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Characterization of Glycerol Nonutilizing and Protoperithecial Mutants of *Neurospora*

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Summary. Mutants defective in polyol metabolism and/or in protoperithecial development were selected in *Neurospora tetrasperma*, a species in which protoperithecial development occurs at nonpermissively high temperature if certain polyols are used in lieu of sucrose as carbon source. Mutants selected for nonutilization of one of the four polyols tested, glycerol, mannitol, sorbitol, or xylitol, were usually found to be nonutilizers of the other three polyols as well. Mutants blocked at various stages of protoperithecial development complemented pairwise to produce more advanced developmental stages, usually mature protoperithecia and, when of opposite mating type, mature perithecia. About one-third of the mutants manifested both polyol auxotrophy and defective protoperithecial development upon initial isolation, but protoperithecial defectiveness in such mutants usually showed erratic segregation in crosses and/or instability to repeated vegetative transfer, whereas polyol auxotrophy usually did not and was, therefore, studied further. Two glycerol nonutilizing strains were introgressed into *N. crassa* to facilitate genetic analysis. One, *glp-4*, lacked both inducible and constitutive glycerol kinase and mapped to linkage group VI, between *ad-1* and *rib-1;* the other, *glp-5*, lacked glyceraldehyde kinase and mapped to linkage group I, proximal to *ad-9*. Another mutant, *gly-u*(234), has been reported by other investigators to lack inducible glycerol kinase but to map to linkage group I, distal to *ad-9*.

Introduction

The sequential development of protoperithecia in *Neurospora crassa* (Rothschild and Suskind, 1966) and *Neurospora tetrasperma* (Viswanath-Reddy and Turian, 1972, 1975) and the subsequent maturation of these structures into perithecia have provided opportunities for a variety of morphogenetic studies in this genus (Tan and Ho, 1970; Srb et al., 1973; Howe and Johnson, 1976). Additional studies revealed a protein specifically associated with developmental events during the sexual cycle (Viswanath-Reddy and Turian, 1972; Nasrallah and Srb, 1973), as well as the development of protoperithecia at 37° C, a nonpermissive temperature, when certain polyols replaced sucrose as carbon source in the culture medium (Viswanath-Reddy and Turian, 1972, 1975). The present study examined further the possible relationship between protoperithecial development and polyol, particularly glycerol, metabolism. Such a relationship might not be unexpected in view of the known involvement of polyols in the reproductive tissues of other forms (Horecker, 1969; Bischoff, 1976).

Three pathways, one phosphorylative and two oxidative, have been found in various organisms for the dissimilation of glycerol (Fig. 1). The phosporylative pathway, which leads to glycerol-3-phosphate and dihydroxyacetone phosphate, was demonstrated in Neurospora by North (1973, 1974) and Courtright (1975a, 1975b). We report here the occurrence in Neurospora of the NADP-linked oxidative pathway leading to glyceraldehyde, but the apparent absence of the NAD-linked oxidative pathway leading to dihydroxyacetone that has been found in procaryotes by other investigators (Lin, 1976).

Mutants unable to grow on certain polyols as carbon source and/or defective in protoperithecial development were isolated. Protoperithecial defectiveness tended to be vegetatively unstable and/or to segregate abnormally in crosses, whereas polyol auxotrophy more commonly was stable, segregated as a single-gene effect, and included a strain lacking glycerol kinase and another lacking glyceraldehyde kinase in the glycerol metabolic pathways.

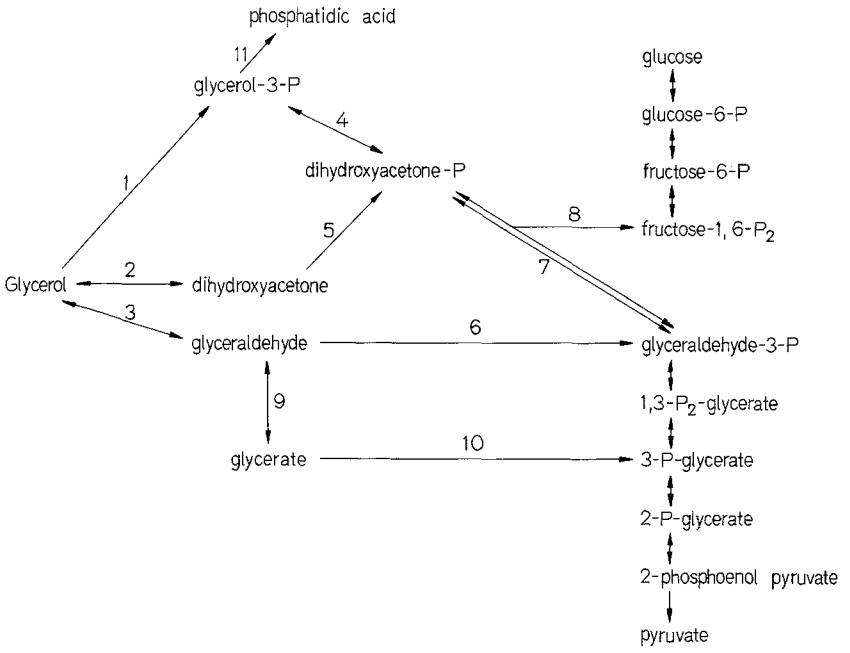

Fig. 1. Pathways of glycerol metabolism in various organisms and the relationship of these pathways to glycolysis. *1* glycerol kinase, *2* NAD-linked glycerol dehydrogenase, *3* NADP-linked glycerol dehydrogenase, *4* NAD- or FAD-linked glycerol-3-P dehydrogenase, *5* dihydroxyacetone kinase (triokinase), *6* D-glyceraldehyde kinase (triokinase), *7* triose-P isomerase, *8* aldolase, *9* aldehyde dehydrogenase, *10* glycerate kinase, *11* glycerol-3-P acyl transferase

Materials and Methods

Strains. Strains of *N. crassa* used in this study and obtained from the Fungal Genetics Stock Center, Arcata, California, were: *ad-1* (3254), *ad-9* (Y154M37), alcoy (multiple translocation linkage tester), *cr-1:* crisp (B123), *fl:* fluffy (fl^p), *pan-2* (Y153M96), *pan-2* (Y154M64), *rib-1* (51602t), *trp-2* (75001), *ylo-1:* yellow (Y30539y), and wild types Em 5256A, Em 5256a, 1A, 25a, SY7A, SY4f8a, 74-OR23-1A and 74-OR8-1a. *N. crassa – N. tetrasperma* interspecific hybrids, C_1T_3 and C_3T_1, were obtained from Dr. R.L. Metzenberg, University of Wisconsin. *N. tetrasperma* wild types used were 85A and 85a (Howe, 1964) and 180.27, a strain heterokaryotic for mating type and obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands. Wild type 180.27, previously referred to as strain GN4 (Howe, Viswanath-Reddy and Bennett, 1975), was used for the isolation of mutant strains, as described below. For use of wild type 180.27 in crosses, the *A* and *a* unisexual components were isolated from this strain.

Media. The basic medium used was that of Westergaard and Mitchell (1947). For selecting and growth-testing polyol mutants, Westergaard and Mitchell medium (W-M) was modified by singly replacing the carbon source, 2% sucrose, with 2% (w/v) concentrations of the polyols glycerol, mannitol, sorbitol, or xylitol.

Isolation of Mutant Strains. Twenty-ml suspensions containing 2×10^7 conidia/ml from heterokaryotic *N. tetrasperma* wild type 180.27 in 0.1 M phosphate buffer, pH 7, were treated with N-methyl-N'-nitro-N-nitrosoguanidine (100 µg/ml condial suspension) on a rotary shaker for a period of 15 or 30 min at 25°C. The conidia were then washed 3 times in 0.1 M phosphate buffer, pH 7. Conidial survival ranged from about 1 to 4 per cent. Ten-ml samples of the treated suspensions containing 2×10^7 conidia/ml were subjected to the filtration-concentration procedure (Woodward, DeZeeuw and Srb, 1954), with glycerol, mannitol, sorbitol, or xylitol, singly, as the selective carbon source. The conidia were then plated on W-M sucrose-sorbose agar. Colonies which showed neither perithecia nor protoperithecia were isolated to W-M slants, then growth-tested on W-M slants containing the corresponding selective polyols, singly, as sole carbon source, and examined for perithecia and protoperithecia on both slants.

Enzymology. Conidia were inoculated into 200 ml W-M medium in 500 ml Erlenmeyer flasks (10^6 conidia/ml medium). The flasks were incubated on a reciprocating shaker at 25°C for various periods, after which the mycelia were harvested on Whatman No. 40 filter paper in a Buchner funnel and washed repeatedly with cold distilled water. The mycelial pads were dried between layers of fresh filter paper. About 5–10 g of mycelial pad was cut into small pieces with scissors, frozen with liquid nitrogen in a mortar, and powdered with one-half weight of 2 mm glass beads. The mycelial powder was extracted with 2 volumes of 0.1 M Tris buffer, pH 7.4, containing 10^{-4} M Dithiothreitol. The extract was centrifuged at $25{,}000 \times g$ in a Sorvall refrigerated centrifuge for 30 min, and the supernatant was used for enzyme assays.

Enzyme activities were measured by following pyridine nucleotide oxidation or reduction with a Hitachi Perkin-Elmer 139 UV-VIS Spectrophotometer at 340 nm in quartz cuvettes of 1 cm light path. All assays were performed with freshly prepared extracts in reaction mixtures of 2 ml total volume.

Glycerol dehydrogenase (glycerol-$NADP^+$ oxidoreductase, D-glyceraldehyde forming, E.C. No. 1.1.1.72). The standard assay mixture for the forward reaction contained: 100 mM bicarbonate buffer, pH 10.0; 0.3 mM NADP; 100 mM glycerol; and 0.1 or 0.2 ml of enzyme extract. The reaction was initiated by the addition of extract, and activity was linear up to 5 min. The standard assay for the reverse reaction contained 40 mM acetate buffer, pH 5.6; 0.25 mM NADPH; 100 mM D-glyceraldehyde; and 0.1 or 0.2 ml of enzyme extract. The reaction was initiated by the addition of extract, and activity was linear up to 3 min. NAD-linked glycerol dehydrogenase was not detected by using either glycerol or dihydroxyacetone in the forward and reverse reactions.

Glycerol kinase (ATP: glycerol phosphotransferase, E.C. No. 2.7.1.30). This was measured according to the procedure of Bublitz and Wieland (1962), using 0.2–0.4 ml enzyme extract. The reaction was initiated by the addition of glycerol.

Glycerol-3-phosphate dehydrogenase (sn glycerol-3-phosphate: NAD oxidoreductase, E.C. No. 1.1.1.8). This enzyme was measured according to Lowry and Passoneau (1972), using 0.1

to 0.2 ml enzyme extract. Dihydroxyacetone phosphate was used as substrate, and the reaction mixture without substrate served as a blank.

D-glyceraldehyde kinase (triokinase) (ATP: D-glyceraldehyde-3-phosphotransferase, E.C. No. 2.7.1.28). The reaction mixture contained 50 mM imidazole buffer, pH 7.5; 50 mM D-glyceraldehyde; 3 mM ATP; 3 mM $MgCl_2$; 0.6 mM NAD: 0.1 mM sodium arsenate; 2 U/ml glyceraldehyde-P-dehydrogenase; and 0.1 to 0.2 ml of enzyme extract. The reaction was initiated by the addition of D-glyceraldehyde. After a lag of 1 min, activity was linear up to 5 min. The reaction mixture without ATP served as a blank.

Dithiothreitol present in the buffer used in extraction of the enzymes interferred with the protein determination. Therefore, the proteins in the enzyme extract were precipitated with 10% TCA, washed once with fresh 10% TCA and finally with ethyl ether in order to eliminate this interference. The amount of protein present in the extract was determined by the Biuret method (Gornall et al., 1949).

Results

Characteristics of Strains upon Isolation. Following filtration-concentration and plating of conidia of *N. tetrasperma* strain 180.27, 3102 colonies were isolated to W-M slants. On the basis of growth tests on corresponding selective polyols and scoring for perithecia and protoperithecia, as described in Materials and Methods, the isolates were found divisible into the following four groups. Fifty-seven isolates (group 1) gave negative growth tests and did not develop protoperithecia on W-M slants. An additional 107 isolates gave positive growth tests, but were defective in protoperithecial development, as follows: two (group 2) developed protoperithecia only on the growth-test slants, whereas 105 (group 3) developed protoperithecia on neither slant. All of the remaining isolates (group 4) behaved as wild type, i.e., grew and developed protoperithecia on both slants. No isolates were found which gave negative growth tests but developed protoperithecia on W-M slants.

Stage of Blockage of Protoperithecial Development. Seventy-nine isolates from groups 1 and 3, initially scored as lacking protoperithecia, were further examined microscopically to determine at which stage protoperithecial development was blocked. The stages were scored in accordance with the morphogenetic sequence of protoperithecial development described by Viswanath-Reddy and Turian (1975), viz., bud, ascogonial hook, young ascogonial coil, ascogonial coil with envelope, and protoperithecium. Fifteen of the 79 isolates showed no protoperithecial development, whereas the remainder showed partial development to one of the following stages: four developed buds; three, ascogonial hooks; five, young ascogonial coils; and 52, ascogonial coils with envelopes.

Table 1. Protoperithecial stages produced by complementation between five mutant strains blocked at two different stages of protoperithecial development

Most advanced proto-perithecial stage of mutant	Mutant strain	Mutant strain			
		X228*A*	X793*A*	M108*a*	M1051*a*
None	G660*A*	hook	young coil	protoper.[a]	protoper.[a]
None	X228*A*		protoper.	protoper.[a]	protoper.[a]
None	X793*A*			protoper.[a]	protoper.[a]
Young coil	M108*a*				protoper.
Young coil	M1051*a*				

[a] These six complementation tests involved strains of opposite mating type, and subsequently produced fertile perithecia

Selection of 12 Group 1 Isolates for Further Study. Twelve group 1 isolates, each initially unable to grow on one of the four selective polyols, glycerol, mannitol, sorbitol or xylitol, as sole carbon source, were chosen for further studies and were designated by a letter indicating the selective polyol, followed by an isolation number (Tables 2 and 4). Subsequent growth tests showed that these 12 mutant strains were unable to utilize most of these four polyols as carbon source rather than only the single selective polyol in the particular filtration-concentration experiment from which each strain was obtained.

Complementation Tests for Protoperithecial Development. Three mutant strains which showed no protoperithecial development and two which produced young ascogonial coils were tested for complementation in petri dishes containing W-M medium (Table 1). All 10 pairings of the five strains showed complementation, as judged by the production of a more advanced stage of protoperithecial development than in the control cultures of the five strains grown separately. Moreover, six of the pairings involved strains of opposite mating type and subsequently produced perithecia that ejected black ascospores. Unlike *N. crassa,* strains of both like and unlike mating type of *N. tetrasperma* are heterokaryon-compatible.

Isolation of Self-Sterile Ascospores for Genetic Analysis. The 12 mutant strains chosen for further study were crossed to *N. tetrasperma* wild types 180.27 and 85 (Table 2). Crosses were made by inoculating both parents onto crossing medium simultaneously, or by using the wild types as protoperithecial parents. Attempts were made to recover self-sterile ascospores (homokaryotic for mating type) from these crosses, for purposes of genetic analysis, by selectively isolat-

Table 2. Frequencies and characteristics of dwarf-ascospore isolates from crosses of 12 mutant strains × *N. tetrasperma* wild types 180.27 and 85, respectively[a]

Mutant strain	Number of dwarf ascospores				Number of viable isolates						Mating type of self-sterile isolates			
	isolated		viable		self-fertile		cryptically self-fertile		self-sterile		A		a	
	180.27	85	180.27	85	180.27	85	180.27	85	180.27	85	180.27	85	180.27	85
G27a	100	206	6	4	2	0	1	0	3	4	3	4	0	0
G66a	–	214	–	36	–	3	–	2	–	31	–	30	–	1
G660A	398	50	54	26	31	3	15	0	8	23	7	17	1	6
M108a	345	51	83	13	57	1	10	0	16	12	11	3	5	9
M234a	50	–	6	–	6	–	0	–	0	–	0	–	0	–
M1051a	318	50	66	14	36	0	2	0	28	14	27	6	1	8
S14a	51	–	8	–	4	–	3	–	1	–	0	–	1	–
S204a	–	50	–	3	–	1	–	1	–	1	–	1	–	0
S277a	526	101	47	18	36	5	4	0	7	13	6	11	1	2
X228A	404	100	53	48	33	9	11	0	9	39	9	8	0	31
X583a	53	–	2	–	2	–	0	–	0	–	0	–	0	–
X793A	276	–	108	–	82	–	21	–	5	–	4	–	1	–
Total	2521	822	433	162	289	22	67	3	77	137	67	80	10	57

[a] Dashes indicate infertile crosses

Table 3. Allelic ratios and parental:recombinant ratios among the self-sterile progeny from crosses of six mutant strains × *N. tetrasperma* wild type 85

Mutant strain	Allelic ratios						Parental		Recombinant		
	Polyol utilizaton			Protoperithecial production			poly$^-$ proto$^-$	poly$^+$ proto$^+$	poly$^-$ proto$^+$	poly$^+$ proto$^-$	
	+	−	p[a]	+	−	p[a]					p[a]
G66a	17	14	>0.05	21	10	<0.05	9	16	5	1	<0.01
G660A	11	12	>0.05	23	0	<0.05	0	11	12	0	>0.05
M108a	5	7	>0.05	3	9	>0.05	7	3	0	2	<0.05
M1051a	8	6	>0.05	7	7	>0.05	6	7	0	1	<0.01
S277a	6	7	>0.05	10	3	<0.05	3	6	4	0	>0.05
X228A	2	37	<0.01	25	14	>0.05	13	1	24	1	>0.05

[a] Probability of a chance deviation from the 1:1 random expectation

ing the smaller (dwarf) ascospores under a stereomicroscope (Howe, 1964). Two of the crosses to wild type 180.27 and four of the crosses to wild type 85 were infertile. Ascospore germination frequencies from the remaining 18 crosses were low (17.2 and 19.7 per cent with wild types 180.27 and 85, respectively). Additionally, isolates were often self-fertile (heterokaryotic for mating type) or cryptically self-fertile (nonselfing, but nevertheless shown to be heterokaryotic for mating type because of fertility with both *A* and *a* mating type testers), despite our attempts to select self-sterile ascospores on the basis of dwarfness. However, crosses to wild type 85 yielded a much higher percentage of self-sterile isolates (137/162 = 84.6%) than did crosses to wild type 180.27 (77/433 = 17.8%), and hence were used subsequently in genetic analysis. Segregation of the mating type alleles in crosses to both wild types differed significantly from a 1:1 chance expectation. These allelic ratios were 67:10 (Chi-square = 42.2, $p < 0.01$) in the crosses to wild type 180.27, and 80:57 (Chi square = 3.86, $p < 0.05$) in the crosses to wild type 85.

Genetic Analysis with Self-Sterile Ascospores. The self-sterile progeny from crosses of six of the mutant strains to wild type 85 were scored with respect to allelic ratios and parental:recombinant ratios for the two traits under study, polyol utilization and protoperithecial production (Table 3). Crosses of the other six mutant strains to wild type 85 were either infertile or yielded too few self-sterile ascospores to be meaningful (Table 2).

Table 4. Production (+) or non production (−) of viable ascospores in crosses between 12 mutant strains derived from *N. tetrasperma* wild type 180.27 and five strains having, from left to right, increasing genetic relatedness to wild type 180.27

Mutant strain derived from wild type 180.27	Strain crossed to mutant strains				
	N. crassa wild type	Interspecific hybrids		*N. tetrasperma* wild types	
	74	C_3T_1	C_1T_3	85	180.27
G27a	−	−	−	+	+
G66a	−	−	−	+	−
G660A	−	+	+	+	+
M108a	−	−	+	+	+
M234a	−	−	+	+	+
M1051a	−	+	+	+	+
S14a	−	−	−	−	+
S204a	−	−	−	+	−
S277a	−	−	+	+	+
X228A	−	−	+	+	+
X583a	−	−	−	−	+
X793A	−	−	+	−	+
Total+	0	2	7	9	10

Allelic ratios for polyol utilization showed no significant deviation from a 1:1 random expectation in any of the mutant strains except X228 (Table 3). Protoperithecial production, however, behaved more erratically, for three of the six mutant strains, G66, G660, and S277, showed significant deviations from a 1:1 allelic ratio.

All of the parental:recombinant ratios with respect to polyol utilization and protoperithecial production showed recombinants, indicating that these two traits were not determined by the same locus in any of the six strains analyzed (Table 3). In three of the six strains, G66, M108, and M1051, the two traits appeared linked.

Preliminary Introgressive Crosses. Because of the difficulties encountered in isolating viable, homokaryotic ascospores, introgression of genetic traits from the 12 mutant strains into *N. crassa* was attempted so as to facilitate genetic analysis by means of the homokaryotic ascospores and the well-marked linkage groups of that species (Howe and Haysman, 1966). The 12 mutant strains were first crossed to *N. crassa* wild type 74, but none of the 12 crosses produced viable ascospores (Table 4). The 12 mutant strains were, therefore, crossed to interspecific hybrids C_1T_3 and C_3T_1, which were developed by Metzenberg and Ahlgren (1973) to facilitate gene transference on those occasions when direct interspecific crosses prove infertile. In the nomenclature of those authors, hybrid C_1T_3 has a greater degree of *N. tetrasperma* than of *N. crassa* ancestry; hybrid C_3T_1, the converse. Production of viable ascospores by use of the two hybrids was then compared to that obtained in crosses using *N. tetrasperma* wild types 180.27 and 85, and also *N. crassa* wild type 74. Crosses were made by inoculating both parents onto crossing medium simultaneously, or by using the nonmutant strains as protoperithecial parents. The results clearly showed that fertility, as measured by production of viable ascospores, increased as genetic relatedness between these various parental strains increased, i.e., from left to right in Table 4.

Further effects of introgression became evident when progeny from the seven fertile crosses to hybrid C_1T_3 and from the two fertile crosses to hybrid C_3T_1 (Table 4) were scored (Table 5). Asci tended to be 4-spored, 8-spored, or intermediate types in crosses to hybrid C_1T_3, but 8-spored in crosses to the more *N. crassa*-like hybrid C_3T_1. Only nine of the 241 viable isolates were self-fertile, and eight of these nine involved the more *N. tetrasperma*-like hybrid C_1T_3 (Table 5). The allelic ratios, however, indicated a ten-

Table 5. Introgressive crosses between seven mutant strains and the hybrids C_1T_3 and C_3T_1, with the self-sterile progeny scored as parental or recombinant

Cross	Number of dwarf ascospores			Parental		Recombinant	
	isolated	viable	self-sterile	polyol$^-$ proto$^-$	polyol$^+$ proto$^+$	polyol$^-$ proto$^+$	polyol$^+$ proto$^-$
G660A × C_1T_3a	100	76	73	11	29	26	7
G660A × C_3T_1a	60	21	20	0	12	8	0
M108a × C_1T_3A	52	19	19	10	4	4	1
M234a × C_1T_3A	74	2	2	1	0	1	0
M1051a × C_1T_3A	62	32	32	0	2	30	0
M1051a × C_3T_1A	52	27	27	3	8	16	0
S277a × C_1T_3A	48	23	23	5	6	10	2
X228A × C_1T_3a	53	15	13	1	5	7	0
X793A × C_1T_3a	64	26	23	3	11	9	0
Total	565	241	232				

Table 6. Three- and four-point crosses for mapping *glp-4*(G660) and *glp-5*(M1051), after introgression into *N. crassa*, with respect to various *N. crassa* markers

Genotype of zygote and percentage recombination[a]	Parental combinations		Recombinations								Total and percentage germination
			Singles, region 1		Singles, region 2		Singles, region 3		Doubles, regions 1 & 2		
$\frac{+\ b\ c\ d}{a\ +\ +\ +}$	$+bcd$	$a+++$	$++++$	$abcd$	$+b++$	$a+cd$	$+bc+$	$a++d$	$++cd$	$ab++$	
$\frac{ylo\text{-}1\ +\ pan\text{-}2\ trp\text{-}2}{+\ glp\text{-}4\ +\ +}$	33	21	1	0	2	1	7	7	0	0	72
1.4 4.2 19.4											72.0
$\frac{+\ ad\text{-}1\ +\ pan\text{-}2}{ylo\text{-}1\ +\ glp\text{-}4\ +}$	125	129	7	9	1	0	9	8	0	0	288
5.6 0.35 5.9											88.0
$\frac{ad\text{-}1\ glp\text{-}4\ +}{+\ +\ rib\text{-}1}$	178	179	0	0	8	8	–	–	0	0	373
0 4.3											96.1
$\frac{ad\text{-}1\ glp\text{-}4\ +}{+\ +\ rib\text{-}1}$	158	129	2	4	4	4	–	–	0	0	301
2.0 2.7											75.3
$\frac{ad\text{-}1\ +\ pan\text{-}2}{+\ rib\text{-}1\ +}$	82	78	2	2	2	2	–	–	0	1	169
3.0 3.0											85.4
$\frac{+\ cr\text{-}1\ +}{glp\text{-}5\ +\ ad\text{-}9}$	20	19	5	4	3	13	–	–	1	0	65
15.4 26.2											65.0

[a] Cross 3 was incubated at 25°C; cross 4, at 18°C

dency for diminution or loss of the protoperithecial trait, as was observed earlier in the intraspecific crosses. Again, as in the intraspecific crosses, only the smaller ascospores (dwarfs) were isolated from these hybrid crosses, since self-sterile isolates for genetic analysis were desired.

Chromosome Mapping Following Further Introgression. Introgression was most effective with mutant strains G660 and M1051, which were the only strains that produced viable ascospores in crosses with hybrid C_3T_1 (Tables 4 and 5). Therefore, a G660 and an M1051 isolate from these two fertile crosses to hybrid C_3T_1 were each used to initiate three successive backcrosses to *N. crassa* wild type 74, followed by two successive backcrosses to marked strains of *N. crassa*. During this further introgression into *N. crassa* genetic background, the protoperithecial trait, which behaved erratically in the preliminary introgressive crosses, as noted above, was lost, whereas the polyol trait consistently segregated 1:1. Therefore, only the polyol trait in mutant strains G660 and M1051 was studied further.

Isolates of mutant strains G660 and M1051 from the third successive backcrosses to *N. crassa* wild type 74 were crossed to alcoy, which allowed pairwise-testing of six of the seven linkage groups simultaneously (Perkins et al., 1969). Strain G660 showed linkage to *ylo-1* (parental:recombinant ratio = 111:46; $X^2=27$, $p<0.01$), indicating a location in either group III or group VI. A follow-up cross using *rib-1* as a marker placed G660 in linkage group VI (parental:recombinant ratio = 95:1; $X^2=92$, $p<0.01$). Strain M1051 showed linkage to *al-1* (parental:recombinant ratio = 104:65; $X^2=9$, $p<0.01$), indicating a location in either group I or group II. A follow-up cross using mating type as a marker placed M1051 in linkage group I (parental:recombinant ratio = 49:7; $X^2=31.5$, $p<0.01$).

Further mapping was done using isolates of mutant strains G660 and M1051 derived from three to five successive introgressive backcrosses to *N. crassa*. G660 and M1051 were designated *glp-4* and *glp-5*, respectively, because of nonutilization of glycerol as sole carbon source. The first four crosses, collectively, with these isolates (Table 6) localized *glp-4* between

Table 7. Specific activities (nanomoles/min/mg protein) of certain enzymes of glycerol metabolism in two wild types and in two glycerol non-utilizing mutant strains

Enzyme	Wild type		Mutant strain	
	180.27	74A	*glp-4A*	*glp-5a*
Glycerol dehydrogenase, NAD-linked	[a]	[a]	[a]	[a]
Glycerol dehydrogenase, NADP-linked	34	44	51	31
D-glyceraldehyde kinase	11	12	16	[a]
Glycerol kinase	3	5	[a]	2.4
Glycerol-3-P dehydrogenase, NAD-linked	4	5.9	3.4	9[b]

[a] Not detected

[b] Crude extract was diafiltered through an Amicon UM 10 filter before the activity was determined

ad-1 and *rib-1*, while the fifth cross verified the order of the critical markers as *ad-1 – rib-1 – pan-2*. The centromere is located between *ad-1* and *rib-1* (Stadler, 1956), but the position of *glp-4* relative to the centromere has not yet been determined. The order *ad-1-glp-4-rib-1* was determined as a result of using an incubation temperature of 18° C (cross 4), after finding no recombination in region 1 at 25° C (cross 3). Stadler (1956) showed that lowering the temperature of incubation increased recombination frequency, especially near the centromere.

Cross 6 (Table 6) placed *glp-5* left of both *cr-1* and *ad-9*. Since another glycerol nonutilizing mutant, *gly-u* (234), was mapped 2.2 units to the right of *ad-9* (Holm et al., 1976), further verification of the location of *glp-5* was sought by analysis of 26 tetrads from the cross *ad-9a* × *glp-5A*. The following gene order and map distances were obtained: mating type 7.7 centromere 17.3 *glp-5* 26.9 *ad-9*.

Enzymological Analysis of Strains glp-4 and glp-5. NAD-linked glycerol dehydrogenase activity was not detected in either *N. crassa* or *N. tetrasperma* wild types. On the contrary, NADP-linked glycerol dehydrogenase activity was present, and both forward and reverse reactions could be demonstrated with the crude extracts of both wild types and both mutants (Table 7).

In both *N. crassa* and *N. tetrasperma* wild types, the specific activities of NADP-linked glycerol dehydrogenase and D-glyceraldehyde kinase were considerably higher than the specific activities of glycerol kinase and NAD-linked glycerol-3-phosphate dehydrogenase. The activity of NADP-linked glycerol dehydrogenase was about ten-fold greater than that of glycerol kinase, and the activity of D-glyceraldehyde kinase was at least two-fold greater than that of NAD-linked glycerol-3-phosphate dehydrogenase (Table 7).

In mutant *glp-4*, no glycerol kinase activity was detected (Table 7). Although both NADP-linked glycerol dehydrogenase and D-glyceraldehyde kinase activities were present in this mutant strain in levels comparable to those in the two wild types, *glp-4* failed to grow on glycerol as sole carbon source. The activity of NAD-linked glycerol-3-phosphate dehydrogenase in *glp-4* was also comparable to those in the two wild types.

In mutant *glp-5*, no D-glyceraldehyde kinase activity was detected. The activities of NADP-linked glycerol dehydrogenase, glycerol kinase, and NAD-linked glycerol-3-phosphate dehydrogenase in *glp-5* were similar to those in the two wild types (Table 7).

Discussion

Polyols were implicated as possibly playing a role in the sexual cycle of *Neurospora tetrasperma* when it was found that substitution of glycerol, mannitol, sorbitol or xylitol for sucrose in the culture medium allowed protoperithecial development to occur at 37° C, a nonpermissive temperature for protoperithecial development when sucrose was the carbon source (Viswanath-Reddy and Turian 1972, 1975). In the present study, tests with these polyols on *N. crassa* strains *fl A*, *fl a*, and eight wild types did not enable protoperithecial development at 35° C, and therefore *N. tetrasperma* was used.

The concomitant occurrence of polyol auxotrophy and defective protoperithecial development in about one-third of the mutant strains isolated (57/164) indicated a possible relationship between polyol metabolism and protoperithecia development, while the high frequency of such isolates suggested pleiotropic effects rather than double mutations. Alternatively, induction of only single mutations to polyol auxotrophy would have been necessary in these 57 isolates, if *N. tetrasperma* wild type 180.27 were already heterokaryotic for defective protoperithecial development at the time this strain was used in the mutation experiments.

Twelve of the 57 strains were selected for further study, and crosses of six of these twelve strains to *N. tetrasperma* wild type 85 yielded sufficient homokaryotic ascospores for genetic analysis. In these six crosses, defective protoperithecial development tended to segregate erratically, whereas polyol utilization usually segregated 1:1. Nevertheless, recombination occurred between the polyol and protoperithecial traits in all six crosses, indicating that the two traits were not determined by the same locus. When attempts were made to introgress both traits in two

of the strains into *N. crassa* for further genetic analyses, the protoperithecial trait was lost. Loss or diminution of markers during interspecific hybridization in Neurospora has been observed previously (Howe and Haysman, 1966). The instability of the protoperithecial trait to crossing and, in some instances, to repeated vegetative transfer, suggested temporary, nongenetic changes in at least some of the strains. Fitzgerald (1963) suggested that failure to develop protoperithecia in a strain of *N. crassa* was caused both by genetic factors and by an epigenetic cellular control system. Itoh and Morishita (1971) found that a cytoplasmic factor was responsible for protoperithecial formation in *N. crassa.*

Despite the instability of the protoperithecial trait, complementation was observed between five strains in all combinations. Some complementing pairs were blocked at apparently identical stages of protoperithecial development, suggesting the possible occurrence of additional, intermediate stages. Further assessment of the significance of the complementation was difficult, however, because, in the absence of recombination tests between these five strains, it was not known whether the complementation observed was intergenic or interallelic. Mutants blocked at progressive stages of protoperithecial development have not been previously identified in Neurospora, although female-sterile mutants have been studied (Mylyk and Threlkeld, 1974). Not all female-sterile mutants may be equally suitable for studies of protoperithecial development, however, since some female sterility may arise from causes other than protoperithecial defectiveness. Esser and Straub (1958) reported the occurrence of at least 5 loci operating in protoperithecial development in *Sordaria macrospora,* mutation at any one of which blocked further differentiation.

Polyol mutants isolated on the basis of nonutilization of a single polyol were generally found to be nonutilizers of other polyols as well, perhaps as a result of structural similarities of these sugar alcohols. Moreover, hexitols, pentitols and glycerol are metabolized via glycolysis. As many as three pathways (Fig. 1) have been demonstrated for the dissimilation of glycerol among animals, higher plants, fungi, and bacteria (Bublitz and Kennedy, 1954; Toews, 1966; Sillero et al., 1969; Yamada, 1960; Bergmeyer et al., 1961; Gancedo et al., 1968; Lin, 1976). In the first pathway glycerol is phosphorylated by an ATP-dependent kinase to sn glycerol-3-phosphate, which is then converted to dihydroxyacetone phosphate by either a flavin- or NAD-linked dehydrogenase. In the second pathway, glycerol is converted to dihydroxyacetone by an NAD-linked dehydrogenase. Dihydroxyacetone is then phosphorylated to dihydroxyacetone phosphate by an ATP-dependent kinase. In the third pathway glycerol is converted to glyceraldehyde by an NADP-linked dehydrogenase. Glyceraldehyde is then incorporated into the glycolytic pathway at two locations: (i) phosphorylation by means of an ATP-dependent triokinase into glyceraldehyde-3-phosphate; (ii) conversion into 3-phosphoglycerate through the sequential action of an aldehyde dehydrogenase and a glycerate kinase. In wild type strains of *N. crassa* and *N. tetrasperma*, we detected the first and third pathways but not the second pathway, although the latter occurs in procaryotes.

Mutants of Neurospora altered in glycerol utilization have been reported previously. One, *gly-u* (234), was deficient in inducible glycerol kinase and mapped to the right arm of linkage group I, 2.2 units distal to *ad-9* and 10.9 units proximal to *nit-1* (Nilheden et al., 1975; Holm et al., 1976). A second mutant, *glp-2*, lacked flavin-linked glycerol-3-phosphate dehydrogenase and mapped to linkage group II (Denor and Courtright, 1975). A third mutant, *glp-3* (744), utilized glycerol as sole carbon source with high efficiency, was female-sterile and altered in conidial production, and behaved as if allelic to the female-sterile mutant, *ff-1*, of Tan and Ho (1970) in linkage group II, between *arg-5* and *fl* (Courtright, 1975).

Two of our glycerol-nonutilizing mutants, *glp-4* (G660) and *glp-5* (M1051), introgressed into *N. crassa* background, as well as wild types *N. crassa* 74 and *N. tetrasperma* 180.27, were assayed for enzymes involved in the pathways of glycerol metabolism. Wild type strains of both species of Neurospora were analyzed, because the two mutants had been induced in *N. tetrasperma* 180.27 prior to introgression into *N. crassa.* Combined enzymological and genetic analyses indicated that *glp-4* lacked both constitutive and inducible glycerol kinase and was located in linkage group VI, between *ad-1* and *rib-1*. Similarly, *glp-5* was found to lack D-glyceraldehyde kinase and to be located in linkage group I, proximal to *ad-9*.

It has been postulated that in Neurospora, there are two glycerol kinases, one constitutive and the other inducible (North, 1974; Holm et al., 1976). North (1973, 1974) found that glycerol kinase activity could be induced in *N. crassa* by placing the cultures at 4° C after a period of growth at 26° C and that such induction required protein synthesis. We could demonstrate neither constitutive nor inducible glycerol kinase activity in *glp-4* at either 4° C or 26° C. Denor and Courtright (1974) suggested that glycerol was the only inducer of glycerol kinase at 26° C and that cold induction of the enzyme may result from the generation of an internal inducer, possibly glycerol-3-phosphate. However, North (1976) showed that at 26° C glycerol kinase activity was induced by several carbon sources, which included different sugars

and polyols. Attempts were made to differentiate the two glycerol kinases by use of antibodies raised against partially purified constitutive glycerol kinase from wild type *N. crassa* (Courtright, 1975b; Holm et al., 1976). Protein extracts from *gly-u* (234) which was grown under induced as well as noninduced conditions cross reacted with these antibodies, suggesting the presence of constitutive glycerol kinase; in addition, a radiochemical assay indicated that *gly-u* (234) had at least a low level of constitutive glycerol kinase but lacked inducible glycerol kinase.

Moreover, three types of kinases have been reported with interesting substrate affinities for glycerol, glyceraldehyde and dihydroxyacetone: (i) highly purified glycerol kinase from different organisms was shown to phosphorylate not only glycerol, but also D-glyceraldehyde, L-glyceraldehyde and dihydroxyacetone (Thorner and Paulus, 1973); (ii) purified triokinase (D-glyceraldehyde kinase) was shown to phosphorylate both D-glyceraldehyde and dihydroxyacetone at almost the same rate, whereas glycerol was not phosphorylated by this enzyme (Hers, 1962; Heinz and Lamprecht, 1961); and (iii) there is some evidence for the presence of a specific triokinase (dihydroxyacetone kinase) with higher affinity for dihydroxyacetone than for glycerol (Lin, 1976).

Genetic regulation of glycerol metabolism in eucaryotes is not well understood, although in *Escherichia coli* a *glp* regulon was found which was composed of three operons and three kinds of control mechanisms (Freedberg and Lin, 1973). A role assigned to one of the products of glycerol dissimilation, glycerol-3-phosphate, is the regeneration of NAD in the cytoplasm for the continuation of glycolysis by the so-called glycerophosphate shuttle. Glycerol-3-phosphate is also a basic component of glycerophosphatides, the building blocks of neutral fats and phospholipids (Fig. 1). Indeed, Mindich (1970) reported the cessation of phospholipid synthesis and a slowing of fatty acid synthesis as the most striking effects in glycerol nonutilizing strains of *Bacillus subtilis*. Morphologically, such mutants exhibited a slowing of cell division. In an inositol-requiring morphological mutant of *N. crassa*, phospholipids were reduced to one-fifth of the levels found in wild type (Fuller and Tatum, 1956).

Lipid synthesis occurs with very low amounts of glycerol-3-phosphate, owing to the low Km of glycerol-3-phosphate acyl transferase. If the concentration of glycerol-3-phosphate rises and oxygen is available, the mitochondrial flavin-linked glycerol-3-phosphate dehydrogenase will oxidize glycerol-3-phosphate to dihydroxyacetone phosphate. The multiplicity of pathways involved in glycerol dissimilation in various organisms appears to be geared to replenish dihydroxyacetone phosphate, normally a byproduct of glycolysis. Thus the glycerol metabolic pathways may be considered anaplerotic, a suggestion consistent with our finding that the *glp-4* and *glp-5* mutants, defective in two different pathways of glycerol dissimilation, grew well on sucrose.

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