REGULATION OF PHOSPHATE METABOLISM IN *NEUROSPORA CRASSA*: IDENTIFICATION OF THE STRUCTURAL GENE FOR REPRESSIBLE ACID PHOSPHATASE

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ABSTRACT

A mutant of *Neurospora crassa* with an altered repressible acid phosphatase has been isolated. The enzyme is much more thermolabile than that of wild type, and has an increased Michaelis constant. Tests of allelic interactions (in partial diploids) and *in vitro* mixing experiments were consistent with the mutation being in the structural gene for the enzyme. This gene, *pho-3*, was found to be located in the right arm of Linkage Group IV (LG IV). Thus, *pho-3* and the structural gene for repressible alkaline phosphatase, *pho-2* (LG V), map in separate linkage groups and cannot be part of the same operon. Neither of these structural genes is linked to the known regulatory genes, *nuc-1* (LG I), *nuc-2* (LG II), and *preg* (LG II).

A family of enzymes in *Neurospora crassa* functions to acquire phosphorus for the organism when inorganic phosphate is in short supply in the environment. In a milieu with ample phosphate, these enzymes are highly repressed. Under conditions of phosphate starvation, members of the family are derepressed in a roughly parallel fashion (LEHMAN, *et al* 1973). Enzymes that have been identified as members of the family are: (i) a repressible alkaline phosphatase (NYC, KADNER and CROCKEN, 1966); (ii) a repressible acid phosphatase that is both a phosphomono- and diesterase (NYC 1967); (iii) a high-affinity phosphate permease (LOWENDORF and SLAYMAN 1975); (iv) an O-phosphorylethanolamine permease (METZENBERG, unpublished results), and (v) a number of nucleases (HASUNUMA 1973; HASUNUMA, TOH-E, and ISHIKAWA, personal communication). Genetic studies indicate that a system of interacting positive and negative control elements causes enzymes of the family to be repressed when phosphate concentration is high, and expressed when it is low (METZENBERG, GLEASON and LITTLEWOOD 1974; LITTLEWOOD, CHIA and METZENBERG 1975). The structural gene for repressible alkaline phosphatase has been identified (LEHMAN and METZENBERG 1976). In this paper, we describe the identification of the structural gene for *N. crassa* repressible acid phosphatase.

MATERIALS AND METHODS

Strains and culture conditions. Strains of *Neurospora crassa* used in this study are listed in Table 1. Strains diploid for segments of the right arm of Linkage Group IV (LG IV R) were used

for dominance tests. These strains were constructed and characterized for genetic stability according to principles described previously (PERKINS 1972; METZENBERG, GLEASON and LITTLEWOOD 1974).

The composition of media and growth conditions were as described previously (GLEASON and METZENBERG 1974).

Analytical technique. Mycelia from air-sparged liquid cultures were collected on filter paper, washed twice with cold distilled water and lyophilized. The dry mycelial pads were finely powdered using a cold mortar and pestle and then extracted with cold 0.05 M sodium succinate buffer (pH 5.0). Extracts were centrifuged for 15 minutes at 17,000 × g and the pellets discarded. Where indicated, extracts were dialyzed twice against 100–200 volumes of the succinate buffer at 4° for six hours.

It was determined that repressible acid phosphatase is the only enzyme in extracts of *N crassa* with measurable bis-(*p*-nitrophenyl) phosphate phosphodiesterase activity as judged by the following criteria: (i) Extracts of wild-type strains grown on repressing levels of phosphate have no measurable phosphodiesterase activity. (ii) Extracts of wild-type strains grown on low, derepressing levels of phosphate have phosphodiesterase activity and all of this activity can be localized by electrophoresis in the species of repressible phosphatase (phosphomonoesterase) that has an acidic pH optimum. (iii) This acid phospho(mono-,di-)esterase is always detected in strains that should be producing catalytically-active repressible acid phosphatase (e.g.—*pho-3* (221t) strains grown on low phosphate at 25°, *pho-2* (MKG 1) strains grown on low phosphate at 25°, and $pcon^{c-6}$ and $preg^{c-2}$ strains grown on low or high phosphate at 25°), and is never detected in strains that should not be producing active repressible acid phosphatase (e.g.—*pho-3* (221t) strains grown on low phosphate at 37° and *nuc-1* (A1) and *nuc-2* (B1) strains grown on low phosphate at 25°). Therefore, crude extracts were specifically assayed for repressible acid phosphatase using bis-(*p*-nitrophenyl) phosphate (Sigma) as the substrate of choice. Each assay tube (1.1 ml) contained 4.4 μmoles of substrate (4 mM), 330 μmoles of sodium succinate, pH 5.0 (300 mM), and an appropriate amount of extract. The tubes were incubated at 25° for 10 minutes and then stopped and deproteinized by the addition of 1 ml of a mixture of ethanolamine (6.8 parts, v/v), syrupy (85%) phosphoric acid (3 parts), ethanol (45 parts) and water (45 parts). The quantity of free *p*-nitrophenol released was determined by the absorptivity of the deproteinized mixtures at 405 nm. Absorbance was found to be directly proportional to enzyme concentration between 1–15 units per assay tube and linear with time for 30 minutes. Extracts were assayed for repressible alkaline phosphatase by a method described previously (LEHMAN *et al* 1973), except that assay tubes were incubated at 25°C[1] instead of 37°. For both enzymes, a unit of activity was defined as the quantity of enzyme that catalyzes the release of one nmole of *p*-nitrophenol per minute at 25°. Specific activity was defined in units per mg protein. Protein concentration was estimated by the method of LOWRY *et al.* (1951).

Electrophoresis of cell extracts was performed on 2.5 × 15 cm Sepraphore III[R] cellulose polyacetate strips by electrophoresis (200 volts for 60 minutes at 4°) in each of the following buffers: (i) 0.05 M sodium citrate pH 4.0. (ii) 0.05 M sodium succinate, pH 5.0. (iii) 0.05 M MES (2[N-morpholino]ethane sulfonate), pH 6.0. (iv) 0.05 M imidazole-HCl, pH 7.0. (v) 0.05 M Tris-HCl, pH 8.1. Repressible acid phosphatase was located by placing the strips on agar-solidified reaction mixtures (2 mM bis-(*p*-nitrophenyl) phosphate, 300 mM sodium succinate buffer, pH 5.0, and 1% (w/v) Difco agar) in 100 × 15 mm square petri dishes. After incubation at 33° for 15–30 minutes, the dishes were opened and inverted over concentrated ammonium hydroxide to deprotonate the *p*-nitrophenol released during the reaction to the yellow-colored nitrophenolate ion. In some experiments (as indicated in RESULTS), p-nitrophenyl phosphate (CalBiochem.) or napthol AS-MX phosphate (Sigma) were used as the phosphatase substrate. After exposure to ammonia, *p*-nitrophenol was located by its color under visible light as indicated above, and free napthol AS-MX was located by its fluorescence under an ultraviolet lamp.

[1] The unit of enzyme activity in this paper is defined for assays at 25° instead of 37° as in previous publications from this laboratory. This was necessary because a highly thermolabile protein was being assayed.

TABLE 1

Neurospora crassa *strains**

Strain	Mating type	Genotype†	Source
UW-R103	A	*al-2*(15300)	FGSC; inbred to standard wild-type strain, 74-OR-1
UW-R401	A	*al-2*(15300)	FGSC; inbred to standard wild-type strain, 74-OR-1
UW-R402	a	*al-2*(15300)	FGSC; inbred to standard wild-type strain, 74-OR-1
253-R-4	A	*al-2*(15300); *pho-3*(221t)	UV-induced derivative of UW-R103; inbred to UW-R402
253-R-1	a	*al-2*(15300); *pho-3*(221t)	UV-induced derivative of UW-R103; inbred to UW-R402
linkage-tester	a	*al-2*(15300); *trp-3*(td 37); *tyr-1*(Y-6994)*pdx-1*(37803); *inl*(37401); *chl-2*(47904); *thi-3*(18558), *ars*(101)	R. L. METZENBERG, inbred to 74-OR-1
79-R-34	a	*ad-6*(Y175M30)	R. E. NELSON, inbred to 74-OR-1
FGSC 335	a	*leu-2*(37501), *pan-1*(5531), *mat*(B 57)	FGSC
267-R-89	A	*al-2*(15300); *leu-2*(37501), *pan-1*(5531), *pho-3*(221t), *mat*(B 57)	253-R-4 × FGSC 335
DP 46–939	a	*ylo-1*(Y30539y); T(IV→VI) ALS159 *cot-1*(c102t), *uvs-2*(no #)	D. PERKINS via E. BARRY
FGSC 1752	A	T(IV→I) NM152	FGSC
270-U3-4	A	*al-2*(15300); Dp[*pho-3*(221t)/T(IV→VI) ALS159 *cot-1*(c102t), *uvs-2*(no #)]	253-R-4 × DP 46–939
271-U8-4	A	Dp[*pho-3*(221t)/T(IV→I) NM152]	253-R-4 × FGSC 1752

* Abbreviations: FGSC, Fungal Genetics Stock Center (Humboldt State Univ. Foundation, Arcata, Calif.); *al*, albino; *pho*, phosphatase; *UV*, ultraviolet light; *trp*, tryptophan; *tyr*, tyrosine; *pdx*, pyridoxine; *inl*, inositol; *chl*, choline; *thi*, thiamine; *ars*, aryl sulfatase; *ad*, adenine; *leu*, leucine; *pan*, pantothenate; *mat*, mat; T, translocation; *ylo*, yellow; *cot*, colonial-temperature-sensitive; Dp, partial diploid.

† Each locus designation, in italics, is immediately followed by an allele number in parentheses.

Mutagenesis and detection of acid phosphataseless mutants. Conidia from two-week-old cultures of strain UW-R103 (*al-2*) were suspended in distilled water (1×10^6 cells/ml) and irradiated with ultraviolet light to about 10% survival. Treated conidia were plated on a low phosphate (0.05 mM) medium that also induces colonial growth (GLEASON and METZENBERG 1974) and incubated at 37° for three days. Each plate, which contained ca. 75 colonies, was then gently overlayed with 10 ml. of molten (43°) soft agar containing 1–2 mM bis-(*p*-nitrophenyl) phosphate and 300 mM sodium succinate, pH 5.0. After 1–1.5 hours, plates were opened and briefly inverted over ammonium hydroxide. Colonies were scored for repressible acid phosphatase activity by the presence or absence of the yellow color of free *p*-nitrophenol.

Colorless colonies were transferred to separate culture tubes containing liquid minimal medium and permitted to form individual cultures. Isolates were again scored for repressible acid phosphatase activity and for repressible alkaline phosphatase activity (LEHMAN *et al.* 1973) after growth on low phosphate medium at 37° and 25°. Isolates were also scored for the high-affinity phosphate permease by their ability or inability to grow at high pH and low phosphate concentration ("MOPS" medium—GLEASON and METZENBERG 1974).

RESULTS

Isolation of repressible acid phosphataseless mutants. Three mutants deficient in this enzyme were isolated from among 18,000 survivors of mutagenesis. Two of these were temperature-sensitive for the expression of both repressible acid phosphatase and repressible alkaline phosphatase, and were found to bear a single mutation in or close to the *nuc-1* locus. These will not be described further in this report. The third mutant was found to be temperature-sensitive for the appearance of repressible acid phosphatase only, and this phenotype was found to be due to a single-gene mutation [henceforth referred to as *pho-3* (221t)]. Table 2 shows the specific activities of repressible acid phosphatase and repressible alkaline phosphatase in derepressed cultures of *pho-3* (221t) and *pho-3*$^+$ strains. It is apparent that acid phosphatase activity was severely reduced in *pho-3* (221t) cultures a high temperature (37°) as compared with *pho-3*$^+$ (wild-type) cultures at that temperature. On the other hand, *pho-3* (221t) and

TABLE 2

Specific activities of repressible acid phosphatase and repressible alkaline phosphatase in cultures of siblings pho-3*(221t) and sibling* pho-3+ *strains**

Strain	Acid phosphate		Alkaline phosphatase	
	Growth at 37°	Growth at 25°	Growth at 37°	Growth at 25°
253-R-4 [*al-2*; *pho-3*(221t)]	8.9	122	119	821
253-R-1 [*al-2*; *pho-3*(221t)]	5.4	78	96	475
UW-R401 (*al-2*; *pho-3*+)	502	440	122	630
UW-R402 (*al-2*; *pho-3*+)	746	580	78	437

* Cultures were grown on low (0.05 mM) phosphate liquid medium by sparging with sterile air for 24 hours at 37° or 25°. Specific activity equals the total activity in the medium plus the total soluble activity in the mycelium divided by the total soluble protein in the mycelium.

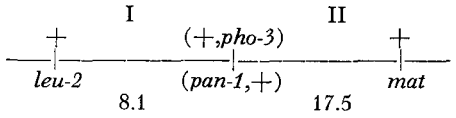
pho-3+ strains grown under derepressing conditions displayed identical patterns of alkaline phosphatase expression at high and low (25°) temperatures. *pho-3* (221t) strains grew on MOPS medium at either 37° or 25°, indicating that the repressible phosphate permease was functioning normally. Finally, when *pho-3* (221t) and *pho-3*+ strains were grown on high phosphate (7.5 mM) medium at 25°, the acid and alkaline phosphatase activities were repressed to undetectable levels (data not shown).

Localization of pho-3 *in the right arm of Linkage Group IV (LG IV).* Strain 253-R-4 [*al-2*; *pho-3* (221t)] was crossed to a linkage-tester strain bearing markers on all seven linkage groups (*al-2*; *trp-3*; *tyr-1*; *pdx-1*; *inl*; *chl-2*; *thi-3*, *ars*). Analysis of random spore progeny indicated that *pho-3* was linked to *pdx-1* and, therefore, located in LG IV. Strain 253-R-4 was subsequently crossed to strain 79-R-34 (*ad-6*); among 1192 prototrophic progeny, 35 were positive for acid phosphatase activity, indicating that *pho-3* was located about three map units from *ad-6* in the right arm of LG IV. Table 3 shows the results of a four-point cross involving three LG IV markers and *pho-3* (221t). The gene order deduced from this cross along with previous mapping data (RADFORD 1975) was: centromere, *leu-2*, (*pan-1*, *pho-3*), *mat*, where the precise order of *pan-1* and *pho-3* relative to the other markers remains uncertain.

The pho-3 *gene specifies the structure of repressible acid phosphatase.* Enzyme activities from mycelia of *pho-3* (221t) and *pho-3*+ (wild-type) strains were examined for temperature sensitivity. As shown in Figure 1, repressible acid phosphatase from *pho-3* (221t) strains was rapidly inactivated at 50° (curve

TABLE 3

Linkage of pho-3 *to* leu-2, pan-1, *and* mat*
Zygote genotype, per cent recombination, and inferred gene order



Crossover regions†	Relevant genotype of progeny	No. progeny scored
Parental	+,(+,*pho-3*),+	76
	leu-2,(*pan-1*,+), *mat*	82
I	+,(*pan-1*,+), *mat*	7
	leu-2,(+,*pho-3*),+	9
II	+,(+,*pho-3*), *mat*	15
	leu-2,(*pan-1*,+),+	21
I and II	+,(*pan-1*,+),+	0
	leu-2,(+,*pho-3*), *mat*	1

* Strain 253-R-4[*al-2*;*pho-3*(221t] was crossed to strain FGSC 335 (*leu-2*,*pan-1*,*mat*). Of 240 randomly isolated black ascospores, 212 (88%) germinated after heat shock and formed cultures.

† No progeny were isolated that were the result of a *single* crossover between *pan-1* and *pho-3*. However, one progeny was isolated that was the result of a crossover between *pan-1* and *pho-3* and a second crossover either in region I or II, depending on the order of the genes. The order of *pan-1* and *pho-3* within the *leu-2*-*mat* region cannot be deduced from the data.

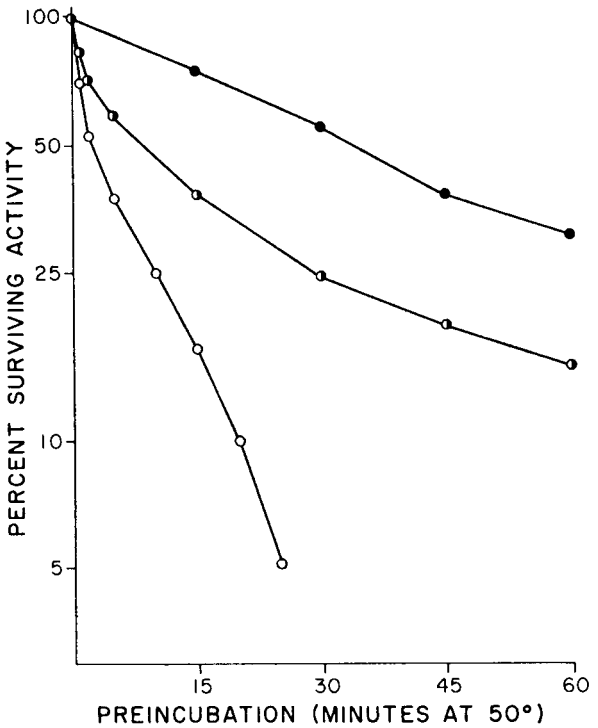


FIGURE 1.—Relative heat stability of repressible acid phosphatase in dialyzed cell-free extracts from *pho-3*(221t) and *pho-3*+ strains. Extracts were prepared from mycelia grown on low phosphate (0.05 mM) medium at 25°. ○, strain 253-R-4[*al-2*; *pho-3*(221t)]; ●, strain UW-R401 (*al-2*; *pho-3*+); ◑, 1.4:1.0 mixture of extracts from strain 253-R-4 and strain UW-R401. The protein concentration of each extract or extract mixture was ○, 1.86 mg per ml; ●, 1.43 mg per ml; ◑, 1.68 mg per ml.

○: $t_{1/2} = 2.5$ minutes) whereas the enzyme from *pho-3*+ strains was relatively stable at that temperature (curve ●: $t_{1/2} = 34$ min.). In addition, the residual activity of heat-treated enzyme mixtures was strictly additive (Figure 1, curve ◑). In contrast, the stabilities of the repressible alkaline phosphatase from all strains were identical (data not shown). These observations indicated that the *pho-3* (221t) lesion affected the heat-stability of repressible acid phosphatase only and that the effect was not due to a dissociable modifier of the enzyme.

The Michaelis constants (Km) and Arrhenius activation energies (E) of repressible acid phosphatase for the substrate, bis-(*p*-nitrophenyl) phosphate, are shown in Table 4. The Km of the phosphatase from strains bearing the *pho-3* (221t) lesion was significantly and reproducibly elevated (six fold) over that of the phosphatase from *pho-3*+ strains. The E of the phosphatase from *pho-3* (221t) strains differed from that of the phosphatase from *pho-3*+ strains by an average of one kcal. This difference is probably not significant, as the standard deviation of E among *pho-3*+ strains was equal to or greater than the difference in E between *pho-3*+ strains and *pho-3* (221t) strains in repeated experiments.

TABLE 4

Michaelis constants and activation energies of repressible acid phosphatase from sibling pho-3 *(221t) and sibling* pho-3+ *strains**

Strain	Genotype	Km (M)	E (kcal)
253-R-4	*al-2; pho-3* (221t)	1.49×10^{-3}	14.3
253-R-1	*al-2; pho-3* (221t)	1.30×10^{-3}	14.1
UW-R401	*al-2; pho-3+*	2.20×10^{-4}	14.7
UW-R402	*al-2; pho-3+*	2.43×10^{-4}	15.7

* Dialyzed cell-free extracts were prepared from mycelia grown on low (0.05 mM) phosphate media for 24 hours at 25°. Michaelis constants (Km) were calculated from initial velocities with 0.016 to 8.18 mM bis-(*p*-nitrophenyl) phosphate at 25° in 300 mM sodium succinate (pH 5.0). Activation energies (E) were calculated from initial velocities at 5°, 15°, and 25° with 4 mM bis-(*p*-nitrophenyl) phosphate in the above buffer. Calculations were made with the aid of a least squares computer program for slopes and intercepts. In all cases, coefficients of regression were greater than 0.997.

Undialyzed enzyme extracts from *pho-3* (221t) and *pho-3+* strains were subjected to electrophoresis on cellulose polyacetate strips at pH 4.0, 5.0, 6.0, 7.0, and 8.1. Repressible acid phosphatase was identified by phosphodiesterase activity as a single electrophoretic species (band) with a pI near pH 7. The electrophoretic behavior of this band was found to be identical when electrophoretograms of *pho-3* (221t) and *pho-3+* extracts were compared to one another. However, the activity of this band with both *p*-nitrophenyl phosphate and napthol AS-MX phosphate was visibly reduced when electrophoretograms of *pho-3* (221t) extracts were exposed to substrate concentrations below 1 mM. In contrast, the phosphomonoesterase activities of this band in electrophoretograms of *pho-3+* extracts was noticeably reduced only at substrate concentrations below 0.05 mM. These observations are consistent with the conclusion that the *pho-3* (221t) lesion affected the Michaelis constant of repressible acid phosphatase for these phosphate monoesters in addition to that for bis- (*p*-nitrophenyl) phosphate, a phosphate diester.

Apparent co-dominance of pho-3 *(221t) and* pho-3+ *in partial diploids.* Heterozygous partial diploids were isolated from among the progeny of crosses between insertional translocation and Normal Sequence strains. Strains 270–U3–4 and 271–U8–4 contain a Normal Sequence LG IV with the *pho-3* (221t) lesion and a duplicated piece of the right arm of LG IV carrying *pho-3+*. In strain 270–U3–4, the duplicated segment has been translocated to the right end of LG VI, and in strain 271–U8–4, the duplicated segment has been inserted in LG I (D. D. PERKINS, personal communication, and cf. Table 1). Examination of these strains for genetic stability indicated that fewer than 3.5% of the nuclei had lost the translocated segment carrying the *pho-3* locus after two cycles of vegetative growth (data not shown).

Figures 2 and 3 show the heat-inactivation curves for the repressible acid phosphatase activity in dialyzed extracts from strains 270–U3–4 and 271–U8–4, and from their respective parent strains. *A priori*, we would expect *pho-3* (221t)

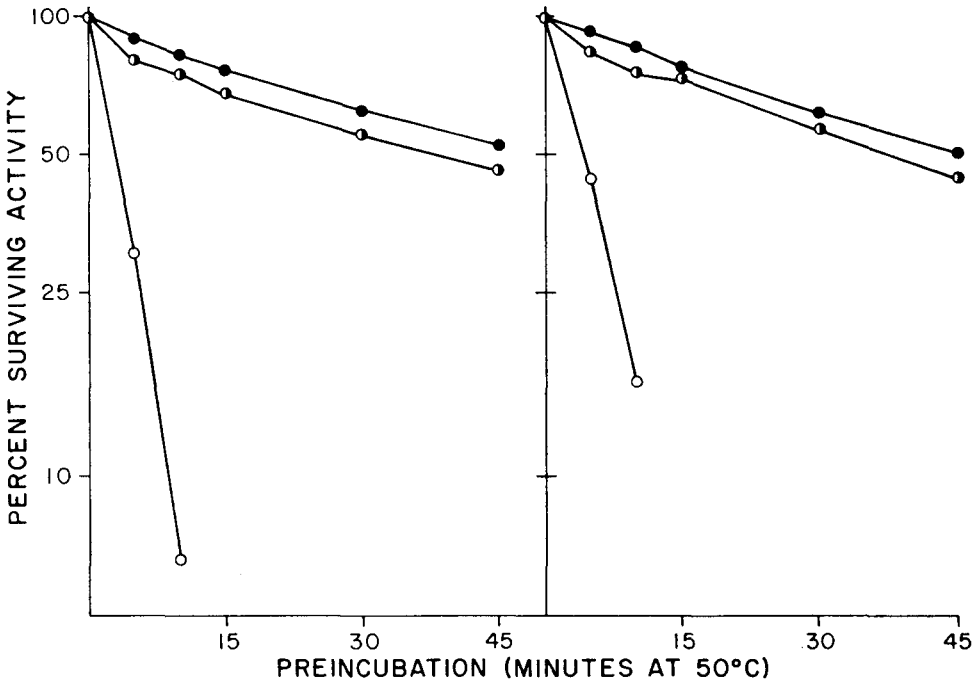


FIGURE 2 (left) and 3 (right).—Relative heat stability of repressible acid phosphatase in dialyzed cell-free extracts from *pho-3*(221t)/(T)*pho-3*+ partial diploids and their parent strains. Extracts were prepared from mycelia grown on low phosphate (0.05 mM) medium at 25°. The protein concentration of each extract during preincubation was 2.07 ± 0.13 mg per ml.

FIGURE 2: ○, strain 253-R-4[*al-2;pho-3*(221t)]; ●, strain DP46-939 [*ylo-1*,T(IV→VI)-ALS159 *cot-1,uvs-2*]; ◑, strain 270-U3-4[*al-2*; Dp [*cot-1*+,*pho-3* (221t),*uvs-2*+/T(IV→VI) ALS 159 *cot-1,pho-3*+,*uvs-2*]].

FIGURE 3: ○, strain 253-R-1. [*al-2;pho-3* (221t)]; ●, strain FGSC1752[T(IV→I) NM152]; ◑, strain 271-U8-4 Dp[*pho-3*(221t)/T(IV→I)NM152 *pho-3*+].

to be co-dominant with *pho-3*+ in the partial diploid strains in the sense that approximately equal molar amounts of the two gene products should be synthesized *in vivo*. Yet the results in Figures 2 and 3 indicate a large excess of the product of the wild-type gene. This apparent contradiction probably has a trivial basis. We have repeatedly observed that the mutant enzyme activity is much less stable than the wild-type enzyme activity even at "permissive" temperatures, and especially during dialysis. In addition, it is possible that the intrinsic activity of the mutant enzyme is less than that of the wild-type enzyme. (cf. Table 2). Hence, the apparent excess of wild-type enzyme activity in heterozygous partial diploids most likely reflects a loss of mutant enzyme activity during extraction and dialysis.

DISCUSSION

It is likely that the *pho-3* gene is the only gene that specifies the primary structure of *N. crassa* repressible acid phosphatase. JACOBS, NYC and BROWN (1971)

purified this enzyme to apparent homogeneity and found its physical-chemical properties to be consistent with that of a homo-dimer. The native molecule contained an even number of half-cysteine residues and could be dissociated into half-molecules by treatment with guanidine plus mercaptoethanol.

The structural gene for *N. crassa* repressible alkaline phosphatase has now been identified as being within the *pho-2* locus (LEHMAN and METZENBERG 1976). This gene was previously mapped in the right arm of Linkage Group V (GLEASON and METZENBERG 1974). Clearly, *pho-2* (LG V) and *pho-3* (LG IV) are not cistrons in a operon-like cluster of structural genes. In addition, genes that have been identified as controlling the simultaneous expression of *pho-2* and *pho-3* are located in linkage groups other than LG IV and LG V, i.e.—*nuc-1* (LG I), *preg* (LG II), and *nuc-2* (LG II). Therefore, these regulatory genes are not control sites adjacent to the structural genes, and would be expected to exert their control over the structural genes via the synthesis of regulatory macromolecules.

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