Effects of inositol starvation on the levels of inositol phosphates and inositol lipids in *Neurospora crassa*

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An inositol-requiring strain of *Neurospora crassa* was labelled during growth in liquid medium with [^3H]inositol, and the levels of inositol phosphates and phosphoinositides were determined under inositol-sufficient and inositol-starved conditions. Because the mutant has an absolute requirement for inositol, the total mass of inositol-containing compounds could be determined. Inositol-containing lipids were identified by deacylation and co-migration with standards on h.p.l.c.; PtdIns3P, PtdIns4P, and PtdIns(4,5)P₂ were found in approximately equal amounts, in addition to large amounts of PtdIns. Inositol starvation decreased the level of PtdIns to 10% of the sufficient level, and decreased the levels of the other phosphoinositides to about 25%. A number of inositol phosphates were found, including several

InsP₃s, InsP₄s and InsP₅s and phytic acid. Ins(1,4,5)P₃ was identified by co-migration with standards on h.p.l.c. and by digestion with inositol phosphomonoesterase. High concentrations of all inositol phosphates were found in the extracellular medium in inositol-starved cultures. Inositol starvation on both liquid and solid agar media decreased the intracellular levels of some inositol phosphates, but increased the levels of phytic acid and several other inositol phosphates which may be its precursors and/or breakdown products. These results may indicate that inositol starvation induces phytic acid synthesis as a protection against the free-radical production and lipid peroxidation characteristic of inositol-less death.

INTRODUCTION

The phosphoinositide signalling system which is well-characterized in animal cells (Berridge, 1987) has also been shown to operate in several eukaryotic micro-organisms, including *Dictyostelium* (Firtel, 1991), *Chlamydomonas* (Musgrave et al., 1992; Quarumby et al., 1992), and yeast (Kaibuchi et al., 1986; Uno and Ishikawa, 1990). Many of the components of this signalling pathway are present in the filamentous fungus *Neurospora crassa*: phosphoinositides (including PtdInsP₂), inositol phosphates [including Ins(1,4,5)P₃] and appropriate enzymes such as inositol phosphate phosphatases and phosphoinositide kinases (Hanson, 1991). A Ca²⁺- and phospholipid-dependent protein kinase similar to protein kinase C has also been described in *Neurospora* (Turian and Favre, 1990). In addition, it has been demonstrated that Ins(1,4,5)P₃ will release Ca²⁺ from isolated organellar stores (Cornelius et al., 1989). However, the activation of this pathway in response to an extracellular signal has not yet been reported in *Neurospora*.

I have been investigating the possibility that the phosphoinositide signalling pathway may be activated by blue light in *Neurospora*. Blue light has a number of physiological effects in this organism, including resetting the phase of the circadian oscillator. I have found that inositol depletion of an inositol-requiring mutant growing on solid agar medium alters the response of the circadian oscillator to light (Lakin-Thomas, 1992). Contrary to predictions, however, inositol depletion does not decrease sensitivity to light but rather increases sensitivity. In the present paper I report the effects of inositol depletion on the levels of the putative components of the signalling pathway in an attempt to explain these unexpected results.

MATERIALS AND METHODS

Materials

myo-[2- ^3H]inositol was purchased from Amersham International

(Amersham, Bucks., U.K.), D-[1- ^3H]Ins(1,4,5)P₃ was purchased from New England Nuclear/du Pont (Stevenage, Herts., U.K.), and [^3H]Ins(1,3,4,5)P₄ was provided by Dr. L. R. Stephens, AFRC Biochemistry Department, Babraham, Cambridge, U.K. Partisphere 5 SAX columns for h.p.l.c. were purchased from Whatman International, Maidstone, Kent, U.K.

Culture and labelling methods

The strain used was *bd csp inl*, as described in Lakin-Thomas (1992). Stocks were maintained on solid-agar slant cultures [Vogel's medium N with 2% (w/v) glucose, 2% (w/v) agar and 50 µg/ml (278 µM) *myo*-inositol]. A liquid culture system was developed to allow either biochemical or chronobiological analyses [the following paper (Lakin-Thomas, 1993)] to be performed on cultures under identical growth conditions. Cultures were inoculated from conidial suspensions into liquid medium [Vogel's medium N with 2% (w/v) glucose, either 25 µM or 200 µM unlabelled *myo*-inositol and *myo*-[^3H]inositol at 10 or 20 µCi/ml], and 1 ml aliquots were incubated in microtitre wells (15 mm diameter). Inoculation conditions were standardized by preparing conidial suspensions in the same volume from stock cultures of the same age. After 48 h under white light (21 µmol·s⁻¹·m⁻² in the range 400–700 nm) at 22 °C, cultures were transferred to constant darkness at 22 °C. At 77 h after inoculation (29 h after the light-to-dark transition) the mycelial discs were removed from the microtitre wells and processed under a red safelight. For analysis of inositol phosphates in cultures at 25 µM inositol, discs were washed with 50–100 ml of fresh unlabelled medium to remove extracellular radioactivity. For all experiments, discs were frozen in liquid N₂ and stored at –70 °C.

The final levels of inositol in the liquid growth medium at the time of harvesting were determined as described below in the Results section. The final free inositol concentration was found to be about 5 µM for cultures inoculated at 25 µM and about

73 μM for cultures inoculated at 200 μM . The initial high level of inositol (200 μM) is enough to fully supplement the cultures; the dry weight of cultures inoculated at 200 μM is the same at the time of harvesting as cultures inoculated at 2 mM inositol, while the dry weight of cultures inoculated at 25 μM inositol is about 60 % of the dry weight of 200 μM cultures.

For some experiments (Tables 1b and 2b below) cultures were grown on solid agar medium [Vogel's medium N with 0.5 % (w/v) maltose, 2 % (w/v) agar, 0.01 % (w/v) arginine and *myo*-[^3H]inositol at various concentrations as required]. A 10 ml portion of medium was used per 90 mm-diameter Petri dish, and the solidified medium was overlain with a circle of a single layer of dialysis tubing sterilized by autoclaving. Cultures were inoculated on top of the dialysis tubing at a single point with conidia from stock slant cultures, exposed to white light (21 $\mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ in the range 400–700 nm) at 22 °C for 24 h and transferred to constant darkness at 22 °C. Mycelia were harvested from the dialysis tubing under red safelight after either 29 h in darkness ('2-day' cultures) or 97 h in darkness ('5-day' cultures). Cultures at 25 and 250 μM inositol formed a single band of conidiation in the middle of the dark period, and only the youngest region between the end of the band and the growth front was harvested. Cultures at 7.5 μM inositol did not conidiate, and because the total mass produced was low compared with 25 μM and 250 μM inositol, the entire culture was harvested. In all cases, the harvested mycelia were immediately frozen in liquid N_2 .

Because the *inl* mutant has an absolute requirement for exogenous inositol, and because the cultures on both solid and liquid medium were labelled during growth from a negligibly small inoculum, essentially all cellular inositol was derived from the exogenous pool and the total mass of inositol-containing compounds could be determined. The specific activity of the [^3H]inositol in the cultures was calculated from the radioactivity in the medium and the molarity of the unlabelled inositol, and this value was used to calculate the total mass (in pmol) of inositol-labelled compounds in the extracts.

Inositol lipid analysis

Frozen mycelia (approx. 2–6 mg dry wt.) were pulverized in liquid N_2 with a pre-cooled mortar and pestle and transferred frozen to tubes containing 1.5 ml of chloroform/methanol (1:2, v/v) at 0 °C. Extraction was exactly as described by Boss (1989). The aqueous phase was discarded and the debris was washed with methanol and dried at 60 °C overnight for determination of residual dry weight (RDW). For comparison with the assays of inositol phosphates, which are reported per mg dry weight, four unlabelled samples grown in liquid medium were freeze-dried, weighed and extracted as described above and the RDWs after extraction were found to be about 70 % of the dry weights before extraction.

The combined organic phases were dried under N_2 and deacylated as described by Hawkins et al. (1986). The aqueous fraction after deacylation was freeze-dried, and the glycerophosphoinositol phosphates were redissolved in water and analysed by h.p.l.c. using a 25 cm Partisphere 5 SAX column and a gradient of 1.25 M $(\text{NH}_4)_2\text{HPO}_4$, pH 3.8, as described by Brearley and Hanke (1992) and Stephens et al. (1989). Samples contained 1 mM EDTA and 2.5 μM each ATP, ADP and AMP as standards. Radioactivity in h.p.l.c. fractions was determined by liquid-scintillation counting. The organic fraction after deacylation was dried under vacuum, and the total radioactivity in this fraction was determined as a measure of inositol-

containing lipids other than phosphoinositides, as reported in Table 1.

Inositol phosphate analysis

Frozen mycelia (approx. 8–16 mg dry wt. for liquid cultures and 1–5 mg dry wt. for solid cultures, based on phytic acid content as described below) were pulverized in liquid N_2 and transferred frozen to tubes containing 300 μl of 10 % (v/v) HClO_4 at 0 °C. Samples were extracted at 0 °C for 10 min and centrifuged. The supernatant was neutralized with 2 M KOH/0.1 M Mes/20 mM Na_2EDTA as described by Stephens (1990) with the addition of 5 μl phytate hydrolysate (0.8 mg of *P* per ml) prepared by Dr. Charles Brearley by the method of Wreggett et al. (1987). Inositol phosphates were analysed by h.p.l.c. on a Partisphere SAX column using a linear gradient of 0–100 % 2.5 M NaH_2PO_4 (Stephens and Irvine, 1990). Samples contained 1 mM EDTA and 2.5 μM each ATP, ADP and AMP as standards. Radioactivity in h.p.l.c. fractions was determined by liquid-scintillation counting.

Endogenous phytic acid (InsP_6) was used as an internal control in each sample, and the levels of the other inositol phosphates were calculated relative to the phytic acid peak. To quantify the amount of phytic acid per mg dry weight, samples of labelled frozen mycelia were freeze-dried, weighed, pulverized with a mortar and pestle while dry, extracted with HClO_4 and analysed by h.p.l.c. Three samples grown in 25 μM and four samples grown in 200 μM inositol were assayed for phytic acid per mg dry weight for liquid cultures and the mean values are included in Table 2(a) as the InsP_6 levels; one culture each at 7.5 μM , 25 μM and 250 μM inositol on solid medium were assayed and are included in Table 2(b). These estimates of phytic acid per mg dry weight were used to calculate the amounts (pmol) of other inositol phosphates per mg dry weight in samples which were grown at the same inositol concentrations but which were neither freeze-dried nor weighed before extraction. This method of calculating inositol phosphate levels relative to InsP_6 was chosen for two reasons: (1) to allow the use of InsP_6 as an internal control to compensate for any differences in extraction efficiency and recovery between experiments designed to look for relatively small changes in the levels of the other inositol phosphates [as in the following paper (Lakin-Thomas, 1993)] and (2) to allow the extraction of frozen tissue without the danger of thawing during weighing or during the freeze-drying process. The validity of this calculation relies on the assumption that the levels of phytic acid do not change as a result of freeze-drying. To test this assumption, a sample of frozen mycelium grown in liquid medium at 200 μM was divided into two, and half was pulverized in liquid N_2 while the other half was pulverized after freeze-drying. Both samples were extracted with HClO_4 and analysed by h.p.l.c., and the profiles of inositol phosphate peaks were very similar relative to their respective InsP_6 peaks. This result indicates that the relative levels of inositol phosphates do not change on freeze-drying, and it seems unlikely that the total levels of all inositol phosphates would change in parallel.

RESULTS

Identification of phosphoinositides

Lipid extracts of frozen mycelial discs were initially analysed by the h.p.l.c. method of Coté et al. (1989). At least three peaks were found in the PtdIns region, and small peaks ran in the positions expected for PtdInsP and PtdInsP_2 (results not shown). A sample of authentic [^{32}P] PtdInsP_2 (generously provided by Dr.

Table 1 Effects of (a) inositol starvation on the levels of inositol lipids in liquid culture and (b) inositol depletion on the levels of inositol lipids on solid medium

(a) Extracted lipids of mycelia were deacylated, and the water-soluble deacylation products were analysed by h.p.l.c. as described in Figure 1. Levels of phosphoinositides were determined from the areas of the peaks identified in Figure 1. Values for other inositol-containing lipids were calculated from the radioactivity remaining in the organic fraction after deacylation. Values reported are means $\pm$ S.E.M. for three samples. *Significantly different from 200 μ M at the $P = 0.05$ level of significance in a two-tailed unpaired t test; **significantly different from 200 μ M at the $P = 0.01$ level of significance. (b) Extracted lipids of mycelia were deacylated and the water-soluble deacylation products were analysed by h.p.l.c. as in Figure 1. Levels of phosphoinositides were determined from the areas of the peaks identified in Figure 1. Values for other inositol-containing lipids were calculated from the radioactivity remaining in the organic fraction after deacylation. Values are reported from two experiments: in the first, samples were pulverized and extracted while frozen and the RDW was determined after extraction; in the second, samples were freeze-dried and weighed before extraction and RDW was estimated from dry weight. In both experiments, cultures were harvested after 2 days of growth.

Inositol lipid	[Inositol] (μ M) ...	Composition (pmol/mg RDW)	
		200	25
PtdIns		2500 $\pm$ 560	240 $\pm$ 46*
PtdIns3P		29.5 $\pm$ 4.2	7.3 $\pm$ 1.0**
PtdIns4P		29.5 $\pm$ 7.3	5.0 $\pm$ 2.0*
PtdIns(4,5)P ₂		24.3 $\pm$ 5.1	6.9 $\pm$ 1.0*
Other		408 $\pm$ 55	136 $\pm$ 36*

Inositol lipid	[Inositol] (μ M) ...	Composition (pmol/mg RDW)	
		250	25
PtdIns		4823, 4021	3765, 3810
PtdIns3P		44.6, 47.6	24.8, 26.0
PtdIns4P		35.6, 23.0	39.2, 29.6
PtdIns(4,5)P ₂		6.2, 3.8	12.0, 5.1
Other		502, 483	898, 600

L. R. Stephens) co-migrated with the putative PtdInsP₂ peak. Individual peaks from a separation of a lipid extract of mycelia grown at 25 μ M inositol were dried and deacylated. Greater than 95 % of the radioactivity in the PtdInsP₂ peak, and about 90 % and 60 % of the radioactivity in the PtdIns and PtdInsP peaks respectively, was found in the aqueous phases. This indicates that inositol-containing lipids resistant to deacylation co-migrated with PtdIns and PtdInsP, but not PtdInsP₂. These resistant lipids are likely to be members of the three classes of inositol-containing sphingolipids which have been found in *Neurospora* (Lester et al., 1974; Hanson and Lester, 1980).

When the deacylation products were analysed by h.p.l.c. using the method of Stephens et al. (1989), the deacylation product of the putative PtdInsP₂ was found to co-migrate with a sample of authentic [³²P]GroPIns(4,5)P₂ and to be eluted after a sample of authentic [³²P]GroPIns(3,4)P₂, both prepared from *Spirodela* by Dr. Charles Brearley (Brearley and Hanke, 1992) (GroP = glycerophospho). The deacylation products of the putative PtdInsP peak were resolved into two peaks which co-migrated with authentic [³²P]GroPIns3P and [³²P]GroPIns4P, and the deacylation product of the putative PtdIns co-migrated with authentic [³²P]GroPIns, also prepared by Dr. Charles Brearley. A typical h.p.l.c. separation of the deacylation products of a whole lipid extract is shown in Figure 1.

Table 2 Effects of inositol starvation on the levels of inositol phosphates (a) in liquid culture and (b) on solid medium

(a) HClO₄ extracts of mycelia were analysed by h.p.l.c. as described in Figure 2. Levels of inositol phosphates were calculated from the areas of the peaks designated by letters in Figure 2. Levels of inositol monophosphates (peaks A and B) could not be determined in these samples owing to the presence of these compounds in the extracellular medium. InsP₆ levels per mg dry weight were determined in freeze-dried samples, which were weighed before extraction. All other inositol phosphates were determined in samples which were not freeze-dried or weighed before extraction, and their levels are calculated relative to the mean InsP₆ level. Values reported are means $\pm$ S.E.M. (n). N.D., not detected. **Significantly different from 200 μ M at the $P = 0.01$ level of significance in a two-tailed unpaired t test. (b) HClO₄ extracts of mycelia were analysed by h.p.l.c. as described in Figure 2. Levels of inositol phosphates were calculated from the areas of the peaks designated by letters in Figure 2. InsP₆ levels per mg dry weight were determined in freeze-dried samples which were weighed before extraction. For cultures at 25 μ M and 250 μ M inositol, all other inositol phosphates were determined in samples which were neither freeze-dried nor weighed before extraction, and their levels are calculated relative to the mean InsP₆ level. For cultures at 7.5 μ M, all values were determined from freeze-dried samples. The number of days of growth before harvesting is indicated in parentheses next to the concentration of inositol in the medium. Values reported are from single experiments. N.D., not detected.

Peak	Identity	[Inositol] (μ M) ...	Composition (pmol/mg dry wt.)	
			200	25
C + D	InsP ₂		2.8 $\pm$ 0.4 (5)	2.3 $\pm$ 0.1 (4)
E + F	InsP ₃		3.7 $\pm$ 1.3 (6)	1.2 $\pm$ 0.1 (4)
G	InsP ₃		0.30 $\pm$ 0.06 (6)	1.9 $\pm$ 0.3 (4)**
H	Ins(1,4,5)P ₃		0.57 $\pm$ 0.16 (6)	0.30 $\pm$ 0.05 (4)
I	InsP ₃		2.2 $\pm$ 0.5 (6)	1.5 $\pm$ 0.1 (4)
J	InsP ₄		0.24 $\pm$ 0.02 (5)	0.78 $\pm$ 0.08 (4)**
K	InsP ₄		N.D.	0.09 $\pm$ 0.03 (4)
L	InsP ₄		0.30 $\pm$ 0.07 (5)	0.32 $\pm$ 0.02 (4)
M	InsP ₅		0.17 $\pm$ 0.03 (5)	1.0 $\pm$ 0.1 (4)**
N	InsP ₅		0.14 $\pm$ 0.02 (5)	0.73 $\pm$ 0.06 (4)**
O	InsP ₅		0.30 $\pm$ 0.04 (5)	1.1 $\pm$ 0.04 (4)**
P	InsP ₆		9.9 $\pm$ 0.7 (4)	27.9 $\pm$ 4.1 (3)**
Q	InsP ₇		0.54 $\pm$ 0.07 (4)	1.1 $\pm$ 0.03 (3)**

Peak	Identity	[Inositol] (μ M) ...	Composition (pmol/mg dry wt.)			
			250 (2)	25 (2)	7.5 (2)	7.5 (5)
A	InsP		365	224	102	6.4
B	InsP		204	161	65.6	15.4
C + D	InsP ₂		9.3	6.5	4.1	2.0
E + F	InsP ₃		2.6	1.1	0.32	0.08
G	InsP ₃		1.3	1.5	2.2	1.2
H	Ins(1,4,5)P ₃		7.1	6.1	8.3	1.5
I	InsP ₃		4.0	3.3	2.5	0.67
J	InsP ₄		2.6	2.6	2.1	0.84
K	InsP ₄		N.D.	0.14	0.12	0.15
L	InsP ₄		0.70	0.56	0.52	0.56
M	InsP ₅		2.1	1.6	1.6	2.1
N	InsP ₅		1.2	1.6	1.4	2.0
O	InsP ₅		0.73	0.92	1.3	4.4
P	InsP ₆		49.2	44.5	33.6	76.1
Q	InsP ₇		2.1	2.2	2.1	10.8

Effects of inositol starvation on the levels of inositol lipids

The levels of inositol-containing lipids were compared in cultures grown at either 25 μ M or 200 μ M inositol in liquid medium, and the results are summarized in Table 1(a). The level of PtdIns was

Figure 1 H.p.l.c. separation of deacylated inositol lipids

[^3H]inositol-labelled glycerophosphoinositol phosphates from a culture (2.3 mg RDW) grown in liquid medium at $25\ \mu\text{M}$ inositol ($10\ \mu\text{Ci/ml}$) were separated on a Partisphere SAX column with a gradient of $(\text{NH}_4)_2\text{HPO}_4$, as described in the Materials and methods section. The flow rate was $1\ \text{ml/min}$; $0.5\ \text{min}$ fractions were collected from 15 to 35 min, and $1\ \text{min}$ fractions were collected at other times. The positions of ^{32}P -labelled standards are indicated. The inset shows the same separation on a different scale.

reduced in the $25\ \mu\text{M}$ cultures to about one-tenth the level on $200\ \mu\text{M}$ inositol. The three phosphorylated phosphoinositides, $\text{PtdIns}3\text{P}$, $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$, were found in approximately equal amounts, and the levels of all three were reduced on inositol starvation to about a quarter of their levels on high inositol. The levels of other inositol-containing lipids were estimated from the radioactivity remaining in the organic fraction after deacylation. It is assumed that this radioactivity represents mainly the inositol-containing sphingolipids, and this fraction also declined on inositol starvation to about one-third of its level on high inositol.

Table 1(b) presents data from two experiments on solid medium, which indicate that $25\ \mu\text{M}$ inositol did not lead to severe depletion of the inositol lipids compared with $250\ \mu\text{M}$. Levels of $\text{PtdIns}3\text{P}$ decreased by about half, and levels of PtdIns decreased slightly, but levels of the remaining lipids remained the same or increased on $25\ \mu\text{M}$ as compared with $250\ \mu\text{M}$ inositol. Note also that the level of PtdInsP_2 was substantially lower on solid medium at $250\ \mu\text{M}$ inositol as compared with liquid medium at $200\ \mu\text{M}$ inositol.

Inositol metabolites in the extracellular medium

HClO_4 extracts of frozen mycelial discs grown in liquid medium were analysed by h.p.l.c. using a linear gradient of NaH_2PO_4 , and a number of peaks were resolved, as shown in Figure 2. Initial work was done with unwashed mycelial samples grown at $25\ \mu\text{M}$ inositol, and several of the peaks were found to be highly variable between samples. The used growth medium was sampled after harvesting the mycelial discs and was extracted and analysed by h.p.l.c. All of the peaks found in mycelial extracts were also found in the medium, to greater or lesser extents. The amount of growth medium carried along when the mycelial discs were harvested was an uncontrolled variable in these initial

Figure 2 H.p.l.c. separation of inositol phosphates

The HClO_4 extract from a [^3H]inositol-labelled culture (3.5 mg dry weight) grown in liquid medium at $25\ \mu\text{M}$ inositol ($10\ \mu\text{Ci/ml}$) was separated on a Partisphere SAX column with a gradient of NaH_2PO_4 , as described in the Materials and methods section. The flow rate was $1\ \text{ml/min}$, and $0.5\ \text{min}$ fractions were collected. The positions of standards are indicated. The inset shows the same separation on a different scale. Note that the mycelial sample was not washed after harvesting, and therefore both intracellular and extracellular inositol metabolites are present.

experiments, and accounts for the variable levels of some of the peaks in unwashed samples. The various inositol phosphates were not equally represented in the medium as compared with the mycelial extracts: peaks H [$\text{Ins}(1,4,5)\text{P}_3$], L and C+D were relatively enriched in the medium, while relatively little of peaks G, I, J and M–Q (including InsP_6) were found extracellularly. The amount of contaminating medium in unwashed samples was estimated by briefly incubating unlabelled discs in labelled medium at 0°C before freezing and extracting for inositol phosphate analysis, and it was calculated that, in unwashed mycelial samples, as much as 50% of the radioactivity in the $\text{Ins}(1,4,5)\text{P}_3$ peak could be contributed by extracellular contamination. Cultures grown at $25\ \mu\text{M}$ inositol were therefore washed with fresh unlabelled medium before freezing and extraction. This wash procedure was shown to remove greater than 95% of the extracellular radioactivity. A comparison of washed and unwashed samples at $25\ \mu\text{M}$ indicated that the difference between the two samples could be accounted for by the loss of extracellular material. That is, the washing procedure apparently did not induce any large changes in the intracellular levels of inositol phosphates. A sample of medium from cultures grown at $200\ \mu\text{M}$ inositol contained significant radioactivity in the free inositol, InsP and GroPIns regions, but no radioactivity was found co-migrating with later peaks. The washing procedure was found to interfere with the chronobiological analyses reported elsewhere [the following paper (Lakin-Thomas, 1993)], and therefore samples grown at $200\ \mu\text{M}$ were not washed after harvesting.

The levels of free inositol remaining in the growth medium at the time of harvesting were estimated by assuming that the first peak of radioactivity eluted from the SAX column is free inositol. The final levels of free inositol in samples of used growth medium were found to be about $5\ \mu\text{M}$ in cultures inoculated at $25\ \mu\text{M}$, and about $73\ \mu\text{M}$ in cultures inoculated at $200\ \mu\text{M}$. Cultures

inoculated at 25 μ M and at 200 μ M will be referred to as '25 μ M' or '200 μ M' cultures, although it should be remembered that the actual inositol concentrations at the time of analysis were lower.

A sample of used growth medium from a 25 μ M culture was freeze-dried, subjected to the lipid extraction procedure and analysed by the h.p.l.c. method of Coté et al. (1989). No PtdIns P_2 was found, and only trace amounts of radioactivity co-migrated with PtdIns or PtdIns P . It was calculated that less than 0.1 % of the PtdIns and less than 2 % of the PtdIns P in the unwashed mycelial extracts could be accounted for by contamination from the growth medium.

Identification of inositol phosphates

A typical h.p.l.c. separation of inositol phosphates is presented in Figure 2. Peak P was identified as phytic acid (Ins P_6) by co-migration with a 32 P-labelled standard isolated from *Spirodela polyrrhiza* L. by Dr. Charles Brearley. Peaks H, J and M were tentatively identified as Ins(1,4,5) P_3 , Ins(1,3,4,5) P_4 , and Ins(1,3,4,5,6) P_5 by co-migration with 3 H-labelled authentic standards.

The identity of Ins(1,4,5) P_3 was confirmed by two criteria: (1) the peak co-migrated with the standard on an isocratic h.p.l.c. separation at 550 mM NaH $_2$ PO $_4$ (results not shown); (2) the putative Ins(1,4,5) P_3 peak from an isocratic separation of a 3 H-labelled mycelial extract was desalted by the method of Stephens et al. (1988) and incubated with the inositol trisphosphate 5-phosphomonoesterase activity of human erythrocyte ghosts in the presence of MgCl $_2$ by the method of Stephens et al. (1991). The incubations were extracted with HClO $_4$ as described above and analysed by the isocratic h.p.l.c. method. The fraction of the starting material remaining was determined at several time points until about 60 % of the starting material had been metabolized. An unlabelled sample of frozen mycelia was spiked with an authentic [3 H]Ins(1,4,5) P_3 standard and processed in parallel with the labelled sample. The two samples were metabolized at the same rate and to the same extent: after 30 min, 74 and 70 % remained of the standard and unknown respectively, and, at 150 min, 61 and 59 % remained. The products of the reactions had similar retention times in the isocratic h.p.l.c. separation.

Other tentative identifications of the peaks in Figure 2 are based on relative retention times: peaks A and B, Ins P ; C and D, two peaks of Ins P_2 which were not always clearly separated and were therefore pooled in the calculations for Table 2; E, F, G, H and I, at least six peaks of Ins P_3 (peaks E and F were not always clearly separated, and peak I could sometimes be resolved into two components); J, K and L, three peaks of Ins P_4 ; M, N and O, three peaks of Ins P_5 . Peak E was found to be highly variable in the 200 μ M samples grown in liquid medium: in four of six experiments it was found at a level higher than peak I, but in two experiments it was nearly absent and found at a level similar to peak G. A small peak which was eluted later than Ins P_6 (Peak Q) was consistently found and has been designated 'Ins P_y ' by analogy with the similar peak found in *Dictyostelium* (Europe-Finner et al., 1989).

Effects of inositol starvation on the levels of inositol phosphates

The effects of inositol starvation on the intracellular levels of inositol phosphates in cultures grown in liquid medium are summarized in Table 2(a). On low inositol, the levels of peaks H [Ins(1,4,5) P_3] and peak I were somewhat lower than on high inositol, but were not significantly different, and peaks C + D and L were about the same. Surprisingly, the levels of the remaining

peaks were significantly higher on low inositol: peaks J, O, P (Ins P_6) and Q were about 2–3-fold higher, and peaks G, M and N were 5–6-fold higher. Peak K was clearly present at 25 μ M, but was not detected at 200 μ M. Peaks E + F were too variable to evaluate accurately. The effects of inositol starvation on the levels of inositol monophosphates could not be determined due to the presence of these compounds in the medium in unwashed 200 μ M cultures.

The results of four experiments on solid medium are presented in Table 2(b) in order of increasing severity of inositol starvation. After 2 days of growth at 25 μ M inositol, the cultures showed some decline in the Ins P s and Ins P_2 s as compared with 250 μ M inositol, but little change in the other fractions, in agreement with the results of the lipid analysis (Table 1b), which indicated that these cultures were not severely starved for inositol. After 2 days at 7.5 μ M there was a greater decrease in these fractions, but the most dramatic effects were not seen until the cultures had aged for 5 days at 7.5 μ M: levels of Ins P s, Ins P_2 s, most Ins P_3 s, and peak J had substantially declined, peaks G, L, M and N remained about the same, while O, P and Q increased. These results confirm the observation that the levels of Ins P_6 and several other inositol phosphates increase on inositol starvation.

Inositol metabolites in the *bd csp* strain

The inositol-independent strain *bd csp* was grown in liquid medium with labelled inositol at 25 μ M, and both inositol-containing lipids and inositol phosphates were analysed as described above. The patterns of radioactive peaks were very similar to those of the *bd csp inl* strain on 200 μ M inositol (results not shown): the same peaks were found, and in the same relative proportions, indicating that none of the peaks found in the *bd csp inl* strain are artifacts of the *inl* mutation, and that 200 μ M inositol is sufficient to produce a wild-type phenotype with respect to the pattern of inositol labelling. The total mass of the inositol-labelled compounds in the *bd csp* strain could not be determined by this method owing to the unknown size of the unlabelled pool of endogenous inositol.

DISCUSSION

Identification of inositol lipids and phosphates

PtdIns P and PtdIns P_2 were previously reported in *Neurospora* by Hanson (1991), who identified them by co-migration with known standards on paper chromatography. The results reported here extend this work by identifying PtdIns(4,5) P_2 , and by resolving two PtdIns P s, which are likely to be PtdIns3 P and PtdIns4 P . The existence of 3-phosphorylated phosphoinositides has not previously been reported in *Neurospora*. No evidence of any PtdIns(3,4) P_2 or PtdIns(3,4,5) P_3 was found. The levels of the two PtdIns P s are similar, indicating that the metabolism of these lipids in *Neurospora* may differ from that in mammalian cells dual-labelled with [3 H]inositol and [32 P] P_i (Stephens et al., 1989, 1991) and in a higher plant, *Spirodela*, labelled with [32 P] P_i (Brearley and Hanke, 1992), in which the levels of labelling in PtdIns3 P are much lower than those of PtdIns4 P . These results with *Neurospora* are similar to those reported for *Saccharomyces cerevisiae* labelled with [3 H]inositol (Auger et al., 1989) in that the level of PtdIns3 P is about the same as the level of PtdIns4 P . Quarumby et al. (1992) also reported approximately equal levels of label in PtdIns3 P and putative PtdIns4 P after long-term labelling of *Chlamydomonas* with [32 P] P_i .

A complex pattern of inositol phosphates has been resolved, including at least six species of Ins P_3 and three each of Ins P_4 and

Ins P_5 . Hanson (1991) reported the presence of Ins1 P , Ins4 P , Ins(1,4) P_2 , Ins(4,5) P_2 , Ins(1,4,5) P_3 , and Ins(2,4,5) P_3 , identified by co-migration with known standards on paper chromatography, but the relative levels of these compounds were not reported. Ins P_4 , Ins P_5 and Ins P_6 have not previously been reported in *Neurospora*.

It should be emphasized again that the levels of inositol metabolites reported here represent the true mass, rather than relative labelling, due to the absolute requirement of the mutant for exogenous inositol. The intracellular concentrations of the inositol phosphates can therefore be calculated by making the assumption that dry weight is approximately one-tenth of the wet weight, and therefore pmol/mg dry weight = pmol/9 mg of intracellular water. Using this conversion and assuming uniform distribution of these compounds throughout the intracellular water space, the concentration of Ins(1,4,5) P_3 in cultures grown in liquid medium can be estimated as 33 nM on 25 μ M inositol, and 67 nM on 200 μ M inositol. Cornelius et al. (1989) estimated the K_m for Ins(1,4,5) P_3 -mediated release of Ca^{2+} from isolated *Neurospora* organelles to be about 5 μ M; the K_m values for Ca^{2+} release from animal-cell-membrane fractions range from 0.5 μ M to 5 μ M (Berridge, 1987). If Ins(1,4,5) P_3 acts as a signal to release intracellular Ca^{2+} in *Neurospora*, one would therefore expect to find a 10–100-fold increase in its level during a stimulus. The intracellular concentration of Ins P_6 can be estimated to be about 3.1 μ M at 25 μ M inositol, and 1.1 μ M at 200 μ M inositol in liquid medium. These levels are much lower than the level of 700 μ M estimated for *Dictyostelium* (Martin et al., 1987), 140 μ M in *Paramecium* (Freund et al., 1992) and 54 μ M in HL-60 cells (Pittet et al., 1989), and are closer to the levels of 5 to 15 μ M found by Szwergold et al. (1987) in a variety of mammalian tissues.

Effects of inositol starvation on the levels of inositol lipids and phosphates

As expected, inositol starvation was found to deplete PtdIns (Table 1a), as has been previously reported (Hubbard and Brody, 1975; Hanson and Brody, 1979; Gabrielides et al., 1983). The values for PtdIns in pmol/mg of RDW reported here for cultures at 25 μ M and 200 μ M inositol in liquid medium (final concentrations of about 5 μ M and 73 μ M inositol) are similar to those reported by Hanson and Brody (1979) for cultures at 3.3 and 55.4 μ M inositol. A decline in total inositol-containing sphingolipids was also reported by Hanson and Brody (1979).

It has been suggested (Hubbard and Brody, 1975) that, in *Neurospora*, the function of PtdIns can be at least partially replaced by other anionic phospholipids and that the organism can therefore tolerate large changes in lipid composition. The results presented here indicate that the levels of the phosphorylated inositol lipids seem to be maintained in spite of reductions in PtdIns levels, suggesting that they may be regulated independently of the level of PtdIns and may be more critical to viability. A similar conclusion was reached by Drummond and Raeburn (1984) who found that treatment of GH $_3$ cells with Li $^+$ and thyrotropin-releasing hormone could deplete cellular pools of inositol and PtdIns without depleting PtdIns(4,5) P_2 . Downes and Stone (1986) also reported that, in rat parotid-gland slices, the rate of synthesis of PtdIns P_2 was maintained when synthesis rates of PtdIns and PtdIns P were inhibited by Li $^+$ -induced inositol depletion. Pfeilschifter et al. (1988) reported that in the retinas of rats treated with lithium, the levels of PtdIns P and PtdIns P_2 increased, while PtdIns decreased slightly.

The inositol phosphates fall into two classes with respect to the effects of inositol starvation: those that are slightly depleted or

remain the same [including Ins(1,4,5) P_3] and those that are elevated 2–6-fold (including Ins P_6). An explanation for this pattern could be found if it is assumed that inositol starvation stimulates phytic acid synthesis. Those compounds found at elevated levels may be among the precursors and/or breakdown products of phytic acid, while those compounds that are depleted may be derived from the lipids which decline on inositol starvation.

This apparent increase in phytic acid synthesis on inositol starvation was an unexpected finding. The function(s) of phytic acid are not yet clear; if it acts as an inositol reserve, it should be mobilized and therefore depleted on low inositol, and this is clearly not the case. It has been suggested by Graf et al. (1987) that phytic acid may act as an intracellular antioxidant by chelating iron and suppressing lipid peroxidation. One of the effects of inositol starvation in *Neurospora* is excess production of free radicals and lipid peroxidation (Rana and Munkres, 1978a; Munkres, 1979). It has been shown (Munkres, 1976) that exogenous antioxidants can protect against 'inositol-less death' in which hyphae deprived of inositol lose viability, lyse and release the intracellular contents (Rana and Munkres, 1978b; Hanson, 1980). The presence of inositol phosphates in the medium of cultures grown in liquid medium on low inositol is likely to be an indication that the cultures had reached the stage of inositol-less death and had begun to lyse. Increased phytic acid synthesis may therefore be an emergency response to oxidative stress during inositol starvation. Note also that the level of phytic acid is higher in cultures grown on solid medium as compared with liquid medium; this may correlate with the more aerobic character of metabolism in aerial hyphae as compared with submerged hyphae.

Although the levels of many of the inositol phosphates respond in an unexpected way to inositol starvation, it is clear from these results that in liquid medium the putative signalling precursor PtdIns(4,5) P_2 is depleted as expected, to less than one-third of its level on high inositol. These results therefore do not correlate with the effects of inositol starvation on sensitivity to light, as described in the following paper (Lakin-Thomas, 1993): inositol starvation has no major effect on sensitivity in liquid medium. It may be that the decrease in the level of PtdIns(4,5) P_2 reported here is not sufficient to affect the efficiency of the signal-transduction mechanism in liquid medium; however, as reported in the following paper (Lakin-Thomas, 1993), no effect of light on the levels of Ins(1,4,5) P_3 can be detected. On solid medium, 25 μ M inositol does not substantially deplete the signalling components; in fact, the level of PtdIns(4,5) P_2 increases by about 70%. The changes seen in inositol metabolite levels on solid medium do not seem sufficient to account for the 1000-fold increase in light sensitivity seen on 25 μ M inositol as compared with 250 μ M (Lakin-Thomas, 1992). These results are consistent with the hypothesis that a phosphoinositide signalling system is not directly involved in the transduction of light signals in *Neurospora* and that the change in light sensitivity on solid medium is due to indirect effects of inositol depletion.

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