THE LOCALISATION OF ALKALINE PHOSPHATASE

IN

Neurospora crassa

by

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Presented to the Faculty of Graduate Studies

University of Manitoba

In partial fulfilment

of

The requirements for the degree

MASTER OF SCIENCE

OCTOBER, 1973

FOR MY LATE MOTHER - ALICE BUCHANAN

Some would climb the mountain,
yet stumble over the pebble in
their path.

original.

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ABSTRACT

The location of the non-specific alkaline phosphomonoesterase [E. C. 3.1.3.1] in cells in *Neurospora crassa* wild type 533, was determined by a variety of procedures. It was found that the enzyme was located in the cytoplasm, and not apparently associated with any identifiable cell organelle.

ACKNOWLEDGEMENT

My gratitude to Dr. H. Lees, for his encouragement and evaluation of this thesis, and to Dr. F.W.J. Davis, who introduced the problem.

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HISTORICAL

The inter and intracellular distribution of alkaline phosphatase has been investigated in a large number of organisms. Its general distribution in normal animal tissues has been described by Bourne and Gomori (1941) and in neoplastic tissue by Furth and Kabat, (1941). Gutman and Knitzler, (1941) investigated its distribution in the kidneys; Corner, (1944) and Leckie, (1955) that in the ovaries. Placental alkaline phosphatase has been studied by Hard, (1946); Strauss, (1960) and Deane (1947). Triantaphyllopoulos and Tuba (1959) described the enzyme's distribution in rat small intestine, while its intracellular distribution in the small intestine was described by Krugelis (1946) and Emmel (1946). Danielli (1946) concluded that alkaline phosphatase seldom occurred in animal cytoplasm, except in rapidly regenerating tissue. The distribution of alkaline phosphatase has also been studied in blood and bone marrow, the nervous system, skin and hair, cartilage and bone, teeth, and cells in tissue culture.

The purification and properties of alkaline phosphatase from Hela cells has been described by Tan and Aw (1970). Comparatively little work has been done on its localisation in procaryotes. Ghosh, Wouters and

Lampen, (1971) found that in Bacillus subtilis alkaline phosphatase was present in clusters attached to the peripheral plasma membrane, with smaller sites of activity in the cytoplasm where it was unattached to any discernible structure. An alkaline phosphatase has been found at the external surface of the cytoplasmic membrane, i.e., in the periplasmic space in Myxococcus xanthus. Enzyme dissociated from the membrane migrated into the cytoplasm where it was mainly associated into cytoplasmic aggregates.

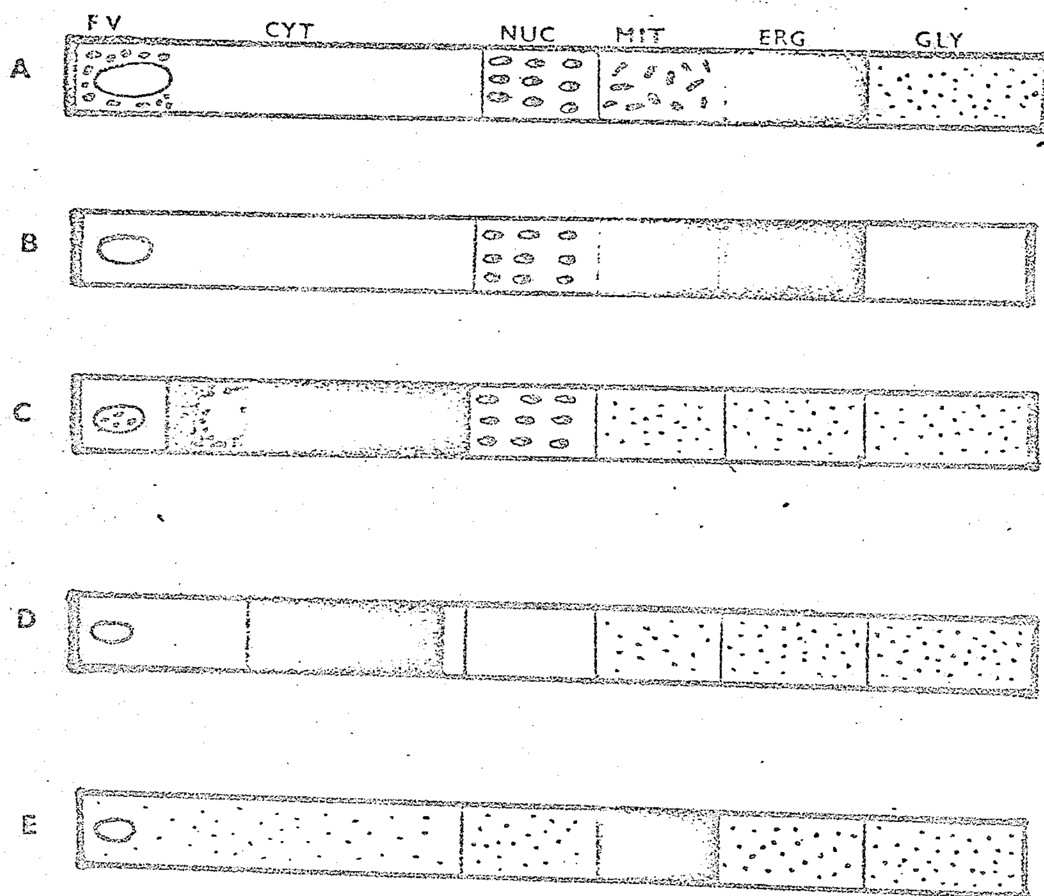
In Escherichia coli, the enzyme has been found in the periplasmic space, external to the cytoplasmic membrane (Neu and Heppel 1965). This distribution in E. coli has been confirmed by electron microscopy [Brockman and Heppel (1965) and Done, Shorey, Loke and Pollard (1965)].

Periplasmic localisation has also been shown in Pseudomonas aeruginosa (Cheng, Ingram and Costerton 1970). Zalokar (1959) demonstrated alkaline phosphatase in the cytoplasm of centrifuged hyphae of N. crassa [See Figure I, page 3].

There are no apparent references to any non-specific alkaline phosphatases being found in green plants.

FIGURE I. CYTOCHEMICAL REACTIONS ON CENTRIFUGED HYPHAE

Zalokar, M. (1960).



A. DIAGRAM OF CENTRIFUGED HYPHAE

B. PAULY'S REACTION FOR PROTEIN

C. ALKALINE PHOSPHATASE - GOMORI.

D. " " DIAZO SALT

E. ACID PHOSPHATASE

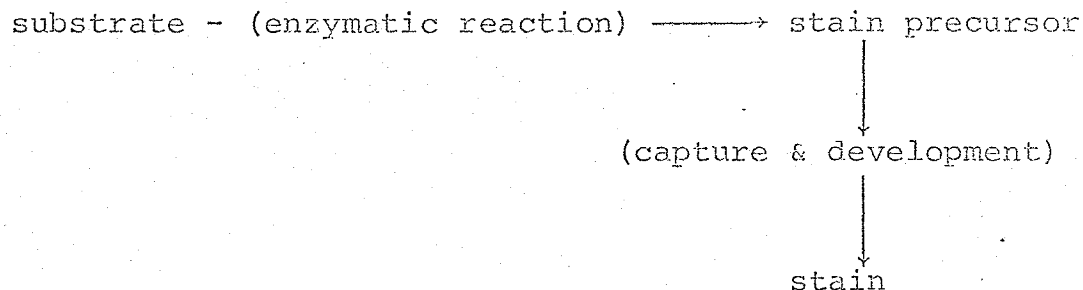
F -fat; V-vacuole
CYT- supernatant
NUC- nucleus
MIT- mitochondria
ERG- ergastoplasm
GLY- glycogen

INTRODUCTION

Two types of non-specific alkaline phosphatases have been shown to be present in *N. crassa*; one constitutive, the other repressible. (Nyc, Kadner and Crocken, 1966; Nyc, Kadner and Brown 1968; Davis and Lees 1972).

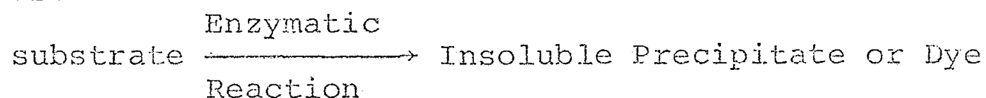
Repressible strains of *B. subtilis* , subsequently derepressed, have been observed to produce alkaline phosphatase (Ghosh, Wouters, Lampen 1971).

In general, histochemical methods depend on the following reactions:-



(Holt and Sullivan 1958)

The mechanism for the more recent post-coupling (Burstone 1962) non-coupling and fluorescence techniques is indicated thus:-



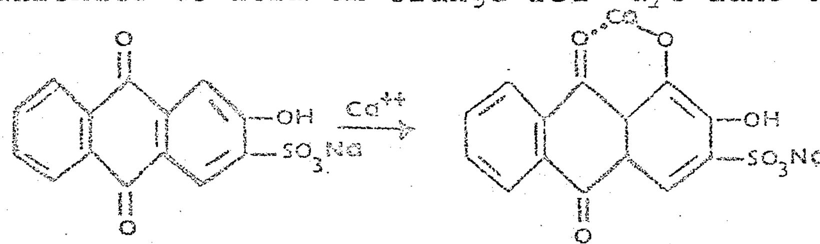
Two cytochemical techniques are most often used, (1) the Gomori technique (2) the azo-dye method.

1. The Gomori technique, independently developed by Takamatsu (1939), depends on the enzymic release of phosphate ions which are precipitated as insoluble calcium phosphate at sites of enzymic activity. The precipitated calcium is then replaced by lead, cobalt, or silver, forming the phosphate of the respective metal

$$Ca_3(PO_4)_2 + 6\ Ag^{+} \longrightarrow 2\ Ag_3PO_4 + 3\ Ca^{++}$$

The silver salt, on reduction to metallic silver, then reveals the sites of enzyme activity as blackened areas.

Fixed (or unfixed) tissue is usually incubated in a buffered medium containing a substrate for the enzyme plus calcium and magnesium salts. Silver is not the only metal that can replace calcium; cobalt, copper etc. can also be used. Reactions of insoluble calcium salts, and metal substitution tests for calcium, are given in Table I. A different technique was employed by Bourne (1943) who rendered the calcium phosphate visible by using sodium alizarin sulfonate to form an orange red "dye-lake".



SODIUM ALIZARIN SULFONATE. ALIZARIN DYE-LAKE

TABLE 1.

METAL SUBSTITUTION TESTS FOR CALCIUM

Metal		Substitution Reagent		Colour Reactions		Counterstains*	
Ag	Remove paraffin, bring to water, and rinse thoroughly in distilled water.	5% aq.$AgNO_3$ > 10 mins.	Rinse thoroughly in distilled water	0.5% aq.hydroquinone, 2 mins. Rinse in distilled water. 2% aq. sodium thiosulphate, 5 mins.	Rinse thoroughly in distilled water	Safranin Tartrazine Neutral red	Dehydrate, clear, and mount in balsam
Co		5% aq.$Co(NO_3)_2$ 5 mins.		Yellow ammonium sulphide (diluted 1 : 4 with water, if desired), 30 sec.		Toluidine blue Eosin Safranin	
Fe^{III}		5% aq.$FeCl_3$ 10 sec.**		5% potassium ferrocyanide freshly acidified with HCl, 1-5 mins.		Eosin Safranin Xylidine red	
Fe^{II}		5% aq. $FeSO_4$ 5 mins.		5% potassium ferricyanide, freshly acidified with HCl, 1-5 mins.		Eosin Safranin Xylidine red	
Cu		5% aq. $CuSO_4$ 5 mins.		Saturated solution of dithiooxamide (rubeanic acid) in ethanol, containing 2 drops conc. NH_4OH per 100 ml., 1 min. Rinse in 50% ethanol		No	

* Sections should be studied carefully before counterstaining in case the staining obscures some deposits or causes dislocation.

** Timing important.

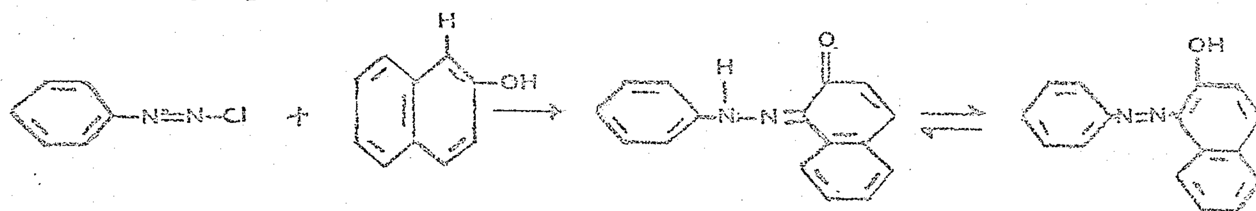
Casselman, W. G. B. (1959)

In addition to light microscopy, several other methods can be used for examination of results.

The enzymically produced calcium phosphate can be viewed by polarised light (Belanger 1951), or by phase contrast microscopy (Hancox and Nicholas, 1956). A number of commonly used phosphatase substrates are shown in Table 2.

2. <u>Azo-dye Technique</u>

β-naphthyl phosphate in the presence of a diazotised amine, e.g. nitro aniline or naphthylamine is commonly used as substrate in this method (Menten et al 1944). Enzymic hydrolysis of phosphate by phosphatase releases naphthol, which then immediately couples with the diazonium salt to form a highly coloured insoluble dye.



Benzene diazonium chloride β-naphthol Dye

TABLE 2

SUBSTRATES OF ALKALINE PHOSPHATASE

COMPOUND	RELATIVE RATE OF HYDROLYSIS	COMPOUND	RELATIVE RATE OF HYDROLYSIS
5'-AMP	0.8 - 1.0		
3'-AMP	1.0	Polymetaphosphate	0.9
2'-AMP	1.0	Ribose 5-phosphate	0.7
ATP	0.8 - 1.05	β-glycerophosphate	0.9 - 1.0
d-ATP	1.05	p-nitrophenylphosphate	1.0
d-AMP	1.1	Riboflavin 5'-phosphate	0.7
2'-3'-GMP	0.9	α-naphthyl phosphate	1.5
d-CMP	0.9	β-naphthyl phosphate	1.0
2'-3'-CMP	1.1	2-4-dinitrophenyl phosphate	1.0
5'-CMP	0.8-1.2		
d-CTP	1.05	Fructose 1-6-diphosphate	0.6
UDP	1.0	Phosphoenolpyruvate	0.42
5'-UMP	0.8-1.3		
d-TTP	1.0	Cysteamine-S-phosphate	0.73
PP_i	1.0		
PP_i	1.0		
PPP_i	0.9		

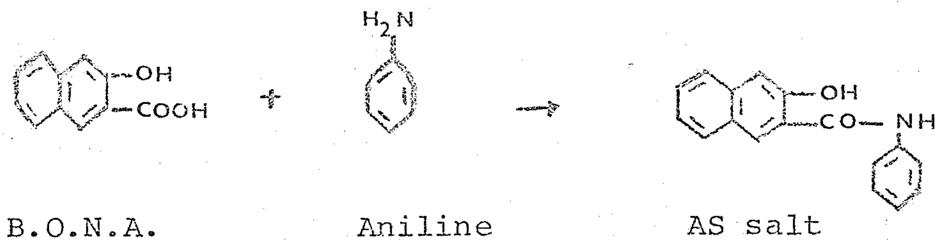
[From Reid and Wilson 1971]

Loveless and Danielli (1949) used a phosphorylated azo dye, which released the coloured insoluble dye on enzymic hydrolysis. Burstone (1962) has reported successful demonstration of alkaline phosphatase by post-coupling techniques. The substrates employed were phosphorylated derivatives of complex naphthols obtained from 3-hydroxy-2-naphthoic acid (β-oxynaphthoic acid - B.O.N.A.) and some aryl amines. On enzymic hydrolysis, many of the naphthols released remained _in situ_, and could then be coupled with a wide variety of diazonium salts to produce exceedingly sharp cytochemical localisations.

Post-coupling techniques apparently represent an ideal type of histochemical procedure, since extraneous and potentially inhibitory substances are eliminated from the incubation medium. The localisations obtained with the post-coupling techniques agreed with the results obtained with immediate or simultaneous coupling.

A larger number of naphthol AS phosphates, as the acid or sodium salt, may be used for localisation. (Burstone 1962). [The AS phosphates are phosphate esters of complex acrylamides or acrylides of 3-hydroxy-2-naphthoic acid or acylacetic acids. The latter are not naphthols, but compounds containing reactive methylene groups capable of coupling with diazonium compounds] AS salts can be

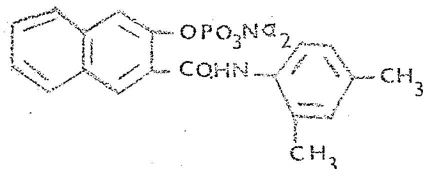
prepared by refluxing BONA and aniline with phosphorus trichloride.



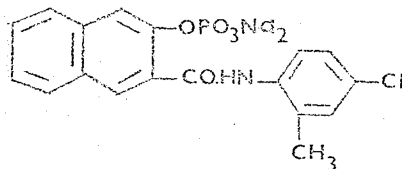
B.O.N.A. Aniline AS salt

Commonly used AS substrates are:-

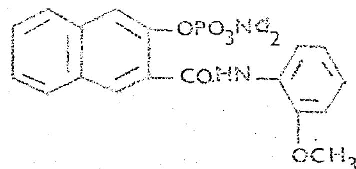
Naphthol AS-MX



Naphthol AS-TR



Naphthol AS-BI



ACTIVATORS AND INHIBITORS

Enzyme activation facilitates the procedure of localisation. Alkaline phosphatase is usually activated by magnesium and/or zinc. Zinc was shown to be essential for activity of *N. crassa* alkaline phosphatase (Davis and Lees, 1972). Manganese and cobalt may also serve as co-activators in very low concentrations. The *E. coli* alkaline phosphatase is reputedly activated by zinc at 10^{-6} M, but inhibited by 10^{-4} M zinc (Reid and Wilson, 1971). Alanine and histidine have also been described as activators, although Morton, (1957) claims that this activation effect is non-specific, being related to the removal of metal inhibitors.

Inhibitors of the constitutive alkaline phosphatase are relatively non-specific, and include metal-complexing agents such as ethylenediaminetetracetate (EDTA). The enzyme may also be competitively inhibited by inorganic and organic phosphates.

Magnesium possibly forms an essential part of the active center of the enzyme, or it may act as a binding agent between enzyme and substrate, thus metal chelators would inactivate the enzyme by preventing such binding. Moreover, the metal might directly activate the enzyme by altering the surface charge on the protein.

INTERPRETATION OF RESULTS

The imaginative interpretation of any cytochemical staining procedure is possibly its most cogent flaw. Reviews of conflicting arguments abound.

The chemical and physico-chemical properties of calcium phosphate solutions make interpretation even more difficult.

Johansen and Linderstrom-Lang (1953) concluded that calcium phosphate exhibits a marked tendency to form supersaturated solutions, so that by the time that the critical concentration of phosphate ions required for precipitation is achieved at the enzyme centres, diffusion of phosphate from production sites will have caused saturation to prevail in a wide zone around such areas of initial precipitation. A preformed crystal nucleus, they claimed, could initiate calcium phosphate precipitation, even in the absence of any enzyme. Gomori and Benditt (1953) partially agreed that spurious staining may occur under "non-optimal conditions", but that it is hardly likely under optimal conditions. Calcium phosphate, however, on precipitation by the addition of phosphate to solutions similar to the Gomori histochemical mixture, does not display any marked tendency to undergo supersaturation.

Simultaneous azo-dye methods confer some advantage, in that they are single-step procedures in which the dye

can be seen on formation. In addition, most naphthols are not specifically adsorbed by tissues. Defendi (1957) showed that naphthol AS derivatives do not display great affinity for tissues, though simpler naphthol derivatives e.g. 6-bromo, or 6-benzoyl-2-naphthol (Rutenberg and Seligman, 1956) do.

Despite obvious criticism of cytochemical methods, some reliance can be placed on the results obtained. Van Duijn, Pascoe, and Vander Ploog (1967) showed that incubation conditions can be obtained for which the amount of azo dye precipitated in a given time of reaction is proportional to the enzyme activity present, and that quantitative cytochemical determination of enzyme activity in an individual cell is possible.

Electron microscopy is now being widely used in enzyme histochemistry. Sabatini, Bensch and Barnett (1963) and Burstone (1962) have reviewed the application of electron microscopy to enzyme cytochemistry. Zetterquist, Brandes and Sheldon, (1955) have demonstrated applications of the Gomori procedure to electron microscopy. Adequate fixation methods have been developed permitting preservation of significant enzyme activity, yet revealing enough structural information to facilitate interpretations of cytochemical reactions.

As always, the delicate balance must be achieved between minimal disturbance of naturally existing conditions, and the generation of sufficient end-product to make its

detection possible, yet the examination must be made with the requisite sensitivity to provide some valid indication of normal activity.

FUNCTION

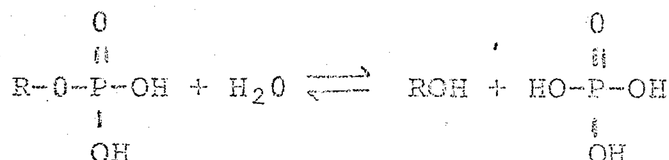
Location of enzyme activity in cells and tissues is closely linked with the metabolic roles of such enzymes at their respective sites. What role has been assigned to alkaline phosphatase? Robinson and Soames (1924) suggested that alkaline phosphatase was significant in tooth and bone formation based on the observation that hydrolysis of calcium hexose monophosphate yielded insoluble calcium phosphate. This is supported by the occurrence of large quantities of alkaline phosphatase in the long bone epiphyses, and the teeth of young animals.

Placental tissue is also extremely rich in alkaline phosphatase, the latter increasing during pregnancy reaching a maximum just prior to term (Anagnostopoulos and Matsudaira 1958). They concluded that the enzyme may take part in the absorption of maternal glucose by the placenta.

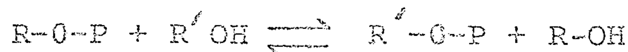
Meyerhoff and Green (1950), and others have demonstrated that purified intestinal alkaline phosphomonoesterase can exhibit synthetic activity under certain conditions, even catalysing phosphorylation of a number of alcohols and

and sugar alcohols. The rate of synthesis of these phosphorylated alcohols was significantly increased when phosphate compounds of higher energy content, e.g. phosphocreatine, were added. When P^{32} labelled donors or P^{32} labelled orthophosphate were used it was found that an increased rate of synthesis was caused by direct trans-phosphorylation mechanisms operative between phosphate donors and acceptors. Subsequent studies by Morton, (1965) indicated that only the non-specific phosphomonoesterases catalyse these transphosphorylation reactions. Thus, the reactions catalysed by alkaline phosphatase are:-

1. General hydrolysis



2. Transphosphorylation with high alcohol concentrations



in which a different alcohol substitutes for water as phosphate acceptor.

In bacteria, the enzyme may be involved in supplying phosphate from phosphate esters under conditions of phosphate deprivation (Heppel, Harkness and Hilmoe (1962). Reid and Wilson, (1971) have also suggested that even though alkaline phosphatase could catalyse non-specific hydrolysis of

phosphate esters, other functions - such as phosphate transport - were possible.

Examination of substrates hydrolysed by E. coli alkaline phosphatase [Table 2.] shows rapid hydrolysis of a large number of nucleotides, including ATP. What metabolic significance does this imply? Consider that (1) alkaline phosphatase hydrolyses a large number of mononucleotides. (2) alkaline phosphatase can - under specific conditions - catalyse transphosphorylation. (3) in animals the enzyme is associated with tissues in which active division is occurring. (4) An exciting observation has very recently been noted for at least one alkaline phosphatase - that from brush border intestinal mucosa of rat. Russel, Monod, Bonjour and Fleisch (1972) have provided evidence to indicate that alkaline phosphatase and calcium activated ATPase may be part of a single enzyme complex. Heat inactivation, changes in dietary calcium and treatment with ethane-1-hydroxy-6-diphosphonate (EHDP) - all had similar effects on both calcium ATPase and alkaline phosphatase. Haussler, Nagode and Rasmussen (1970) and Norman, Mircheff, Adams and Spielvogel (1970) had previously recorded close relation between the two activities.

What then are the conclusions to be derived? If the processes of replication, cell division and cell multiplication are viewed as *inter alia* examples of

increased nucleotide turnover and incorporation, is it not possible that non-specific alkaline phosphatase has a significant role in all these activities - whether such activities are initiated or mediated by the enzyme? Consider the apparent lack of specificity, wide occurrence, tissue association - is it not tenable that an enzyme produced in such abundance has a more dynamic role than has hitherto been ascribed to it?

The localisation of enzymes in organisms is but a clue to elucidating function and assigning significance. Of such import is the present work.

MATERIALS AND METHODS

1. Chemicals

Snail gut enzyme [100 units β-glycuronidase (Fishmann), 800 units sulfatase (Roy) - per ml] was obtained from Industrie Biologique Francaise, 35, Quai du Moulin de Cage - 92 - Gennevilliers, France. All other chemicals were obtained from the Sigma Chemical Company.

2. Growth conditions

The organism was grown on Vogel's medium (Vogel, 1956) with 2% sucrose as carbon source. Conidia, washed from 2% agar slants, were used to inoculate 2 litre flasks of sterile medium. Growth was also effected on agar plates with 1.5% agar, supplemented with 20% glucose, as required. Incubation was at room termperature or 37°C on a rotary shaker.

3. Enzyme assay

p-nitrophenylphosphate was used as substrate for assay of alkaline phosphatase activity. The reaction was followed by measuring the increase in optical density at 410 mμ. All O. D changes were recorded on a Gilford model 2000 recording spectrophotometer with a timed chart drive. Each cuvette, with a path length of 1 cm., contained

12 μmoles $MgCl_2 \cdot 6H_2O$, 30 μmoles p-nitrophenylphosphate
150 μmoles Bicine [N.N.bis(2 hydroxy-ethyl) glycine]
in a total volume of 3 ml.

One unit of enzyme activity is defined as that producing an increase in optical density of 0.01 per minute at 410 mμ under the conditions specified.

Protein was determined by the method of Lowry et al (1951)

Sonication was effected with an Insonator Model 1000, (manufactured by Ultrasonic Systems Inc.) with a current of 3 amps. A. C. maximum.

Electron microscopy was done using an AEI EM6B electron microscope. Sections for examination were cut with glass knives on a Reichert Om U2 ultramicrotome.

Mutant M16 The osmotic mutant M16 (OS-1 locus) was obtained from the Fungal Genetics Stock Centre, New Hampshire.

EXPERIMENTAL PROCEDURE AND RESULTS

I. Cytochemistry

A. Light Microscopy

Unfixed mycelium was incubated in the following incubation reaction mixtures, with the respective substrates being omitted from the controls. Slides were examined with a phase contrast microscope after incubation and washing in tap water for several minutes.

(i) Azo dye method (Burstone, 1962)

Ten mg α-naphthyl phosphate [$C_{10}H_7PO_4Na_2$] was dissolved in 40 ml distilled water; 4 ml 0.05 M magnesium chloride and 0.05 gm sodium barbital were added. Twenty-five mg diazonium salt - Blue RR - were added to the solution, and the latter was shaken vigorously; the solution was filtered and the filtrate used as the incubation medium. Mycelium was immersed for 1 hour in incubation mix.

(ii) Post-coupling Technique [Burstone 1962]

Ten mg naphthol AS-TR phosphoric acid (sodium salt - Sigma) was dissolved in 4 ml dimethylformamide. Four ml glass distilled water were added, and the pH of the solution was adjusted to 8 with a few drops of 1M Na_2CO_3. One hundred-fifty ml glass distilled water were added and the volume made up to 200 ml with 0.2M Tris buffer pH 8.3. This stock solution was stable at

room temperature for several months.

Fifty ml of the above stock solution were shaken with 30 mg red violet LB salt. The resulting mixture was filtered, and the filtrate was used as incubation mix for mycelium for 1 hour.

(iii) Cobalt method [Gomori 1952]

Twenty mg Naβ-glycerophosphate and 20 ml 0.2 M $CaCl_2$ were mixed, and 10 ml 0.05 M magnesium chloride were added to the mixture. Mycelium was incubated in reaction mix for 30 minutes at room temperature, washed in distilled water, immersed in 5% $Co(NO_3)_2$ solution for 10 minutes, washed, transferred to a dilute solution of ammonium sulfide for 2 minutes, followed by vigorous washing in tap water.

Results [see plates 3 (i), (ii), (iii), pages 23, 25, 27]
(i) With the Azo dye method cytoplasm stained red-brown in experimental slides; (ii) yellow-red with post-coupling technique (iii) and dark brown with cobalt indicating that alkaline phosphatase was located in the cytoplasm.

3. Electron Microscopy

Cells washed with 7% glutaraldehyde or 0.2 M magnesium chloride, were incubated in a mixture containing the following:-

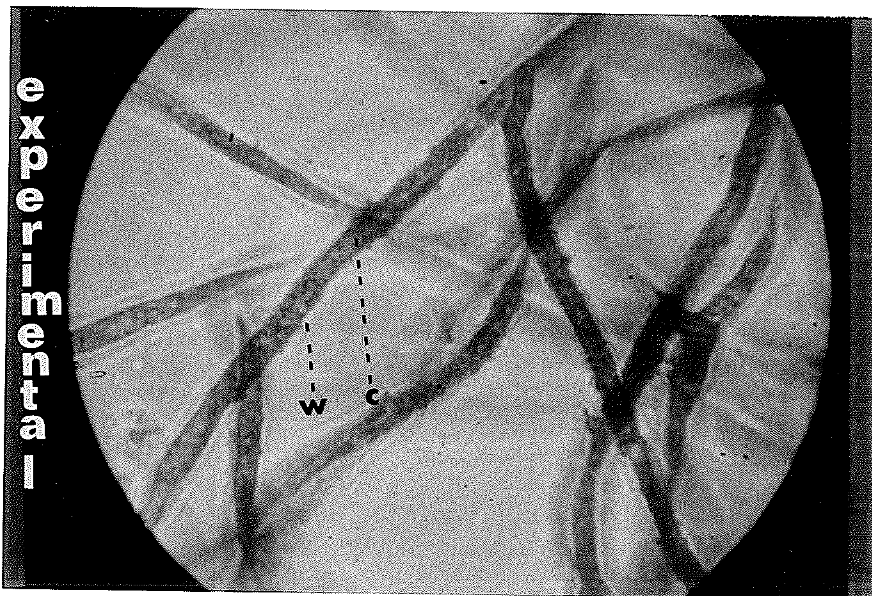
0.5% Naβ-glycerophosphate; 0.5% Na-barbital; 1 ml of 0.5 M $Ca(NO_2)_2$ solution; 8 mls of 0.05 M Tris pH 8.4. The pH of the solution was adjusted to pH 9.0, cells were incubated

Plate 3 (i) - light micrographs of *N. crassa* hyphae incubated in reaction mix with enzyme substrate (experimental), without substrate (control) using

azo dye technique.

w-wall, c-cytoplasm, with red-brown deposits at sites of alkaline phosphatase activity

Magnification 300 X



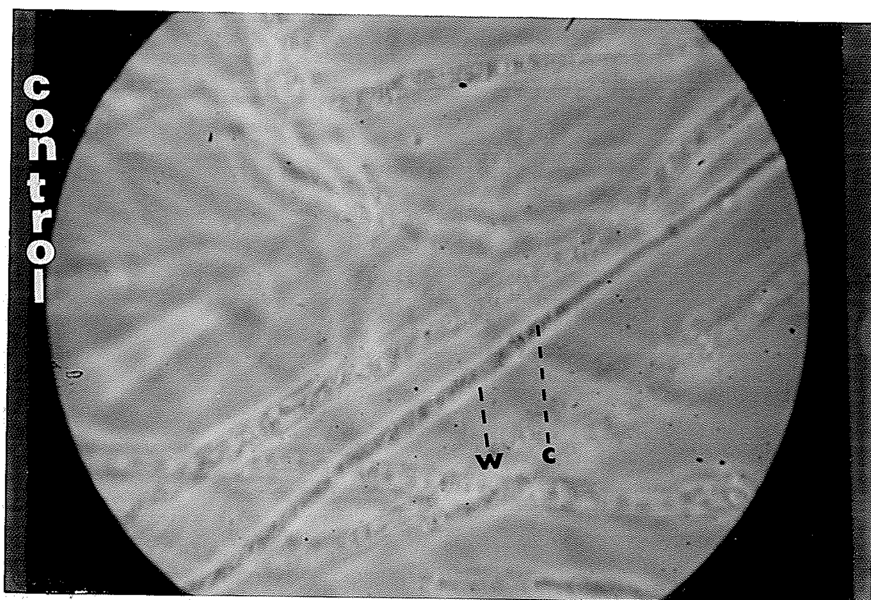
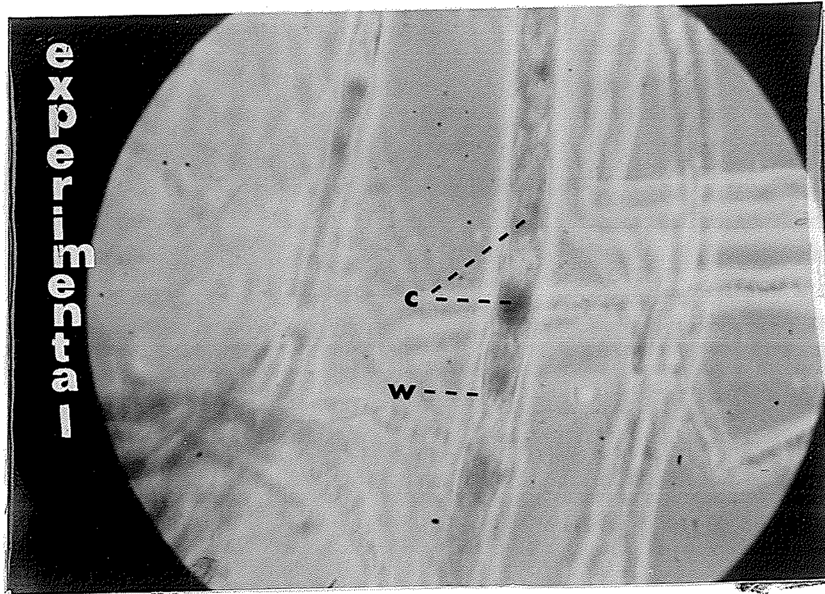


Plate 3 (i)

Plate 3 (ii) - light micrographs of *N. crassa* hyphae incubated in reaction mix with enzyme substrate (experimental); without substrate (control) using post-coupling technique.

w-wall, c-cytoplasm with yellow red deposits at sites of alkaline phosphatase activity.

Magnification 300 X



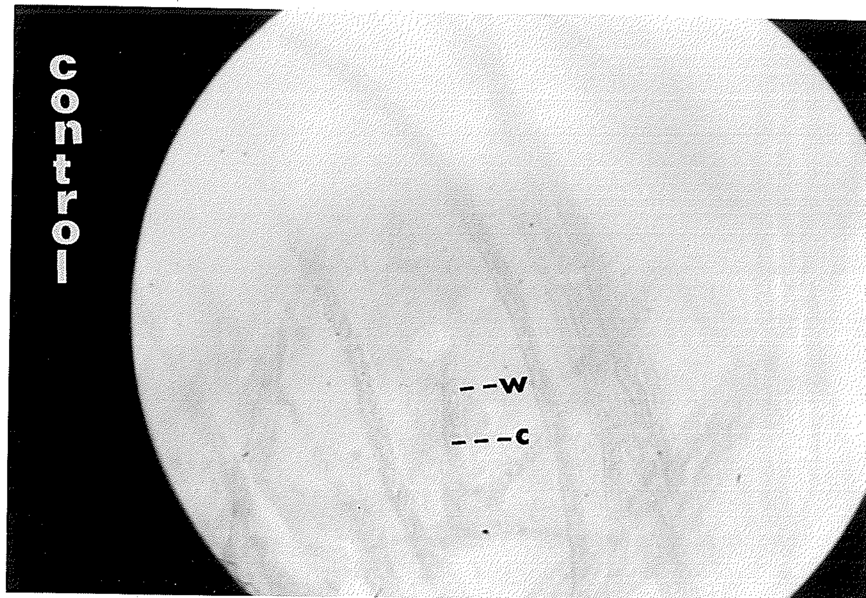
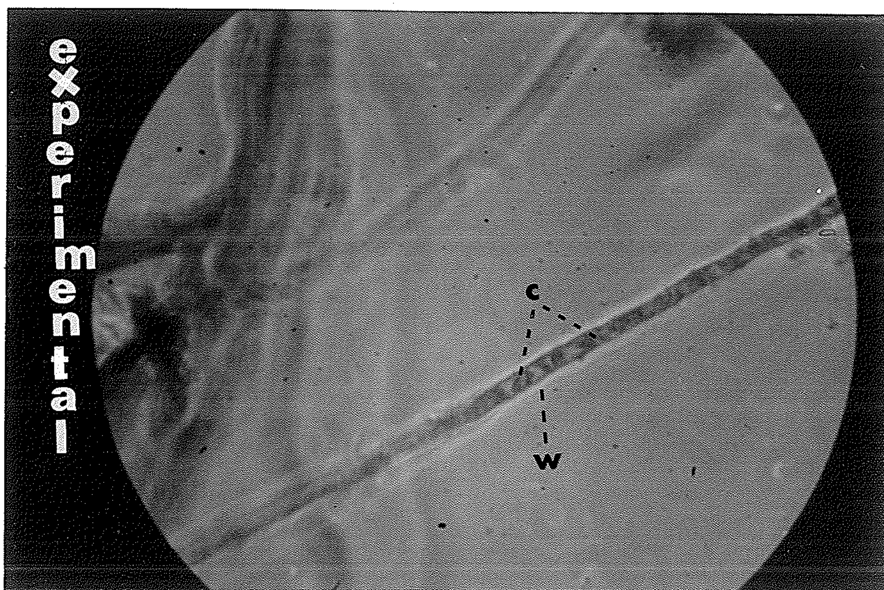


Plate 3 (ii)

Plate 3 (iii) - light micrographs of *N. crassa* incubated in reaction mix with enzyme-substrate (experimental), without substrate (control) cobalt technique; c-cytoplasm with dark brown deposits at sites of alkaline phosphatase activity, w-wall Magnification 300 X



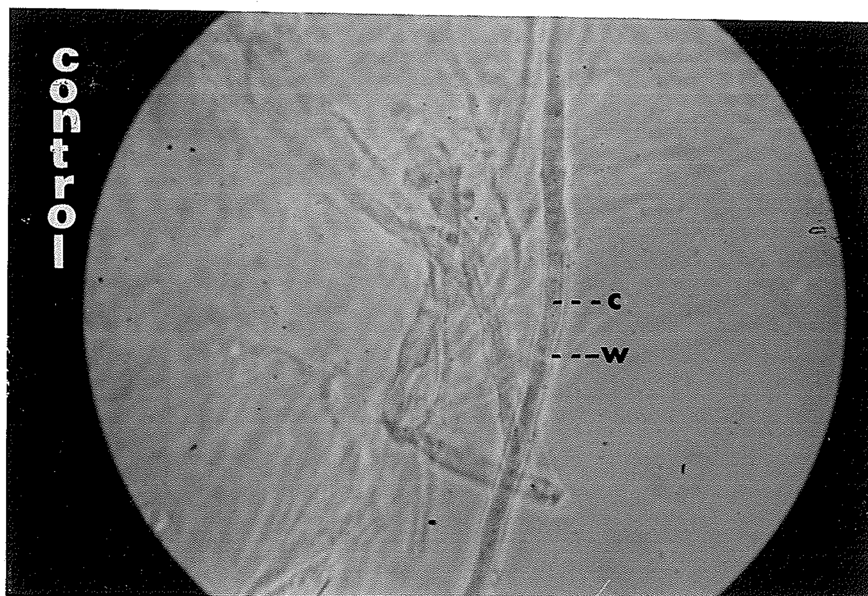


Plate 3 (iii)

for 1 hour. Substrate was omitted from the control. The incubated mycelium was treated with 5% $Co(NO_3)_2$ for 30 minutes at room temperature; then washed in 0.05 M Tris buffer (pH 8.4) for 2 hours. This was followed by staining in 2% osmium tetroxide containing 0.01 M $MgCl_2$ for 2 hours. The mycelium was then removed and washed overnight in Tris buffer pH 7.5 (0.05M). Samples were then dehydrated by passage through 50%, 70%, 90% and absolute ethyl alcohol, embedded in "Spur" or methacrylate, sectioned, mounted on carbon-coated 300 mesh grids, stained with lead citrate solution for 3 minutes, and examined with an electron microscope.

Light microscope examination showed yellow-brown "spots" scattered throughout the cytoplasm.

Electron micrographs

Experimental micrographs showed significant deposits in the cytoplasm, with some deposition (in 1 micrograph) in the peripheral area near to the cytoplasmic membrane. Control micrographs, by comparison, lacked these deposits. [See plates 4, 5 (i), (ii) pages 30, 32, 34]

II. Fractionation [Emmet, Williams, LaFayette 1972]

Fragmentation and analysis of cell fractions was effected by separating (i) nuclear, (ii) mitochondrial, (iii) cytoplasmic components to determine the location

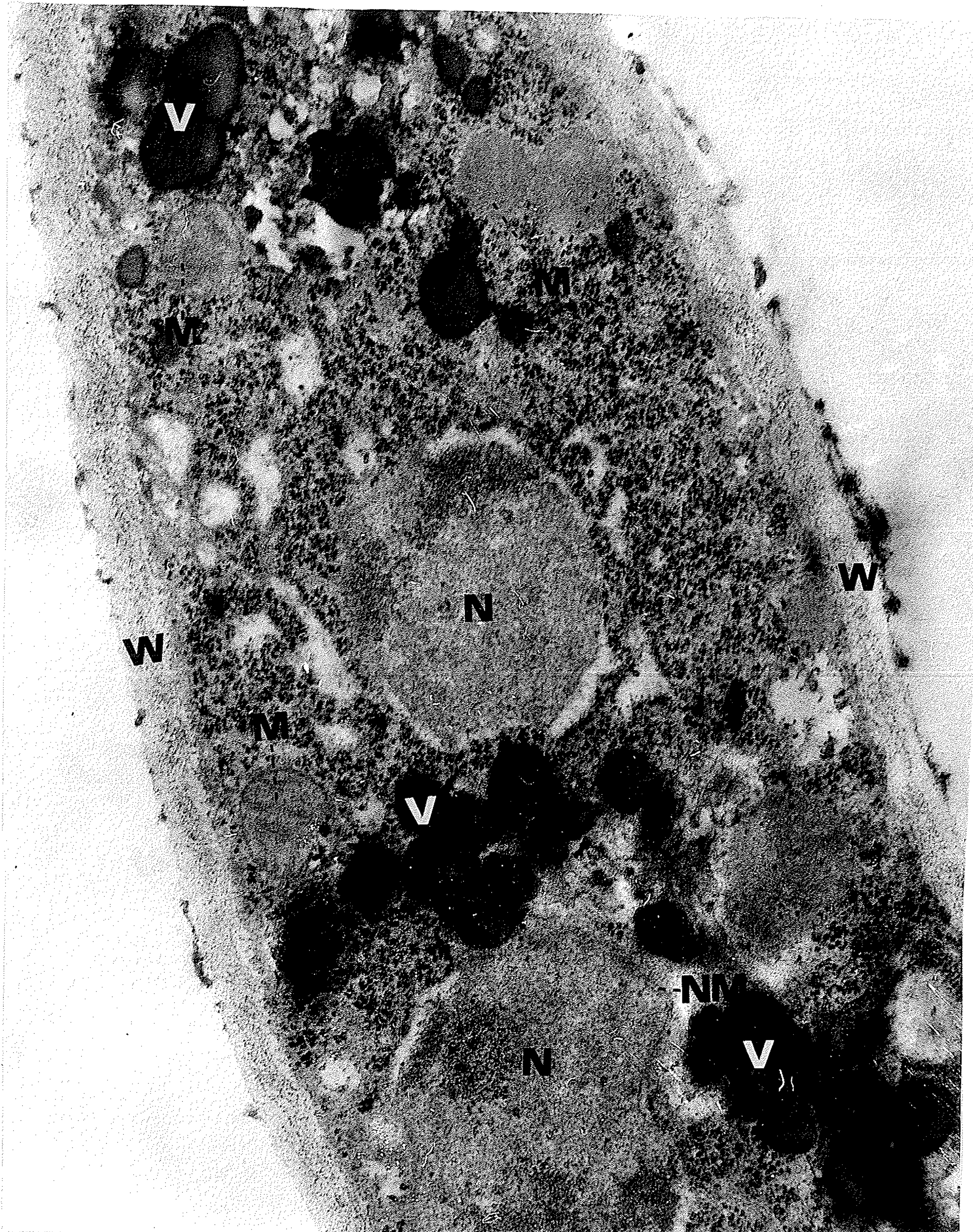


Plate 4

Plate 5 (i) - Experimental

electron micrograph of *N. crassa* hypha incubated in reaction mix with substrate for alkaline phosphatase. Sites of activity (P) in cytoplasm.

CM-cytoplasmic membrane

Magnification 138,400 X

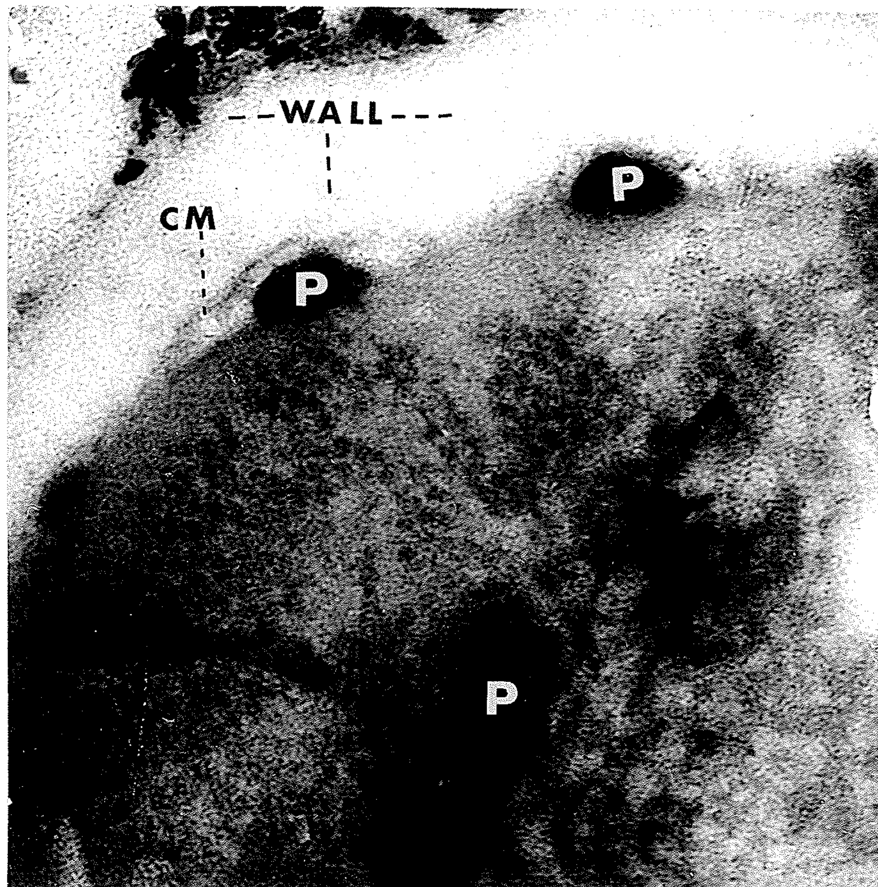


Plate 5 (i)

Plate 5 (ii) - Experimental

electron micrographs of *N. crassa* hypha,

showing sites of alkaline phosphatase

activity (P)

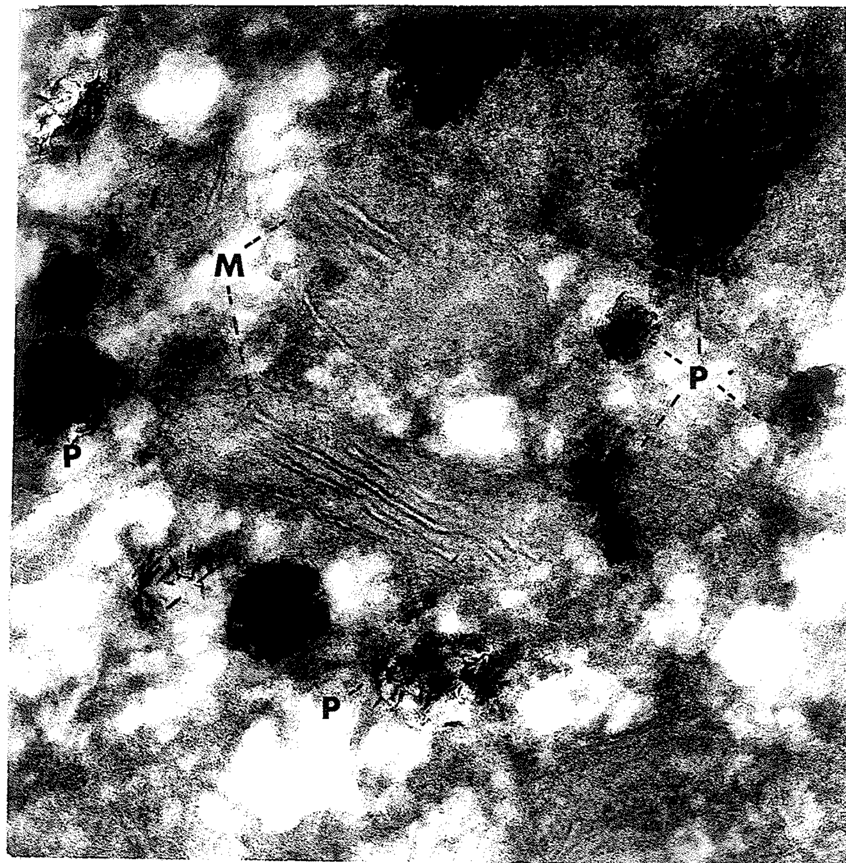
M-mitochondria

W-wall

CM-cytoplasmic membrane

Top plate magnification 76,950 X

Bottom plate magnification 102,750 X



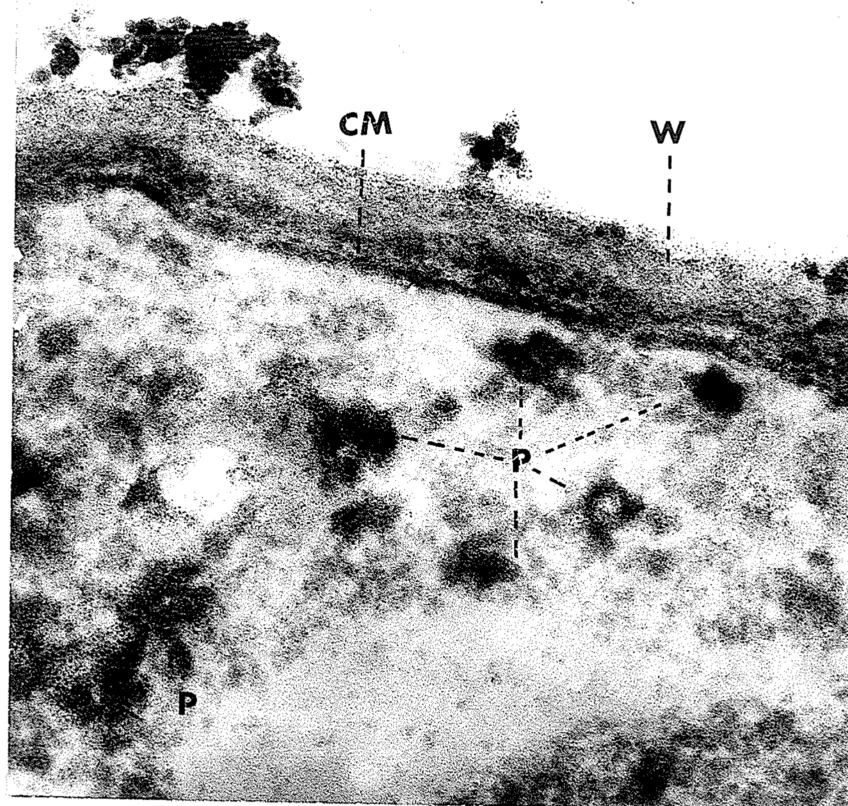


Plate 5(ii)

of the enzyme, alkaline phosphatase, within the hyphae, of strain 533 wild type.

(i) Nuclei

Harvested mycelium, washed with glass distilled water, was squeezed partially dry between filter paper, then frozen. Two gm frozen mycelium were then homogenised in a chilled mortar with 10 ml 0.25 M sucrose, with the mortar in an ice bath. The resultant homogenate was sonicated in a Model 1000 Insonator for 3 minutes; the resulting sonicate was centrifuged at 300 x g for 10 minutes at 0°C to sediment nuclei, unbroken cells and cell debris. The 300 x g residue was resuspended in 0.05M Tris pH 7.5, with 0.25M sucrose and 10^{-3}M $MgCl_2$, and was filtered through 3 layers of gauze, recentrifuged at 300 x g for 10 minutes at 0°C. The supernatant was assayed, the pellet resuspended and washed repeatedly in 0.05 M Tris pH 7.5, with 0.25 M sucrose and 10^{-3}M $MgCl_2$. The pellet from the washings was resuspended in 10 ml of the above buffer containing 2 ml 10% Triton X 100. The resulting suspension was centrifuged at 0°C for 5 minutes at 250 x g. The final pellet was resuspended in 10 ml 0.006 M phosphate buffer pH 7.3. Electron microscopic examination showed intact nuclei in the pellet. The pellet was homogenised in a glass homogeniser (Potter), and the centrifuged residue was examined for alkaline phosphatase.

(ii) Mitochondria were obtained by centrifuging the 300 x g supernatant (obtained in the nuclear isolation above) for 10 minutes at 8,500 x g. The pellet obtained was then resuspended in 10 ml 0.25 M sucrose, centrifuged for 10 minutes at 48,000 x g. Centrifugation at 48,000 x g at 0°-5°C was repeated thrice. Electron microscopic examination showed intact mitochondria. The mitochondrial pellet was finally homogenised in a glass Potter homogeniser in 2 ml 0.006 M phosphate buffer. The resulting homogenate was centrifuged as previously, the supernatant and resuspended residue were examined for alkaline phosphatase activity.

(iii) Cytoplasm

Frozen mycelium homogenised in 0.10 M Tris buffer pH 8.0; 0.01 M $MgCl_2$; 0.01 M mercaptoethanol, 0.5% deoxycholate, in a chilled mortar. The homogenate was sonicated as for nuclear separation and the resulting sonicate centrifuged at 300 x g for 20 minutes; the supernatant was then decanted and recentrifuged. The resulting supernatant was sonicated for 3 minutes and the sonicate centrifuged at 34,800 x g at 0°C for 30 minutes to yield the cytoplasmic extract.

Results

Homogenised nuclear and mitochondrial fractions displayed no alkaline phosphatase activity - indicating that alkaline phosphatase was absent from such cell fractions. Cytoplasm - extracted by the method described - had a total activity of 110 units/ml, whereas the centrifuged residue of the 300 x g sonicate had 40 units/ml, thus 74% of the enzyme was associated with the cytoplasm. Incomplete washing of the residue could explain the quantities of alkaline phosphatase found apparently associated with the residue. The results obtained under the conditions specified indicate alkaline phosphatase, was predominantly associated with the cytoplasm, and not detectable in mitochondrial and nuclear fractions.

III. Solubilisation (Anderson, Green 1967)

Experiment I and II

Frozen mycelium, of differing weights, was homogenised (in 10 ml 0.006 M phosphate buffer pH 7.5) by grinding in a chilled mortar. The homogenate was then sonicated for 3 minutes, and the sonicate was filtered through 3 layers of cheese cloth, then centrifuged for 30 minutes at 121 x g. The supernatant was decanted, the residue was washed once with 5 ml phosphate buffer and the washing from the residue was added to the supernatant (in Expt. I). The residue was

then homogenised in a Potter homogeniser with a glass pestle, resuspended in phosphate buffer and the supernatant assayed for alkaline phosphatase activity. In Expt. II. the residue was not washed after removal of the supernatant, but was homogenised in buffer, centrifuged as above and the supernatant assayed. Activity present in the supernatants was expressed as percentages of total activity, i.e., in resuspended residues and supernatants.

The results obtained [Table 3] indicated that on extrapolation to 100% tissue concentration (solubilised under the conditions described), the enzyme will be predominantly localised in the cytoplasm of the hyphae. [see page 39, 40]

IV. Osmotic Shock (Heppel, 1967, Wood and Tristam 1970]

A. Wild Type 533

Harvested mycelium was vacuum filtered, then stirred with the following solutions, in an effort to effect enzyme release by osmotic shock.

1. Thirty volumes ice cold 0.1M $MgCl_2 6H_2O$
2. Thirty volumes ice cold 0.5M sucrose (for comparison and control)

The supernatant fluids were collected by centrifugation at 10,500 x g at 0°C for 30 minutes, and assayed for alkaline phosphatase. The mycelial pellet was then lyophilised, homogenised in Tris buffer (0.02M) pH 7.5,

Table 3. Enzyme activity of varying concentrations of mycelium homogenised in 0.006 M phosphate buffer pH 7.5.

EXPERIMENT 1

Tissue Concentration Percentage weight/volume	5	10	20	30	40	50
Activity units/ml Supernatant	50	110	220	300	410	530
Activity units/ml Residue-Resuspended	2	4	9	11	17	22
Percentage Activity in Supernatant	96.1	96.5	96.1	96.4	96.1	97.7

EXPERIMENT 2

Tissue Concentration Percentage weight/volume	20	30	40	50	60
Activity units/ml Supernatant	40	130	170	200	240
Activity units/ml Residue-Resuspended	1	2	6	8	16
Percentage Activity in Supernatant	98.9	98.5	96.6	96.7	94

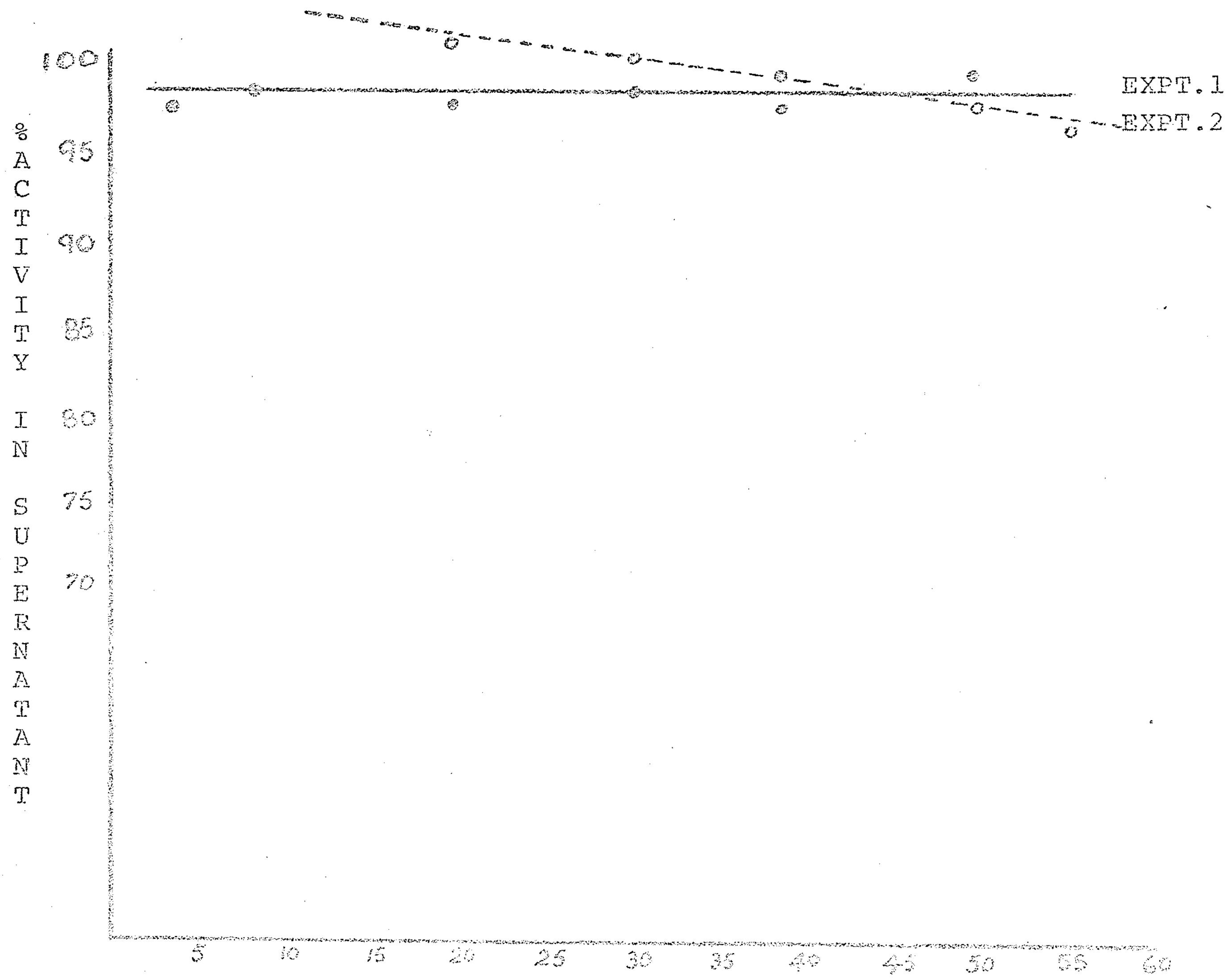
Percentage activity in supernatant represents percentage of total activity (combined supernatant and resuspended residue) present in the supernatant.

FIGURE 2

Plot of Supernatant Activity

VS.

Tissue Concentration



TISSUE CONCENTRATION (weight/volume)

Supernatant activity is expressed as the percentage of total activity present in supernatant and resuspended residue.

containing 4 x 10^{-3} M $MgCl_2$; and 1 x 10^{-4} M $ZnCl_2$ per litre of buffer, the total enzyme activity of the mycelium was then determined. The results obtained with the wild type strain 533 are shown in Table 4.

Table 4. Magnesium and sucrose wash of mycelium to effect enzyme release by osmotic shock.

Wash	Activity units/ml	Mycelial homogenate (centrifuged)	Activity units/ml
0.1M $MgCl_2 6H_2O$	4	Supernatant Residue	140 20
0.5M Sucrose	2	Supernatant Residue	140 20

Microscopic examination of hyphae revealed that they were apparently intact; osmotic shock was not effected under the conditions specified, thus there was no significant release of enzyme and no difference between the two treatments.

B. Osmotic mutant M16

This experiment was prompted by the observation that mutant M16 conidia, after several days growth on agar plates, exuded an intensely coloured orange-red liquid. Mutant conidia, grown on agar plates, were washed, after removal from the plates, by centrifuging in 30 ml glass distilled H_2O at 10,800 x g for 30 minutes. The washing process was repeated 3 times to remove all the exudate. The water-washed conidia were then suspended for 30 minutes in 30 ml 0.05 M Tris pH 6.5 and 20% sucrose, to see if the enzyme would be extracted or secreted. The resulting suspension was centrifuged and the supernatant kept for assay.

The conidia were then vacuum filtered and agitated by vigorous shaking in 30 ml ice-cold 5 x 10^{-4} $MgCl_2 6H_2O$. The mixture was centrifuged at 10,800 x g and the supernatant assayed. Total activity of the conidia was determined by homogenising the lyophilised conidia, followed by centrifugation and analysis for alkaline phosphatase activity. Microscopic examination prior to homogenising showed conidia intact. The results obtained were as follows:-

Table 5. Magnesium and sucrose wash of M16 conidia to effect enzyme release by osmotic shock.

H_2O	20% Sucrose	5 x 10^{-4} $MgCl_2 6H_2O$	Homogenised Conidia
1 96	4	7	94
2 24	-	-	-
3 12	-	-	-

Numbers in columns show units of activity/ml alkaline phosphatase. Conidia were washed 3 times in distilled water, then once in 20% sucrose; followed by washing once in magnesium chloride solution.

It may therefore be concluded that osmotic shock and significant enzyme release were not effected under the conditions specified although the mutant M16 conidia do apparently secrete alkaline phosphatase over several days when growing on agar plates.

Similar experiments to the above were carried out on wild type 533 conidia. Triple washed wild type conidia had a total of 120 units alkaline phosphatase compared with a total conidial activity of 380 units, in a

10 ml volume.

Thus, it seems likely that some quantity of enzyme can be secreted by both the wild type and osmotic mutant conidia, more so in the latter than the former. Conidial structure and alkaline phosphatase association were apparently different from that of the hyphae.

V. Ionic Influence

Two types of experiments were carried out to determine the possible influence of ionic variation on enzyme activity and liberation. The experiments were carried out to show the effect of uni and bivalent cations, and pH variation on enzyme activity.

1. The effect of uni and bivalent cations.

Harvested mycelium was lyophilised, ground in a mortar, further fragmented in a Waring Blender; then homogenised in 40 mls of the following:- (A) glass distilled H_2O, (B) 0.05 M Tris pH 7.5, (C) 0.01 M $MgCl_2 6H_2O$ in Tris 0.05 M pH 7.5, (D) 0.1 M NaCl in Tris 0.05 M pH 7.5.

The homogenates were centrifuged at 105,000 x g for 2 hours at 0°C, to sediment all fragments; the respective supernatants and resuspended residues were assayed. The results are shown in Table 6.

Table 6. The effect of various ionic solutions on the quantity of alkaline phosphatase extracted from homogenised mycelium.

Alk. Phosphatase Activity	H_2O	0.05 M Tris pH 7.5	0.1 M NaCl in 0.05 M Tris pH 7.5	0.1 M $MgCl_2 6H_2O$ in 0.05 M Tris pH 7.5
Supernatant	230	220	230	200
Residue (resuspended)	75	80	60	80
Total Activity	305	300	290	300
Protein mg/ml Supernatant	6.5	6.5	7.4	7.5
Residue	0.8	0.4	2.4	4.5
% Act. in Supernatant	75.4	73.3	79.3	71.1

A combination of higher concentrations of univalent and bivalent salts was also used, with the results shown in Table 7.

Table 7. The effect of combined uni-and bivalent salts on the quantity of alkaline phosphatase extracted from homogenised mycelium.

Ionic Composition	Activity units/ml	% Activity in Supernatant
5.0 M NaCl	370 (Supernatant) 110 (Residue)	78
5.0 M NaCl + and 0.5 M Mg acetate	400 (Supernatant) 150 (Residue)	73

There was no apparently significant difference noted in the relative quantities of alkaline phosphatase solubilised by the various ionic solutions, thus ionic variation from uni to bivalent etc. - excercised no markedly discernible effect on enzyme solubilised.

2. pH Variation

The wild type strain 533 was grown for several days in Vogel's medium of pH's ranging from 6 - 11. The growth media were examined for alkaline phosphatase after removal of the mycelium. The total alkaline phosphatase of the mycelium was then determined. The results are shown in Table 8.

Table 8. The effect of pH on (a) alkaline phosphatase release into the growth medium by hyphae (b) alkaline phosphatase extracted from homogenised mycelium.

pH of medium	6	7	8	9	10	11
Medium (a) Activity units/ml	-	4	10	2	-	-
Homogenised (b) Tissue	163.6	490	450	690	-	-
Weight Homogenised (gm)	0.67	0.5	0.34	0.65	-	-
Activity units/ml in 1 gm Tissue.	270	980	1500	1150	-	-

Activity present in homogenised tissue is the sum of activity present in supernatant and resuspended residue. Weight of mycelium homogenised was the total growth in the medium.

The results indicated that varying the pH of the growth medium did not produce secretion of significant quantities of enzyme into the growth medium. (The minimal activity observed in some media may have been due to enzyme liberated by older ruptured hyphae). However, on the basis of alkaline phosphatase extracted as units of enzyme per gram of mycelium, significantly greater quantities of enzyme were produced by mycelium in media of higher pH, e.g. pH 9.0 compared with pH 6.0, although no growth was observed at pH 10 and 11. Growth media of pH 8 may therefore be possibly used to maximise enzyme production.

VI. Protoplast Production and Enzyme Secretion

(Trevithick and Metzenberg 1964)

It is to be expected that peripherally located enzymes, i.e., those confined to the periplasmic or apparent free space between wall and cytoplasmic membrane, would be released on conversion to protoplasts, provided that the enzyme is not tightly bound to the cytoplasmic membrane or wall.

The mutant M16 was grown on agar plates containing 20% sorbose (or sucrose or dextrose). Conidia were washed from agar slants with sterile liquid media (containing 20% sucrose) and used to inoculate aseptically the sucrose-agar plates. The plates were incubated at 37°C. Osmotically sensitive protoplasts were produced after 24 hours growth. Lysis was effected (as observed microscopically) on addition of water to the protoplasts. On lysis the protoplasts tended to break into a number of rounded vacuole-like structures before final rupture.

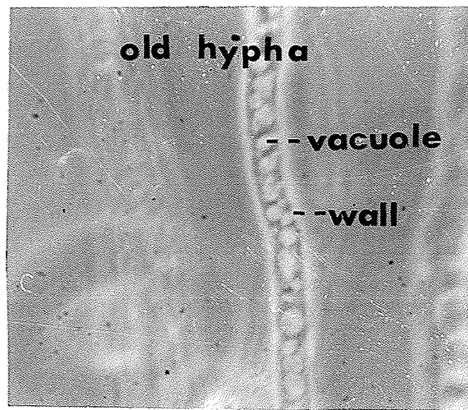
Agar plates containing actively growing protoplasts were washed with 5 ml 20% sucrose added to the plates which were then rotated frequently over a 30 minute period. The sucrose was poured off, and assayed for alkaline phosphatase. This sucrose washing was repeated three times. The plates were then washed with 5 ml glass distilled water, and the procedure with sucrose repeated with water in place of sucrose. [see plate 8, page 49; Table 9, page 50] The protoplasts were collected by centrifugation for 10 minutes at 35,000 x g. The sucrose and water supernatants were assayed for the enzyme. Protoplasts, after final washings, were homogenised in Buffer 'A' [0.02 M Tris buffer pH 7.5, with 4×10^{-3} M $MgCl_2 6H_2O$, and 1.0×10^{-4} M $ZnCl_2$]; and centrifuged at 43,000 x g for 20 minutes. Supernatants and resuspended residues were subsequently assayed.

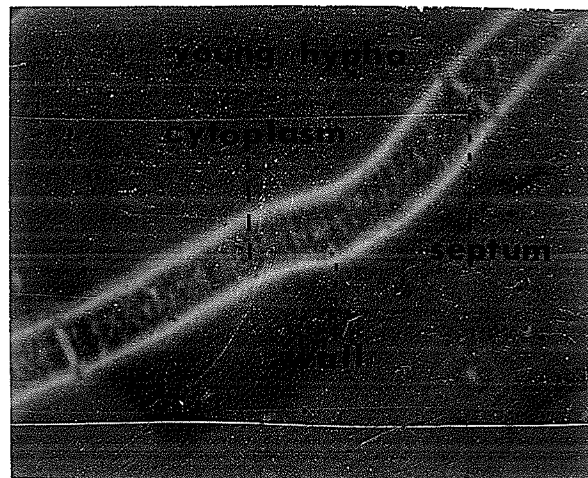
PLATE 8

Osmotic Mutant M16

light micrographs of hypha and protoplasts

400 X





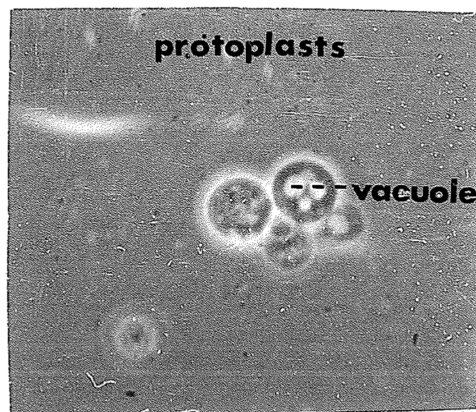


Plate 8

Protoplasts were dispersed in 20% sucrose and incubated over a 22 hour period. Two samples of the supernatant fluid were removed at 1 hour intervals for assay. The protoplasts were lysed, after removal of sucrose, by addition of 10 ml glass distilled water. The lysis fluid was decanted after centrifugation, and assayed. The protoplasts were then homogenised in Buffer "A" and assayed for total alkaline phosphatase.

The results obtained were:-

(1) Sucrose wash:-No activity was detected in any of the washings, though 77 units/ml phosphatase were present in the homogenised protoplasts.

Table 9. Alkaline phosphatase released from M16 protoplasts by washing in distilled water.

Wash	Alkaline phosphatase Activity/ml	Homogenised Protoplasts Activity units/ml
1	2	70
II	4	-
III	9	-

1 unit of activity is that producing an O.D change of 0.01 at 410 mμ. Protoplasts were washed 3 times in glass distilled water, the washings were assayed for alkaline phosphatase activity. The protoplasts were then homogenised and their total activity (of residue and supernatant) was determined.

Lysis was not total, since the removal of the sucrose was not complete.

(2) Twenty-two hour incubation in 20% sucrose produced minor trace quantities of phosphatase in the supernatant fluid. The results obtained indicated that the osmotic mutant does not, apparently, actively secrete alkaline phosphatase. Incubation in sucrose solutions of differing ionic composition did not result in detectable phosphatase being produced. The location of the enzyme on the outside of the protoplasts is therefore doubted.

DISCUSSION

Groups of enzymes in eucaryotic cells are frequently compartmentalised into characteristic membrane-bound organelles. Although morphologically identifiable sub-cellular organelles are not so obvious in procaryotic cells, there might be functionally differentiated areas in the cell which behave like compartments. The existence of such compartmentalisation has been shown theoretically to be necessary for efficient and economic functioning of enzyme systems within a bacterial cell. (Ghosh et al 1971) Such conclusions may also be applied to the fungal cell. The making visible of enzyme deposits by a variety of cytochemical methods is thus possible and has been effected here with N. crassa by both light and electron microscopy. The repeated visualisation of deposits within hyphae, after cytochemical staining designed to detect phosphate deposits produced by enzyme action, indicated that this enzyme was localised in the cytoplasm.

Peripherally located enzymes, in bacteria at least, are released by osmotic shock after the removal of the cell wall or by protoplast or spheroplast formation. The lack of influence by factors (ions, pH variation etc.) which normally affect the binding of proteins (enzymes) to cell components may indicate easy displacement and/or

the lack of such binding. The absence of any appreciable effect on alkaline phosphatase production by Neurospora mutant M16 protoplasts by such factors thus indicated that the enzyme was not bound with any appreciable tenacity, if at all, to the cytoplasmic membrane of the protoplasts, under the conditions specified. No active liberation of enzyme occurred on production of protoplasts, as would be expected if the enzyme was located in the "apparent free space". Periplasmic location of the enzyme is therefore improbable.

Ionic variation had little or no effect on the quantities of enzyme solubilised, as would be expected if the enzyme was predominantly associated with any major cell component - other than the cytoplasmic or soluble phase of the hyphae. Solubilisation of the enzyme from varying concentrations of tissue gave the above conclusion added cogency. Recovery of intact nuclei and mitochondria also support a cytoplasmic locale, since no activity was associated with these cell fractions.

The available evidence derived from this work therefore suggests that the non-specific constitutive alkaline phosphomonoesterase of *N. crassa* 533 is cytoplasmic.

However, more exhaustive experimentation is required to provide results of greater clarity. The basic assumption was made that the repressible enzyme, requiring a particular growth medium for active expression, was absent or inactive under conditions conducive to production and/or expression of the constitutive enzyme. Other techniques, such as autoradiography etc. - should prove quite valuable in precise localisation. Of necessity, such work will only acquire true significance when a definite role for the enzyme has been established.

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