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Genetic analysis of the phosphatases in *Aspergillus nidulans*

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1. INTRODUCTION

The objective of the present work was the investigation of the genetics of a group of enzymes, the phosphomonoesterases, in *Aspergillus nidulans*, and the development of a system for the combined biochemical and genetic analysis of these enzymes. The phosphomonoesterases (hereafter referred to as phosphatases, for short), are characterized by their catalysis of the reaction:

$$RO—PO_3H_2 + R'OH \rightleftharpoons R'O—PO_3H_2 + ROH$$

Selection of this particular system was influenced by the following considerations:

1. Present knowledge suggests that in each of a variety of organisms there exists a number of phosphatases, differing from each other in their pH optima and in other enzymatic characteristics. (For a general review, see Roche, 1950.)

2. Histochemical techniques are available (review: Pearse, 1960) for the detection of phosphatases which differ in their pH optima.

3. Extensive investigations have been conducted on the alkaline phosphatase of *Escherichia coli* (Garen, 1960; Garen & Levinthal, 1960; Torriani, 1960). A comparison of this system with what has turned out to be a much more complex one in *Aspergillus* should prove of value.

2. METHODS AND MATERIALS

With the exception of the strain *bi1*; *orn9 cha* (kindly supplied by E. Käfer, McGill University), all the strains were from the stock held in the Department of Genetics, Glasgow University. For details of the strains, media, symbols and routine methods employed in the genetics of *Aspergillus nidulans*, reference should be made to Pontecorvo, Roper, Hemmons, MacDonald & Burton (1953), Pontocorvo & Käfer (1958), Roberts (1963) and Apirion (1963).

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(i) *Media*

(*a*) *Minimal medium*

$NaNO_3$ 6 g., $MgSO_4$, $7H_2O$ 0·52 g., KCl 0·52 g., KH_2PO_4 1·52 g., dextrose 10 g., unwashed Oxoid Agar No. 3 10 g., zinc and iron traces, singly distilled water 1 l.; pH adjusted to 6·5 with 4 N NaOH before sterilization; autoclaved at 10 lb. pressure for 15 min.

(*b*) *Limiting phosphate medium* (*solid*)

Identical to the minimal medium with the exception that the KH_2PO_4 is omitted. The wild-type grows to a limited extent on this medium owing to the traces of phosphate in the agar.

(*c*) *Sodium fluoride medium*

Minimal medium containing 0·05 M NaF; pH adjusted to 6·7 with 1 N HCl. Fluoride is known to be an inhibitor of phosphatase activity in certain organisms.

(*d*) *Glycerophosphate medium*

$NaNO_3$ 6 g., $MgSO_4$, $7H_2O$ 0·52 g., KCl 0·52 g., dextrose 10 g., trishydroxymethylaminomethane 2·4 g., β-glycerophosphate 12 g., Oxoid Agar No. 3 10 g., zinc and iron traces, distilled water 1 l.; pH adjusted to 8·2 with 4 N HCl; autoclaved at 10 lb. pressure for 15 min. The β-glycerophosphate (Sigma Grade 1 disodium DL-salt) is added to the solid medium and the medium melted at 100°C. This ensures reasonable sterilization without breakdown of the β-glycerophosphate.

(*e*) *Limiting phosphate medium* (*liquid*)

$NaNO_3$ 6 g., $MgSO_4$, $7H_2O$ 0·52 g., KCl 0·52 g., dextrose 10 g., trishydroxymethylaminomethane 1 g., 0·03% (w/v) Difco casamino acids, zinc and iron traces, distilled water 1 l.; pH adjusted to 7·4 with 4 N HCl before sterilization at 10 lb. pressure for 15 min.

Unless specified to the contrary, all ingredients are of analytical reagent standard.

(ii) *Histochemical staining*

The method extensively used for the detection of alkaline phosphatase variants in *E. coli* involves the spraying of colonies with a buffered solution of *p*-nitrophenylphosphate. Enzyme activity is indicated by the release of the product *p*-nitrophenol which is intensely yellow at alkaline pH. This product is colourless at acid pH, and therefore this technique is not suitable for the detection of acid phosphatase activity in single colonies. Furthermore, because of the poor wetting properties of fungal mycelia and the rapid diffusion of *p*-nitrophenol, this technique is not even very suitable for the detection of alkaline phosphatase activity in *Aspergillus* colonies.

In view of the above difficulties it seemed plausible that a minor modification—described below—of the customary histochemical azo dye coupling methods for phosphatase activity (review, Pearse, 1960) would prove more suitable for the

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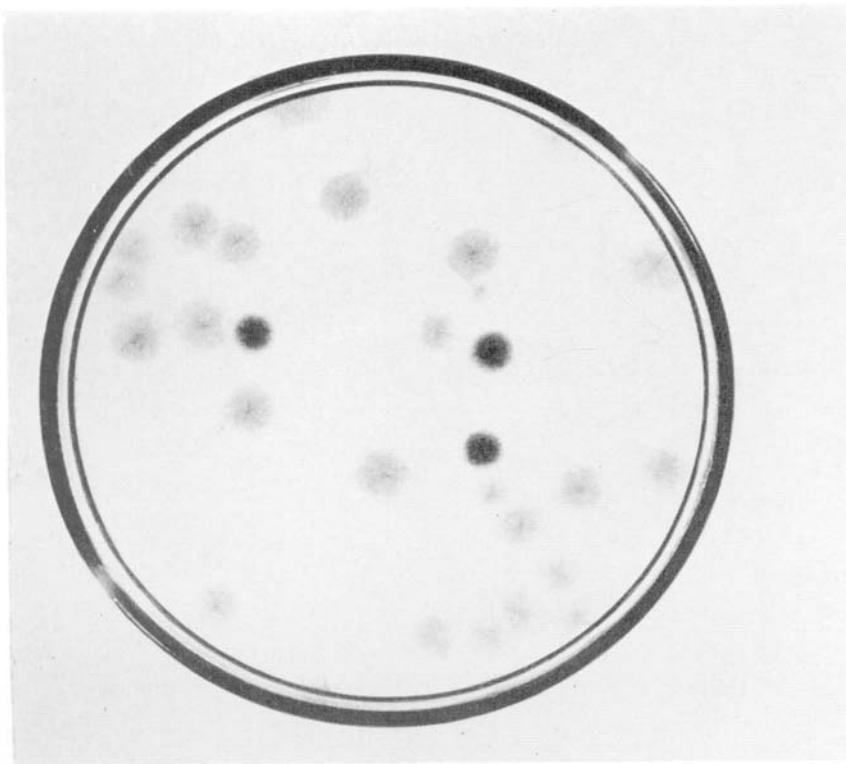

Fig. 1. Comparison of the alkaline phosphatase activity in *rA1* and wild-type colonies on limiting phosphate medium flooded with α-naphthyl phosphate and fast violet salt B in tris buffer (pH 8·3). *rA1* = dark colonies; wild-type = light colonies.

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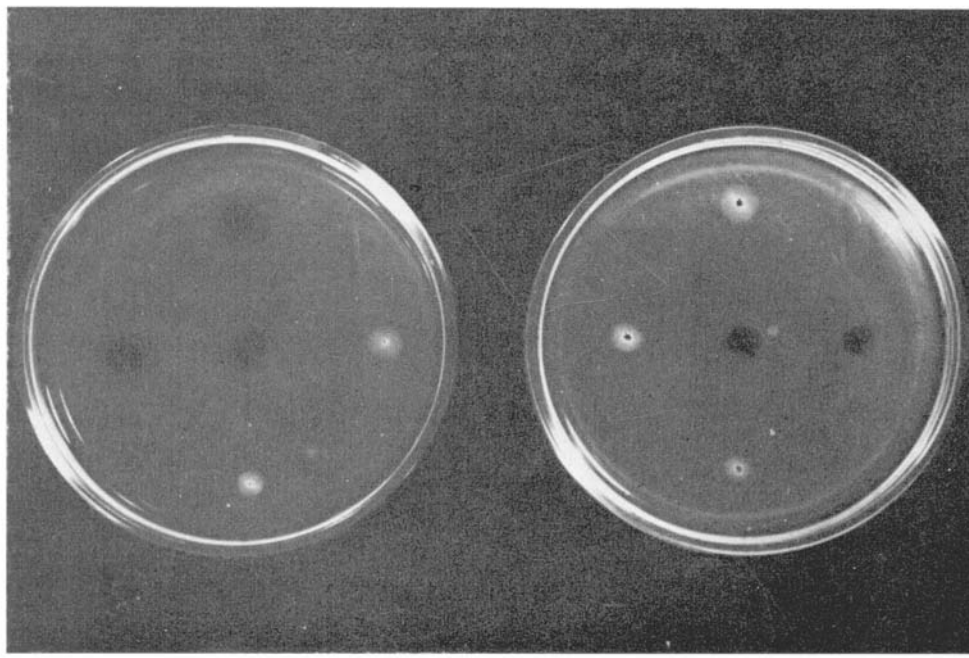

Fig. 2. Alkaline and acid phosphatase activity in wild-type, *palD8*, *palB7*, *pacA1* and *palcA1* colonies on limiting phosphate medium. Left: flooded with α-naphthyl phosphate and fast garnet G.B.C. salt in acetate buffer (pH 4·8). Right: flooded with α-naphthyl phosphate and fast violet salt B in tris buffer (pH 8·2). On each plate: centre, wild-type; top, *palD8*; left, *palB7*; right, *pacA1*; bottom, *palcA1*.

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detection of *both* acid and alkaline phosphatase activity in *Aspergillus* colonies (Plate I, Fig. 1; Plate II, Fig. 2).

After preliminary tests the technique routinely used became the following. Conidia are plated on dishes containing 20 ml. of limiting phosphate medium (solid) and the plates are incubated until the colonies are 5 to 10 mm. in diameter (24 to 36 hours). The colonies are then flooded with the appropriate buffer which contains the reagents, namely α-naphthylphosphate and a diazonium salt. In the presence of most phosphatases, the substrate (α-naphthylphosphate) is split with the release of α-naphthol and inorganic phosphate: the α-naphthol couples with the diazonium salt to form an insoluble coloured complex which precipitates *inside* the mycelium. The appropriate selection of diazonium salts enables one to detect phosphatase activity over a wide pH range (4·0–10·0).

The employment of limiting phosphate medium is essential as inorganic phosphate turned out to inhibit phosphatase *activity* in *Aspergillus*, as it is known to do in other organisms. Furthermore, it is known that phosphate is a specific repressor of alkaline phosphatase *synthesis* in *E. coli* (Garen & Levinthal, 1960).

(*a*) *Detection of alkaline phosphatase activity*

For one Petri dish 2 mg. sodium α-naphthyl acid phosphate (Sigma) and 20 mg. fast violet salt B (George Gurr) are dissolved in 4 ml. of 0·6 M tris-HCl buffer (pH 8·3). The dish is flooded with this solution at room temperature and the formation of a reddish-brown precipitate inside the mycelium indicates alkaline phosphatase activity.

(*b*) *Detection of acid phosphatase activity*

For one Petri dish 2 mg. sodium α-naphthyl acid phosphate (Sigma) and 20 mg. fast garnet G.B.C. salt (George Gurr) are dissolved in 4 ml. of 0·6 M acetate buffer (pH 4·8). As above, the dish is flooded with the solution at room temperature; enzyme activity is indicated by a brown precipitate inside the mycelium.

Routinely, the plates stained for alkaline or acid phosphatase activity were scored after 10 min. and 20 min., respectively. Classification at a fixed time is important for reasons which will become apparent later on in this paper.

(iii) *Liquid cultures and enzyme extraction from the mycelium*

Four 500 ml. Erlenmeyer flasks containing 200 ml. of limiting phosphate liquid medium are each inoculated with approximately $1{\cdot}5 \times 10^6$ conidia of the desired strain. The cultures are put on a wrist-action shaker for 3 days at 37°C. Forty-eight hours after inoculation the flasks are sealed to induce partial anoxia.

The resulting mycelium—in pellets about 2 mm. in diameter—is collected on a Buchner funnel, blotted, and roughly weighed (average yield between 6 and 8 g.). The mycelial pad is resuspended in 25 ml. of 0·05 M veronal buffer (pH 8·2) and disrupted at 4°C. in a high-speed homogenizer. The debris is removed by centrifugation at 2500 g for 20 min. at 4°C. For electrophoresis the crude extract (supernatant) is concentrated to about 1 ml. by collodion membrane ultra-filtration.

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(iv) *Electrophoresis*

The electrophoretic pattern of the phosphatases in crude extracts of *Aspergillus* was examined after horizontal, one-dimensional starch gel electrophoresis. Poulik's (1957) modification of Smithies' (1955) system has been adopted. Each sample is soaked onto a strip of Whatman 3 MM chromatography paper and inserted into the gel. Electrophoresis is conducted at room temperature with a stabilized d.c. potential of 6 V./cm. for 5 hours.

Phosphatase activity is detected by flooding each gel slice (approximate volume, 60 cm^3) with 25 ml. of a 0·6 M acetate buffer (pH 4·8) containing 25 mg. sodium α-naphthyl acid phosphate and 50 mg. fast garnet G.B.C. salt. The slices are incubated at room temperature for 60 min. and the reaction is stopped by immersing them in a methanol–acetic acid–water solution (parts per volume 4:1:5). During the incubation period, the interaction between the alkaline buffer of the gel and the acid buffer of the treatment results in a *gradual* decrease in pH from approximately 8·5 to 5·0. This technique enables one to detect *on the same gel* (Plate IV, Fig. 4) both alkaline and acid phosphates and, more generally, phosphates with differing pH optimum within that range.

(v) *Isolation of mutants*

(*a*) *Forward mutations*

Suspensions of conidia from two translocation-free strains (*bi1* and *bi1 rA1*) were exposed to 2650 Å ultra-violet irradiation to give about 5% survival and plated on limiting phosphate medium (supplemented with biotin) to yield between 100 and 150 colonies per dish. The plates from a given irradiation were flooded with the histochemical reagents for either acid or alkaline phosphatase activity. Colonies which showed either a substantial increase or decrease in staining intensity were isolated. Each set of twenty dishes analysed yielded an average of one to two clearly distinguishable mutants. As the reagents are toxic to this organism, isolation of the mutant colony was done within 20 to 30 min. after commencing the staining procedure. The purified strains were retested for both alkaline and acid phosphatase activity.

To make sure that each mutant used in the present work was of independent origin, only one mutant was taken from each irradiation and the conidia for each irradiation were harvested from a separate colony which almost certainly had arisen from a single conidium (conidia of *Aspergillus nidulans* are uninucleate).

(*b*) *Revertants*

The majority of the mutants which failed to stain at alkaline pH (for short, alkaline phosphataseless) are capable of only very poor growth on the β-glycerophosphate medium (Plate III, Fig. 3). In order to select revertants, dishes of this medium were heavily inoculated by stabbing at twenty-six points per dish with conidia from alkaline phosphataseless mutants. After 5 days' incubation at 37°C., a few rapidly growing sectors were observed and isolated. The revertant

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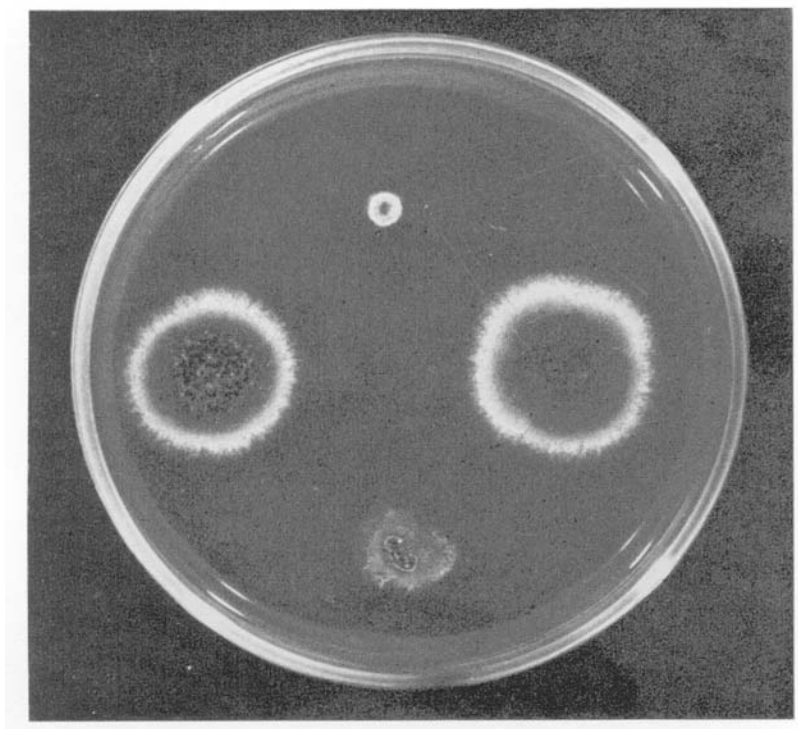

Fig. 3. Growth response of wild-type, and three mutants on β-glycerophosphate medium. Top, wild-type; left, *palB7*; right, *suA1palB7 palB7*; bottom, *suB2palB7 palB7*.

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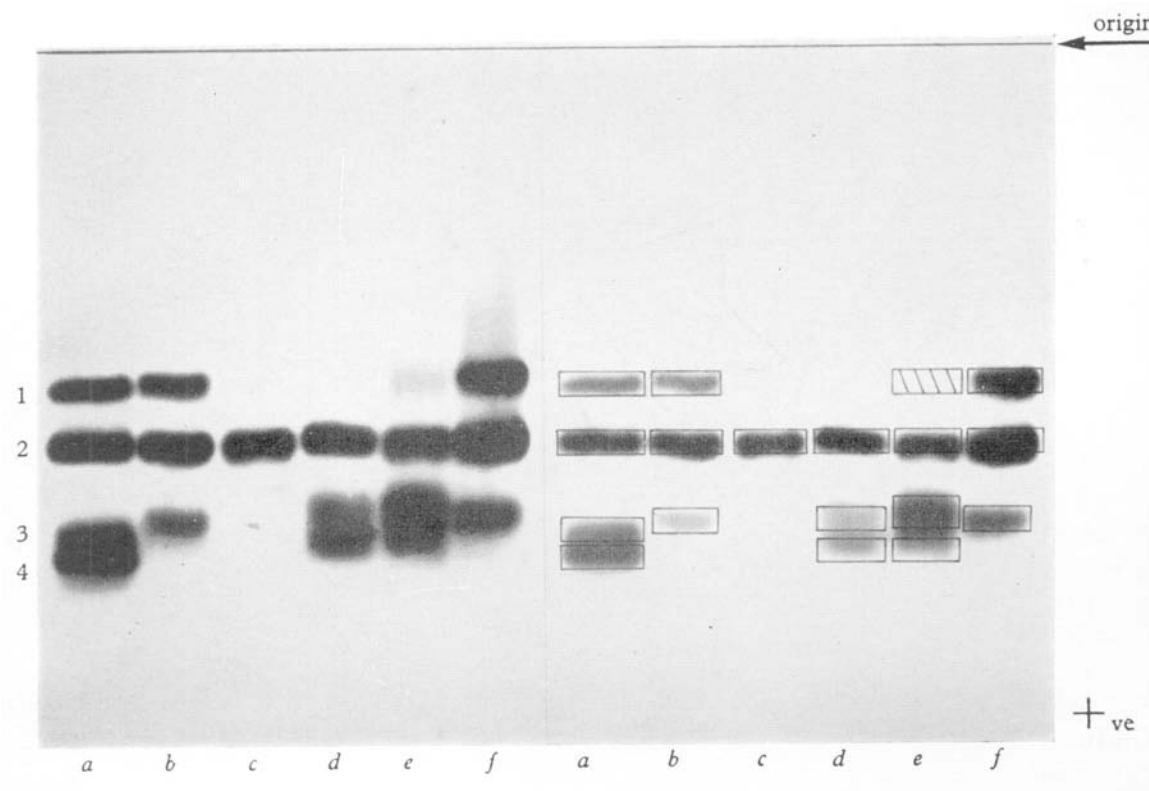

Fig. 4. Left: electrophoretic pattern of the alkaline (bands 1 and 2) and acid (bands 3 and 4) phosphatases in crude extracts of wild-type, *pacC5*, *palcA1*, *palB7*, *suA1palB7 palB7*, and *suB2palB7 palB7*. Right: the same, with the phosphatase bands outlined. (*a*) wild type; (*b*) *pacC5*; (*c*) *palcA1*; (*d*) *palB7*; (*e*) *suA1palB7 palB7*; (*f*) *suB2palB7 palB7*.

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strains were purified and retested for growth on β-glycerophosphate medium and for phosphatase activity. In this paper only two such revertants are further discussed.

(vi) *Genetic analysis*

Location of the mutants was usually accomplished in the following manner: a mutant was first assigned to a particular linkage group and then located with respect to other markers in that group. The assignment of a mutant to a linkage group is made possible by the use of the parasexual cycle (Pontecorvo, 1956) and the technique of mitotic haploidization (Pontecorvo, Tarr Gloor & Forbes, 1954; Pontecorvo & Käfer, 1958; Forbes, 1959). The employment of this method is simplified by the availability of a 'tester strain' (symbol: M.S.D.) with a marker on each of the eight linkage groups (Forbes, 1963) and the use of *p*-fluoro-phenylalanine, which facilitates the isolation of haploids from diploid strains (Lhoas, 1961; Morpurgo, 1961).

Perithecium analysis (Pontecorvo *et al.*, 1953) was employed for the location of a mutant with respect to other markers in the same linkage group.

(vii) *Notations*

The following symbols—followed by the isolation number—have been employed to designate the various phosphatase mutants:

r: substantial increase (as compared with the wild-type *bi1* strain) in phosphatase activity at alkaline pH but normal phosphatase activity at acid pH.

pal: loss or reduction in activity at alkaline pH but normal or increased activity at acid pH.

pac: loss or reduction in activity at acid but not at alkaline pH.

palc: simultaneous loss or reduction in activity at both alkaline and acid pH.

supal: recessive suppressor of a *pal* mutant.

The assignment of a mutant to a particular locus is indicated by the addition of a capital letter between the mutant symbol and the isolation number. For example, after *pal7* was shown from genetic analysis to be one of the mutants at the *palB* locus, its complete symbol became *palB7*.

3. RESULTS

(i) *Isolation and characterization*

With the aid of the azo dye coupling method (see Methods and Materials) twenty-seven mutants have been isolated. All of the mutants turned out to have a recessive phenotype in heterozygous diploids. On the basis of phosphatase activity and growth response on two selective media—β-glycerophosphate medium and sodium fluoride medium—the mutants (Table 1) have been classified into nine categories, as follows:

Class I. Three mutants (*rA1, r2, r3*) characterized by high alkaline but normal

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acid phosphatase activity (Plate I, Fig. 1). All three mutants produce a very reduced number of conidia, which makes them visibly distinguishable from wild-type.

Table 1. *Isolation and characterization of phosphatase mutants in* Aspergillus nidulans

Strain	Treatment	Mutant* symbol	Phosphatase activity		Growth response		Linkage group**
			pH 4·8	pH 8·3	β-glycerophosphate	Sodium fluoride	
bi1	U.V.	*rA1*					
,,	,,	*r2*	+	+ + +	+	+	I
,,	,,	*r3*					
bi1 rA1	,,	*palA1*	+ + +	−	−	0	III
,, ,,	,,	*palA3*					
,, ,,	,,	*pal2*					
,, ,,	,,	*pal12*	+ + +	−	−	−	III
,, ,,	,,	*pal14*					
,, ,,	,,	*palB5*					
,, ,,	,,	*palB7*					
,, ,,	,,	*palB9*	+ + +	−	−	−	VIII
,, ,,	,,	*palB10*					
,, ,,	,,	*palB13*					
,, ,,	,,	*palC4*	+ + +	−	−	−	IV
,, ,,	,,	*palC6*					
,, ,,	,,	*palD8*	+	−	+	+	VII
,, ,,	,,	*palE11*	+ + +	−	−	+	VIII
,, ,,	,,	*palF15*	+ + +	−	−	−	VII
,, ,,	,,	*pacA1*	−	+	+	+	IV
bi1	,,	*pacB4*	(±)	+	+	+	VIII
,,	,,	*pacC5*	−	+	+	+	VI
bi1 rA1	,,	*palcA1*	−	−	+	+	II
bi1	,,	*palcA2*	−	(±)	+	+	II
,,	,,	*palcB3*	(±)	(±)	+	+	III
,,	,,	*palcC4*	−	(±)	+	−	VIII
bi1 palB7	Spontaneous	*suA1palB7*	+ + +	(±)	(±)	(±)	VIII
,, ,,	,,	*suB2palB7*	−	+	+	+	VI

\+ = wild-type.
(±) = partial enzyme activity or growth.
− = no enzyme activity or growth.
+ + + = enzyme activity much greater than wild-type.
0 = not tested.

* The properties of the *supalB7* mutants were determined in the presence of *palB7*. Any diploid carrying any one of the above mutant alleles and the corresponding wild-type allele is phenotypically wild-type, i.e., all the mutants are recessive.

** Each mutant was assigned to a particular linkage group by means of mitotic haploidization of diploids. At least twenty-five haploids were analysed for each location. See text (genetic analysis).

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Class II. One mutant (*palD8*) showing reduced alkaline phosphatase activity, normal acid activity (Plate II, Fig. 2), and normal growth on both selective media.

Class III. Fourteen mutants showing reduced alkaline phosphatase activity and substantially increased acid phosphatase activity (Plate II, Fig. 2). Growth tests subdivide this class into two categories:

(*a*) Reduced growth on both β-glycerophosphate and sodium fluoride media (*palA1*, *3*; *pal2*, *12*, *14*; *palB5*, *7*, *9*, *10*, *13*; *palC4*, *6*, and *palF15*).

(*b*) Reduced growth on β-glycerophosphate medium but normal growth on sodium fluoride medium (*palE11*).

Class IV. Three mutants (*pacA1*, *pacB4* and *pacC5*) showing reduced acid phosphatase activity, normal alkaline activity (Plate II, Fig. 2), and normal growth on both selective media.

Class V. Four mutants showing a simultaneous reduction in alkaline and acid phosphatase activity (Plate II, Fig. 2), these four falling into two categories:

(*a*) Normal growth on both selective media (*palcA1*, *2*; *palcB3*).

(*b*) Normal growth on β-glycerophosphate medium and reduced growth on sodium fluoride medium (*palcC4*).

Class VI. Two second-step mutants ('suppressors'), revertants of the *palB7* (Class III*a*) mutant, selected on the basis of their ability to grow on β-glycerophosphate medium (Plate III, Fig. 3). Each falls into a separate class:

(*a*) Partial restoration of alkaline phospharase activity with corresponding partial growth response on both selective media (*suA1palB7*; *palB7*). Like the original mutant—*palB7*—this strain shows a level of acid phosphatase activity higher than wild-type.

(*b*) Full restoration of alkaline phosphatase activity and normal growth response on both selective media (*suB2palB7*; *palB7*). Unexpectedly, this strain shows a concomitant *reduction* in acid phosphatase activity.

(ii) *Electrophoresis*

In order to characterize the phosphatases of the wild-type *Aspergillus nidulans* and the ways in which the mutants differ from it, crude extracts of mycelia were subjected to starch gel electrophoresis. When the *bi1* strain is grown on limiting phosphate in the manner described in 'Methods and Materials', crude extracts show four electrophoretically distinct components. By staining at alkaline or acid pH, the slower components (bands 1 and 2, Plate IV, Fig. 4) turn out to be alkaline phosphatases (pH optimum greater than 7·0), while the two faster components (bands 3 and 4) are acid phosphatases (pH optimum less than 6·0).

Results obtained from the electrophoretic analysis of extracts from the wild-type (*bi1* strain) and *pacC5*, *palcA1*, *palB7*, *suA1palB7 palB7*, or *suB2palB7 palB7* are consistent with the staining reactions of their colonies on agar plates. To be specific, *palB7* shows a loss of activity of an alkaline phosphatase component (band 1); *pacC5*, a loss of activity of an acid phosphatase component (band 4) and *palcA1*, a loss or reduction in activity of both acid and alkaline phosphatase components (bands 1, 3 and 4).

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Electrophoretic analysis of extracts of the two 'suppressor' strains reveals two most striking situations. Strain *suB2palB7 palB7* has regained complete activity of the (alkaline) component originally absent in *palB7* (band 1) but this is coupled with a loss of acid phosphatase activity in band 4. Strain *suA1palB7 palB7* has regained only partially the (alkaline) activity in band 1 but this is coupled with an electrophoretic alteration of an acid phosphatase component, this time in band 3.

A preliminary electrophoretic analysis of a number of other *pal* mutants (*palA1*; *palC4, 6*; *palD8*; *palE11* and *palF15*) has been undertaken. In every case, the mutant extracts showed a loss of enzyme activity in band 1. To date, no mutant has been isolated which affects either the enzyme activity or the electrophoretic mobility of the faster of the two alkaline phosphatase components, i.e. band 2.

In view of the fact that none of the *pal* mutants has lost this faster alkaline phosphatase, how is it that these mutants are at all detectable by histochemical staining on agar plates at pH 8·2 (Plate II, Fig. 2)? The simplest hypothesis is that the two alkaline phosphatases differ in their pH optima (say 8·5 and 7·5). If this were the case a loss of the pH 8·5 enzyme alone would result in a substantial reduction, though not a complete loss, of phosphatase activity in intact colonies at pH 8·2; and, furthermore, this would result in a detectable difference in the staining rates of the mutant and wild-type colonies. A similar hypothesis can account for the detection of acid phosphataseless mutants which have lost only one of the two acid phosphatases.

(iii) *Location of mutants*

The various phosphatase mutants were first assigned to their linkage groups by the routine technique of haploidization of diploids, combining each of these mutants and a tester strain MSD (Forbes, 1963) marked in each linkage group. The mutants so assigned were then crossed with strains multiply marked in the relevant linkage group. The results of these two procedures are shown on Text-fig. 1. In computing the recombination percentages in crosses involving a *pal* and a *pac* or *palc* mutant, it was taken that in every case the double mutant condition is lethal *on limiting phosphate medium*, an assumption which has been verified in one case (*palB7* × *palcA2*). Of the 15 loci which have been identified, 12 have been assigned to a particular region in their linkage group, though for 3 of them the order is only tentative.

All three *r* mutants are on linkage group I. The various *pal*, *pac* and *palc* mutants fall into 6, 3 and 3 loci, respectively. Furthermore, the two suppressor mutants define two additional loci.

The loci at which two or more mutants have been located are *palA*, *palB*, *palC* and *palcA*. Meiotic analysis demonstrates that *palA1* is between the linkage group III markers *arg2* and *gal1*, showing about 23% recombination with the former and about 14% recombination with the latter. The mutants *pal2, 3, 12* and *14* also map in this interval (Table 2). A complementation test (Table 3) involving a diploid heterozygous for *palA1* and *palA3* confirmed the allelism of these two.

The *palB* locus includes the following mutants: *palB5, 7, 9, 10, 13*. It is situated

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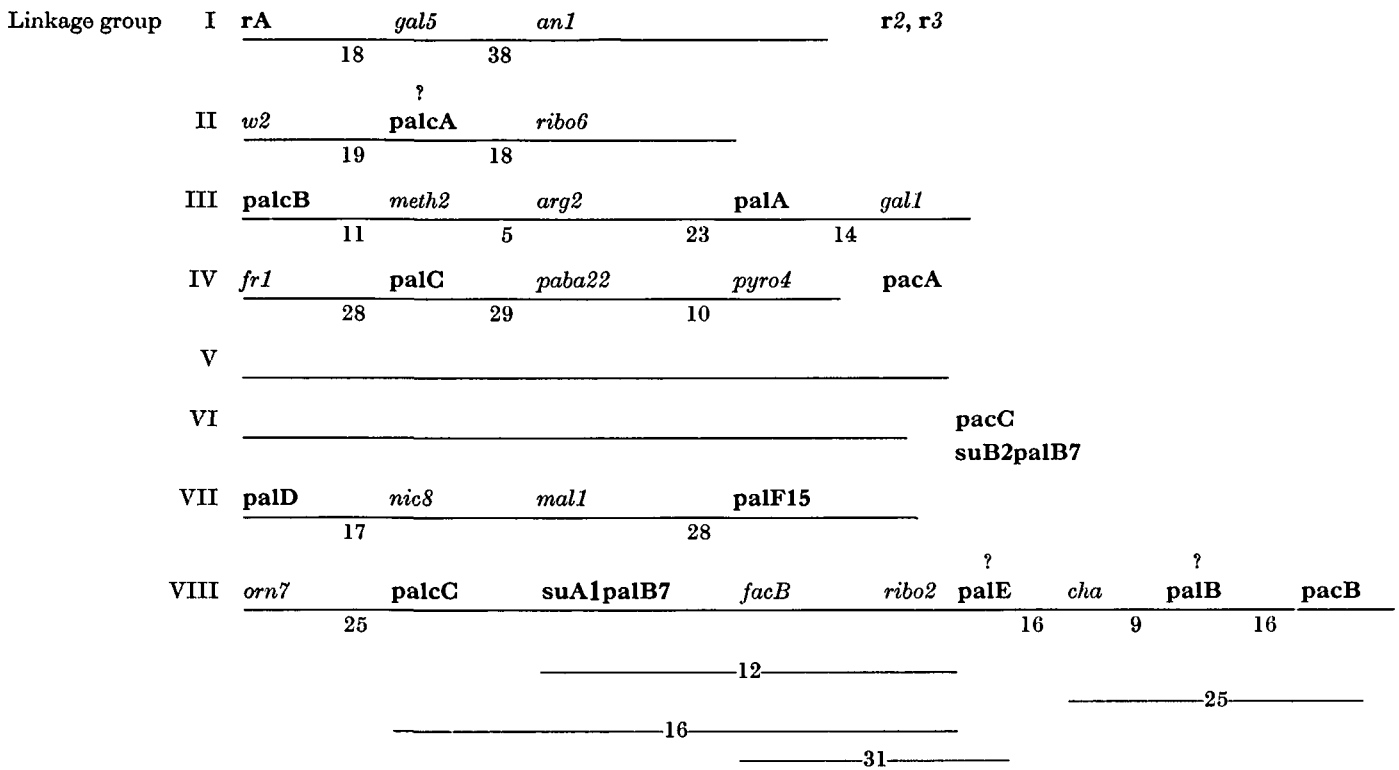

Text-fig. 1. Location of the phosphatase mutants (heavy-type) on the linkage map of *Aspergillus nidulans*. Distances are given in meiotic recombination percentages as found in the present work. In some cases, indicated by '?', the order given is the most likely but not certain.

Mutants placed to the right of a particular linkage group are those for which location is based solely on mitotic haploidization.

between *cha* and *pacB* on linkage group VIII, giving about 9% and 16% recombination with these two, respectively. Diploids involving *palB7* and each of the four other *palB* mutants are non-complementing (Table 3). Furthermore, all mutants show approximately the same degree of linkage to *cha* (Table 4).

Table 2. *Comparison of recombination percentages of* palA1, 3, pal2, 12 *and* 14 *with respect to* arg2 *and* gal1

Type of cross: $\frac{arg2\ +\ gal1}{+\ pal\ +}$

Phosphatase mutant	Tested segregants	Percentage recombination in the interval: *arg—pal*	*pal—gal*
palA1	143	23·1	14·0
palA3	52	17·3	9·6
pal2	50	22·0	14·0
pal12	87	20·7	19·5
pal14	90	16·7	11·1

The results between crosses are homogeneous.

$arg2—pal$: $\chi^2_4 = 1{\cdot}51$; $p > 0{\cdot}80$
$pal—gal1$: $\chi^2_4 = 3{\cdot}34$; $p > 0{\cdot}50$

Locus *palC* is in linkage group IV between *fr1* and *paba22*, giving 28% and 29% recombination with these, respectively. Allelism of *palC4* and *palC6* has been established by complementation tests (Table 3).

Table 3. *Complementation pattern of twelve alkaline phosphataseless mutants in diploids carrying pairs of* pal *mutants in the* trans *configuration*

Mutants *pal*	3	4	6	7	8	9	10	11	13	15
1	—	+	+	+	+	0	0	+	0	+
3	.	0	0	0	+	0	0	0	0	0
4	.	.	—	+	+	0	0	+	0	+
5	.	.	0	—	+	0	0	0	0	0
6	.	.	.	+	+	0	0	+	0	+
7	.	.	.	.	+	—	—	(+)	—	+
8	.	.	.	.	.	+	+	+	+	+
11	.	.	.	.	.	.	.	.	0	+

The degree of complementarity was determined by the histochemical method for alkaline phosphatase activity (see Methods and Materials).

\+ = wild-type activity.
— = mutant activity.
(+) = activity less than the wild-type but greater than either mutant.
0 = not tested.

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Repeated mitotic analyses have consistently shown that *pacA1* is situated on linkage group IV; however, this mutant recombines freely with various widely-spaced markers in this linkage group, namely, *meth1*, *fr1*, *paba22* and *pyro4*.

Table 4. *Comparison of recombination percentages of* palB5, 7, 9, 10 *and* 13 *with respect to* cha

Type of cross: $\frac{cha\ +}{+\ pal}$

Phosphatase mutant	Tested segregants	Percentage recombination in the interval: *cha*—*pal*
palB5	144	9·0
palB7	164	9·1
palB9	204	12·0
palB10	204	10·6
palB13	256	10·5

The results between crosses are homogeneous.

$\chi^2_4 = 0{\cdot}99; p > 0{\cdot}90.$

The mutants *palcA1* and *palcA2* are alleles; the *palcA1* + / + *palcA2* diploid is non-complementing for acid phosphatase activity and ambiguous in respect of alkaline phosphatase because *palcA2* is leaky at alkaline pH. A cross involving these two mutants gave no wild-type recombinants out of 3449 ascospores.

Table 5. *Segregation of the* suB2 *suppressor of* palB7

Type of cross: $\frac{+\ \ suB2palB7}{lac1\ \ +}\ \frac{palB7}{+}$

Genotype of progeny	No.	Phosphatase activity	
		pH 4·8	pH 8·3
lac + +	7	+	+
+ + +	6	+	+
lac + *pal*	5	+ + +	—
+ + *pal*	5	+ + +	—
+ *supal pal* + *supal* +	11	—	+
lac supal pal *lac supal* + *	16	—	+
Total	50		

* Two strains from this class were crossed to the *bi1* strain. One of the crosses showed segregation for both alkaline and acid phosphatase; the other segregated for only acid phosphatase. This shows that the phenotype of *suB2palB7* by itself is acid phosphataseless.

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The *suA1* suppressor of *palB7* is in linkage group VIII and shows about 12% recombination with *ribo2*. There are indications that *suA1palB7* and *ribo2* may be loosely linked to *cha*.

As mentioned previously, the *suB2palB7*; *palB7* strain is characterized by the complete restoration of alkaline phosphatase activity with a concomitant loss of acid phosphatase activity. The suppressor by itself—separated from *palB7* by outcrossing to wild-type—shows a loss of acid phosphatase activity (Table 5).

4. CONCLUSIONS

The most striking features of these enzyme systems are (1) the number of types of phosphatase variants and (2) the large number of loci controlling enzyme activity. It has been possible to isolate mutants which affect either separately or simultaneously alkaline and acid phosphatase activity. In several instances this loss or reduction in enzyme activity involves one or more of the four electrophoretically separable phosphatase components present in crude extracts of the wild-type. Of these four components, two have alkaline and two acid phosphatase activity.

Furthermore, of the two suppressor strains so far investigated, one shows a complete restoration of alkaline phosphatase activity with a concomitant loss of an acid phosphatase component; the other shows a partial restoration of alkaline phosphatase activity with an electrophoretic alteration of the other acid phosphatase component.

One possible explanation of these results is that the electrophoretic components with phosphatase activity represent four distinct heteropolymeric enzymes (i.e., each is made up of two or more different polypeptide chains) and that some of the polypeptide chains are common to more than one of these enzymes. Consider only the three enzymes, mutants of which have been identified: one alkaline and two acid phosphatases. The alkaline phosphatase might be composed of the chains $abcd$ and the two acid phosphatases of the chains $abef$ and $acfg$, respectively. A mutation in the d, e or g chains could result in the loss of activity in a single alkaline or acid component; a mutation in the b, c or f chains could result in the simultaneous loss of activity of an alkaline and acid or both acid phosphatase components; and finally, a mutant in the a chain could result in the loss of activity of all three enzymes.

In a similar manner one can account for the unusual properties of the two suppressor strains. On can imagine that in the original *palB7* mutant the d chain is affected and causes the loss of alkaline phosphatase activity ($abcd'$). The *suB2* suppressor affects the c (common) chain in such a way that it compensates for the effect of the mutation in the d chain with respect to alkaline phosphatase activity, i.e., $abc'd'$ has alkaline activity like $abcd$. But this altered c chain causes loss of activity of one of the acid phosphatase components, namely, $ac'fg$.

On the other hand, the *suA1* suppressor affects the b (common) chain in such a way that it partially compensates for the original mutation in the d chain but results in an electrophoretic alteration of the other acid phosphatase component, namely, $ab'ef$.

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The minimum number of loci affecting the phosphatases in *Aspergillus nidulans* is fifteen. Of these loci, at least *nine* control the activity of an electrophoretically single alkaline phosphatase (band 1). This result becomes particularly striking when compared with *E. coli*. In this organism the alkaline phosphatase is controlled by three known loci, two regulatory and one structural (Echols, Garen, Garen & Torriani, 1961).

As in other cases (Jacob & Monod, 1961) one or more of the fifteen loci in *Aspergillus* may be regulator genes, permease genes, or operator sections of structural genes, and thereby control the rate of synthesis of the enzymes. In fact, the *r* mutants, which are characterized by a raised level of alkaline phosphatase activity, could well be regulatory in nature. Obviously, the precise functions of the various loci are a matter for more detailed investigations.

SUMMARY

1. A histochemical method has been applied to the detection of alkaline and acid phosphatase mutants in single colonies of *Aspergillus nidulans*.

2. With the above method it has been possible to isolate mutants in which the alkaline and acid phosphatase activities are affected either separately or simultaneously.

3. Crude extracts of wild-type *A. nidulans* contain four electrophoretically distinct phosphatase components, two with activity at alkaline pH and two with activity at acid pH. Genes affecting three of the four components have been identified.

4. Two suppressor mutants of an alkaline phosphataseless mutant (*palB7*) have been isolated. In a strain carrying *palB7* and one of these suppressors, the restoration of an alkaline phosphatase component is accompanied by loss of the faster acid phosphatase component. In a similar strain carrying the other suppressor, the partial restoration of the alkaline phosphatase component goes with an electrophoretic alteration of the slower acid phosphatase component.

5. Genetic analysis of twenty-seven mutants has resulted in the identification of fifteen loci affecting the phosphatases. All these loci have been assigned to linkage groups, and twelve of them were also mapped meiotically in relation to other loci.

6. One possible model (based on heteropolymeric proteins) has been proposed to account for the electrophoretic and genetic data on the various phosphatase and suppressor mutations.

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