lexington.

Dialysis tubing. In all studies except for the liquid medium experiments, the *Neurospora* mycelia were grown on dialysis tubing overlying the solid medium prepared in the following manner (35). Circles (90 mm each; VWR Scientific, St. Louis, Mo.) were cut from 45-mm-diameter dialysis tubing. After rinsing in distilled water, the circles were sterilized for 1 h in 95% ethanol. The sterilized tubing was rinsed in sterile distilled water, centered on the surface of the solid medium, and smoothed gently to remove any air pockets. The plates were inverted and allowed to dry overnight. Excess water was shaken from the plates. Alternatively, dialysis tubing was sterilized with germicidal UV light at 254 nm. Autoclaved dialysis tubing is not suitable for this type of experiment.

Inositol-containing lipids and soluble cellular inositol and inositol phosphate. Inositol-containing lipids and soluble cellular inositol and inositol phosphate, which compose 90 to 95% of the total cellular inositol (12, 12a), were extracted by the procedures of Hanson and Brody (12) and Hanson and Lester (12a). Inositol and inositol phosphate in the extracts were resolved by paper chromatography with Whatman no. 1 paper with *tert*-butyl alcohol–methyl ethyl ketone–water–concentrated NH_4OH (40:30:20:10) (8). The lipids in the extracts were resolved by one-dimensional paper chromatography on EDTA-dipped Whatman SG81 paper (4, 12a, 33). The inositol-containing lipids, inositol, and inositol phosphate were identified by cochromatography with authentic standards. The radioactivity on the chromatograms was analyzed as previously described (1, 7).

Inositol-limited growth. The *inl* strain was grown in 1,000 ml of VM supplemented with inositol (3.3 to 55.5 μM) and [2-^{3}H]inositol (0.06 mCi; specific activity, 1 to 5 Ci/μmol; New England Nuclear Corp., Boston, Mass.). The inositol incorporated by the mutant was not metabolized further in the cell (12, 22). These early stationary phase cultures showing no cell lysis were harvested by filtration through Whatman no. 1 paper and washed twice with cold VM without inositol. The mycelial pad then was rapidly frozen, lyophilized overnight, weighed, and stored at −20°C. Samples of lyophilized mycelia were hydrolyzed, and the total inositol content was determined by the procedures of Hanson and Brody (12). Inositol-containing lipids and soluble cellular pools of inositol and inositol phosphate were extracted as previously described.

Liquid medium repair experiments. The *inl* mutant was grown for 48 h in 500 ml of VM supplemented with inositol (3.3 μM). At this time, 5 μCi of *myo*-[2-^{3}H]inositol and enough nonradioactive inositol to bring the total inositol concentration to 33.0 μM were added to the medium. Growth was allowed to continue for an additional 24 h. Several times during this period, samples of mycelia were harvested and the total inositol content was determined as previously described.

Solid medium repair experiments. The *inl* mutant was inoculated on dialysis tubing overlying VM supplemented with inositol (16.7 or 111.1 μM) in petri dishes. When the mycelium had grown partially across the plate (24 h), the mat was transferred on the dialysis tubing to VM supplemented with inositol (111.1 μM) and [2-^{3}H]inositol (0.15 μCi/ml) (Fig. 1A). Growth was allowed to continue for 2 to 16 h after transfer. Samples of mycelium were harvested, and the total inositol, inositol-containing lipids, and soluble cellular inositol and inositol phosphate were determined as previously described. Higher inositol concentrations were used in all solid medium experiments because of the partial impermeability of the dialysis membranes. It was found that inositol at a concentration of 16.7 μM was growth limiting for the mutant.

Pulse-chase repair experiments. The *inl* mutant was grown and transferred as described above for solid medium experiments with the following exception. After 2 h of growth on VM with inositol (111.1 μM) and [2-^{3}H]inositol (0.15 μCi/ml), the mycelium was transferred on the dialysis membrane to the same medium without radioactive inositol and incubated for an additional 2 h. Samples of the growing hyphal tips and older hyphal areas were taken after both the 2-h pulse and the 2-h pulse-chase. The inositol-containing lipids and soluble cellular inositol and inositol phosphate in these samples were determined as previously described.

Mycelial translocation. In these experiments, special petri dishes with a partition dividing the plate in half were used to allow the use of two different media in one plate (Falcon Plastics, Oxnard, Calif.). For measurement of the backward translocation of [2-^{3}H]inositol from the growing tips to the older mycelial areas, the *inl* strain was inoculated on VM supplemented with inositol (16.7 or 111.1 μM) (Fig. 1B).

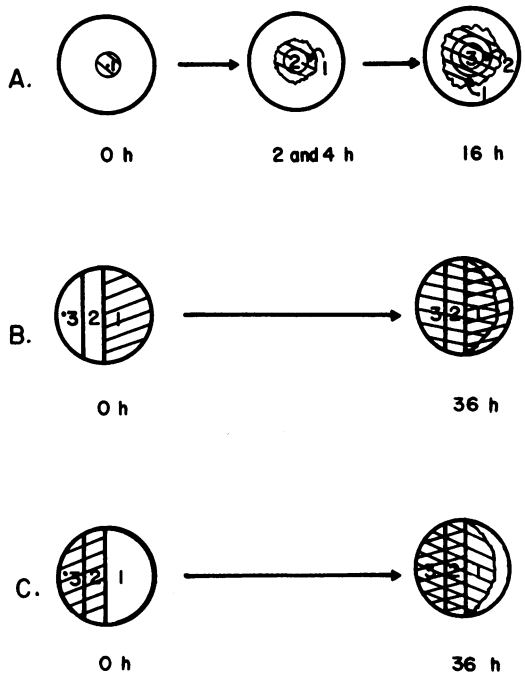

FIG. 1. *Points of inoculation and areas of mycelial growth during solid medium repair experiments. (A) Mycelial repair: mycelial growth after transfer to medium with a high concentration of inositol (111.1 μM) and [2-^{3}H]inositol (0.15 μCi/ml). Areas 1, 2, and 3 were harvested at 2, 4, and 24 h, respectively, after transfer. (B and C) Mycelial translocation: mycelial growth across two media with different concentrations of inositol (16.7 and 111.1 μM). The plate was inoculated in area 3 (0 h), and mycelium grew across the plate to cover areas 1 and 2 (24 h). The medium in areas 2 and 3 contained 16.7 or 111.1 μM inositol, whereas the medium in area 1 contained 111.1 μM inositol. In (B), the medium in area 1 contained [2-^{3}H]inositol (0.15 μCi/ml); in (C), the medium in areas 2 and 3 contained [2-^{3}H]inositol (0.15 μCi/ml). Symbols: ⊡, point of inoculation; ▨, [^{3}H]inositol in medium; ▧, area of growth.*

When the mycelium had grown across the partition to the VM supplemented with inositol (111.1 μM) and [2-^{3}H]inositol (0.15 μCi/ml), samples of mycelium from specific areas (Fig. 1B) were harvested. For measurement of the forward translocation of [2-^{3}H]inositol from the older mycelial areas to the growing tips, the *inl* strain was inoculated on VM supplemented with inositol (16.7 or 111.1 μM) and [2-^{3}H]inositol (0.15 μCi/ml) (Fig. 1C). When the mycelium had grown across the partition to the VM supplemented with nonradioactive inositol (111.1 μM), samples of mycelium from specific areas (Fig. 1C) were harvested. The inositol-containing lipids and soluble cellular inositol phosphate in the samples were determined as previously described.

RESULTS

Inositol-limited growth in liquid medium.

In the *inl* mutant, the cellular levels of the inositol-containing lipids and inositol were proportional to the concentrations of inositol in the growth medium (Fig. 2). The inositol phosphate levels remained relatively constant at about 0.1 μmol/g (dry weight) (Fig. 2). These results are consistent with the hypothesis of Williams (38) which suggests that the biochemical lesion in the *inl* mutant which renders it dependent for growth upon exogenous inositol involves loss of D-*myo*-inositol-1-phosphatase, an enzyme which cleaves D-*myo*-inositol-1-phosphate to form *myo*-inositol (26).

Cell leakage during inositol-limited growth in liquid medium. The hypothesis that osmotically stabilizing the *inl* mutant would prevent cell lysis and leakage was tested by measuring the level of protein released into the medium as an indicator of cell lysis during inositol-limited growth. The *inl* mutant, after 72 h of growth on limiting inositol, released 57-fold more protein per milligram (dry weight) into the medium than did the control (Table 1). Similar results were reported by Rana and Munkres with *inl* mutant RL 1256A (31). Their mutant on limiting inositol released 12-fold more protein per milliliter of culture medium, whereas the 89601 *inl* mutant used in this study released 3- to 6-fold more protein. Both results are consistent with cell lysis occurring in the *inl* mutant grown on limiting inositol because of a structurally weakened cell wall and membrane deterioration.

The *inl* mutant also was grown on either 6% sorbitol (432 mosm), 1% NaCl (320 mosm), or 3% NaCl (960 mosm) to try to osmotically stabilize the lysing mycelium. Sorbitol and NaCl at 6 and

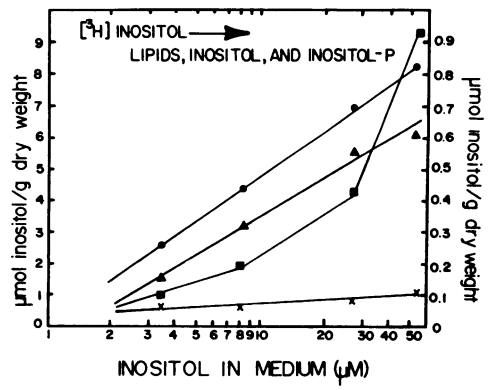

FIG. 2. *Distribution of inositol-containing compounds in the mycelium of the inl mutant grown on various levels of inositol. The micromoles of each compound per gram was determined from specific activity of [2-^{3}H]inositol in the medium. Symbols: ●, total inositol; ▲, inositol-containing lipids; ■, extractable inositol; ×, inositol phosphate.*

TABLE 1. *Release of protein into medium by the inl mutant grown on various levels of inositol*[a]

Time after inoculation (h)	Growth phase	mg of protein released per ml of medium		mg of protein released per mg (dry wt)	
		277.7[b]	3.3	277.7	3.3
0		0	0	0	0
25.0	Logarithmic	0	0	0	0
40.0	Early stationary	0	0.03	0	0.06
50.0	Stationary	0.05	0.14	0.01	0.27
72.0	Stationary	0.06	0.38	0.01	0.57
88.0	Stationary	0.12	0.52	0.03	1.03

[a] Erlenmeyer flasks (500 ml each) containing 250 ml of VM supplemented with either 3.3 or 277.7 μM inositol were inoculated with *inl* mutant conidia. Flasks were incubated at 25°C. Samples (1 ml each) were removed at various times after inoculation and filtered on membrane filters (Millipore Corp., Bedford, Mass.; RAWP; pore size, 1.2 μm). The protein in the filtered medium was determined by the method of Lowry et al. (23) and by spectrophotometric measurements at 260 and 280 nm (2).

[b] Micromolar concentration of inositol in medium.

1%, respectively, are hypotonic, but growth morphology became abnormal at higher concentrations of these compounds. The isoosmolar point for wild-type *Neurospora* is 600 mosM (18, 19). After 72 h, 1% NaCl, 3% NaCl, or 6% sorbitol in the medium reduced the amount of protein released by 33, 74, or 54%, respectively. The amounts of protein released in the osmotically stabilized mutant were still significantly higher than those released by the control. Although osmotically stabilizing the mutant reduced release of cell protein, it did not lessen the changes in cell morphology or growth resulting from limiting inositol.

Inositol repair in liquid medium. Experiments were designed to determine whether an *inl* mycelium grown on limiting inositol and transferred to medium supplemented with high concentrations of inositol was capable of incorporating and utilizing inositol.

The kinetics of inositol incorporation after transfer of an *inl* mycelium to a medium with high concentrations of inositol (33.0 μM) and [2-^{3}H]inositol are shown in Fig. 3A. Incorporation by the mycelium as measured by total inositol content was extremely rapid after transfer, occurring within 2 h and without a significant increase in mycelial dry weight. The total inositol content doubled about 1.2 h after transfer, whereas the dry weight doubled after about 14 h. For comparison, hypothetical values for total inositol content based on either immediate or no incorporation of inositol after transfer are given in Fig. 3A. The calculations for immediate incorporation of inositol were based on the assumption that incorporation can occur without growth and that, upon transfer to high concentrations of inositol, the inositol levels will rise immediately to normal (about 8 to 11 nmol/g of mycelium). The "no incorporation" values were based on the assumption that incorporation of inositol cannot occur without growth of the mycelium. The hypothetical inositol content values were determined in the following manner: for immediate incorporation, total inositol content (nanomoles) per sample flask = [mycelial dry weight (milligrams) at x h after transfer] [normal inositol content, 11 nmol/mg (dry weight)] and for no incorporation, total inositol content (nanomoles) per sample flask at x h after transfer = [mycelial dry weight at x h after transfer − mycelial dry weight at transfer] [normal inositol content, 11 nmol/mg (dry weight)] + [inositol content, sample flask at transfer]. The experimental values for inositol content corresponded more closely with the hypothetical values based on immediate incorporation of inositol. Within

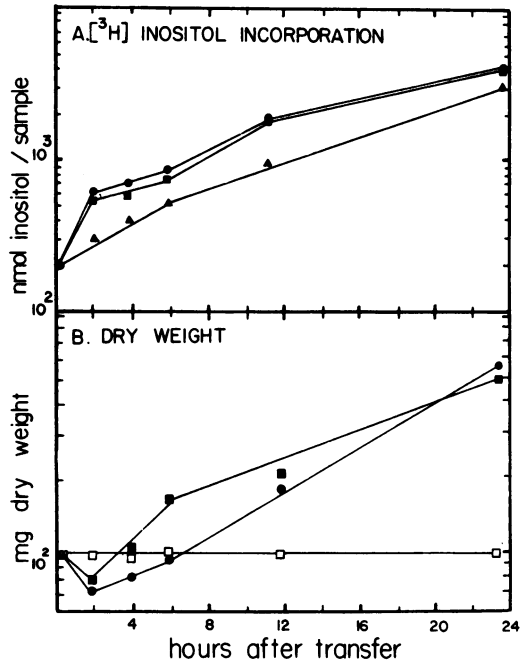

FIG. 3. *Liquid repair experiments. (A) Comparison of the total inositol content (nanomoles per flask) of mycelia grown on limiting inositol (3.3 μM) after transfer to medium with high concentrations of inositol (33.3 μM)* (■). *Hypothetical values based on immediate* (●) *or no* (▲) *incorporation of inositol into existing areas were calculated as described in the text. (B) Total dry weight per flask of wild type (RL3-8A)* (■) *and inl 89601* (●) *after transfer to a medium with a high concentration of inositol (33.3 μM) and inl 89601* (□) *maintained on limiting inositol (3.3 μM). Mycelial dry weight is normalized for both strains to 100 mg (dry weight) at 0 h.*

2 h, the experimental value was already 85% of the immediate incorporation value. The growth with time of the *inl* and wild-type strains is shown in Fig. 3B. After a lag period, the *inl* mutant appeared to resume a normal growth rate.

Inositol-limited growth on solid medium. The distribution of inositol-containing lipids in the *inl* mycelium was measured to determine whether the growing hyphal tips differed in lipid composition as compared with the older mycelial areas. After 24 h, about 38.8 and 51.0% of the total extractable [2-^{3}H]inositol from *inl* mycelium grown under inositol-limited (16.7 μM) and control (111.1 μM) conditions, respectively, were found as PI (Table 2). The total extractable [2-^{3}H]inositol in the mutant grown on limiting inositol was reduced by about 2.5-fold (Table 2). After 39 h, the distribution of these lipids in specific areas of the mycelium was found to be similar, except for about a 17% decrease in PI at the growing tips (Table 2). The distributions of PI and IPC in the growing tips of the inositol-supplemented mutant were 20% less and 4.2-fold higher, respectively, than in other mycelial regions.

Inositol repair in specific mycelial areas. Additional experiments were designed to determine whether specific mycelial areas were still capable of transporting and incorporating inositol and resuming a normal growth rate when transferred to medium supplemented with a high concentration of inositol. This was accomplished by allowing the mycelium to grow partially across a solid medium overlaid with dialysis tubing (Fig. 1A). The dialysis membrane with the mycelium growing on its surface then was transferred to a medium supplemented with a high concentration of unlabeled and radioactive inositol (111.1 μM). About 3.2 and 5.6 nmol of inositol per mg (dry weight) were incorporated by the *inl* mycelium at 2 and 4 h, respectively, after transfer. This incorporation occurred without a significant increase in mycelial dry weight (Table 3). The amount of inositol incorporation at 4 h represented 40% of the total inositol incorporated after 16 h.

Specific areas of the mycelium showed preferential incorporation of [2-^{3}H]inositol. After transfer, the older mycelial areas incorporated 30 to 40% more [2-^{3}H]inositol than did the growing hyphal tips. However, after 16 h, the growing tips had incorporated 40 to 50% more [2-

TABLE 3. *Inositol repair: dry weights of inl and wild-type (RL3-8A) mycelia after transfer from a solid medium with limiting inositol (16.7 μM) to a medium with a high concentration of inositol (111.1 μM)*[a]

Time after transfer of mycelium (h)	Mycelial sample area[b]	mg (dry wt) per petri dish	
		89601 *inl*	RL3-8A
0	1	3.4	5.0
2	1	1.3	2.9
	2	2.0	2.9
4	1	1.8	2.9
	2	1.8	2.9
16	1	11.6	7.4
	2	12.8	11.1
	3	15.6	13.9

[a] Experimental details are given in the text and the legend to Fig. 1A.
[b] 1, Hyphal growing tips; 2 and 3, older hyphal areas.

TABLE 2. *Distribution of inositol-containing compounds extracted from the mycelium of the inl mutant*[a]

Inositol in medium (μM)	Mycelial sample area[b]	Time after inoculation (h)	Total extractable [2-^{3}H]inositol (%) in following compound:				nmol of extractable [2-^{3}H]inositol per mg (dry wt)
			IP + I[c]	$(IP)_2C$	IPC	PI	
16.7	1, 2, 3	24	14.6	37.3	8.9	38.8	4.4
111.1	1, 2, 3	24	15.1	29.0	4.6	51.0	10.7
16.7	1	39	16.7	38.9	16.7	27.7	3.6
	2		13.9	41.7	13.9	30.5	3.6
	3		13.3	36.7	16.7	33.3	3.0
111.1	1	39	20.5	29.0	9.7	40.8	9.3
	2		18.2	35.5	5.0	41.4	9.9
	3		18.6	27.9	2.3	51.2	8.6

[a] Values are the average of two experiments. Experimental details are given in the text.
[b] 1, hyphal growing tips; 2 and 3, older hyphal areas.
[c] IP, inositol phosphate; I, inositol.

^{3}H]inositol than had any of the older mycelial areas.

The distribution of inositol-containing lipids in specific areas of the mycelium was examined after a 2-h pulse with [2-^{3}H]inositol and also after a 2-h pulse followed by a 2-h chase with nonradioactive inositol (Table 4). In the *inl* mutant grown on both limiting and high concentrations of inositol, the distribution of [2-^{3}H]inositol in lipids was similar in the growing tips and older hyphal areas. There appeared to be no preferential incorporation of [2-^{3}H]inositol into a specific lipid by the *inl* mutant grown on either a limiting or a high concentration of inositol (Table 4).

The distribution of [2-^{3}H]inositol incorporated into lipid changed after the mycelium was chased by a 2-h exposure to nonradioactive inositol (Table 4). The predominant lipids in the growing tips were PI after the pulse and $(IP)_2C$ after the pulse-chase. Lester et al. (22) and Brennan and Lösel (5) suggested that $(IP)_2C$ is synthesized from PI; this is consistent with about 40% of [2-^{3}H]inositol in PI being chased into $(IP)_2C$ (Table 4). In the region of the *inl* mycelium most limited in inositol, the PI remained the predominant lipid and the [2-^{3}H]inositol did not chase into $(IP)_2C$ (Table 4). The PI levels may be so reduced that little is available for synthesis of $(IP)_2C$.

Inositol repair and mycelial translocation. The intracellular translocation of inositol was measured to determine whether inositol from the growing tips could reverse the effects of limiting inositol in the older mycelial regions. This movement was monitored by measuring the migration of [2-^{3}H]inositol from one area of the mycelium to another. The backward translocation of [2-^{3}H]inositol from the growing tips to the older mycelial areas was measured in the *inl* mycelium (Table 5). Of the total [2-^{3}H]inositol incorporated at the growing tips, 5 and 2.5% translocated backward to the inositol-limited (16.7 μM) and control (111.1 μM) mycelial areas, respectively. The individual [2-^{3}H]inositol-containing compounds in the older regions of the mycelium were not analyzed because of the low level of translocation.

The forward translocation of [2-^{3}H]inositol from the older mycelial areas to the growing hyphal tips also was measured (Table 5). Of the total incorporated [2-^{3}H]inositol in the older mycelial areas grown on limiting (16.7 μM) and high (111.1 μM) concentrations of inositol, 10 and 16%, respectively, were translocated forward to the growing tips. In the *inl* mutant grown initially on limiting inositol, 38 and 37.3% of the translocated [2-^{3}H]inositol were found in $(IP)_2C$ and PI, respectively, whereas in the mutant grown on sufficient inositol, 45.2 and 31.2%, respectively, of the [2-^{3}H]inositol were found in these compounds (Table 6).

The inositol-supplemented mutant translocated about sevenfold more inositol toward rather than away from the growing tips, whereas the inositol-limited mutant translocated only about twofold more inositol in the same direction (Table 5).

DISCUSSION

In this study, two strategies were used to try to reverse the cellular and chemical changes

TABLE 4. *Inositol repair: distribution of [2-^{3}H]inositol in inositol-containing compounds extracted from the inl mycelium after a 2-h pulse with [2-^{3}H]inositol and a 2-h chase with nonradioactive inositol*[a]

Inositol in medium (μM)[b]	Type of labeling with [2-^{3}H]inositol	Mycelial sample area[c]	Total extractable [2-^{3}H]inositol (%) in:			
			IP + I[d]	$(IP)_2C$	IPC	PI
16.7	Pulse	1	11.8	32.2	10.1	43.8
		2	12.4	37.5	7.0	43.3
16.7	Chase	1	8.4	52.7	8.9	30.8
		2	6.5	38.8	10.8	44.5
111.1	Pulse	1	8.9	25.5	7.3	57.5
		2	11.5	25.5	4.3	58.2
111.1	Chase	1	3.3	60.1	1.8	36.6
		2	2.8	55.6	4.7	36.1

[a] Values are the average of three experiments. Experimental details are given in the text.

[b] Inositol concentration of the growth medium before transfer of *inl* mycelium to medium with 111.1 μM inositol during the pulse and pulse-chase.

[c] 1, Hyphal growing tips; 2, older hyphal areas.

[d] IP, Inositol phosphate; I, inositol.

TABLE 5. *Mycelial translocation: forward and backward translocation of [2-^{3}H]inositol between the areas of the mycelium growing on either a limiting (16.7 μM) or a high (111.1 μM) concentration of inositol*[a]

Inositol in medium (μM)[b]	Mycelial sample area[c]	Total extractable [2-^{3}H]inositol (%) incorporated by the entire mycelium	
		Forward translocation	Backward translocation
16.7	1	10.2	95.2
	2	44.9	2.8
	3	44.9	2.0
111.1	1	15.8	97.5
	2	34.7	1.4
	3	49.5	1.1

[a] Forward translocation is from the older hyphal areas to the growing tips, and backward translocation is from the growing tips to the older hyphal areas. Values are the average of three experiments, and experimental details are given in the text and the legend to Fig. 1B and C.

[b] Inositol concentration of growth medium at point of inoculation.

[c] 1, Hyphal growing tips; 2 and 3, older hyphal areas.

TABLE 6. *Mycelial translocation: distribution of [2-^{3}H]inositol in inositol-containing lipids translocated forward to the hyphal growing tips*[a]

Inositol in medium (μM)[b]	Mycelial sample area[c]	Total extractable [2-^{3}H]inositol (%) in:			
		IP + I[d]	$(IP)_2C$	IPC	PI
16.7	1	15.4	38.0	9.3	37.3
	2	13.8	45.4	10.2	30.6
	3	12.4	44.9	11.3	31.4
111.1	1	14.6	45.2	9.0	31.2
	2	12.5	26.8	7.2	53.5
	3	14.5	26.5	6.4	52.6

[a] Values are the average of three experiments. Experimental methods are described in the text and the legend to Fig. 1B and C.

[b] Inositol concentration of the growth medium at point of inoculation.

[c] 1, Hyphal growing tips; 2 and 3, older hyphal areas.

[d] IP, Inositol phosphate; I, inositol.

which occur during inositol-limited growth. First, agents such as NaCl and sorbitol, which raise the osmolality of solutions, were added to the growth medium in an attempt to prevent cell leakage during inositol-limited growth. Raising the osmolality of the growth medium did reduce the release of protein; however, it did not reverse the abnormal mycelial morphology or restore the growth rate. Munkres and co-workers also have tried to use protective agents added to the growth medium to reduce or reverse the effect of inositol-limited growth. They found that exposure of the *inl* mutant to antioxidants such as nordihydroguariaretic acid reduces cell leakage (31) and lipid peroxidation (26, 30) as measured by the formation of malondialdehyde in isolated mitochondria.

Second, high concentrations of inositol were supplied to the *inl* mutant in an attempt to reverse the effects of inositol-limited growth. The studies showed that after the mutant was transferred to a medium supplemented with sufficient inositol for normal growth, there was a rapid incorporation of inositol, raising the cellular inositol to normal levels (8 to 11 nmol/mg [dry weight]) and returning the mutant to normal mycelial morphology and growth. This phenomenon has been named "inositol repair." This repair process occurred throughout the entire mycelium at both the growing hyphal tips and the older areas behind the tips.

Translocation of inositol from the growing hyphal tips to the older inositol-depleted mycelial areas does not appear to be a major mechanism for reversing the effects of inositol limitation since the predominant direction of translocation was in the opposite direction. This direction is the same as for cytoplasmic streaming, i.e., toward the growing tips (40). The most direct interpretation of the data is that inositol or inositol phosphate is translocated by the movement of the cytoplasm toward the growing tips. Inositol or inositol phosphate then is incorporated into PI from which the other inositol-containing lipids are synthesized (4). Inositol also may be translocated by exchange of lipid head groups. For example, choline rapidly exchanges into phosphatidylcholine in choline-deficient mycelia of *Neurospora* (16). An analogous situation may occur with inositol, wherein the inositol incorporated in the mycelium is rapidly exchanged into PI.

The predominant lipid in the growing tips which incorporates [2-^{3}H]inositol from the older mycelial areas was $(IP)_2C$ rather than PI. The translocated [2-^{3}H]inositol probably is not preferentially incorporated into $(IP)_2C$; instead, nonradioactive inositol incorporated by the growing tips chases the translocated [2-^{3}H]inositol from PI into $(IP)_2C$. This is suggested by the fact that the distribution of [2-^{3}H]inositol-containing lipids was similar for growing tips which have translocated [2-^{3}H]inositol (Table 6) and for the mycelium in which the [2-^{3}H]inositol after a 2-h pulse has been chased for 2 h with nonradioactive inositol in the mycelium (Table 4). The fact that $(IP)_2C$ was not the predominant lipid synthesized in the growing tips of a mycelium uniformly labeled with [2-^{3}H]inositol would also suggest that $(IP)_2C$ is not synthesized preferentially (Table 2).

What remains unclear from this and other studies is why inositol limitation in the *inl* mutant results in the drastic cellular and chemical changes which eventually lead to cell death. Matile and others have suggested that inositol limitation in the mutant causes membrane deterioration (9, 25, 26, 30–32, 34). Most of this work has been done with cells in the stationary phase of growth; this type of cell may show the effects of both inositol limitation and the stationary phase of growth. Studies on the phospholipid composition of choline- and inositol-requiring mutants of *Neurospora* actually suggest that in logarithmic-phase cells the membranes remain intact and that there are compensatory mechanisms for conserving phospholipid membrane charge and content (16). This work showed that a decrease in the content of acidic PI is compensated for by an increase in another acidic phospholipid, phosphatidylserine. As a result, the total phospholipid content and charge were conserved. Similar findings were subsequently reported for inositol starvation in *Saccharomyces cerevisiae* (4, 13). Studies by Hanson and Lester (13) with yeast also showed that a dramatic drop in cell wall glucan and mannan synthesis occurred before any changes in general phospholipid content and synthesis. These results suggest that a shutoff of cell wall synthesis preceded any general membrane deterioration except for a reduction in PI synthesis and that the inhibition of cell wall synthesis may be important to the onset of inositolless death. Significant membrane deterioration occurred, but only after the cessation of cell wall synthesis.

The auxotrophic lipid mutants of *Neurospora* are ideal for experiments such as those discussed above. These mutants make possible extensive manipulation of phospholipid composition without drastic variation of growth conditions, a situation unique among eucaryotic systems (16). In addition, one can measure the spatial distribution of phospholipids within the mycelium, as well as the overall lipid composition. Such studies can be done only in the myceliated fungi. These advantages make these mutants an ideal tool for studying the mechanism of inositol-limited growth and intramycelial transport of inositol-containing lipids.

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