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Mutants of the Pentose Phosphate Pathway in *Aspergillus nidulans*

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Mutants of the pentose phosphate pathway have been isolated in *Aspergillus nidulans*. These fail to grow on a variety of carbohydrates that are catabolized through the pentose phosphate pathway. They also grow poorly on nitrate and nitrite as sole nitrogen sources. The pentose phosphate pathway mutations have been assigned to two unlinked genes. Mutants with lesions in the *pppB* locus have reduced activities of four enzymes of the pentose phosphate pathway, of glucose-phosphate isomerase, and of mannitol-1-phosphate dehydrogenase. *pppA*$^-$ mutants have elevated activities of these same enzymes except for transaldolase, for which they have much reduced activity. Both classes of mutants accumulate sedoheptulose-7-phosphate to an extent that is increased considerably when nitrate is present in the medium. Nitrate does not cause an increase in accumulation of sedoheptulose-7-phosphate in double mutants which, in addition to the *pppA1* mutation, carry a mutation that leads to the lack of nitrate reductase activity. These last results suggest that nitrate stimulates the flux through the oxidative pentose phosphate pathway, but that this stimulation depends upon the metabolism of nitrate.

Aspergillus nidulans utilizes nitrate and nitrite as nitrogen sources by reducing them to ammonium at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (14). The two dehydrogenases of the pentose phosphate pathway (PPP) probably afford the major means whereby the organism converts oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) back to NADPH.

It has been reported previously (O. Hankinson and D. J. Cove, J. Biol. Chem., in press; Can. J. Microbiol., in press) that the presence of nitrate in the growth medium affects the activities of several enzymes of the PPP and the activities of some other enzymes of carbohydrate metabolism in *A. nidulans*. Thus, when wild-type *A. nidulans* is grown with urea and nitrate, it possesses twofold higher activities of four enzymes of the PPP, threefold higher activity of glucose-phosphate isomerase (an enzyme which is common to the PPP and the Embden-Meyerhof-Parnas pathway), and sixfold lower activity of mannitol-1-phosphate dehydrogenase than when it is grown with urea alone. Furthermore, it was shown that the *nirA* gene product which is responsible for mediating nitrate induction of the enzymes of the nitrate reduction pathway is also responsible for mediating the effect of nitrate on the activities of the four PPP enzymes and glucose-phosphate isomerase.

The research reported in this paper developed from an investigation into the characteristics of several mutants of *A. nidulans* that had been isolated by D. J. Cove and J. A. Pateman (unpublished data). These mutants do not grow on nitrate or nitrite, but preliminary analysis indicated that they did not belong to any of the previously known (14) classes of mutants of the nitrate reduction pathway. It will be shown here that one of these mutants is blocked in the PPP, and this paper describes some properties of this mutant and the isolation and characterization of additional mutants of the PPP.

MATERIALS AND METHODS

Strains. The strains of *A. nidulans* carried markers that, except for the pentose phosphate pathway mutations (*ppp*$^-$ mutations) to be described in this paper, are in general use (A. J. Clutterbuck and D. J. Cove. *In* A. I. Laskin and H. Lechevalier [ed.], C.R.C. handbook of microbiology, in press). Characteristics of the mutants of the nitrate reduction pathway are summarized in Table 1. The mutant PC 570 (*pppA1*) had been obtained by J. A. Pateman and D. J. Cove (unpublished data) by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis of strain *biA1* and had been selected as being unable to utilize nitrate. The strain *biA1*, which is normal with respect to carbon and inorganic nitrogen metabolism, was

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TABLE 1. *Some properties of mutants of the nitrate reduction pathway of A. nidulans*

Strain	Growth characteristics	Basis	Reference
Wild type	Able to use NO_3^-, NO_2^-, and NH_4^+ as nitrogen sources		
niaD$^-$	Unable to use NO_3^- as nitrogen source	Lack nitrate reductase activity	13
cnx$^-$ (five loci)	Unable to use NO_3^- as nitrogen source	Lack nitrate reductase activity	13
niiA$^-$	Unable to use NO_3^- and NO_2^- as nitrogen sources	Lack nitrite/hydroxylamine reductase activity	14
nirA$^-$	Unable to use NO_3^- and NO_2^- as nitrogen sources	Nitrate reductase and nitrite/hydroxylamine reductase not inducible by nitrate	12, 14

considered as the wild type in the studies to be reported here.

Chemicals. Analytical grade chemicals were used wherever possible.

Media and growth tests. Growth tests were performed on the nitrogenless minimal medium of Cove (4) containing the appropriate supplements and nitrogen sources and solidified with 1.5% agar. Media containing carbon sources other than glucose were made by substituting the carbon source for the glucose in the minimal medium. Carbon sources were used at 1% except for acetate which was added as a mixture of sodium acetate and acetic acid at pH 6.5 to give a final concentration of 144 mM acetate. Ammonia was added as a solution of ammonium chloride buffer to pH 6.5 with 100 mM phosphate buffer.

Unless otherwise stated, nitrogen sources were tested with glucose as carbon source and carbon sources were tested with urea as nitrogen source. The amount of growth attained by the wild type and the pentose phosphate pathway mutants after two days of incubation at 37 C on the various carbon sources was scored according to a scale from 0 to 5. An attempt was made to take account of both the diameter of the colony and the density of growth when scoring.

Induction and isolation of mutants. Conidia of strain *biA1* were treated with NTG at 300 μg/ml as described by Adelberg et al. (1), and mutant colonies were identified by the replica-plating technique of Mackintosh and Pritchard (10).

Genetic analysis. Genetic techniques were modified after Pontecorvo et al. (15) and McCully and Forbes (11).

Preparation of extracts. Mycelium was grown at 25 C in liquid minimal medium containing the appropriate supplements, 1% glucose, and the indicated nitrogen sources, harvested after 21 h unless otherwise stated, and extracted, all as previously described (O. Hankinson and D. J. Cove, J. Biol. Chem., in press).

Enzyme assays. NADPH-nitrate oxidoreductase (EC 1.6.6.3) was assayed by the method of Cove (4) and NADPH-nitrite oxidoreductase (EC 1.6.6.4) and NADPH-hydroxylamine reductase (EC 1.6.6.4) were assayed by the methods of Pateman et al. (14). The other enzyme assays have been described previously (O. Hankinson and D. J. Cove, J. Biol. Chem., in press; Can. J. Microbiol., in press). Soluble protein was determined by the method of Lowry et al. (9).

The unit of activity of ribose-phosphate isomerase is given as change in absorbancy at 520 nm per min at 37 C. The activities of all other enzymes are expressed in nanomoles of substrate (NADPH in the case of nitrite reductase and hydroxylamine reductase) utilized per minute at 25 C. Specific activities are given as units per milligram of protein.

Measurement of heptose in mycelium. A portion of mycelium was extracted with water. The dry weight of this portion was estimated from the value obtained for another. A 0.4-ml amount of the extract, or of a suitable dilution of it, was added to 1.4 ml of an ice-cold aqueous solution of 5% (wt/vol) trichloroacetic acid, and the mixture was centrifuged. The amount of heptose in the supernatant was determined by the cysteine-sulfuric acid method of Dische (5), using sedoheptulose anhydride (hydrate) (Sigma Chemical Co.) as standard.

RESULTS

It was found that the mutant PC570, which had been isolated as a mutant unable to grow with nitrate as nitrogen source, was unable to grow with D-xylose, L-arabinose, and D-glucuronate as carbon sources. Since these three carbohydrates are most probably catabolized by two distinct pathways which lead into the PPP—D-xylose and L-arabinose along one pathway and D-glucuronate along another (Hankinson, unpublished data)—it was concluded that mutant PC570 was blocked in the PPP.

Isolation of additional mutants of the PPP. Mutagenized conidia of strain *biA1* were spread on minimal medium containing glucose and urea. Some of the resulting colonies were replicated onto the same medium except that D-xylose replaced the glucose, some were replicated onto L-arabinose and some were replicated onto D-glucuronate. Colonies which failed to grow on the replica plates were isolated and tested for growth on D-xylose, L-arabinose, and D-glucuronate. If they failed to grow on all three, they were designated as mutants blocked in the PPP (*ppp*$^-$ mutants). Twelve *ppp*$^-$ mutants were isolated. In addition, 52 mutants were iso-

lated that failed to grow on one or two of the above three carbon sources. Some characteristics of these last mutants will be the subject of a later report.

All the *ppp*$^-$ mutants grew less well than the wild type on nitrate and nitrite as nitrogen sources, but all but one grew as well as the wild type on urea and ammonia. All of the 52 mutants which were unable to grow on one or two of D-xylose, L-arabinose, and D-glucuronate grew as well as the wild type on nitrate and nitrite.

Outcrosses of the ppp$^-$ mutants. Strain *biA1 PC570* was crossed to *yA2 pyroA4 lacA1* and the other *ppp*$^-$ mutant strains were crossed to either *wA4 puA2 pyroA4 hxA1* or to *yA2 nicB8 malA1*. Inability to use D-xylose, L-arabinose, and D-glucuronate as carbon sources and poor growth on nitrate and nitrite segregated together in all but three of the outcrosses. Analysis of these three crosses showed that the strain originally isolated as carrying the *ppp*$^-$ mutation carried another mutation, besides the *ppp*$^-$ mutation, which affected growth on nitrate. Strain *biA1 pppB1* carried a mutation leading to complete lack of growth on nitrate and nitrite as well as the *pppB1* mutation. The former mutation was not allelic with either *niiA4* or *nirA*$^-$*1* and showed a recombination frequency with the *pppB1* mutation of 26%. Strain *biA1 pppB7* carried a *niiA*$^-$ mutation which was not linked to *pppB7*, and strain *biA1 pppA5* carried a *cnx*$^-$ mutation which was not linked to *pppA5*. *ppp*$^-$ progeny from these last three crosses which did not carry the independently segregating mutation affecting nitrate metabolism themselves grew poorly on nitrate and nitrite.

Tests of allelism among the ppp$^-$ mutants. Heterokaryons made from various pair-wise combinations of the *ppp*$^-$ mutants were tested for growth on D-xylose. Crosses between *ppp*$^-$ mutants were also performed, and progeny from the crosses were scored on D-xylose. The results from these complementation and recombination tests were in complete agreement and showed that mutations at only two loci had been isolated. Six of the *ppp*$^-$ mutations were assigned to the *pppA* locus and seven were assigned to the *pppB* locus. The PC570 mutation was renamed *pppA1*.

The fact that heterokaryons made between *pppA*$^-$ and *pppB*$^-$ mutants were able to grow on D-xylose showed that the *pppA*$^-$ and *pppB*$^-$ mutations are recessive to their wild-type alleles in heterokaryons. These mutations were also shown to be recessive in diploids.

pppA$^-$ pppB$^-$ double mutants. Approximately 25% of the progeny from crosses between *pppA*$^-$ and *pppB*$^-$ mutants grew less well than either parent on medium containing glucose and urea and grew less well than either parent on nitrate and nitrite and on D-xylose, L-arabinose, and D-glucuronate (in those cases where the *pppA*$^-$ parent grew at all on these carbon sources). Crosses carried out with two of these segregants showed that they were *pppA*$^-$ *pppB*$^-$ double mutants.

Mapping of the ppp$^-$ mutations. It was shown by the method of haploidization that the *pppA* and *pppB* loci are located on linkage groups VIII and III, respectively. The position of *pppA1* on linkage group VIII was determined by carrying out appropriate crosses. Analysis of 87 progeny from the cross *biA1 pppA1* × *yA2 pyroA4 uX3 nirA*$^-$*1* gave a linkage of 26 centimorgans (i.e., a frequency of recombination of 26%) between *pppA1* and *nirA*$^-$*1* with a standard deviation of 5 centimorgans. Analysis of 368 progeny from the cross *yA2 pyroA4 lacA1 pppA1* × *biA1 uZ4 palB7* (cross 2) and 834 progeny from the cross *yA2 pppA1 palB7* × *biA1 uZ4 chaA1* (cross 3) gave the following gene order and the following map distances (in centimorgans) with their standard deviations:

Cross 2 ←1.6 ± 0.7→ ←1.4 ± 0.6→

chaA1 *uZ4* *pppA1* *palB7*

Cross 3 ←2.6 ± 0.6→←1.3 ± 0.4→←1.1 ± 0.4→

Growth of the ppp$^-$ mutants on nitrate, nitrite, and various carbon sources. The *pppA*$^-$ mutants grew poorly on nitrate and nitrite and either did not grow or grew poorly on all those carbon sources tested whose pathways of catabolism lead into the PPP (Table 2). These mutants grew less well on nitrate than they did on nitrite. They were heterogeneous. They could be ranked according to the amount of growth they attained on nitrate, nitrite, and the above carbon sources as follows: *pppA2* < *pppA4* < *pppA1* < *pppA3* < *pppA5* = *pppA6*. The *pppB*$^-$ mutants were very similar to one another. These mutants grew poorly on those of the carbon sources whose pathways of catabolism lead into the PPP which supported good growth of the wild type but grew as well as the wild type on the poorer of these carbon sources (Table 2). The *pppB*$^-$ mutants grew to the same extent on nitrate and nitrite. They grew less well than the wild type on these nitrogen sources but better than any of the *pppA*$^-$ mutants.

TABLE 2. *Growth of the ppp⁻ mutants on various carbon sources*[a]

Carbon source	Growth of		
	Wild type	*pppA1*	*pppB⁻* mutants
D-Xylose[b]	5	0	2
L-Lyxose	5	0	2
L-Arabinose	5	0	2
L-Arabitol	4	0	2
Myo-inositol (meso-inositol)	4	0	2
D-Glucuronate-δ-lactone	4	0	2
D-Glucuronate (Na^+ salt)	4	0	2
D-Arabitol	3	0	2
Xylitol	3	0	3
D-Ribitol (adonitol)	3	1	3
D-Ribose	1^+	1	1^+
D-Arabinose	1^+	0	1^+
D-Gluconate-δ-lactone	1^+	0	1^+
D-Gluconate (K^+ salt)	1^+	0	1^+
L-Xylose	1^+	0	1^+
D-Erythrose	1^+	1	1^+
No added carbon source	1	1	1
D-Glucose[c]	5	5	5
D-Fructose	5	5	5
DL-Lactate (Na^+ salt at 2%)	4	4	4
D-Galactose	4	4	4
Maltose	4	4	4
Ethanol	3	3	3
Mannitol	3	3	3
Acetate (144 mM)	3	3	3
Glycerol	3	3	3
Lactose	3	3	3
L-Glutamate (Na^+ salt)	2	2	2
No added carbon source	1	1	1
D-Glucose-6-phosphate (di-Na^+ salt)[d]	1	1	1
D-Sedoheptulose monohydrate	1	1	1
L-Gulonate-γ-lactone	1	1	1
D-Galacturonate	0	0	0

[a] Carbon sources were tested at 1% with 5 mM urea as nitrogen source.

[b] The *pppA1* mutant did not grow on carbon sources in this section.

[c] The wild type and *pppA1* and *pppB⁻* mutants grew equally well on carbon sources in this section.

[d] Neither the wild type nor the *ppp⁻* mutants grew on carbon sources in this section.

Some of the growth characteristics of the *pppA⁻ pppB⁻* double mutants have already been described. The double mutants grew less well than the wild type on all media tested.

Photographs showing the growth of *ppp⁻* mutants on various nitrogen and carbon sources are given in Fig. 1.

Poor growth of certain pppA⁻ mutants on carbon sources other than those catabolized through the PPP and on nitrogen sources other than nitrate and nitrite. The *ppp⁻* mutants were tested on the carbon sources listed in Table 2B and on the following nitrogen sources: 5 mM urea, 10 mM NH_4Cl, 1 mM L-arginine, 1 mM hypoxanthine, and 2.5 mM L-glutamate. The *pppB⁻* mutants and the *pppA⁻* mutants, with the exception of those mentioned below, grew as well as the wild type on all these carbon and nitrogen sources. The mutant *pppA2* grew slightly less well than the wild type on medium containing D-glucose as carbon source and either urea or NH_4Cl as nitrogen source. This mutant, and also *pppA4*, grew even less well than the wild type on some

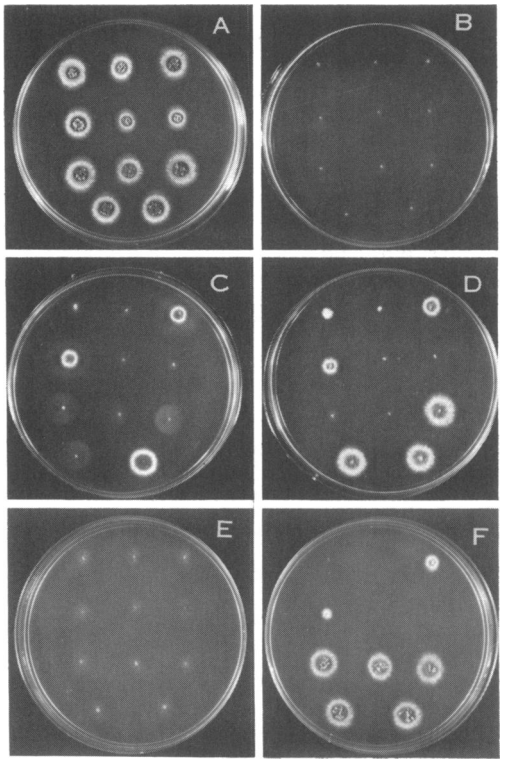

FIG. 1. *Growth of ppp⁻ strains on various carbon and nitrogen sources. All the strains carry the marker biA1. They were grown for 48 h at 37 C on minimal medium containing 10 μg of biotin/liter and the indicated carbon and nitrogen sources. Strains are arranged on the plates (from top to bottom, left to right) as follows. Row 1: pppA1, pppA2, pppB1; row 2: pppB4, pppA1 pppB1, pppA1 pppB4; row 3: nirA⁻1, niiA4, niaD17; row 4: cnxG4, wild type. A, 1% glucose plus 10 mM NH_4Cl; B, 1% glucose (no added nitrogen source); C, 1% glucose plus 10 mM $NaNO_3$; D, 1% glucose plus 10 mM $NaNO_2$; E, 10 mM NH_4Cl (no added carbon source); F, 1% D-xylose plus 10 mM NH_4Cl.*

of the other carbon sources listed in Table 2B, and their poor growth was most marked on L-glutamate of the nitrogen sources. These growth characteristics segregated with the *pppA2* and *pppA4* mutations in crosses. The *pppA2* and *pppA4* mutants did not grow so poorly on the carbon sources mentioned above as they did on the carbon sources listed in section b of Table 2, and they did not grow so poorly on L-glutamate as they did on nitrate and nitrite.

Inhibition of growth of ppp⁻ mutants by certain carbohydrates. The amount of growth of the *pppA*⁻ mutants and of the *pppA*⁻ *pppB*⁻ double mutants on 0.1% glucose was reduced when D-xylose, L-arabinose, or D-glucuronate (each at 1%) was also present in the medium. D-Xylose (1%) also reduced the growth attained by the *pppB*⁻ mutants on 0.1% glucose.

Inhibition of growth of pppA⁻ mutants by nitrate and nitrite. The presence of 10 mM $NaNO_3$ or 10 mM $NaNO_2$ in the medium reduced the amount of growth attained by the *pppA*⁻ mutants and the *pppA*⁻ *pppB*⁻ double mutants on 5 mM urea (10 mM $NaNO_3$ had a slightly greater effect than 10 mM $NaNO_2$). In contrast, the *pppB*⁻ mutants, *niaD15*, *niaD17*, *cnxB11*, *cnxB13*, *cnxG2*, *cnxG4*, and *nirA⁻1*, each attained the same amount of growth on urea plus $NaNO_3$ as on urea, and although the *niiA4* mutant grew less well on urea plus $NaNO_3$ than on urea, it grew better on the former medium than did the mutant *pppA1*. *niaD15 pppA1*, *niaD17 pppA1*, *cnxB11 pppA1*, *cnxB13 pppA1*, *cnxG2 pppA1*, *cnxG4 pppA1*, and *nirA⁻1 pppA1* double mutants each attained the same amount of growth on urea plus $NaNO_3$ as on urea, and the *niiA4 pppA1* double mutant grew as well as the *niiA4* parent on urea plus nitrate. The additional presence of a *niaD*⁻, *cnx*⁻, *niiA*⁻, or *nirA*⁻ mutation therefore repaired the poor growth of the *pppA1* mutant on urea plus nitrate.

Specific activities of enzymes of carbohydrate metabolism in the ppp⁻ mutants. The results (Table 3) can be summarized as follows.

(i) The *pppA1* and *pppA2* mutants possessed approximately 10% of the transaldolase activity of the wild type.

(ii) When they were grown with urea, the *pppA*⁻ mutants had activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, transketolase, and glucose-phosphate isomerase which were two- to threefold higher and activities of mannitol-1-phosphate dehydrogenase and fructose-6-phosphate reductase which were fourfold higher than those in the wild type grown with urea. (Mannitol-1-phosphate dehydrogenase is most probably responsible for the major proportion, if not all, of the fructose-6-phosphate reductase activity [O. Hankinson and D. J. Cove, Can. J. Microbiol., in press].)

(iii) When they were grown with urea, the *pppB*⁻ mutants had activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, transketolase, transaldolase, and glucose-phosphate isomerase which were one-third to one-half, and activity of mannitol-1-phosphate dehydrogenase which was one-quarter of that in the wild type grown under the same conditions.

(iv) In the *pppA1* mutant and in the *pppB*⁻ mutants, the presence of nitrate in the medium caused increases in the activities of some of the PPP enzymes (including glucose-phosphate isomerase) and caused a decrease in the activity of mannitol-1-phosphate dehydrogenase.

(v) The *pppA1 pppB4* double mutant had approximately 1% of the transaldolase activity of the wild type.

(vi) When grown both on urea and on urea plus nitrate, the *pppA1 pppB4* double mutant had activities of the PPP enzymes and of mannitol-1-phosphate dehydrogenase which were more similar to those of the *pppB4* parent than to those of the *pppA1* parent.

(vii) The *pppA1* and *pppB4* mutants and the *pppA1 pppB4* double mutant had normal activities of ribose-phosphate isomerase, pyruvate kinase, aldolase, and nicotinamide adenine dinucleotide phosphate-isocitrate dehydrogenase. The last two strains also had normal activities of phosphofructokinase, whereas mutant *pppA1*, when it was grown into urea, appeared to have elevated activity of this enzyme.

Additivity of the activities of glucose-6-phosphate dehydrogenase in extracts of the pppA1 and pppB4 mutants with that in an extract of the wild type and additivity of the transaldolase activities in extracts of the pppA1 mutant and the wild type. The results presented in Tables 4 and 5 suggest that extracts of mutants *pppA1* and *pppB4* do not contain freely diffusible activators or inhibitors of glucose-6-phosphate dehydrogenase and that extracts of mutant *pppA1* do not contain freely diffusible inhibitors of transaldolase.

Enzyme activities in strains carrying a ppp⁻ mutation and a mutation of the nitrate reduction pathway. The results presented in Table 6 indicate that nitrate induction of the enzymes of the PPP in the *pppA1* mutant and in the *pppB*⁻ mutants is mediated through the *nir* product as it is in the wild type (see O. Hankinson and D. J. Cove, J. Biol. Chem., in press).

It will be noted that the *niaD*⁻ *ppp*⁻ and *cnx*⁻

TABLE 3. *Specific activities of enzymes of carbohydrate metabolism in the ppp⁻ mutants*[a]

Strain	Specific activities of:																					
	Glucose-6-phosphate dehydrogenase		6-Phosphogluconate dehydrogenase		Transketolase		Transaldolase		Ribosephosphate isomerase		Glucose phosphate isomerase		Phosphofructokinase		Pyruvate kinase		Fructose-1,6-diphosphate aldolase		NADP-isocitrate dehydrogenase		Mannitol-1-phosphate dehydrogenase		Fructose-6-phosphate reductase	
	Urea[b]	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$
Wild type	752	1,440	354	626	184	387	447	778	2.2	2.5	2,565	7,810	45	33	1,235	1,570	630	669	111	119	115	13	228	18
pppA1	1,550	2,373	629	582	480	530	50	63	3.2	2.6	6,900	14,594	127	58	1,210	1,515	658	827	82	106	353	84	802	204
pppA2	1,430		688				58																739	
pppB1	267	430	248	329			170	154															34	14
pppB4	298	527	178	288	123	125	150	157	2.2	2.0	1,250	2,555	37	45	1,265	1,545	674	754	120	124	41	11	59	14
pppA1 pppB4	396	1,040	223	318	167	150	5	2	2.1	1.3	1,235	3,010	45	52	1,575	1,830	818	758	114	127	16	7	32	32

[a] Glucose (1%) was used as the carbon source, and either 5 mM urea or 5 mM urea plus 10 mM $NaNO_3$ was used as the nitrogen source. Mycelium was harvested after 21 h of growth at 25 C. The values presented for the wild type and the *pppA1*, *pppB4*, and *pppA1 pppB4* strains are the means of two or three independent determinations. The values presented for the *pppA2* and *pppB1* strains are, in each case, the results of a single experiment.

[b] Nitrogen source.

TABLE 4. *Additivity of the activities of glucose-6-phosphate dehydrogenase in extracts of the pppA1 and pppB4 mutants with that in the wild type*[a]

Extract	Activity	Activity expected from additivity
Wild type	30.4	
pppA1	39.5	
Wild type + *pppA1*	68.7	69.9
Wild type	29.5	
pppB4	47.8	
Wild type + *pppB4*	77.8	77.3

[a] The strains were grown with 5 mM urea plus 10 mM $NaNO_3$ as nitrogen source. The wild type and mutant *pppB4* were harvested after 21 h of growth, and mutant *pppA1* was harvested after 41 h. The specific activities of glucose-6-phosphate dehydrogenase in the extracts were as follows: wild type, 1,470; *pppA1*, 3,479; *pppB4*, 598. An appropriate dilution of the extract of mutant *pppA1* or of mutant *pppB4* was mixed in the assay mixture with a dilution of the extract of the wild type, and after 6 min of incubation at 25 C the reaction was started by the addition of glucose-6-phosphate.

ppp$^-$ double mutants (except for the *cnxG2 ppp*$^-$ double mutants grown on urea) had higher activities of glucose-6-phosphate dehydrogenase than the corresponding *ppp*$^-$ single mutants grown on urea plus nitrate. It is probable that the same unknown process that is responsible for the elevation in activities of glucose-6-phosphate dehydrogenase in *niaD*$^-$ and *cnx*$^-$ single mutants (O. Hankinson and D. J. Cove, J. Biol. Chem., in press) is also responsible for the elevation in activities of glucose-6-phosphate dehydrogenase in the *niaD*$^-$ *ppp*$^-$ and *cnx*$^-$ *ppp*$^-$ double mutants. It will also be seen from Table 6 that the *cnxG2 pppA1* and *niaD17 pppA1* double mutants had activities of transaldolase that were very similar to those in the *pppA1* parent.

Specific activities of the enzymes of the nitrate reduction pathway in ppp$^-$ mutants. The *pppA1* and *pppB4* mutants had activities of nitrate reductase, nitrite reductase, and hydroxylamine reductase which were similar to those of the wild type (Table 7). (Nitrite reductase and hydroxylamine reductase are activities of the same enzyme in *A. nidulans* [14].)

Accumulation of sedoheptulose-7-phos-

TABLE 5. *Additivity of the transaldolase activities in extracts of mutant pppA1 and the wild type*[a]

Extract	Activity	Activity expected from additivity
Wild type	13.0	
pppA1	2.0	
Wild type + *pppA1*	13.9	15.0

[a] Both strains were grown with 5 mM urea and harvested after 21 h. The specific activities of transaldolase in the extracts were as follows: wild type, 423; *pppA1*, 33. Dilutions of the two extracts were mixed together in the assay mixture, and after 5 min of incubation at 25 C the reaction was started by the addition of erythrose-4-phosphate.

TABLE 6. *Enzyme activities in strains carrying a ppp$^-$ mutation and a mutation of the nitrate reduction pathway*[a]

Strains	Specific activities of:					
	Nitrite reductase		Glucose-6-phosphate dehydrogenase		Transaldolase	
	Urea[b]	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$
Wild type	2	181	597	1,260	423	708
pppA1	0	94	1,310	2,420	33	60
cnxG2 pppA1	1	184	1,470	3,450	64	62
cnxG4 pppA1	139	126	3,130	3,100	42	53
nirA$^-$*1 pppA1*	3	8	1,450	1,400		
Wild type	1	133	544	1,360		
pppB4	1	95	265	510		
cnxG2 pppB4	0	144	306	977		
niaD17 pppB4	177	165	1,050	1,130		
nirA$^-$*1 pppB4*	2	5	263	282		

[a] All strains were harvested after 21 h of growth.
[b] Nitrogen source.

TABLE 7. *Specific activities of the enzymes of the nitrate reduction pathway in ppp⁻ mutants*[a]

Strains	Specific activities of:					
	Nitrate reductase		Nitrite reductase		Hydroxylamine reductase	
	Urea[b]	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$	Urea	Urea + $NaNO_3$
Wild type		41		122		188
pppA1	1	34	0	88	9	140
Wild type		40		107		141
pppA1		25		102		121
Wild type		104		89		
pppB4		70		119		
Wild type	2	88	0	142	8	181
pppB4	3	55	1	102	22	149

[a] The initial nitrogen source was 5 mM urea plus 10 mM $NaNO_3$ in the case of the *pppB4* mutant and the wild type assayed with it. In the case of the *pppA1* mutant and the wild type assayed with it, the strains were inoculated into medium containing 5 mM urea, and 10 mM $NaNO_3$ was added after 16 h of growth. All strains were harvested after 21 h of growth.

[b] Nitrogen source.

phate by the ppp⁻ mutants. Extracts of the *pppA*⁻ mutants, of *pppB4*, and of *pppA1 pppB4* gave spectra with sharp peaks at 508 nm when they were assayed for heptose by the method of Dische (5). Absorption at this wavelength is specific for heptoses (5). All extracts (including that of the wild type) had spectra with peaks at 415 and 600 nm, and equivalent dilutions of all the extracts gave approximately the same absorption values at these wavelengths. The 600 nm peak was probably caused by glucose (5). The heptose initially accumulated by the *ppp*⁻ mutants is most probably sedoheptulose-7-phosphate, although it is possible that this substance is hydrolyzed to sedoheptulose by phosphorylases in the cell.

The *pppA1* and *pppB4* mutants and the *pppA1 pppB4* double mutant accumulated considerably more sedoheptulose-7-phosphate when they were grown with urea plus nitrate than when they were grown with urea (Table 8). (Sedoheptulose-7-phosphate [estimated as sedoheptulose anhydride {hydrate}] comprised 10% of the dry weight of the *pppA1 pppB4* double mutant harvested after 41 h of growth with urea plus nitrate.) The presence of a *niaD*⁻, *cnx*⁻, *niiA*⁻ or *nirA*⁻ mutation abolished the increase in accumulation of sedoheptulose-7-phosphate that nitrate normally caused in *pppA1*

DISCUSSION

All the enzymes found to be affected by the *pppA*⁻ and *pppB*⁻ mutations are, in the normal cell, subject to regulation by nitrate. However, it seems unlikely, for the reasons that follow, that the products of the *pppA* and *pppB* genes are involved directly in implementing the effect of nitrate on these enzymes.

(i) Both classes of mutants remain responsive to nitrate in that they are "inducible" by nitrate for some of the enzymes of the PPP and "repressible" by nitrate for mannitol-1-phosphate dehydrogenase.

(ii) The presence of nitrate in the medium causes increases in the activities of the PPP enzymes and a decrease in the activity of mannitol-1-phosphate dehydrogenase, whereas a *pppB*⁻ mutation causes decreases in all these activities and a *pppA*⁻ mutation causes in-

TABLE 8. *Accumulation of heptose by the ppp⁻ mutants*[a]

Strains	nmol of heptose/mg dry weight of mycelium	
	5 mM urea[b]	5 mM urea + 10 mM $NaNO_3$
Harvested after 21 h		
Wild type	0.6	1.3
	0.4	0
pppA1	12	201
	8	
	3	194
pppA2	21	
niaD15 pppA1	5	3
niaD17 pppA1	4	9
cnxG2 pppA1	6	5
cnxG4 pppA1	2	1.2
niiA4 pppA1	5	
nirA⁻1 pppA1	7	9
pppB4	5	24
	4	59
pppA1 pppB4	42	
Harvested after 41 h		
Wild type		1.7
pppA1		196
niiA4 pppA1		6
pppB4		0
pppA1 pppB4		419

[a] The strains were grown with 1% glucose as the initial carbon source and with the indicated initial nitrogen sources. Where more than one value has been given for a particular strain grown under a particular condition, these values were determined in different mycelium samples which were assayed on different occasions.

[b] Nitrogen source.

creases in all these activities (except transaldolase). Thus, a *pppA*$^-$ mutation and a *pppB*$^-$ mutation each has in one case an effect similar to that of nitrate and in another case an effect opposite to that of nitrate.

Nevertheless, it is probable that the *pppB*$^-$ mutants are affected in some regulatory component that is common to the PPP enzymes and mannitol-1-phosphate dehydrogenase. The frequency with which the *pppB*$^-$ mutants were obtained, their uniformity of phenotype, and their recessiveness all suggest that they may be defective in an inducer molecule specified by the *pppB*$^-$ gene.

The most plausible explanation for the properties of the *pppA*$^-$ mutants is that they are defective in the structure of transaldolase and that the elevation in activities of some of the other enzymes of carbohydrate metabolism in these mutants is secondary to their reduction in transaldolase activity.

It therefore appears that the PPP enzymes and mannitol-1-phosphate dehydrogenase are each subject to at least two independent regulatory mechanisms, one of which is effected by nitrate and in another of which the product of the *pppB* gene is involved. Perhaps glucose-6-phosphate, or a closely related substance, is an inducer of the enzymes of the PPP (as has been proposed for the rat mammary gland [7]) and also of mannitol-1-phosphate dehydrogenase, and causes induction by activating the product of the *pppB* gene. The elevated activities of these enzymes in the *pppA*$^-$ mutants might then be explained if it is supposed that these mutants accumulate glucose-6-phosphate as well as sedoheptulose-7-phosphate.

Sedoheptulose-7-phosphate will accumulate in a strain lacking or having reduced activity of transaldolase only when glucose-6-phosphate is metabolized through the oxidative PPP and not when fructose-6-phosphate and glyceraldehyde-3-phosphate are metabolized through the PPP in the reverse direction. Since *pppA1* accumulated 25 times more sedoheptulose-7-phosphate when it was grown with urea plus nitrate than when it was grown with urea, nitrate must stimulate the flux through the oxidative PPP. Nitrate did not cause an increase in the amount of sedoheptulose-7-phosphate accumulated by *niaD*$^-$ *pppA1* and *cnx*$^-$ *pppA1* double mutants. Since two of these double mutants were shown to have similar transaldolase activities to the *pppA1* parent and activities of glucose-6-phosphate dehydrogenase which were at least as great as the activity in *pppA1* when these strains were grown with urea plus nitrate, it can be concluded that the reason the *niaD*$^-$ and *cnx*$^-$ mutations prevent the stimulation by nitrate of the accumulation of sedoheptulose-7-phosphate in the *pppA1* mutant is because they prevent the metabolism of nitrate. The increase in flux through the oxidative PPP must therefore depend upon the metabolism of nitrate. It is very probable that it is in fact a change in the concentration of $NADP^+$, NADPH, or both, produced during the reduction of nitrate to ammonium that stimulates this flux.

Lack of growth on carbon sources which are catabolized solely by pathways that lead into the PPP and poor growth on nitrate and nitrite are pleiotropic effects of both the *pppA*$^-$ and *pppB*$^-$ mutations. Severe *pppA*$^-$ mutations also lead to poor growth on other carbon sources and nitrogen sources.

As the following findings show, all the growth characteristics of the *ppp*$^-$ strains on the various nitrogen sources can be explained as resulting from an inhibition of growth caused either by sedoheptulose-7-phosphate or by a substance that accumulates in parallel with it.

(i) The *pppA1 pppB4* double mutant and the *pppA1* and *pppB4* mutants all accumulated sedoheptulose-7-phosphate when they were grown on urea plus nitrate. The *pppA1 pppB4* double mutant accumulated the greatest amount of sedoheptulose-7-phosphate and grew the least well on nitrate. Mutant *pppB4* accumulated the least amount of sedoheptulose-7-phosphate and grew the best on nitrate.

(ii) The *pppA1 pppB4* double mutant grew poorly on urea and also accumulated a considerable amount of sedoheptulose-7-phosphate when it was grown on this nitrogen source.

(iii) *pppA2* accumulated more sedoheptulose-7-phosphate than *pppA1* when these mutants were grown on urea. Mutant *pppA2* grew slightly less well than the wild type on urea, whereas mutant *pppA1* grew as well as the wild type on this nitrogen source.

(iv) *niaD*$^-$, *cnx*$^-$, *nirA*$^-$, and *niiA*$^-$ mutations repaired the poor growth of *pppA1* on urea plus nitrate and also prevented the increase in accumulation of sedoheptulose-7-phosphate which nitrate normally caused in this mutant.

In not a single case, however, can the possibility be dismissed that a deficiency of NADPH is at least partly responsible for poor growth. Thus, it is not known if the block in transaldolase in *pppA*$^-$ mutants and *pppA*$^-$ *pppB*$^-$ double mutants restricts the flux through the oxidative PPP, and, since *pppB*$^-$ mutants have reduced activities of many enzymes of the PPP, it is quite possible that these mutants produce insufficient NADPH for full utilization of nitrate and nitrite.

It is of interest that several morphological mutants of *Neurospora crassa* have been shown to have either defective glucose-6-phosphate dehydrogenase or defective 6-phosphogluconate dehydrogenase (3, 6, 8, 16; W. A. Scott and E. L. Tatum, Fed. Proc. **28**:468, 1969) and that some of these have been shown to have reduced levels of intracellular NADPH (2). For most studies these mutants have been grown in media containing NH_4NO_3 as nitrogen source (reference 6 is an exception). It would be interesting to compare the effects of growth with $NaNO_3$ and NH_4Cl as sole nitrogen sources on the morphologies of these mutants.

The major conclusions about the regulation of the PPP in *A. nidulans* that have been presented in this and a previous paper (O. Hankinson and D. J. Cove, J. Biol. Chem., in press) can be summarized as follows.

(i) Nitrate causes increases in the activities of several enzymes of the PPP. These increases can occur in the absence of nitrate metabolism and are effected by the same regulatory gene product that is responsible for inducing the enzymes of the nitrate reduction pathway.

(ii) Nitrate causes an increase in flux through the oxidative PPP. This increase in flux depends upon the metabolism of nitrate.

It will be noted that some important questions remain unanswered. Thus, it has not been possible to determine whether the increase in flux through the oxidative PPP that occurs during growth with nitrate can occur in the absence of an increase in the activities of the PPP enzymes. Indeed, it has not been possible to demonstrate that activity of the PPP is needed at all for growth on nitrate.

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