# A STUDY OF SOME ALKALINE PHOSPHATASES OF *Neurospora crassa*

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## ABSTRACT

The activity of the alkaline phosphatase from the mycelium of *Neurospora crassa* was examined in an attempt to learn something of the mechanism of action of the enzyme responsible for that activity.

Product inhibition studies on a relatively impure preparation showed that the catalytic mechanism involved the ordered release of products from the enzyme, the alcoholic or phenolic product being released first, followed by the release of phosphate.

Inorganic phosphate was a linear competitive inhibitor of the hydrolysis of either p-nitrophenyl phosphate or β-glycerophosphate. p-Nitrophenol was a non-linear non-competitive inhibitor of p-nitrophenyl phosphate hydrolysis, whereas the noncompetitive inhibition by glycerol was linear when β-glycerophosphate was the substrate.

It was shown that not one, but two constitutive alkaline phosphatases were present in *Neurospora*. They were separable by DEAE cellulose chromatography and by electrophoresis, and differed in molecular weight, electrophoretic mobility and in their response to some cations. Due to the presence of phosphate in the growth medium neither of the enzyme activities could be attributed to the alkaline phosphatase, repressible by phosphate, known to be present in

*Neurospora* during phosphate deficiency. Both enzymes required $Zn^{++}$, which was tightly bound to the protein and could not be replaced by any other ion tested. A second, loosely bound metal ion was also required for maximum activity, $Mg^{++}$ being by far the most effective of the ions tested.

A purification procedure whereby one of the two activities could be obtained free of the other was devised. It was purified approximately 400 fold as compared to the activity in the crude extract and appeared to be about 50% pure enzyme. The effects of pH on the activity of the purified enzyme were studied in detail, using p-nitrophenyl phosphate and glucose 6-phosphate as substrate. In both cases the optimum pH increased with the substrate concentration and was shown to be a linear function of the logarithm of the substrate concentration. Dixon plots for both substrates were prepared and are qualitatively, but not quantitatively, interpreted.

Product inhibition experiments using p-nitrophenyl phosphate and glucose 6-phosphate with the purified enzyme confirmed and extended the results obtained earlier. The non-linear nature of the inhibition due to p-nitrophenol of p-nitrophenyl phosphate hydrolysis was found to be more complex than was evident in the early work. A two-substrate

mechanism, involving hydroxyl ion as the second substrate, is proposed and appears to offer satisfactory explanations of the observed pH effects and substrate inhibition.

A possible explanation for the competitive inhibition by glucose of p-nitrophenyl phosphate hydrolysis and for the apparent lack of inhibition by p-nitrophenol of glucose 6-phosphate hydrolysis is also presented. The proposed model involves the hypothesis that two substrate binding sites exist in the enzyme.

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*Fig. H1 is part of the Historical Review and is redrawn from the literature.

FIGURE PAGE

FIG. H1

$pK_m$ *versus* pH and Log $V_{max}$ *versus* pH curves from various sources.

Scales have been changed so that all figures are to the same scale.

A. Redrawn from Motzok (1959)
   (a) Fowl plasma enzyme with phenyl phosphate,
   (b) Bone phosphatase from rachitic chicks with β-glycerophosphate.

B. Redrawn from Morton (1957)
   (a) Milk phosphatase with phenyl phosphate,
   (b) Intestinal phosphatase with phenyl phosphate.

C. Redrawn from Anagnostopoulos and Matsudaira (1958)
   (a) Human placental enzyme in ammonium buffer with β-glycerophosphate,
   (b) Human placental enzyme in ethanolamine with β-glycerophosphate.

D. Redrawn from Anagnostopoulos and Matsudaira (1958)
   Human placental enzyme in ethanolamine buffer with phenyl phosphate.

E. Redrawn from Lazdunski and Lazdunski (1966)
   *E. coli* enzyme (ionic strength was 0.4)
   (a) with p-nitrophenyl phosphate at 25°,
   (b) with p-nitrophenyl phosphate at 45°,
   (c) with β-glycerophosphate at 45°,
   (d) with glucose 1-phosphate at 45°.

F. Redrawn from Lazdunski and Lazdunski (1966)
   *E. coli* enzyme with p-nitrophenyl phosphate (ionic strength 0.4 at 25°).

G. Redrawn from Kadner and Nyc (1969)
   Repressible enzyme from *Neurospora crassa* with p-nitrophenyl phosphate, at 37° in 0.2M buffers. Curves prepared in the presence of 1mM EDTA and in its absence are coincident.

H. Redrawn from Kadner and Nyc (1969).
   Repressible enzyme from *Neurospora crassa* with p-nitrophenyl phosphate, at 37° in 0.2M buffers.
   (a) in the presence of 1mM EDTA,
   (b) in the absence of EDTA.

# INTRODUCTION

Numerous individual phosphomonoesterases exist in all cells. They may be divided into two large groups (specific and non-specific phosphomonoesterases) on the basis of substrate specificity. The non-specific enzymes have been divided into two groups; those with pH optima in the alkaline range, known as alkaline phosphatases and the acid phosphatases whose pH optima lie on the acid side of neutrality.

Since only a few non-specific phosphatases have been obtained in high purity, very little is known of the properties of these enzymes. Thousands of scientific papers have dealt with some aspects of the phosphatases, such as their distribution and varying levels in diseased organs or tissues. Despite the volume of the literature on the subject little is understood of the mechanism of action or physiological function of the non-specific phosphatases. It has even been suggested that the phosphatase activity observed *in vitro* is an artifact.

Due to the ubiquitous presence and extreme importance of phosphorylated compounds in living material it is of major academic and probably practical interest to clarify our understanding of those enzymes potentially responsible for massive non-specific dephosphorylation. In addition to

their hydrolytic activity at least some of these enzymes also exhibit phosphotransferase activity. It is possible that phosphate transfer may be a physiological function, but the high concentrations of acceptor required to produce a measurable reaction rate do not encourage the idea.

The present work is an attempt to extend our knowledge of the properties of the alkaline phosphatases of *Neurospora crassa*. Particular attention has been paid to the kinetics of the enzyme-catalysed reaction and to the effects of pH on catalysis. These appear to be extremely complex and the data obtained raise more questions than they answer. No attempt has been made to investigate the potential phosphotransferase ability of the enzyme.

The format of the thesis is unusual in that discussion of the results is not reserved to the end. It seemed more logical to present results in the order in which they were obtained, dividing the body of the work into three phases related to different degrees of purification of the enzymes. Within the three phases, discussion of each topic investigated appears as a natural consequence of the completion of the presentation of experimental results.

# HISTORICAL REVIEW

Over fifty years have elapsed since reports of the occurrence of an enzyme responsible for splitting phosphate esters appeared in the literature (Suzuki *et al*, 1906; McCollum and Hart, 1908). Since that time such enzymes have been shown to act on many substrates and their presence has been observed in a wide variety of organisms.

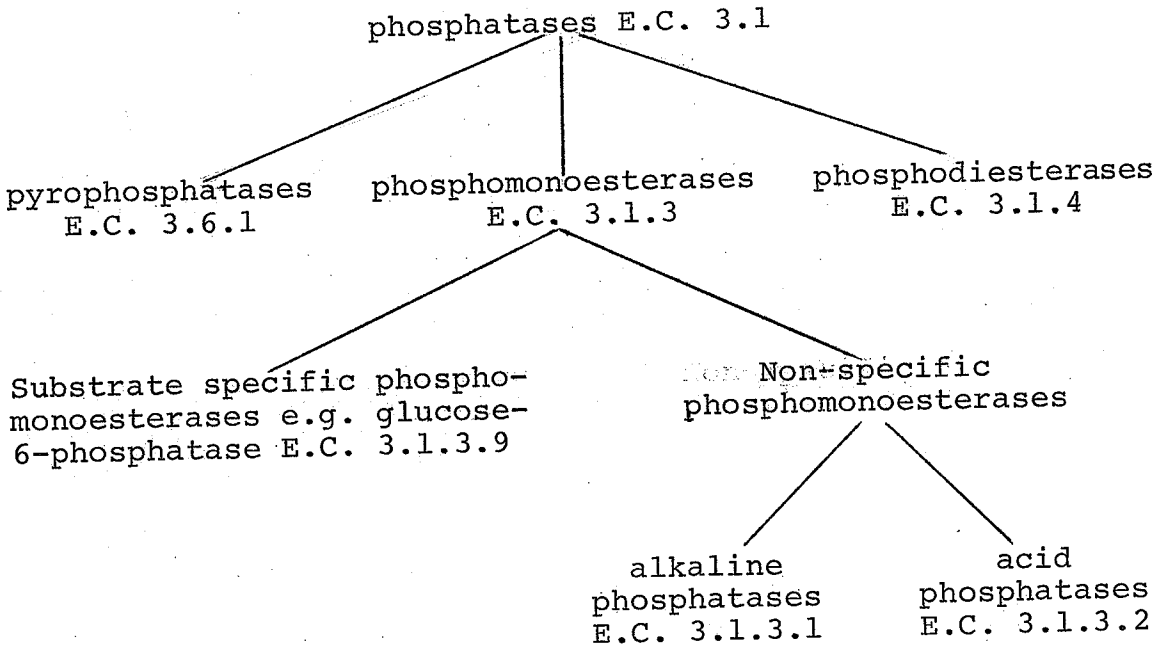
## A. Classification of the phosphatases

Attempts to classify the phosphatases are of two kinds, those based on the types of substrates hydrolysed and those based on such characteristics as the optimum pH for the reaction catalysed. Roche (1950) listed four types of phosphomonoesterases as follows:

Roche's classification of the phosphomonoesterases

| | | |
|---|---|---|
| Type I | optimum pH 8.6 - 9.4 | Activation by magnesium ion, inhibition by -SH. More active on β- than on α-glycerophosphate. Optimal stability at pH 7.5 - 8.5. |
| Type II | optimum pH 5.0 - 5.5 | No activation by magnesium ion. Inhibition by fluoride ion. More active on β- than on α-glycerophosphate. Optimal stability at pH 5.0 - 6.0. |
| Type III | optimum pH 3.4 - 4.2 | Inhibition by magnesium ion. More active on β- than on α-glycerophosphate. Optimal stability at pH 6.5 - 7.5. |

An "organizational chart" of the phosphatases




Many of the non-specific phosphomonoesterases also exhibit the ability to transfer a phosphate group to an alcoholic acceptor (in high concentration) other than water (Anderson and Nordlie, 1967; Wilson *et al*, 1964; Dayan and Wilson, 1964). Such an activity would require these enzymes to be classed as phosphotransferases E.C. 2.7.1, further complicating any attempt at an inclusive scheme of classification.

Only the alkaline phosphatases will be dealt with in detail in the remainder of this review.

B. Distribution of alkaline phosphatase in nature

Alkaline phosphatase has been observed in a list of organisms far too long to enumerate. In man, it has been reported in the blood serum, capillaries, leukocytes, milk, placenta, intestine, liver, urine, bone, teeth and in cell cultures both normal and cancerous. It is practically absent from skeletal muscle and from the heart. Similar lists, which are by no means exhaustive, could be made for other mammals, insects, birds, amphibia and fish. The enzyme has been reported in protozoa, bacteria and fungi. Strangely enough, no reference to the existence of alkaline phosphatase in green plants has been found.

C. Cellular localization and possible function of alkaline phosphatase.

No one has yet been able to assign a specific metabolic role to alkaline phosphatase. Interest in the enzyme was greatly stimulated by the suggestion (Robison and Soames, 1924) that it is involved in the calcification of bone and in tooth formation. The observation that the epiphyses of long bones and the teeth of young animals are rich in alkaline phosphatase, and that the hydrolysis of the calcium salts of phosphorylated hexoses leads to the formation of tricalcium phosphate supported their suggestion

(Stadtman, 1961). The enzyme has been histochemically demonstrated in the chondrocytes of human tibial cartilage (Bona *et al*, 1965).

Anagnostopoulos and Matsudaira (1958) suggested that the enzyme plays a role in the absorption of maternal blood glucose by the human placenta, which has very high alkaline phosphatase activity. The activity was reported to increase during pregnancy and to reach a maximum just before birth. Enzymatic activity in the mammary gland also increases during pregnancy and is maintained at a high level during lactation (Folley and Greenbaum, 1947), again suggesting a possible role for the enzyme in either absorption or secretion. The localization of the enzyme in mouse duodenum has been examined (Hugon and Borgers, 1966a) in considerable detail. Its location in the brush border, in the Golgi zone and in granules below the brush border was demonstrated with the light microscope following specific staining techniques. In the cells of the villi the enzyme occurred on the outer side of the membrane of the microvilli, the inner side and the core of the microvilli being devoid of activity. Small bodies, supposedly pinocytic vesicles derived from the membrane of the microvilli, also contained alkaline phosphatase, located, as one would expect, on the inner side of the membrane. Many Golgi vesicles stained