PHYTASES IN PLANTS AND MICRO-ORGANISMS

LIM PHAIK EE

A THESIS

submitted for admission to the degree of

DOCTOR OF PHILOSOPHY

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South Australia

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PLATE 1

STRUCTURAL MODEL OF *MYO*-INOSITOL HEXAPHOSPHATE

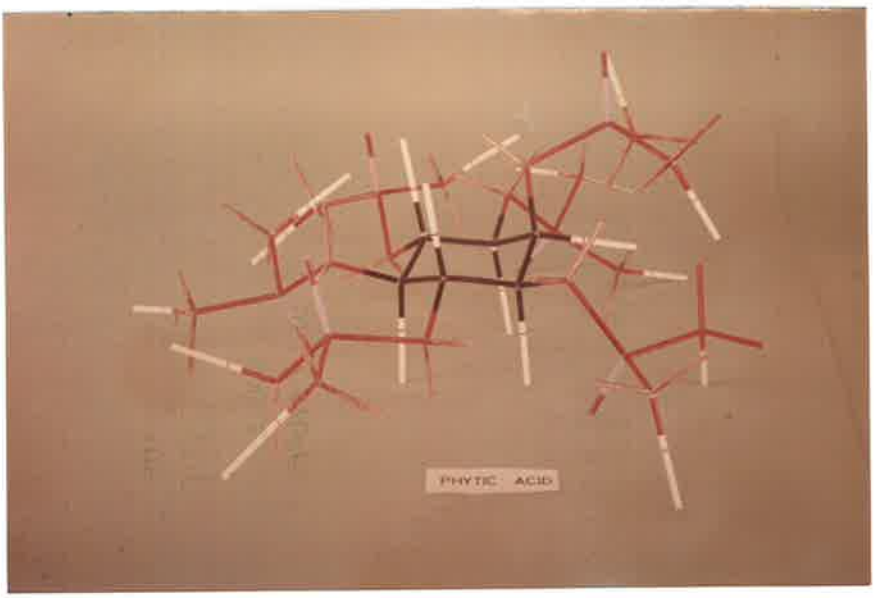
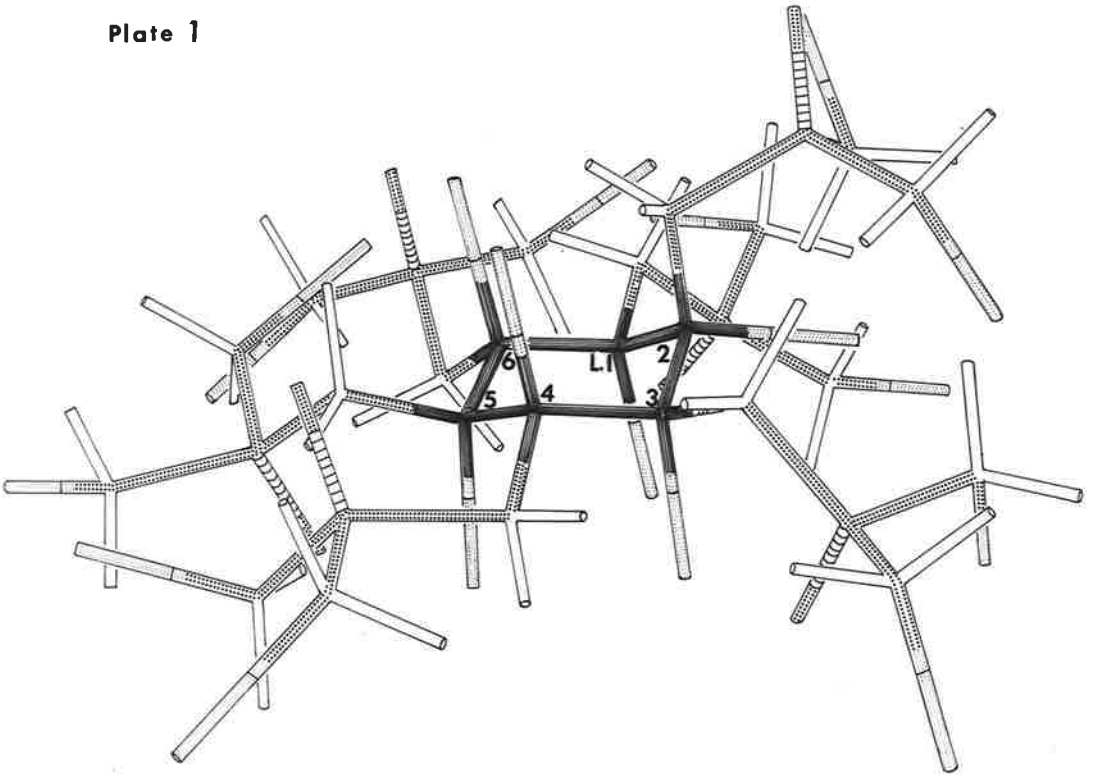


Plate 1



PHYTIC ACID

The numbering system is that recomended by IUPAC–IUB (1968).

The atoms are represented as follows :–

C black

H white

O red

P violet

SUMMARY

1. Area of study

The hydrolysis of *myo*-inositol hexaphosphate [*myo*-inositol hexakis (dihydrogen phosphate)] or phytic acid is carried out in biological systems by enzymes known as *myo*-inositol hexaphosphate phosphohydrolases (EC 3.1.3.8) or phytases. This thesis deals with the fractionation, purification and properties of phytases from plants and the ascomycete, *Neurospora crassa*. Among the properties which have been examined, particular emphasis has been laid upon the stepwise substrate degradation route.

2. New and original results

(a) The phytase from *Neurospora crassa* has been isolated, purified 100-fold and characterised.

(b) The crude phytase from wheat bran has been resolved into two fractions F_1 and F_2. Fraction F_1 contained the bulk of the activity and was shown to contain a lipid activator. The lipid activator was isolated and identified as lysolecithin. Fraction F_2, from wheat bran phytase, was shown to have markedly different properties from the F_1 fraction, including a higher pH optimum (pH 7) and an absence of inhibition by inorganic phosphate.

(c) A comparison of the substrate degradation routes for the purified *Neurospora crassa* enzyme, and the wheat bran Fractions F_1 and F_2 has involved the isolation and characterisation of the

intermediate inositol polyphosphates. The isolation of two minor pentaphosphates with hydroxyl groups at the 2 and 5 positions respectively, from reactions employing the F_2 enzyme fraction, indicates the presence of two new biological pathways for the degradation of phytic acid.

3. Conclusions

The complexity of the enzyme substrate degradation routes of phytic acid suggests a multiplicity of phytases in biological systems and provides another criterion for homogeneity of these phosphatases. The bifurcation of the wheat bran phytase substrate degradation routes previously observed by Tomlinson and Ballou (1962) is explicable in terms of the combined action of enzyme Fractions F_1 and F_2.

Next to phytic acid, lysolecithin is the largest phosphorus component in the wheat grain and the present study appears to be the first account of specific lipid activation of a soluble enzyme system in which it has been possible to characterise the lipid component. It is considered that the lysolecithin activation is unlikely to be a unique situation and hence a new field of investigation into enzyme activation by water soluble lipids has been uncovered.

DECLARATION

I hereby declare that the work presented in this thesis has been carried out by myself unless otherwise stated in the text, and that it has not been submitted in any previous application for a degree. To the best of my knowledge the material described in the thesis has not been previously published by another person, except where due reference is made in the text.

LIM PHAIK EE

February, 1972.

ACKNOWLEDGEMENTS

I would like to acknowledge my supervisors, Professor D.J.D. Nicholas and Dr. M.E. Tate; the former for permission to undertake this project in his Department and for reading the final proof of the thesis.

I sincerely thank my immediate supervisor, Dr. M.E. Tate, for his valuable time, patient guidance, encouragement and advice during the course of this research. His suggestions and profound understanding of the overall chemical and morphological aspects of biochemistry were a constant help and inspiration.

Thanks also are extended to Dr. D.J. Cosgrove (C.S.I.R.O., Division of Plant Industry, Canberra, A.C.T.) who kindly supplied a sample of *Pseudomonas* SB2 phytase; to Mr. B. Palk, who prepared the photographs and to all those who helped in the preparation of the thesis. I particularly thank my sister, Dr. Yvonne Tan, for her encouragement.

The financial assistance of a University Research Grant is gratefully acknowledged.

PREFACE

Part of the work has been presented to the Australian Biochemical Society Conferences (Adelaide, 1969; Brisbane, 1971) and part has been published, or been submitted for publication.

1. Wheat bran and *Neurospora crassa* phytases.
   P.E. Lim and M.E. Tate
   Aust. Biochem. Soc. Conf., Adelaide, 1969.

2. Wheat bran phytases.
   P.E. Lim and M.E. Tate
   Aust. Biochem. Soc. Conf., Brisbane, 1971.

3. The Phytases.
   I. Lysolecithin-activated phytase from wheat bran.
   P.E. Lim and M.E. Tate
   Biochim. Biophys. Acta 250 (1971) 155.

Abbreviations used in this thesis:-

KCN	potassium cyanide
EDTA	ethylene diamine tetraacetic acid (sodium salt)
PCMB	p-chloromercuribenzoate
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ϕ	*myo*-inositol hexaphosphate or phytic acid
PP_i	inorganic pyrophosphate
P_i	orthophosphate
Fr	fructose
Tris	tris-hydroxymethylaminomethane
PPO	2,5-diphenyloxazole
POPOP	1,4-Bis-(5-phenyloxazolyl-2)benzene
$E^{1\ cm}$, $A^{1\ cm}$	optical extinction in path length of 1 cm
$M_{cyto\ c}$	mobility of substance with respect to cytochrome *c* on starch gel
M_{PP_i}	electrophoretic mobility of substance with respect to PP_i
R_{PP_i}	chromatographic mobility of substance with respect to PP_i

All other symbols and abbreviations are listed in Chapters 2 and 7 (Physico-chemical Symbols) of the 'Handbook for Chemical Society authors, 'The Chemical Society Special Publications No: 14, 1961.

All temperatures are expressed in degrees Centigrade.

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1. LITERATURE REVIEW

1.1 Introduction to the study of phytic acid (*myo*-inositol hexaphosphate) and phytases (*myo*-inositol hexaphosphate phosphohydrolases)

Because of its universal distribution as a major organic phosphorus component of plants and soils, phytic acid or *myo*-inositol hexa-kis-dihydrogen phosphate is one of the most abundant organic phosphates in nature. Its degradation by both chemical and enzymic methods (mediated by phytases or *myo*-inositol hexaphosphate phosphohydrolases) is of particular importance to soil-plant phosphorus relationships.

The phytases degrade a wide range of phosphorylated substrates other than phytic acid and hence they are classified as non-specific acid phosphatases. However, the enzymic removal of phosphate groups from the inositol hexaphosphate molecule is very specific indeed. Thus the phytases can be classified according to their mode of attack on this poly-functional substrate and the subsequent formation of one of the six likely isomeric inositol pentaphosphates.

The pioneering work of Posternak (1921) and Courtois (1951*a*) established the stepwise nature of the phytic acid degradation pathway using wheat bran phytase and Tomlinson and Ballou (1962) elucidated the structures of many of the intermediate inositol polyphosphates which were formed. Their results seemed inconclusive since the enzyme used in their study was only purified 20-fold.

The work reported in this thesis is concerned with the fractionation and isolation of a phytase from *Neurospora crassa* and the phytases from wheat bran. Succeeding chapters include an account of a comparative study of their properties, particularly of their degradative pathways.

It is important to note that the nomenclature used in this thesis is the currently accepted IUPAC-IUB nomenclature (European J. Biochem. *5*, 1968, 1) and that this differs markedly from the systems used by earlier workers, e.g. Tomlinson and Ballou (1962).

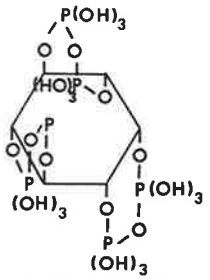
1.2 The structure of phytic acid

1.2.1 Neuberg formula (A)

The structure of *myo*-inositol hexaphosphate or phytic acid (see frontispiece) from cereal grains has been the subject of some controversy (Neuberg, 1908; Anderson, 1914, 1920; Courtois, 1951*a*; Posternak, 1965; Cosgrove, 1966*a*; Johnson and Tate, 1969*a*), centred mainly around the structure proposed by Neuberg (1908) (Formula A, Figure 1) and that suggested by Anderson (1914) (Formula B).

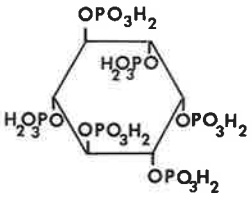
Arguments in favour of the Neuberg formula (A, Figure 1) were mainly based on studies of salts of phytic acid which form trihydrates. These were thought to be incapable of further dehydration without decomposition (Posternak, 1921). Further, the studies included experiments performed on an octadecasodium salt of phytic

STRUCTURE OF PHYTIC ACID



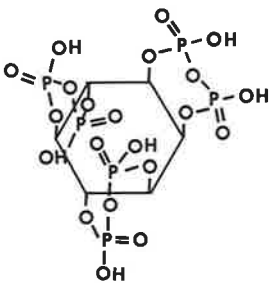
NEUBERG 1908

FORMULA A



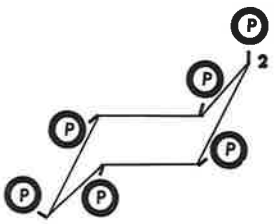
ANDERSON 1914

FORMULA B



PREECE 1962

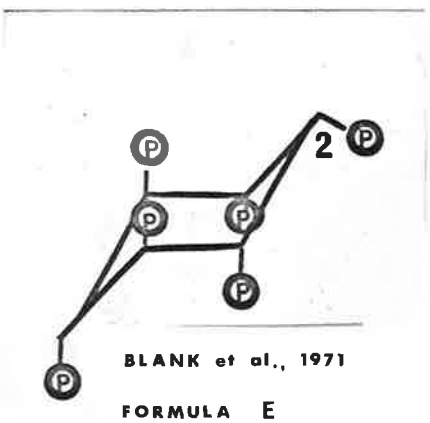
FORMULA C



JOHNSON & TATE 1969a

FORMULA D

Ⓟ = OPO_3H_2



BLANK et al., 1971

FORMULA E

Figure 1.

acid by Brown *et al.* (1961), who based their evidence on the results of a high sodium:phosphorus ratio (3:1) of their material. However, since neither a carbon nor an inositol estimation was given, the subsequent data were inconclusive (Johnson and Tate, 1969*a*). The tri-hydrate configuration of Neuberg (Formula A) was considered an oversimplified model (Preece, 1962). A modified tri-pyrophosphate structure was postulated (Formula C) but Johnson and Tate (1969*a*) synthesised this compound and showed it was electrophoretically separable from phytic acid. It was not detectable in bran extracts prepared under conditions which did not hydrolyse the synthetic triphosphate. The asymmetry of the Neuberg structure suggests that, as a naturally occurring product, phytic acid would exhibit optical activity. However, no significant rotation could be detected in crystalline sodium phytate (Johnson and Tate, 1969*a*).

1.2.2 Anderson structure (B)

Support for the Anderson structure (B) came from the potentiometric titration studies of crystalline sodium phytate by Hoff-Jorgensen (1944), which clearly showed the presence of six well dissociated protons (pK's < 3.5) and six weakly dissociated protons (pK's 4.6-10). Recent ^{31}P nuclear magnetic resonance (n.m.r.) studies (Johnson and Tate, 1969*a*) of crystalline sodium phytate and similar studies of the dodecamethyl ester (Angyal and Russell, 1969*a*) confirmed that the structure of phytic acid is *myo*-inositol hexaorthophosphate (B) rather than the cyclic pyrophosphate

(Formula A). It was also found that there is little significant difference between the enthalpies for inositol hexa-, penta-, tetra- and tri-phosphates, the lower esters of phytate (Raison and Evans, 1968). This study also supports the Anderson formula (B) for phytic acid, as a marked variation in enthalpies among the lower homologues would be expected for structure Formula A. As has been pointed out by Cosgrove (1966*a*) most of the properties and reactions of inositol polyphosphates are explicable in terms of the Anderson structure. Finally, the X-ray structure (Blank *et al.*, 1971) of the crystalline dodecasodium salt shows unequivocally that the correct structure is the Anderson hexaorthophosphate structure.

1.2.3 The conformation of phytic acid

The Haworth formulation for the Anderson structure (B) of *myo*-inositol hexaphosphate can be depicted in two possible chair conformations (D and E). Johnson and Tate (1969*a*) interpreted their ^{31}P n.m.r. spectra in terms of formula D in which the phosphate groups at positions 1,3,4,5,6 of the inositol ring are equatorially orientated whereas the phosphate at position 2 is axially placed. The assignment of axial and equatorial groups was based upon analogy with the chemical shifts of the model inositol-2-phosphate. However, the X-ray crystallographic study of phytic acid (as the dodecasodium salt octatricontahydrate) clearly shows the alternate conformation as in Formula E, in which all the phosphate groups (at C 1,3,4,5,6) except the one on D 2 are axially orientated (Blank *et al.*, 1971).

According to Tate (1971), a re-examination of the ^{31}P n.m.r. spectra (Figure 2) shows that the conformation of phytic acid depends on pH; at pH 1-5 the normal conformation (Formula D) exists but inversion occurs at pH 6-10 to give Formula E. This is in agreement with the finding of Blank *et al.* (1971). Thus the earlier interpretation was in error because of the selection of an inappropriate model (inositol-2-phosphate) which has no phosphate-phosphate interaction. It should be emphasised that the alternate conformation (E) is only stable above pH 10. As the enzyme studies are usually carried out at pH 5 the predominant conformation in solution must therefore be as in Formula D, but the conformation at the enzyme surface is still a matter of conjecture at this stage. In conclusion it must be said that the *myo*-inositol hexaorthophosphate structure proposed by Anderson (1914) is now firmly established, but there is considerable uncertainty as to its exact conformation under various conditions.

1.3 Dephosphorylation routes for phytic acid

Phytic acid can be dephosphorylated by either chemical or enzymic means resulting in a series of lower phosphates, the end products being free inositol and inorganic phosphate (Pizer and Ballou, 1959; Posternak, 1965; Cosgrove, 1966*a*, 1970; Davies, 1968).

1.3.1 Chemical dephosphorylation

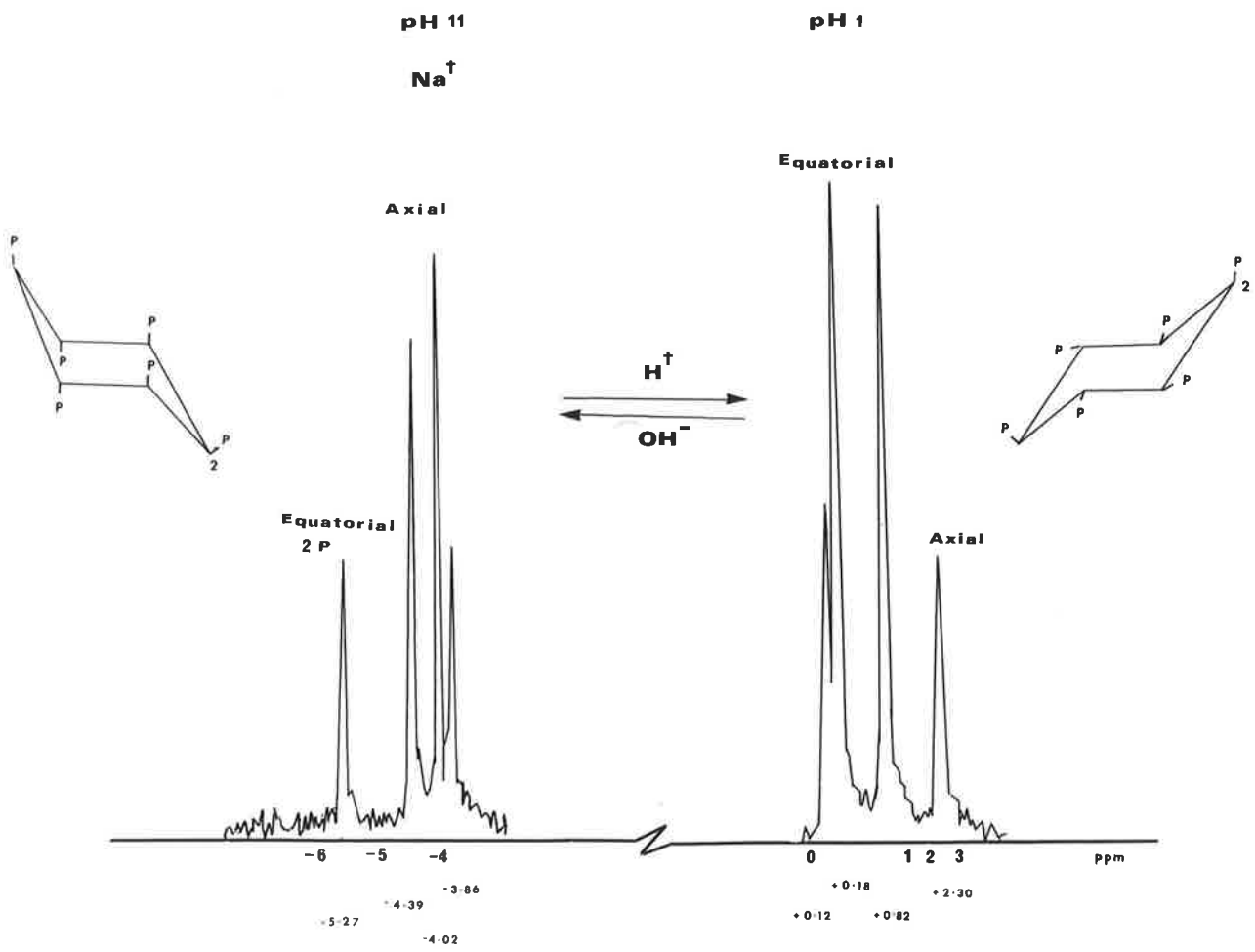
Dephosphorylation of phytic acid in neutral or alkaline medium is slow because the phosphate-ester linkages are very

Figure 2.

^{31}P Nuclear Magnetic Resonance of *Myo*-inositol Hexaphosphate

Figure shows the ^{31}P n.m.r. spectrum of *myo*-inositol hexaphosphate and its conformational inversion as a function of pH.

^{31}P Nmr $MyoIP_6$



stable in strongly alkaline media. Prolonged heating of sodium phytate at pH 8.0 results in *myo*-inositol 2-phosphate (Desjobert, 1954; Schormuller and Bressau, 1960). In acid conditions (pH 3-4) dephosphorylation of phytic acid proceeds with maximum velocity (Fleury *et al.*, 1954; Cosgrove, 1966*a*; Angyal and Russell, 1969*a*,*b*), resulting in a more complex mixture of lower phosphates than with the enzymic hydrolysis (Cosgrove, 1963, 1969; Posternak, 1965; Angyal and Russell, 1969*a*,*b*). Although *myo*-inositol hexaphosphate can give rise to six different pentaphosphates (or penta-O-dihydrogen phosphates) (Figure 3), only two of these have a plane of symmetry (Structures II and V, Figure 3) and are meso compounds. The remaining four are optically active and exist as racemic pairs (Structures I and III, IV and VI, Figure 3) (Angyal and Russell, 1969*a*; Johnson and Tate, 1969*b*).

Only four structurally isomeric pentaphosphates from acid hydrolysates of phytic acid can be identified since the enantiomorphs of each racemic pair are indistinguishable by chromatographic techniques (Cosgrove, 1966*a*, 1969; Tate, 1968; Angyal and Russell, 1969*a*). Acid-catalysed interconversion of two of four pentaphosphate components readily occurs suggesting that migration of a phosphoryl group across *cis*-orientated vicinal hydroxyl groups is involved (Tate, 1968; Cosgrove, 1969). This and other data permitted tentative assignments based on electrophoretic mobility to the four separable pentaphosphates (Tate, 1968) which were later confirmed (Angyal and Russell, 1969*b*; Johnson and Tate, 1969*b*). The four pentaphosphates have electrophoretic mobilities (M_{PP_i}) relative to

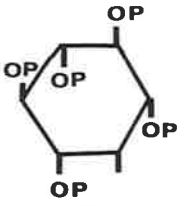
Figure 3.

Structure of *Myo*-inositol Pentaphosphates

This figure shows the different isomers of *myo*-inositol pentaphosphates with their electrophoretic mobilities (M_{PP_i}), see Section 2.4.5).

The number denotes the positions of the phosphate groups on the inositol molecule.

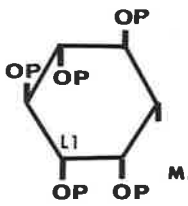
Structures of myo-inositol Pentaphosphates



1 3 4 5 6

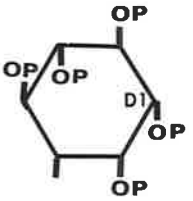
II $M_{PP_i} = 1{\cdot}21$

L 1 2 4 5 6



$M_{PP_i} = 1{\cdot}19$

I



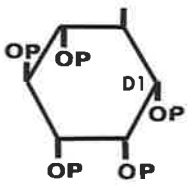
D 1 2 4 5 6

$M_{PP_i} = 1{\cdot}19$

III

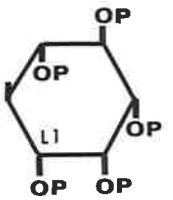
OP = OPO_3H_2

D 1 2 3 4 5



VI

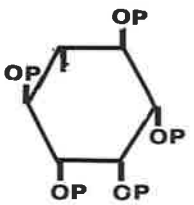
$M_{PP_i} = 1{\cdot}12$



L 1 2 3 4 5

IV

$M_{PP_i} = 1{\cdot}12$



1 2 3 4 6

V

$M_{PP_i} = 1{\cdot}08$

Figure 3.

inorganic pyrophosphate as shown in Figure 3 (Tate, 1968). The inverse order of elution is found for ion-exchange separations (Cosgrove, 1969). The two separation methods are complementary.

Examination of commercial 'phytin' preparations show traces of isomeric *myo*-inositol pentaphosphates (Cosgrove, 1962, 1963*a*,*b*, 1966*a*; Johnson and Tate, 1969*a*; Angyal and Russell, 1969*a*,*b*). It is not certain whether they represent steps in the biosynthesis of phytic acid or are products of its degradation during the isolation procedure (Cosgrove, 1966*a*; Angyal and Russell, 1969*a*). They could be derived from both routes since the pentaphosphate ester has been extracted from natural sources; barley, wheat, cotton and rice (Sobolev, 1962, 1963, 1964, 1966; Posternak, 1965; Asada *et al.*, 1967, 1969). Further hydrolysis of the pentaphosphate fraction results in the formation of mixtures of tetra-, tri-, di-, and mono-phosphate esters (Schormuller, 1960; Cosgrove, 1966*a*; Angyal and Russell, 1969*b*); the only well characterised product is the major monophosphate which is *myo*-inositol 2-phosphate (Desjobert, 1954; Brown and Hall, 1959; Pizer and Ballou, 1959; Tomlinson and Ballou, 1962; Cosgrove, 1963*b*, 1969, 1970; Angyal and Russell, 1969*b*).

1.3.2 Enzymic dephosphorylation

Biodegradation of phytic acid is carried out by phytases (*myo*-inositol hexaphosphate phosphohydrolases) in a stepwise process in which the lower inositol polyphosphates are intermediates and inositol and P_i are the end products (Suzuki *et al.*, 1907,

Posternak and Posternak, 1929; Albaum and Umbreit, 1943; Andrews and Herrarte, 1947; Courtois, 1951*a*,*b*; Desjobert and Petek, 1956; Anderson, 1956; Fowler, 1957; Ergle and Guinn, 1959; Preece *et al.*, 1960; Courtois and Lino, 1961; Preece and Grav, 1962; Tomlinson and Ballou, 1962; Nagai and Funahashi, 1962, 1963*b*; Sobolev, 1962, 1963, 1966; Uehara *et al.*, 1962; Saxena, 1964; Kulaev *et al.*, 1964; Mihailovic, 1965; Anderson, 1965; Cosgrove, 1969, 1970; Greaves *et al.*, 1967; Davies, 1968; Greaves and Webley, 1969; Kurasawa *et al.*, 1969; Johnson and Tate, 1969*b*; Irving and Cosgrove, 1971*a*; Blank *et al.*, 1971; Theodoru, 1971).

The degradative scheme, as shown in Figure 4 was initially elucidated for wheat bran phytase by Tomlinson and Ballou (1962). The entire pathway was demonstrated with a 20-fold purified wheat phytase preparation and a similar degradation scheme has been proposed for peanut phytase (Davies, 1968).

The stepwise dephosphorylation of phytic acid in higher plants begins by hydrolysing a phosphate group at L-*myo*-inositol position 6 (Figure 4). It then apparently proceeds by hydrolysis of another phosphate group which is flanked by a free hydroxyl group. The intermediates in the wheat bran phytase pathway were characterised and identified structurally (Tomlinson and Ballou, 1962; see Figure 4) as D and possibly L-*myo*-inositol 1-monophosphates and *myo*-inositol 2-monophosphate (Structures Ia and Ib, Figure 4), D (and possibly L) *myo*-inositol 1,2-diphosphates (Structure II), *myo*-inositol 1,2,3- (IIIb) and D-*myo*-inositol 1,2,6-triphosphates (IIIa) and

Figure 4.

Scheme of Dephosphorylation of Phytic Acid by Wheat Bran Phytase

(Tomlinson and Ballou, 1962)

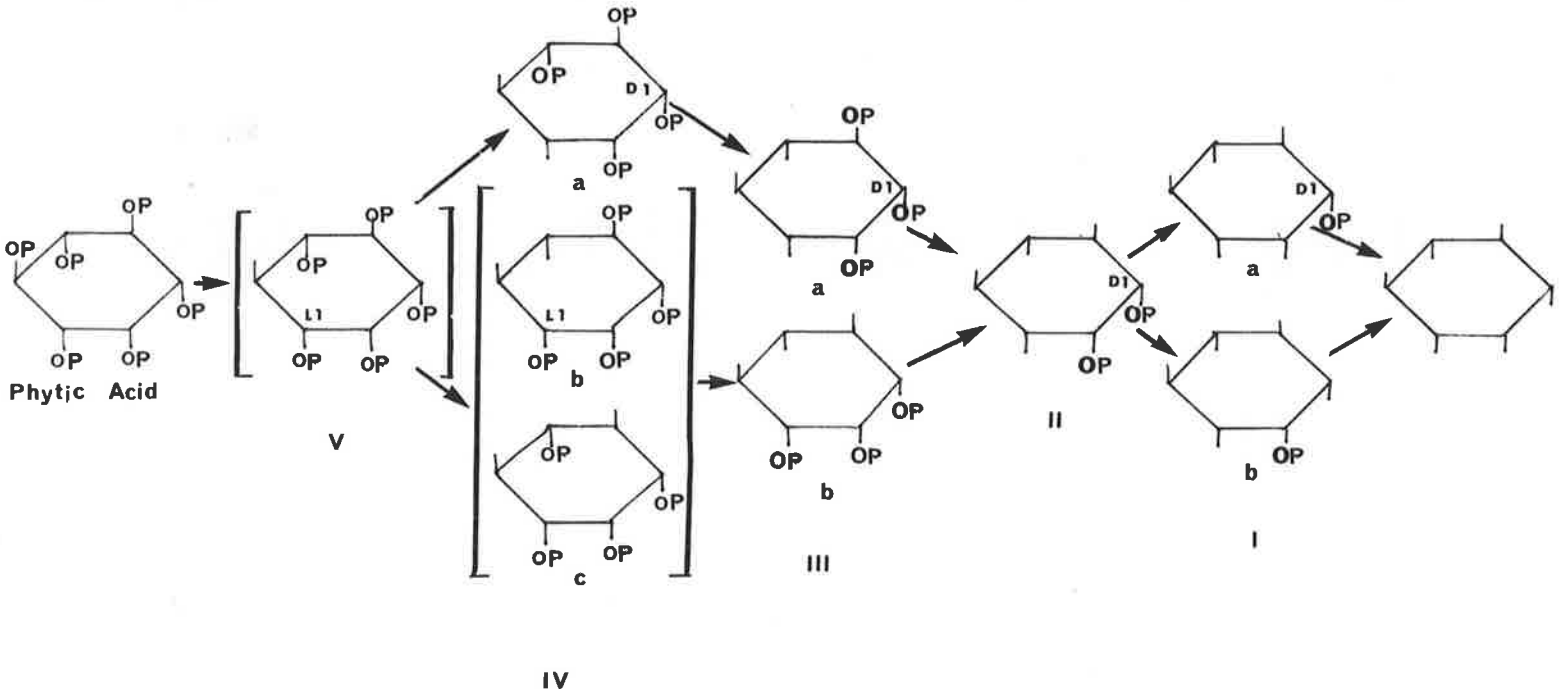
This figure is from Tomlinson and Ballou (1962) who originally used the nomenclature of Fletcher, Anderson and Lardy (1951). In this thesis the currently accepted IUPAC-IUB nomenclature (European J. Biochem. *5*, 1968, 1) is always employed and the relevant D_1 or L_1 positions have been marked for optically active phosphates; for meso compounds no D or L prefix is required.

$$OP = OPO_3H_2$$

unattached bond = OH, throughout

Scheme of Dephosphorylation of Phytic Acid

Tomlinson & Ballou 1962



D-*myo*-inositol 1,2,5,6-tetraphosphate (IVa). The structure of the pentaphosphate as L-*myo*-inositol 1,2,3,4,5-pentaphosphate (V) was deduced, as this fraction was not sufficiently separated from the hexaphosphate by the chromatographic procedure for definitive characterisation. It is also possible that of the tetraphosphates L-*myo*-inositol 1,2,3,4 (IVb) and a symmetrical isomer *myo*-inositol 1,2,3,5 (IVc) may also be present, but these were not separable by the techniques used.

Although the structural configuration of the inositol polyphosphates was not given, the hydrolysis of phytic acid by *Aerobacter aerogenes* (Greaves *et al.*, 1967) was said to be similar to that of phytase from plant tissues (Figure 4). The phosphates were only tentatively identified by their characteristic position in the elution pattern and on paper chromatography based on standards. Chemical analysis of the type carried out by Tomlinson and Ballou (1961, 1962) and Davies (1968) would be necessary to confirm the inositol polyphosphate structures.

Studies of the degradation of phytate in the growth medium of *Neurospora crassa* by Johnson and Tate (1969*b*) showed a different degradation route. A tentative scheme was proposed which involved acceptance of the assignments made by Tomlinson and Ballou (1962). A similar pathway (Figure 5) was found by Cosgrove (1970) for the degradation of phytate by a partially purified enzyme from a soil bacterium, *Pseudomonas* SB2. Again the structure assignments were based on relationships to the phosphates characterised by Tomlinson

Figure 5.

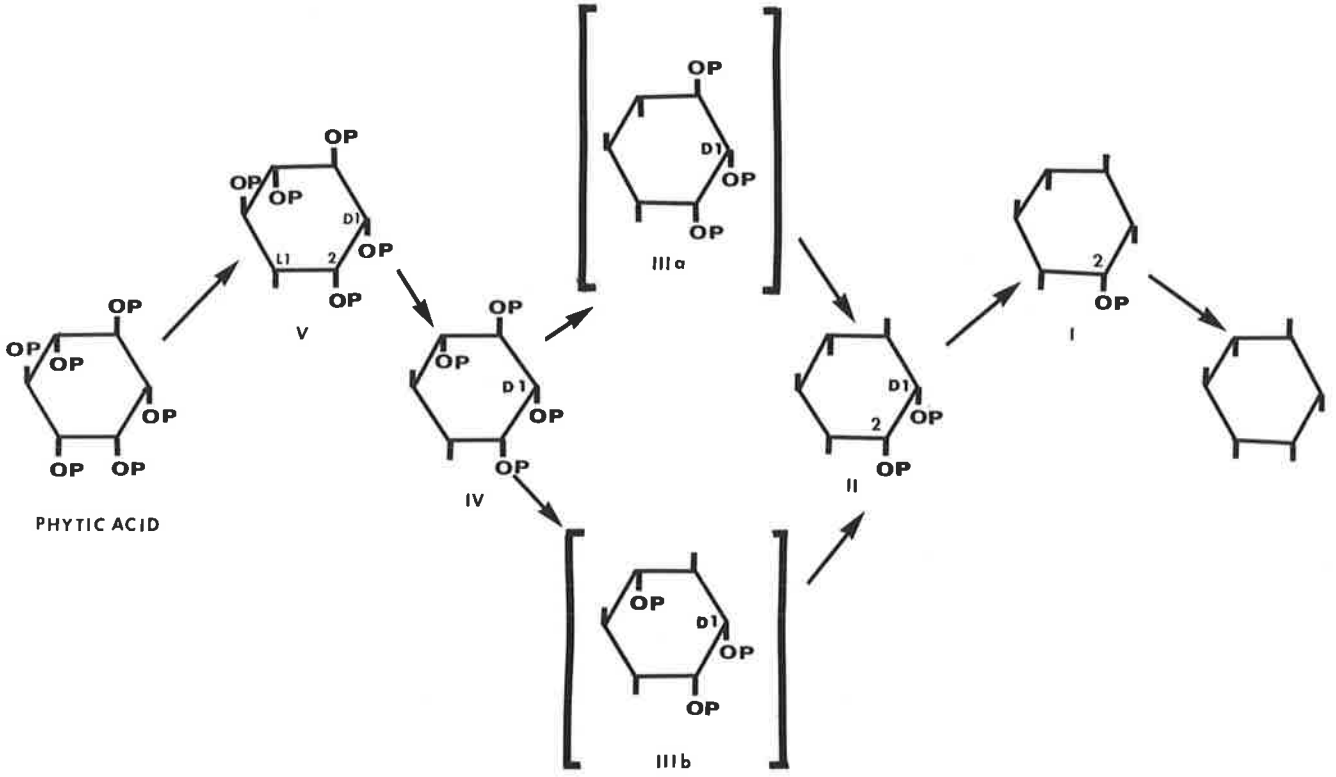
Dephosphorylation of Phytic Acid by *Pseudomonas SB2* enzyme

This figure is from Cosgrove (1970). The relevant D or L 1 positions have been marked for the naming of the inositol polyphosphates.

OP = OPO_3H_2

Dephosphorylation Of Phytic Acid By *Pseudomonas* SB2 Phytase

Cosgrove 1970



and Ballou (1962). All these structural assignments for the optically active inositol polyphosphates essentially require the isolation of D arabitol from the periodate oxidation-borohydride-reduction and hydrolysis sequence (see Section 2.4.10.5) applied to a mixture of inositol triphosphates by Tomlinson and Ballou (1962). The distinctive feature of the fungal and bacterial degradation resides in the initial point of attack at the L 1 position which produces the presumed D-*myo*-inositol 1,2,4,5,6-pentaphosphate (see route 2 of Figure 6). The major pathway of degradation for the plant phytases is via route 1 of the same figure (Figure 6). Most of the lower inositol polyphosphates are common to plant and microbial phytase substrate degradation routes as can be seen by comparing Figures 4 and 5. In both schemes *myo*-inositol 2-monophosphate is formed prior to free inositol. The fact that the chemical (Section 1.3.1) and both the enzymic hydrolysis routes of phytic acid all lead to the same *myo*-inositol 2-monophosphate may be explained by the steric protection of the axial phosphoryl group attached at the C 2 position of the *myo*-inositol hexaphosphate molecule (Baddiley *et al.*, 1958; Pizer and Ballou, 1959; Cosgrove, 1963*b*, 1966*a*, 1969, 1970; Posternak, 1965; Davies, 1968; Tanaka *et al.*, 1971).

1.4 Enzymology of phytases

Phytase (*myo*-inositol hexaphosphate phosphohydrolase, EC 3.1.3.8) is strictly the enzyme responsible for the hydrolysis of one phosphate from phytic acid. However, as shown in Section 1.3.2 the products become substrates for further reaction and the overall

Figure 6.

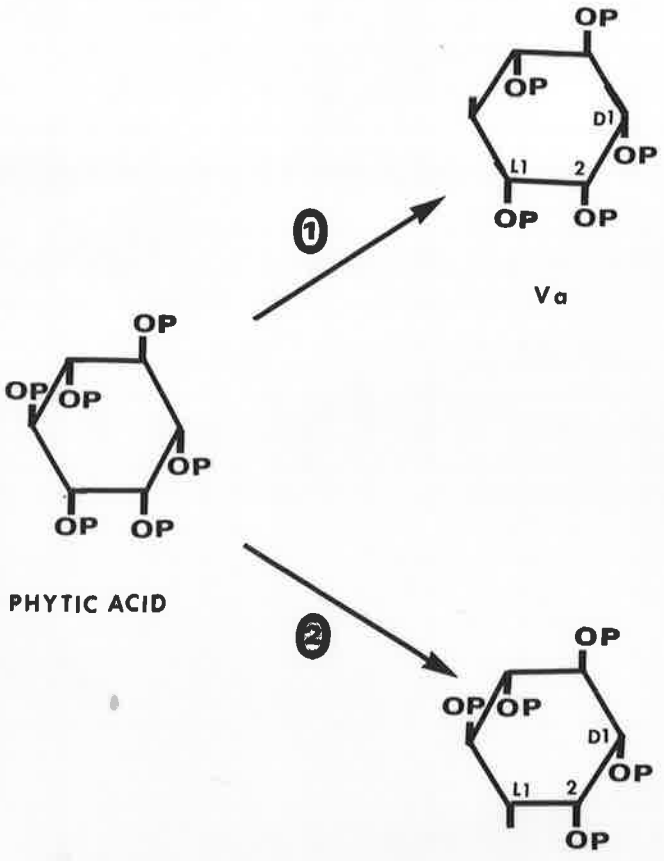
Phytase Routes:-

1 - pathway taken by wheat bran phytase

2 - pathway taken by phytase from micro-organisms

$$OP = OPO_3H_2$$

PHYTASE ROUTES



L-MYO-INOSITOL 1 2 3 4 5 PENTAPHOSPHATE

D-MYO-INOSITOL 1 2 4 5 6 PENTAPHOSPHATE

catalysed reactions may be represented:-

Inositol hexaphosphate ⟶⟶⟶ inositol + 6 inorganic phosphate
(phytic acid)

1.4.1 Distribution

1.4.1.1 Plants

Table 1 shows that phytase is a widely distributed enzyme. Its presence is either known from direct enzyme assay or is implied via the detection of lower inositol polyphosphates; both methods are complementary. Until recently, few inositol phosphate intermediates were recorded in plants. This is probably due to the rapid breakdown of phytate and its lower esters in the tissue. Thus Mellanby (1950) found that wheat phytate disappeared after germinating for 5 to 8 days. Similarly Ashton and Williams (1958) found that phytate decomposed quantitatively to inorganic phosphate and inositol in less than 14 days in the germinating oat grain. The relative abundance of phytase among the cereals (the monocotyledons) is well established and is shown in Table 1.

The importance of the enzyme, phytase, in supplying nutrients to the growing seedling is emphasised by the high concentration (50% of the total phytase activity) which is present in the embryo and the aleurone-scutellum layers of the peanut and also the wheat grain (Peers, 1953; Anderson, 1965; Davies, 1968). The epidermal layer and the cross cells (see Figure 7) do not contain the enzyme

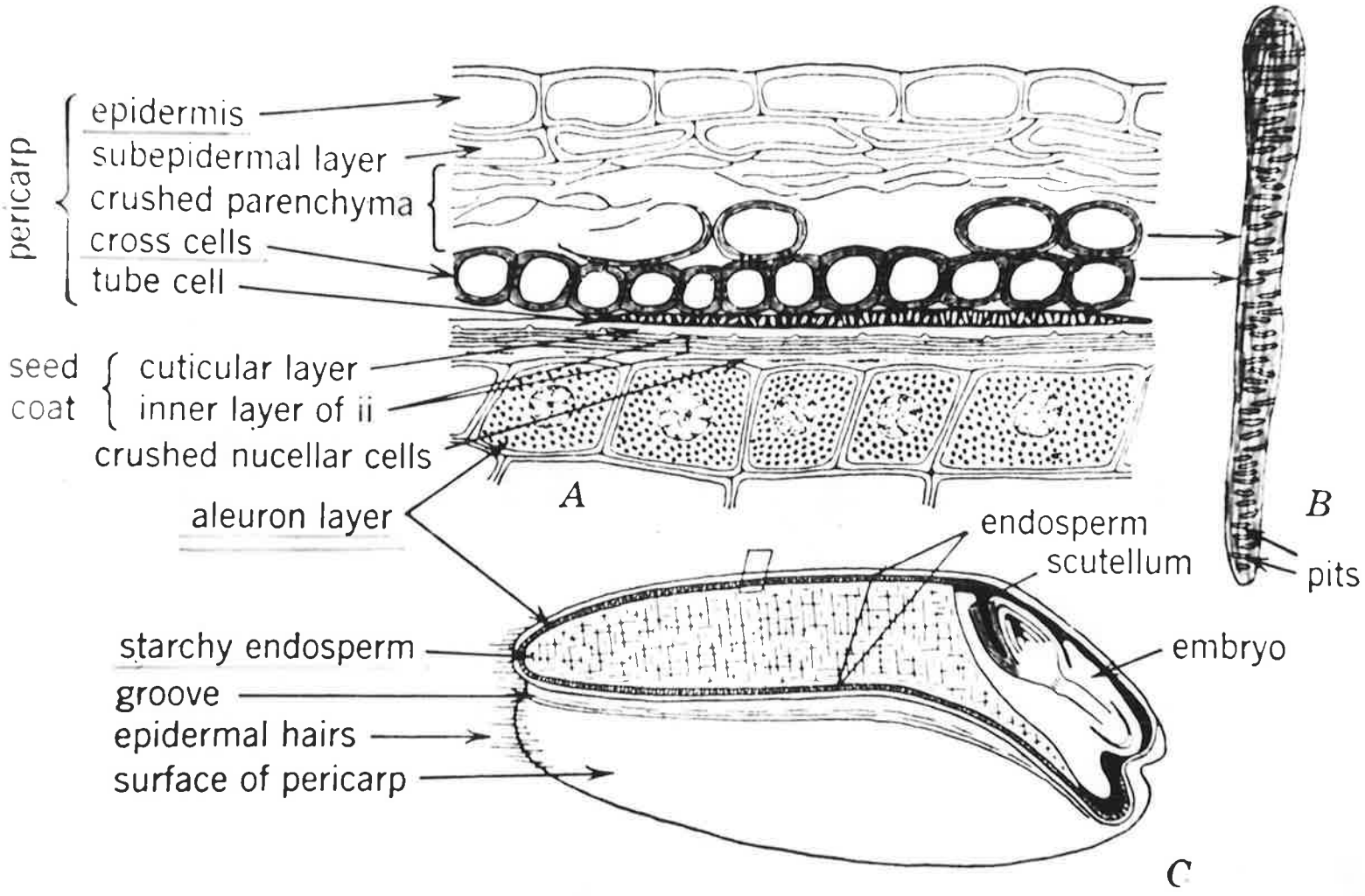
Table 1. Distribution of phytases with reference to pH optimum

pH range	Determined pH	Source	Reference
		Micro-organisms	
2-3	2.2	*Pichia farinosa*	Shimoda, 1927
	2.25	*Escherichia coli*	Courtois & Manet, 1952
	3.0	*Aspergillus niger*	Casida, 1959
4-5	4.4	*Aspergillus oryzae*	Shimoda, 1927
	4.5	*Escherichia coli*	Courtois & Manet, 1952
	4.5	*Aspergillus terreus*	Yamada *et al.*, 1968
		Plants	
	4.0	Cotton seed	Thomas, Walter & George, 1946
	4.9	*Vigna sesquipedalis*	Sugiura & Sunobe, 1961
		Micro-organisms	
5-6	5.4	*Escherichia coli*	Courtois & Manet, 1952
	5.5	*Pseudomonas* sp.	Irving & Cosgrove, 1971.
		Plants	
	5.0	Lettuce seed *(Picris, L)*	Mayer, 1958
	5.0	Wheat bran *(Triticum, L)*	Nagai & Funahashi, 1962
	5.0, 5.2	Peanut *(Arachis hypogaea)*	Anderson, 1965, Davies, 1968
	5.1	Fenugrek (Legume)	Taha & El-Towesy, 1958
	5.15	Wheat *(Triticum L)*	Peers, 1953
	5.1,5.4	Barley *(Hordeum, L)*	Taha & El-Towesy, 1958; Adler, 1916
	5.2	*Phaseolus vulgaris*	Gibbins & Norris, 1963
	5.5	Mustard seed *(Sisymbrium)*	Sumner & Myrback, 1950
	5.5	Soy bean	Sumner & Myrback, 1950
	5.5	Tobacco leaves *(Nicotiana)*	Shaw, 1966
	5.6	Corn *(Zea mays)*	Chang, 1967
		Micro-organisms	
> 6	7.5	*Bacillus subtilis*	Powar & Jagannathan, 1967
		Plants	
	7.0	Lettuce seed *(Picris, L)*	Mayer, 1958
	7.5	*Phaseolus aureus*	Mandal & Biswas, 1970
		Vertebrates	
	6.0	Frog)	Rapoport *et al.*, 1941
	6.6	Goose)	
	6.6	Pigeon)	
	7.2	Turtle)	
	7.3, 7.8	Rat	Patwardhan, 1937; Spitzer & Phillips, 1945; Pileggi, 1959,

Figure 7.

Section of a Wheat (*Triticum*) grain

The small rectangle in C indicates the approximate location of the section shown in A. The letters 'ii' signify 'inner integument'. From ESAU (1953).


pericarp
epidermis
subepidermal layer
crushed parenchyma
cross cells
tube cell
seed coat
cuticular layer
inner layer of ii
crushed nucellar cells
aleuron layer
A
B
endosperm
scutellum
pits
starchy endosperm
groove
epidermal hairs
surface of pericarp
embryo
C

(Vorbrodt, 1910; Peers, 1953; Anderson, 1965; Davies, 1968). In lettuce seeds it has been shown that phytase is not attached to the mitochondria but is a non-particulate enzyme (Mayer, 1958).

Although phytase is a widely distributed enzyme, its activity varies from species to species; it is not always present in all tissues of plants. Of the *Gramineae*, oats, maize and some millets have negligible phytase activity (Rose, 1912; Giri, 1941; Preece and McCallum, 1964); barley has moderate activity (Preece and McCallum, 1964); wheat and rye have high activity (Cosgrove, 1966*a*). The phytase activity from wheat, barley and oats is in the relationship of 3:1:0.02, respectively, on a weight-for-weight basis for each preparation (Preece and McCallum, 1964). Cereals are richer in phytase enzyme than the dicotyledons. The relative phytase activity in barley compared to that in *Vicia faba* and to that of a legume (fenugreck) is in the following ratio:- 10.5:2.3:1 (Taha and El-Towesy, 1958).

It has been suggested that phytase is present wherever phytate occurs (Pringle and Moran, 1942; Mayer and Poljakoff-Mayber, 1963; Davies, 1968; Matheson and Strother, 1969) but this is not necessarily so. While the wheat starchy endosperm tissue does not seem to contain phytate (Williams, 1970), it does have a considerable amount of phytase (Peers, 1953; Koller *et al.*, 1962). In seeds, phytase activity is initially low but increases on germination. This increase is influenced by gibberellin and inorganic phosphate (Srivastava, 1964; Bianchetti and Sartirana, 1967*a*,*b*; Eastwood *et al.*, 1969).

1.4.1.2 Micro-organisms

The presence of phytase activity in the soil is attributed to soil micro-organisms. Although phytase has been known in *Aspergillus niger* for some time (Dox and Golden, 1911), only in recent years have there been attempts to investigate it in fungi and other micro-organisms (such as in *Aerobacter aerogenes* - Greaves *et al.*, 1963, 1967; *Aspergillus terreus* - Yamada *et al.*, 1968; *Bacillus subtilis* - Powar and Jagannathan, 1967; *Neurospora crassa* - Johnson and Tate, 1969*b*; *Pseudomonas* species - Cosgrove, 1969, 1970; Irving and Cosgrove, 1971*a*,*b*; *Rhizopogon luteolus* - Theodoru, 1968, 1971; and other organisms - Greaves and Webley, 1965, 1969; Cosgrove, 1967; Skujins, 1967; Shieh and Ware, 1968; Koichi, 1968; Cosgrove *et al.*, 1970). The earlier findings that members of the genus *Aspergillus*, including some soil isolates, possessed phytase activity (Dox and Golden, 1911; Jackman and Black, 1952*a*,*b*) has now been confirmed by Casida (1959). There are some inconsistencies however in the data of a number of workers concerning the dephosphorylation of phytic acid by micro-organisms. This may be due to solubility problems with substrates. Although sodium phytate is soluble in water throughout the whole pH range the calcium magnesium salt of phytic acid or phytin is not. It is insoluble at alkaline pH; hence the substrate used by Greaves *et al.* (1963) was different from that of Kotelev *et al.* (1962). In addition, commercial samples of phytin and sodium phytate contain considerable amounts of the lower esters (Cosgrove, 1963*b*, 1966*a*, 1967) which were assumed to have resulted from the action of microbial phytases. However, they may well result

from the phosphatase action of the micro-organisms attacking these lower polyphosphates rather than phytase specifically acting on phytic acid (Cosgrove *et al.*, 1970). Furthermore low phytase activity may not be detected in micro-organisms since phytase activity can be inhibited by high concentrations of the substrate. This is because phytate is a protein precipitant and at high concentrations of phytate, substrate inhibition occurs (Gibbins and Norris, 1963; Posternak, 1965).

1.4.1.3 Animals

Although the occurrence of phytase has been reported in calf-liver and blood (McCollum and Hart, 1908) in the intestine of rats (Patwardhan, 1937; Pileggi, 1959) and in the blood of birds, frogs and turtles (Rapoport *et al.*, 1941*a*,*b*), it is absent in the rabbit, guinea pig and man. In contrast, repeated experiments by Patwardhan (1937) and Pileggi (1959) show the absence of phytase in beef and calf-liver, thereby confirming some early work (Scofone, 1904; Plimmer, 1913; Lowe and Steenbock, 1936*a*,*b*).

Consideration of the presence of phytase in plants (Section 1.4.1.1) and micro-organisms (Section 1.4.1.2) leads to the conclusion that the activity of phytase in the rat intestine may be due to intestinal micro-organisms or to phytase in the vegetable part of the animal's diet. However, the presence of large amounts of inositol pentaphosphate in avian blood (Johnson and Tate, 1969*a*) suggests a re-examination of the phytase might be rewarding.

1.4.2 Multiple nature of phytase

Little is known about the number of iso-enzyme systems in phytase although this multiple nature of the enzyme has been found in a number of phytase preparations from plants and micro-organisms. During the purification procedures of barley phytase, Preece and Grav (1962) demonstrated that at least two enzyme fractions could be distinguished by their adsorption behaviour on C γ alumina columns. They found that the first inositol phosphatase fraction was completely eluted from the column by a descending pH gradient while a second enzyme was eluted by an ascending pH gradient. Although details of the properties of the enzyme complexes were not established, Preece and Grav (1962) reported that the second phytase system was different from the first reaction; the former enzyme was more susceptible to substrate inhibition.

Similarly, the phosphatase preparations from rice ear, apricot and *Aspergillus niger* can be resolved into a minor and a major component by ion-exchange chromatography (Ikawa *et al.*, 1964) possibly indicating charge differences. The fact that these fractions were able to hydrolyse phytic acid in addition to phosphomonoesters implied that phytase may consist of more than one enzyme. The presence of iso-enzymes in phosphatases has been reported and found to occur in a number of tissues; soy bean - Mayer *et al.* (1961); *E. coli* - Von Hofstein and Porath (1962); wheat germ - Brouillard and Ouellet (1965), Verjee (1969); roots of *Vicia faba* - Gahan and McLean (1967); bovine liver - Heinrikson (1969) and *Aspergillus oryzae* - Sakurai and Shiota (1970). Courtois and Perez (1949*a*,*b*) fractionated two phosphatase components

from several plant extracts. In addition, they distinguished between the major phytase fraction and a phosphomonoesterase component. The non-detection of phytase activity in the minor phosphomonoesterase fraction does not necessarily prove that it is absent. The workers (1949*a*,*b*) did not assay for phytase activity in the fraction with low concentrations of substrate. It has been shown that low levels of phytase activity cannot be detected unless very small amounts of phytate are used, since at higher concentrations substrate inhibition is prevalent (Yoshida, 1950; Perles, 1955; Gibbins and Norris, 1963; Nagai and Funahashi, 1963*a*). Furthermore, highly susceptible behaviour to substrate concentration has been reported for the second phytase system in barley (Preece and Grav, 1962). Also it would not be surprising if the minor phytase fraction was extremely labile and that during the extraction procedure it was completely inactivated. Moreover, the detection of the enzyme in some tissues and not in others may suggest that the enzyme may be present only at certain stages of the life cycle of the organism or it may be present if induced by particular growth conditions and certain metabolites in a tissue.

Essery (1951) used phytic acid at (>0.05%) similar concentrations to those used by Preece and Grav (1962) for determining the activity of barley phosphatases. Peers (1953) used much greater amounts of phytic acid, which inhibited the barley enzyme preparations. This led to the conclusion that reaction of the barley enzyme depends on the ratio of different enzyme activities, a ratio which may be very different in wheat (Preece and Grav, 1962).

The splitting of the peanut phytase enzyme into several fractions with acetone and methanol during the differential precipitations led Anderson (1965) and later, Davies (1968), to postulate the existence of a mixture of enzymes in peanut phytase. However, no clear distinctions were made among these active peanut phytase fractions.

The highly purified bran phytase of Nagai and Funahashi (1962) exhibited heterogeneity in physical properties. In addition, since the dephosphorylation of phytic acid is a stepwise procedure involving at least six consecutive reactions, the possibility remains that a multi-enzyme complex or a mixture of phosphatases is involved. The kinetics study on barley phytase has shown that the enzyme system which initially attacks phytic acid is quite distinct from the enzyme system acting on the lower inositol phosphates, thus suggesting that more than one distinct group of enzymes may be involved (Preece and Grav, 1962).

More direct data for the presence of two or more enzymes in phytase come from pH optimum studies. The lettuce seed phytase displays a pH optimum at pH 7 as well as at pH 5 (Mayer, 1958). Three pH optima, 2.25, 4.5 and 5.4 have been reported for the *E. coli* enzyme (Courtois and Manet, 1952) suggestive of three iso-enzymes in the bacterial phytase.

The data of Johnson and Tate (1969*b*), Cosgrove (1970) and more recently, Theodoru (1971) on the enzymic formation of isomeric *myo*-inositol pentaphosphates confirm the possibilities of two phytases in biological systems (Section 1.3.2); moreover, the two enzymes can

exist in the one organism. Although isomeric pentaphosphates have been previously observed in enzymic digests of phytic acid in wheat their structures were not established (Schormuller, 1960).

The general conclusion seems to be that there are many types of phytase, some of which may occur as iso-enzymes in the same organism.

1.4.3 Properties

Phytase has been partially purified from a number of organisms, and some of its properties from six sources are presented in Table 2. The general enzymic properties of plant phytases classify it as a non-specific acid phosphatase. It has also been found that phytase and non-specific acid phosphatase (particularly glycerophosphatase) activities of plant extracts are very similar and are inseparable (Courtois, 1951*b*). The properties of acid phosphatase in partially purified preparations from a number of similar phytase sources have been studied:- soy bean - Chia Mu (1954); Campbell *et al.* (1969); wheat - Joyce and Grisolia (1960); lupine seedlings - Newmark and Wagner (1960); *Neurospora crassa* - Kuo and Blumenthal (1961), Kadner and Nyc (1969); dwarf bean - Roussos (1962); Gibbins and Norris (1963); *E. coli* - Von Hofstein and Porath (1962); Tobacco leaves - Shaw (1966); *Aspergillus terreus* - Yamamoto *et al.* (1969).

A comparison of some properties of acid phosphatases from five sources are summarised in Table 3.

Table 2. Some properties of phytase from various sources

Source	Purification	Temp. optimum	pH optimum	K_m value(M)	Substrates hydrolysed in addition to phytic acid	Inhibitor	Activator	Reference
Manitoba wheat	20-fold	55°	5.15	3.3×10^{-3}		KCN, NaF, $ZnSO_4$, $MnSO_4$ (10 mM)	Oxalate, citrate (10 mM) Mg^{++} (2 mM)	Peers, 1953
Triticum (wheat bran)	1,500-fold		5.0	5.7×10^{-4}	ATP, ADP, NADP, αGP, Fr 1,6-di-P, phenyl phosphate	Tartrate (1 mM)	EDTA (1 mM)	Nagai and Funahashi, 1962
Zea mays (L. Var. Marcoss) (Corn - 4 day old)		50°	5.6	9.1×10^{-5}	β-glycerophosphate	NaF (0.05-10 mM)	Ca^{++} (10 mM)	Chang, 1968
Phaseolus vulgaris (dwarf bean)	<50		5.2	1.5×10^{-4}	phenyl phosphate	pCMB, Mg^{++} (10 mM) Ca^{++}, NaF (10 mM)	(Mg^{++}, Ca^{++}) (1 mM)	Gibbins and Norris 1962
Arachis hypogaea (peanut)	17	55°	5.0	1×10^{-1}	ATP, ADP, α and βGP, phenyl phosphate			Davies, 1968
Pseudomonas SB2	25		5.5	1.6×10^{-5}	p-nitro-phenyl-phosphate	Mg^{++} NaF (2 mM)		Irving and Cosgrove 1971

Table 3. Some properties of acid phosphatase from various sources

Source	Purification	pH optimum	K_m value for (GP), glycero-phosphate as substrate	Substrates hydrolysed	Activator	Inhibitor	Reference
Neurospora crassa	1,400-fold	5.6	4.6×10^{-3}M	GP, ADP, nitro-phenyl phosphate		Mo^{++} NaF (+) tartrate	Kuo and Blumenthal, 1961
Pichia farinosa (yeast)	60-fold	6.5	7×10^{-3}M	αGP 1 propane-diol phosphate	Mg^{++}	NaF Cu^{++} Cd^{++} Ca^{++}	Tsuboi and Hudson, 1956
Tobacco leaves	300	5.5-5.7	4.1×10^{-4}M	ATP, ADP, PP_i NADP, GP, phytic acid, nitro-phenyl phosphate, phenyl phosphate		Mo^{++} NaF P_i	Shaw, 1966
Triticum (wheat germ)	800	5.1	2.7×10^{-4}M	ATP, ADP, GP, PP_i, Fr 1,6 di-P, nitro-phenyl phosphate	Mg^{++} Mn^{++}	Pb^{++} Ag^{+} Zn^{++} Cu^{++} P_i	Joyce and Grisolia, 1960
Phaseolus vulgaris (dwarf bean)	100	5.3-6.3		ATP, ADP, GP, PP_i, Fr 1,6 di-P, nitro-phenyl phosphate		NaF P_i PP_i	Roussos, 1962

1.4.3.1 pH optimum value

Most phytase preparations from plants operate in an acid medium around pH 5 (see Table 1) with very few exceptions in the alkaline range. Because of the many and varied reports on the optimal pH values of phytase activity, a pH survey of the phytases from different sources is summarised in Table 1. These pH data emphasize a property of the phytases which is similar to that of the non-specific acid phosphatases. The different pH optima of phytases enabled Courtois (1951*a*) to classify the enzymes into the five principal phosphomonoesterase groups of Folley and Kay (1936); the comparison is given below:-

Phytase I: Phosphomonoesterase I - is very similar to alkaline phosphomonoesterases found in mammalian tissues (Folley and Kay, 1936). It is active in weakly alkaline medium and is activated by magnesium ions. The only example found to date is the rat intestinal phytase (of optimum pH activity at 8.0) (Patwardhan, 1937; Pileggi, 1959).

Phytase II: Phosphomonoesterase II - This group includes the best known phytases of the higher plants, notably the cereals, legumes and brassicas, whose optimum pH is between 4.9 and 5.6. Like phosphomonoesterase II, Phytase II is not activated by magnesium ions (Nagai and Funahashi, 1962; Sugiura and Sunobe, 1962) which at high levels (>10 mM) may be inhibitory (Gibbins and Norris, 1963). The enzyme is strongly inhibited by fluorides and molybdate.

Phytase III: Phosphomonoesterase III - This has a pH optimum around 4.0. Very little is known about this enzyme though it has been found in *Aspergillus* (Casida, 1959; Yamada *et al.*, 1968) and in kidney, liver, muscle and bone (Horiuchi, 1931). Its presence in animal tissues has not been confirmed (see Section 1.4.1.3).

Phytase IV: Phosphomonoesterase IV - The phytase from the plasma and blood corpuscles of lower vertebrates (Rapoport *et al.*, 1941*a*) contribute to the fourth group of enzymes, which seems to correspond to the phosphomonoesterase IV characterised by Roche (1950). The pH of this enzyme is between 6 and 7. It is activated by magnesium and is inhibited by fluorides and oxalates.

Phytase V: Phosphomonoesterase V - exhibits very acid pH optimum (<3.0). Those that have been detected are few and seem to be present only in micro-organisms, e.g. in the yeast *Pichia farinosa*, pH 2.2 (Shimoda, 1927) and in *E. coli*, pH 2.1-2.2 (Courtois and Manet, 1952).

1.4.3.2 Activators and inhibitors of phytase

A property of phytase which is similar to the non-specific acid phosphomonoesterase is the non-requirement of a metal ion, or cofactor for its activity. But under the influence of pH certain divalent cations (Mg and Ca) can either inhibit or activate phytase. Thus Ca^{++} activates phytase from *B. subtilis* (Powar and Jagannathan, 1967). While magnesium at certain concentrations is shown to be an activator for the wheat enzyme at other concentrations

it inhibits (Peers, 1953). It is found to be an inhibitor of the peanut phytase (Davies, 1968) and of the *Pseudomonas* enzyme (Irving and Cosgrove, 1971*a*). Other activators of phytase include thiocyanate and oxalate (Courtois, 1951*a*; Peers, 1953) and even these inhibit at a different pH value and inhibitor concentration. In contrast, Nagai and Funahashi (1962) did not find any effect of these inhibitors on the activity of wheat bran phytase (purified 1,500-fold activity).

Other inhibitors of the enzyme are copper, zinc and iron in the ferric form (Pfankuch, 1936; Jackman and Black, 1951*a*,*b*; Courtois *et al*., 1952; Peers, 1953; Hill and Tyler, 1954; Nagai and Funahashi, 1962). The inhibitors of phytase are probably substrate-precipitants or even a protein precipitant (such as phytate itself) or a denaturation agent. This includes fluoride and mercuric chloride (Peers, 1953; Taha and El-Towesy, 1958; Nagai and Funahashi, 1962; Chang, 1967). Phytate is a potent inhibitor of phytase and acid phosphatase enzyme (Manouvrier, 1953; Perles, 1955; Nagai and Funahashi, 1962; Gibbins and Norris, 1963). Phytate exhibits a non-competitive type inhibition for wheat bran phytase (Nagai and Funahashi, 1963*a*).

Although most acid phosphatases from plants are inhibited by phosphate (Joyce and Grisolia, 1960; Roussos, 1962; Shaw, 1966), this may not affect phytase. End-product inhibition was not shown by the bran phytase preparation of Nagai and Funahashi (1962).

It has been reported that yeast preparations inhibit phytase activity (Peers, 1953) but the nature of this inhibition has not been elucidated.

1.4.3.3 Substrate specificity

The relationship of phytase to the non-specific acid phosphatases has been studied. Thus Fleury and Courtois (1947), Ikawa *et al.*, (1964) and Sloane-Stanley (1961) showed that several plant extracts which were active towards phytic acid invariably possessed phosphatase activity towards other substrates. The 17-fold purified peanut phytase catalyses the hydrolysis of ATP, ADP, glycerophosphate and phenyl phosphate. Even the 1,500-fold partially purified bran phytase extract of Nagai and Funahashi (1962), though devoid of phosphodiesterase activity, catalyses the hydrolysis of ATP, ADP, NADP, pyrophosphate, glycerophosphate, phenyl phosphate and fructose diphosphate in addition to *myo*-inositol hexaphosphate. A constant ratio of activities towards these mixed substrates indicates that these activities are attributed to the same enzyme. This further suggests that the phytase preparation is a non-specific acid phosphomonoesterase. To date, only the enzyme from *B. subtilis* (Powar and Jagannathan, 1967) has been reported to be very specific for phytate as a substrate.

It is not clearly established whether this broad substrate specificity of phytase is due to the presence of iso-enzymes in the preparations or to an inherent lack of specificity (Davies, 1968).

The non-specific acid phosphomonoesterase from wheat germ (Joyce and Grisolia, 1960; Verjee, 1969) hydrolyses adenosine polyphosphates as well as simple phosphate esters. It has been suggested (Davies, 1968) that Nagai and Funahashi's (1962) preparation may contain the

wheat germ enzyme of Joyce and Grisolia (1960) since non-specific acid phosphomonoesterases are abundantly and widely distributed in living tissues. The data that numerous phytase preparations contain phosphatase activity (Courtois and Lino, 1961; Preece and Grav, 1962; Nagai and Funahashi, 1962; Davies, 1968) merely suggests the inseparable nature of these enzyme systems.

The enzymic hydrolysis of *myo*-inositol monophosphate is relatively simple and is of first order kinetics but the reaction of the higher polyphosphates is said to proceed in a complex way (Courtois and Masson, 1950, Preece and Grav, 1962). The affinities of phytase from different sources for phytate have been studied resulting in varying K_m values; 9×10^{-2}M to 1×10^{-1}M for peanut phytase (Anderson, 1965; Davies, 1968); 1×10^{-3}M for goose plasma phytase (Rapoport *et al.*, 1941); 1×10^{-4}M for dwarf bean phytase (Gibbins and Norris, 1963) and 5.7×10^{-4}M for wheat bran phytase (Nagai and Funahashi, 1962) (also see Table 2). It is possible that the protein content in each phytase preparation is different. Accordingly the level of substrate concentration for inhibiting phytase would vary, resulting in the many and varied K_m values reported. Gibbins and Norris (1963) have shown that the K_m for the dwarf bean phytase is 1.5×10^{-4}M but this concentration completely inhibits the phytase from *Vigna sesquipedalis* (Sugiura and Sunobe, 1962).

The variations in the properties exhibited by phytases (see Section 1.4.3.2 and Section 1.4.3.3) may well be due to the presence of iso-enzymes (see Section 1.4.2). This conclusion is also based

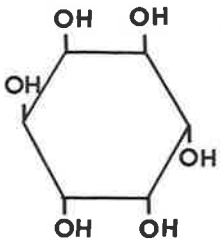
on circumstantial evidence (see Section 1.4.2). Therefore it seemed desirable to investigate in detail the properties of phytases with specific reference to the question of the presence of iso-enzymes. A comparison of the properties of the phytases with those of the enzymes previously reported in the literature as well as with the non-specific acid phosphatase also seemed essential.

1.4.3.4 Mechanism of enzyme action

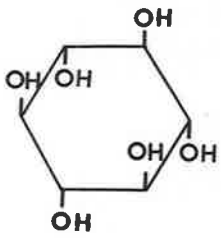
It has been shown that the phosphate either at the L 6 (4) or L 1 (3) position of the inositol hexaphosphate molecule is attacked initially (see Figure 6). Successive phosphate is then removed from positions adjacent to the free hydroxyl (Tomlinson and Ballou, 1962; Johnson and Tate, 1969*b*; Cosgrove, 1970) (Section 1.3).

Phytase seems to have a greater affinity for inositol hexaphosphate and for certain of its stereoisomers. It appears that the presence of an axial group influences the initial attack on the hexaphosphate molecule. In the case of *scyllo*-inositol hexaphosphate, the initial attack is very slow whereas the resulting pentaphosphate is more easily dephosphorylated. The hydrolytic reaction rate of phytase on the isomers of inositol hexaphosphate is in the order of *myo*- > *neo*- > D-*chiro*- > *scyllo*-inositol hexaphosphate (Cosgrove, 1966*b*, 1970). *Scyllo*-inositol hexaphosphate (Figure 8) shows a lack of an axial group which probably prevents the enzyme from attacking this molecule by the normal mode of operation used in *myo*-inositol

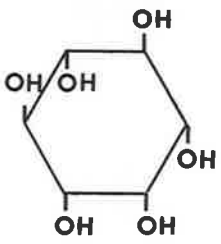
ISOMERS of INOSITOL



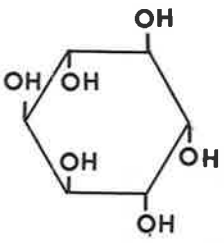
neo-



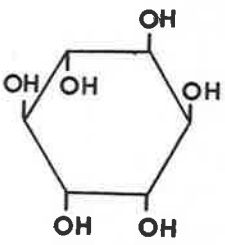
scyllo-



myo-



L chiro-



D chiro-

Figure 8.

hexaphosphate (Cosgrove, 1970).

Basing their results on high substrate inhibition, Gibbins and Norris (1963) indicated that phytase action may operate by a 2-point attachment of the substrate to the enzyme. This would lead to either pathway 1 taken by the bran phytase or pathway 2 of the micro-organism (Figure 6) (Section 1.3.2).

Recently, Irving and Cosgrove (1971) suggested a minimum of 3-point attachment of the enzyme to the substrate. They further proposed the active centre of the *Pseudomonas* phytase on the basis of the structures of *myo*-, D and L *chiro*-inositol pentaphosphates. To be bound in an active complex by the active centre of the enzyme, a substrate requires a nucleophilic group separated by about 2.83 Å units from the ester-bond oxygen atom. *Myo*-inositol hexaphosphate, possessing two pairs of vicinal trans-equatorial phosphate groups has the required stereo-chemistry. This proposed model, a concept similar to that suggested for the human prostatic phosphomonoesterase (London *et al.*, 1958), explains the pattern of biodegradation of inositol hexaphosphates (Irving and Cosgrove, 1971*b*).

1.4.4 Physiological role of phytase

The biological function of phytase is closely related to its substrate, phytate. Its major physiological role is probably to supply inorganic phosphate to the growing plants by its hydrolytic action on phytate (see Section 1.4). Because of the possibility that the *in vivo* concentration of phytate phosphorus and inositol may be

important in nucleotide synthesis and cellular metabolism of seeds it is possible that the activity of phytase may control and regulate DNA and RNA synthesis. However, such a function for the enzyme is still under study.

The hydrolysis of ATP releases 7.3 Kcalories of energy (Lehninger, 1970). Phytic acid contains six phosphate ester bonds, the total energy of which is considerable (Stumpf, 1952). In addition, the close proximity of the phosphate groups to one another could result in a greater ionic repulsion also giving a high phosphoryl transfer potential. For this reason it has been suggested that phytic acid could serve as a phosphagen capable of phosphate transfer to certain nucleotides (Hackett, 1959; Atkinson and Morton, 1959; Morton and Raison, 1963; Jennings and Morton, 1963*a*,*b*) according to this reaction:-

$$\text{Inositol hexaphosphate} + \text{ADP} \xrightleftharpoons{\text{phosphotransferase}} \text{phosphoinositol} + \text{ATP}$$

This idea of the generation of ATP by the phytate-phosphoinositol transferase system is attractive. The enzyme, phytase, could be involved in such a transphosphorylation reaction which could conserve some of the energy of the compound rather than dissipitate it by hydrolysis. Further, the phytate-phosphoinositol system could act as a buffer for the ATP-ADP system in the aleurone grains and maintain the concentration of ATP at an optimum for synthetic functions (Morton and Raison, 1963).

Support for the phosphoinositol transferase system came from the *in vitro* experiments of Biswas and Biswas (1965*a*,*b*) and recently

Mandal and Biswas (1970), who detected the enzyme in germinating mung beans (*Phaseolus aureus*). Although the crude enzyme extract was able to catalyse the transfer of P_i from phytate to all four ribonucleoside diphosphates, ADP, CDP, UDP, GDP, the partially purified enzyme (25-fold) was very specific for GDP. The transfer of phosphate occurred with the addition of magnesium and was twice as effective when manganese was used. Incubating ^{32}P GDP with inositol hexaphosphate (phytate) causes the transfer of ^{32}P to the latter indicating the reversible action of the enzyme and the reaction as:-

$$\text{Inositol hexaphosphate} + \text{GDP} \xrightleftharpoons[+\text{GTP}]{Mn^{++}\ \text{transferase}} \text{inositol pentaphosphate}$$

With high concentrations of GTP the transfer was inhibited (end-product inhibition) and the presence of ATP did not influence the reaction. Consequently, the metabolic significance of this reaction for phytase (and hence of phytase) is an open question.

Phytase may be involved in the transport of cations in the plants. Phytic acid is commonly isolated as the mixed K^+, Mg^{++}, Ca^{++} and Fe^{++} salt or phytin (Fowler, 1956; Posternak, 1965; Sobolev and Radionova, 1966; Johnson and Tate, 1969). A considerable amount of manganese is shown in the oat phytin (Ashton and Williams, 1958). Although little is known about the function of phytic acid as a reserve for metallic elements, these cations are liberated in the course of enzymic hydrolysis (Saio, 1964; Sharma, 1965; Anderson, 1965; Davies, 1968) and thus are placed at the disposal of the plants for their vital activities.

Purpose of the experimental work:

The biodegradation of phytic acid by phytase from wheat bran was studied by Tomlinson and Ballou (1962) and from *Neurospora crassa*, by Johnson and Tate (1969*b*). The latter workers did not use a purified enzyme. Although Tomlinson and Ballou (1962) used a 20-fold purified enzyme from wheat bran they could not account for the presence of all the isomeric inositol polyphosphates detected in the degradation pathway. By distinguishing between the isomeric *myo*-inositol pentaphosphates formed from *myo*-inositol hexaphosphate by the bran phytase and by the *N. crassa* enzyme, it became apparent that these phytases are different enzymes. The object of the investigation described in this thesis was to re-examine the degradation routes of phytic acid by phytases from these two sources. It was hoped to develop an explanation for the formation of different isomeric inositol polyphosphates in the pathway postulated by Tomlinson and Ballou (1962). The presence of these substances may be due to the non-specific removal of the phosphate group from *myo*-inositol hexaphosphate by the enzyme, or to the action of iso-enzymes in phytase. Investigations into the multiple nature of the enzyme were attempted.

Since phytase is involved in the biodegradation of phytic acid, initial studies were concerned with the isolation, purification and characterisation of the enzyme. Furthermore, conditions and factors which would modify the degradation pathway of phytic acid were examined.

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 *Neurospora crassa* and culture methods

2.1.1 Maintenance of stock cultures of *Neurospora crassa*

Routine stock cultures of *N. crassa* (STA 4, wild type) obtained from the Fungal Genetics Stock Centre (Dartmouth College, U.S.A.) were maintained on agar slopes of the appropriate medium.

2.1.2 Culture medium

Unless otherwise stated, the basal culture solution used was the phosphorus-free minimal N medium of Vogel (1956). The medium was prepared and stored at 2^{o} as concentrated stock solutions without the carbon source, sucrose. A few drops of chloroform were added to inhibit microbial growth.

In solid agar media (2% w/v Bacto-sugar), the carbon source was 1% w/v sucrose and 1% w/v glycerol, while the solution cultures contained 2% w/v sucrose.

Culture media and apparatus were sterilised by autoclaving at 120^{o} for 15-60 min depending on the volume of medium in each container. Since phytate was dephosphorylated during autoclaving in culture medium, small volumes (5 ml) of it were sterilised by passing through a millipore filter (25 mm Millipore filter, 0.22 µ; Millipore Corp., Mass., U.S.A).

2.1.3 Culture for the preparation of phytase from *N. crassa*

A thick suspension of conidia in sterile water was prepared from conidiated 3 to 4 day old cultures of wild-type *N. crassa* grown on agar slopes (the method follows essentially that of A.R. Grivell, 1970). The inoculum was added to 18 litre medium in a 20 litre culture bottle. This was incubated for 3 days with vigorous aeration with sterile air through sintered discs at 28^{o}-30^{o}.

Cultures were collected by filtering through a double layer of muslin cloth. After washing with 0.1% KCl the fungus was pressed between absorbent papers to remove surplus liquid. The mycelia were frozen at -15^{o}.

Although enzyme activity has been detected in the medium (Johnson and Tate, 1969b), preliminary studies indicated that the bulk of the enzyme activity was present in the cell-free extracts of the mycelia. Hence, the culture medium was not used as the source of the enzyme. The following results were obtained:-

Source	Specific activity (μmole P_i/mg protein/min)
Cell-free extract	0.075
Culture	0.014

one of the methods described below. The cell debris was sedimented by centrifuging at 7,500 *g* for 20 min. Small quantities of the fungus were usually disrupted in liquid nitrogen. Bulk extracts of *N. crassa* for the fractionation of phytase were prepared in a French pressure cell. Details of the purification procedures for the *N. crassa*

phytase are described in Section 3.1.4.

2.2.1.1 Disruption of cells in liquid nitrogen

The washed felts were frozen in liquid nitrogen (1 g/100 ml) and ground to a fine powder in a mortar and pestle. The powder was suspended in 2 volumes of 0.02 M sodium acetate buffer (pH 5) and the supernatant fraction left after centrifuging at 7,500 *g* for 20 min was the source of the crude phytase.

2.2.1.2 French pressure cell

The washed felts (1 g/2 vols 0.02 M sodium acetate buffer pH 5) were minced in a Waring blendor for 5 min at maximum speed. The homogenate was crushed in a French pressure cell (American Instrument Co., Maryland, U.S.A.) at an operational pressure of approximately 20,000 lb/in^2.

2.2.1.3 Ultrasonication

The minced cell paste suspended in 2 volumes of the acetate buffer as described above was placed in a double-walled glass cell. Ice water was circulated through the outside chamber of the cell to maintain the temperature at 2^{o}. The cells were subjected to ultrasonication vibration at 20 Kcycles/sec for 3 min with an M.S.E. Mullard titanium probe.

2.2.1.4 Glass homogenisation

The minced cells were repeatedly crushed in a glass homogeniser (Kontes Glass Co., Vineland, N.J., U.S.A.) by hand. This procedure was repeated six times and the supernatant fraction left after centrifuging at 7,500 *g* for 20 min was referred to as the crude extract.

2.2.2 Cell-free extracts from plants

2.2.2.1 Aqueous extracts

Aqueous extraction of phytase from wheat bran followed essentially the extraction procedure (Method 1, Purification Table) of Nagai and Funahashi (1962). The ratio of three gram wheat bran (obtained locally from Whiting and Chambers, Adelaide, S. Australia) to 10 ml of distilled water was adopted. The wheat bran which had been soaked for an overnight period was squeezed through a muslin cloth. The 7,500 *g* (20 min) supernatant fraction was the source of crude phytase and was used in the study described in Section 3.2.

2.2.2.2 Water-saturated n-butanol extraction

Normal butanol was saturated with cold double distilled water. The water-saturated n-butanol was stirred into the crude aqueous (1/1, v/v) phytase prepared as described earlier. The mixture was centrifuged at 7,500 *g* for 5 min. The butanol layer was siphoned off and the aqueous layer was further

extracted (9x) with water-saturated n-butanol. The final aqueous phase was used as the butanol extracted phytase in the phospholipid study (see Section 3.2.1) and the butanol layer for the isolation of phospholipid factors (see Section 3.2.1.1).

2.3 Techniques

The following techniques were used in the enzymic study and the preparation of organic compounds.

2.3.1 Electrophoresis

2.3.1.1 Starch gel

Samples of the enzyme material were examined on continuous starch gel electrophoresis of the type described by Graham (1963). The gel, containing 12% starch, was prepared in the appropriate buffer and 2 M urea. Samples (0.1 ml) containing 2-5 mg protein were contained in slots prepared in the gel before it set. Electrophoresis was at 3-5 volt/cm for 3 to 6 hr. The gel was then cut horizontally into 2 slices; one of which was stained in 0.015% w/v of nitrosine for 12 hr and the other half used for the phosphatase stain (the method of Brewer and Sing , 1970) or for the assay of phytase activity as follows:- the sliced gel was cut into (0.5 x 1cm) sections and each was incubated in a reaction mixture as described in Section 2.4.1. Phytase activity was then determined as described.

The buffers normally used were:-

a) 0.017 M aluminium lactate of ionic strength 0.1 and pH 3.1.

b) o.41 M histidine-citrate, pH 7.

2.3.1.2 High voltage

Routine high-voltage paper electrophoresis was used to separate the polyphosphates (Tate, 1968). Standards of ϕ, PP_i, ATP, P_i and Fr were spotted on to the origin of Whatman 3 MM paper alongside the samples to be analysed. The electrophoretograms were dipped in the appropriate buffer and the excess was removed by blotting with dry absorbent Whatman 3 MM paper. Electrophoresis was generally carried out from 1 to 1.5 hr at 1,500 volts.

The buffers used were:-

a) 0.1 M sodium oxalate (pH 1.5) - all inositol polyphosphates were separated.

b) 0.1 M sodium citrate (pH 5) - the tetra, tri, and di phosphates of inositol were more readily separated than the higher polyphosphates.

c) 1 M formic acid (pH 1.9) (for 2 hr) - to distinguish between the monophosphates of inositol.

2.3.1.3 Detection of phosphorus compounds

The nucleotide ATP was initially located under U.V. light and all phosphates by the phosphomolybdate dip method of Harrap (1960). The electrophoretogram was irradiated under U.V. light after being heated at 65^{o} for 15 min.

To avoid a blue background for electrophoretograms which had been run in citrate buffers these were initially dipped in 0.3% w/v ninhydrin in acetone and dried at 110° for 2-3 min. They were then treated with the phosphomolybdate reagent and phosphates became apparent during the heating stage, without exposure to U.V. light.

2.3.2 Column chromatography

2.3.2.1 Gel filtration

Sephadex G-100 beads were swelled in 0.02 M sodium acetate buffer (pH 5) for 24 hr, then packed into a column (1.6 x 52 cm). Appropriate enzyme fractions were loaded and eluted with the same acetate buffer.

Molecular weight determination of phytase was estimated by gel filtration (Sephadex G-100) using the method of Andrews (1965). Samples were loaded, washed into and eluted through the column with the same 0.02 M sodium acetate buffer (pH 5). The fractions were collected in the cold by an automatic collector.

Blue Dextran 2000 (10 mg/ml) and ^{3}H-water were mixed with the sample before loading onto the column to enable the calculation of void volume and the distribution coefficient, K_d value (Section 2.4.4; Flodin, 1962; Steward and Tate, 1969). The enzyme and four known proteins (listed in Figure 14) were individually eluted from the column. Collected fractions were assayed for phytase activity in the enzyme preparation; Blue Dextran by optical extinction measurement at 625 nm; standard proteins by the Folin method and by optical extinction

at 280 nm; and ^{3}H-water as in Section 2.4.7.

2.3.2.2 DEAE-cellulose

DEAE-cellulose (DE-11) was pretreated by precycling with 0.5 M HCl and 0.5 M NaOH as according to the manufacturer's (Whatman Column Chromedia, W. & R. Balston Ltd., England) recommendation. It was finally washed with double distilled water until the pH of the eluant was neutral. Vertical columns were poured at room temperature. Excess bovine serum albumin (20 mg in 2-3 ml) was passed through the column to cover the irreversible adsorption sites of newly prepared cellulose. Reversibly adsorbed protein was eluted with 1 M Tris-HCl (pH 7.3). The column was rewashed thoroughly with water before the appropriate fractions were loaded on. For further runs of the cellulose, it was washed only with 0.1 M NaOH and then with water; any cold solution was degassed using a negative pressure pump before pouring into column.

Linear concentration gradients of buffer were generally used. Effluent fractions were collected by an automatic fraction collector.

2.3.2.3 Dowex Cl^- resin

The *myo*-inositol polyphosphates obtained from a partial enzymic degradation of *myo*-inositol hexaphosphate were resolved by ion-exchange chromatography. A large scale separation of the polyphosphate esters (initial concentration of sodium phytate used, 20-25 g) was achieved on a Dowex 1 (Cl^- form, 200-400 mesh)

resin column (6.2 x 37 cm). Two sequential gradients of lithium chloride were used:- 0.1 M to 0.7 M (2 litres each) and 0.7 M to 1 M (1 litre each).

For a small scale preparation of the polyphosphate products (10-15 g of the starting substrate), a column size of (3.6 x 41.5 cm) was adequate. Alternatively, HCl could be used as the eluant. A gradient of 2 litre of water to the same volume of 2 N HCl was applied.

Fractions of 18 ml per tube were collected by an automatic fraction collector at room temperature. Aliquots (5 µl) were removed from each fraction with a wire loop and spotted on to a grid (10 x 10 cm) ruled on Whatman No. 3 filter paper. Phosphate compounds were detected on the grid by the phosphomolybdate test (Harrap, 1960) and the relevant fractions were examined by paper electrophoresis (Tate, 1968). Homogeneous fractions having the same electrophoretic mobility were pooled.

2.3.2.4 Silica gel H

The thin-layer chromatographic technique (see Section 2.3.4) was adapted to a column procedure using Merck silica H gel for the large scale preparation of pure lecithin and lysolecithin from commercial egg lecithin. Thus 20 g of silica H gel prepared in the solvent system chloroform/methanol/water v/v 65/30/5 (Wagner *et al.*, 1961) was poured into a column (2.6 x 13 cm). The same solvent mixture was used as the eluant. One gram of commercial egg lecithin was used, and eluted fractions (3 ml per tube) were

collected on an automatic fraction collector. Aliquots from each fraction were examined on Eastman chromatogram strips for lecithin and lysolecithin (see Section 2.3.4). Homogeneous fractions which showed the same R_f value as either standard lecithin and lysolecithin were collected separately. The total phosphorus content of the pooled fractions was determined (see Section 2.4.8).

2.3.3 Gas liquid chromatography

The Perkin-Elmer 801 Gas Chromatograph with dual-flame ionisation detector was used. The glass columns (1.84 m x 3.2 mm internal diameter) were packed with 10% ECNSS-M on Embacel (60-100 mesh) or on chromosorb W as the polar support. The carrier gas flow rate was 40 ml N_2/min and the temperatures of the detector and the injection port were 250° and 200°, respectively.

2.3.3.1 Acetates of sugar alcohol

Acetylation was carried out at room temperature using a mixture of acetic anhydride and pyridine (1/1, v/v) for an overnight period. Standards were similarly acetylated and were evaporated to dryness. The acetylated material dissolved in chloroform was analysed by gas liquid chromatography at 220° or at a programmed rate of 1.7°/min from 140° to 220°, an adaptation of the technique developed by Oades (1967).

2.3.3.2 Methyl esters of fatty acids

Methyl esters of fatty acids were prepared with diazomethane and analysed in a Perkin-Elmer 801 Gas Chromatograph using 10% ECNSS on chromosorb W as the polar support. Both polar and non-polar phase separations were temperature programmed at 5°/min from 100° to 200°.

2.3.4 Thin-layer chromatography with Merck silica gel H

Preparative thin-layer chromatograms were carried out on activated (110°/17 hr) silica gel H (0.5 x 10 cm or 10 x 10 cm) plates (0.25 mm thick). Also Eastman chromagram gel strips were used for rapid qualitative examination of lipid extracts. These were developed in an ascending manner using chloroform/methanol/water (v/v, 65/30/5). The plates were dried and examined for the following compounds:- unsaturated and saturated carbon chains using iodine vapour; phospholipids by the method of Vaskousky and Kostetsky (1968); choline residues with the choline spray of Wagner *et al.* (1961); free amino groups with 0.3% w/v ninhydrin in acetone (Toennies and Kolb, 1951).

2.3.5 Paper chromatography

Myo-inositol polyphosphates and sugar alcohols were separated and identified by paper chromatography using Whatman 2 and No. 1 cellulose papers. For reproducible results with inositol phosphates the papers were previously washed for 17 hr with 0.1 M oxalic acid and then with 1.5 M ammonium hydroxide. All samples were

run with authentic standards in the descending manner with one of the solvents listed in Table 4 and were developed at 30°.

For rapid analysis of the sugar alcohols the sample was run in an ascending manner using acetone/water (v/v, 4/1) at room temperature.

As with other methods, the polyphosphates were identified using the Harrap (1960) phosphomolybdate dip procedure. All sugars and inositols were detected by the silver nitrate method of Anet and Reynolds (1954). Chromatographic results were usually taken from an average of identical simultaneous runs; with papers that had been prewashed, dried and run in the same tank under the same conditions.

2.4 Biochemical and chemical determinations

2.4.1 Phytase determination

All enzymes were assayed by measuring the increase in inorganic phosphorus using the method of Watanabe and Olsen (1965). Unless otherwise stated, enzyme activities were determined under conditions such that less than 10% of the substrate was utilised, and hence the initial velocities of the reaction were determined. Suitable controls for non-enzymic activity using boiled enzyme extract were incorporated in each experiment.

The reaction mixture, in a total volume of 1 ml, contained 0.25 M sodium acetate buffer (pH 5) in the presence of 0.02 M sodium phytate (pH 5, unless otherwise stated), 0.1 to 0.5 ml of the enzyme solution and distilled water. The reaction mixture was incubated for 30 min

Table 4. Solvent systems for separating sugars and organic phosphates.

Solvent No	Solvent system	Substance separated	Reference
1	Isopropanol/ammonia/water v/v 60/10/30	sugar alcohol and inositol	Johnson and Tate (1969)
2	Isopropanol/ammonia/water v/v 80/10/10	inositol di- and mono-phosphates	Johnson and Tate (1969)
3	Isopropanol/ammonia/water v/v 70/10/20	inositol mono-phosphates	Pizer and Ballou (1959)
4	Pyridine/ethyl acetate/ water v/v 40/11/6	sugars and sugar alcohol	Jermyn and Isherwood (1949); Grado and Ballou (1961)
5	Acetone/water v/v 4/1	sugar and sugar alcohols	

at 37°. Enzyme activity was stopped by the addition of cold 10% w/v trichloroacetic acid. The precipitated proteins were removed from solutions by centrifuging at 2,000 *g* for 3 min on a M.S.E. centrifuge.

2.4.2 Protein determination

Protein determination was carried out using the Folin method as described by Lowry *et al.* (1951), with reference to a standard solution of bovine serum albumin.. More rapid determinations were made by measuring the optical extinction at 280 nm or at both 260 nm and 280 nm (Kalckar, 1947).

2.4.3 Michaelis constants (K_m)

K_m values were determined from the double reciprocal plots of reaction rates against substrate concentrations, as described by Lineweaver and Burk (1934). Experimental results were also subjected to a statistical assessment with the aid of a CDC 6400 computer at the University of Adelaide, S. Australia. The program written by M. Atkinson (of the same University) follows essentially that of Wilkinson (1961) (see Appendix).

2.4.4 Calculation of distribution coefficient (K_d value) on Sephadex G-100

The distribution coefficient (K_d) of each of the proteins and of the enzyme used in the molecular weight determination experiment (see Section 2.3.2.1) was calculated according to the

formula given below:-

$$K_d = \frac{V_e - V_o}{V_i}$$

where V_e = elution volume of the protein, V_o = void volume and V_i = volume of the stationary phase. ^{3}H-water and Blue Dextran 2000 were used to determine V_i and V_o respectively (see Section 2.3.2.1)

2.4.5 Calculation of the electrophoretic mobility (M_{PP_i}) and the chromatographic mobility (R_{PP_i})

The polyphosphates were characterised by their electrophoretic mobilities with reference to inorganic pyrophosphate (PP_i) and with fructose (Fr) as the non-migratory marker. The electrophoretic mobility (M_{PP_i}) of each substance (X) was calculated according to:-

$$M_{PP_i} = \frac{\text{Distance of X from fructose}}{\text{Distance of } PP_i \text{ from fructose}}$$

The chromatographic value, i.e. the mobility of the substance in a solvent system (see Table 4) with respect to PP_i is given as:-

$$R_{PP_i} = \frac{\text{Distance of substance from origin}}{\text{Distance of } PP_i \text{ from origin}}$$

2.4.6 Concentration of Tris-HCl buffer

The concentration of Tris-HCl buffer in effluents from DEAE-cellulose columns (1.8 x 18 cm) were determined by measuring

the refractive index of the solution at room temperature. This was correlated to a previously prepared calibration graph.

2.4.7 ^{3}H-water determination

For the determination of ^{3}H-water in fractions from Sephadex G-100, 0.1 ml aliquots were counted after adding to 5 ml of dioxane-based scintillating fluid containing (in g/litre of dioxane) 7 g PPO, 0.3 g dimethyl POPOP and 100 g naphthalene.

A Packard Model 3375 Tricarb Liquid Scintillation counter was used.

2.4.8 Phosphorus analysis

Inorganic phosphorus was determined by the method of Watanabe and Olsen (1965).

Total phosphorus was measured after first digesting the substance in perchloric acid (Usher, 1963) with subsequent colour development by the method of Bartlett (1959).

2.4.9 Determination of the % of enzymic degradation of phytic acid

The following methods were employed:-

2.4.9.1 Direct phosphorus analysis

The amount of inorganic phosphate released was expressed as a percentage of the total phosphorus content in the reaction mixture.

2.4.9.2 Electrophoretic analysis

An aliquot (20-25 µl) of the reaction mixture was spotted onto Whatman 3 MM paper and subjected to routine high voltage electrophoresis (Tate, 1968; see Section 2.3.1). The electrophoretogram was dried and developed using the phosphate reagent of Harrap (1960). It was irradiated under U.V. light after heating for 15 min at 65°. Strips (0.5 x 1 cm) were cut out and each was determined for total phosphorus (see Section 2.4.8). A blank reading was taken as a control. The amount of inorganic phosphorus liberated from the phytate phosphorus was calculated.

2.4.10 Structural analysis of *myo*-inositol polyphosphate esters

Homogeneous fractions of *myo*-inositol polyphosphates were used for the tests as set out below:-

2.4.10.1 Determination of the phosphorus to inositol ratio

L-inositol (200 µg/ml) and the same amount of the polyphosphate were hydrolysed in 6 M HCl at 110° for 4 days in teflon sealed tubes. The contents were evaporated to dryness at 100° under air. The dry residue was acetylated using a mixture of acetic anhydride and pyridine (v/v, 1/1). Acetylation was then carried out at 110° for 2 hr. The contents of the tube were again evaporated to dryness as described previously.

The acetylated residue dissolved in an aliquot of chloroform was examined by gas-liquid chromatography. The chromatogram was produced at a programmed rate of 1.7°/min.with a temperature gradient of 140° to 200°. Tracings of the area corresponding to the internal standard L-inositol and to the substance under examination were made, cut out and weighed. The ratio of the μmoles of phosphorus to μmoles of inositol (equivalent to the ratio of their weights) was calculated and listed as in Results (see Sections 3.1.6.1 and 3.2.4.1).

2.4.10.2 Optical activity and molecular rotation

Optical rotation measurements of the polyphosphates were made in a 4 decimeter tube using a Bellingham and Stanley Ltd. (London) polarimeter, with the D-line of sodium as a light source. The solution was first read at room temperature in an acid medium at pH 1 and finally made alkaline with NH_4OH to pH 11 and again examined. The molecular rotation of each inositol phosphate fraction was calculated according to the formula below:-

$$[M]_D = \frac{100\ \alpha \times n \times 3.1}{1 \times c} = 0.01\ \text{M.Wt.} \times [\alpha]_D$$

where $[M]_D$ = molecular rotation with the D line of sodium as light source, $[\alpha]_D$ = specific rotation at the sodium D line, α = optical activity of the substance, n = number of phosphate groups per mole, l = length of the polarimeter tube in decimetres, c = concentration of the substance in μg P_t/ l, and P_t = total phosphorus.

2.4.10.3 Alkaline hydrolysis

Phosphate monoesters were hydrolysed under basic conditions which did not involve a cyclic intermediate (Tomlinson and Ballou, 1962). Thus, partial alkaline hydrolysis of *myo*-inositol diphosphates will yield *myo*-inositol monophosphates which have their phosphate groups in the same positions as the original compound. In this procedure the diphosphate fractions were hydrolysed in concentrated NH_4OH in sealed tubes at 110° for more than 24 hr. The solution was then chromatographed on oxalate washed Whatman No. 1 paper in one of the solvent systems as given in Section 2.3.5.

2.4.10.4 Acid-catalysed phosphate migration across *cis*-hydroxyls

When inositol phosphates are heated in dilute acid, the phosphate groups migrate across *cis*-hydroxyls much more rapidly than across *trans*-hydroxyls (Pizer and Ballou, 1959; Angyal and Tate, 1961). Thus, *myo*-inositol 1,2 diphosphate would be expected under such conditions to form a mixture of *myo*-inositol 1,3 and 2,3 diphosphates in addition to the 1,2 diphosphate (see Figure 9). Using this procedure, samples of the di- and tri- phosphates were heated in 1 N HCl at 100° for 15 min. The solution was neutralised with 1 N NaOH and an aliquot was examined with authentic standards by paper chromatography and routine electrophoresis.

Figure 9.

Acid catalysed phosphate migration across *cis*-hydroxyls

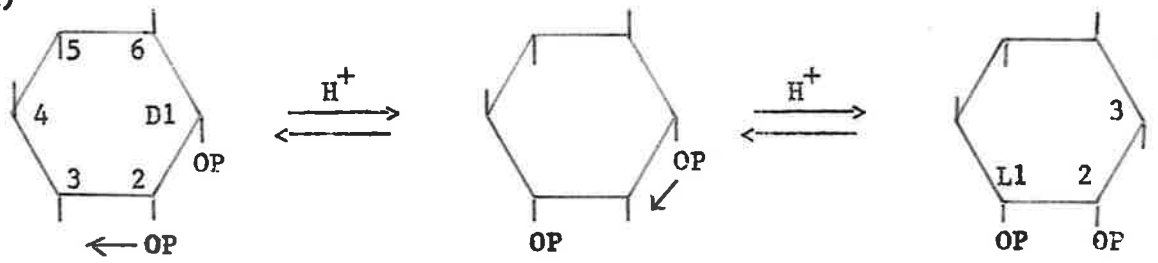
In Figure 9(a), under acid conditions the phosphate group at position 2 in the molecule *myo*-inositol 1,2-diphosphate migrates to the adjacent *cis*-hydroxyl at position 3. This migration converts *myo*-inositol-1,2-diphosphate into a mixute of itself and the 1,3 diphosphate. Further migration results in the enantiomer of the starting material, but migration across positions 4 and 6 is very slow because of the trans disposition of the vicinal hydroxyls to the phosphates.

Similarly D-*myo*-inositol 1,2,6-triphosphate is converted to itself and D-*myo*-inositol 1,3,6-triphosphate and L-*myo*-inositol 1,2,4 triphosphate under acid conditions which induce *cis* migration.

In Figure 9(b), brief acid treatment has no effect on D-*myo*-inositol 1,2,3-triphosphate because the adjacent free hydroxyls at positions 4 and 6 are *trans* to 3 and 1, respectively.

Acid catalysed phosphate migration across *cis*-hydroxyls

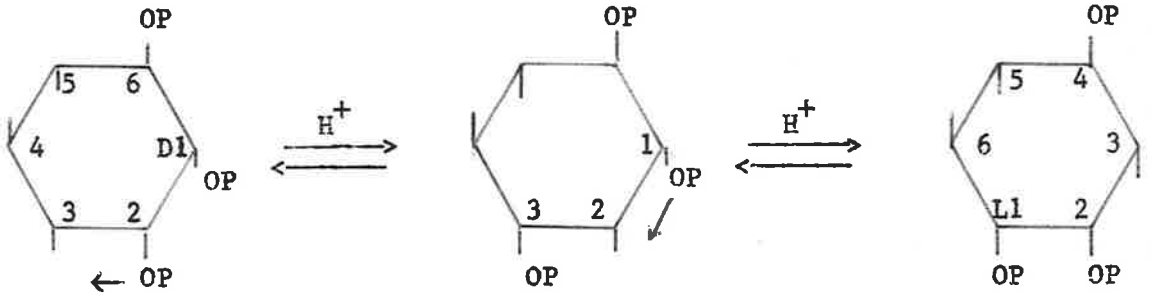
(a)



D-*myo*-inositol
1,2-diphosphate

myo-inositol
1,3-diphosphate

L-*myo*-inositol
1,2-diphosphate

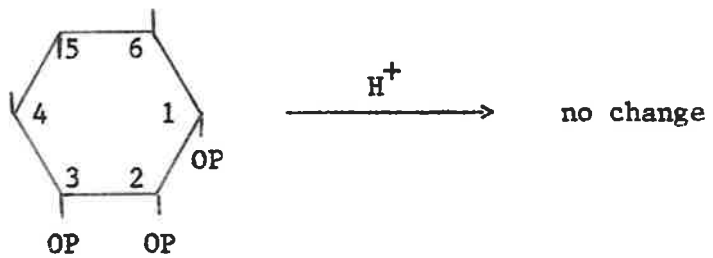


D-*myo*-inositol
1,2,6-triphosphate

D-*myo*-inositol
1,3,6-triphosphate

L-*myo*-inositol
1,2,4-triphosphate

(b)



***myo*-inositol**
1,2,3-triphosphate

2.4.10.5 Periodate-oxidation-borohydride-reduction and dephosphorylation reaction

During the periodate oxidation procedure, periodate cleaves the carbon-carbon bond between adjacent carbon atoms having free vicinal hydroxyl groups to a phosphorylated dialdehyde (Pizer and Ballou, 1959). The dialdehyde groups are reduced to primary alcohol by reduction with excess sodium borohydride. Dephosphorylation of the phosphorylated alcohol is achieved by hydrolysis at pH 5 to yield a sugar alcohol or polyol (Johnson and Tate, 1969*a*) (see Figure 10). The identification of the alcohol and a knowledge of the number of phosphate groups on the original *myo*-inositol polyphosphate molecule enables one to decide which of the possible isomers of the *myo*-inositol phosphate were present in the original material. Certain *myo*-inositol polyphosphate isomers will contain no free vicinal hydroxyl groups and will not be oxidised by periodate. In this case, only *myo*-inositol will be detected. However, under acid conditions phosphate groups will be induced to move across *cis*-hydroxyls (see Section before, 2.4.10.4) giving rise to an isomer. This, then, can be oxidised by periodate and the resulting polyol will indicate the original *myo*-inostiol polyphosphate. If again only *myo*-inositol is obtained, this gives evidence as to the original structure of the *myo*-inositol polyphosphate.

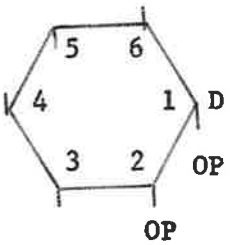
The periodate oxidation reaction was carried out with 0.1 M $NaIO_4$ in the dark at room temperature. The course of the reaction was followed by the consumption of the periodate spectrophotometrically at 260 nm according to the method of Dixon and Lipkin (1954) and by

Figure 10.

Periodate-oxidation-borohydride reduction and hydrolysis sequence for D-*myo*-inositol 1,2-diphosphate

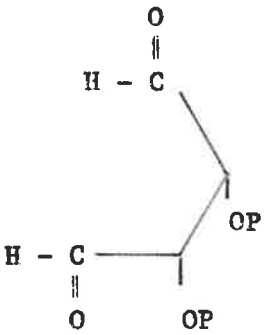
D-*myo*-inositol 1,2-diphosphate is oxidised in two stages to a phosphorylated dialdehyde. Reduction of the dialdehyde with borohydride and dephosphorylation at pH 5 yields erythritol as shown in this Figure.

Periodate-oxidation-borohydride reduction and hydrolysis sequence for D-*myo*-inositol 1,2-diphosphate



D-*myo*-inositol 1,2-diphosphate

$NaIO_4$



phosphorylated dialdehyde

$NaBH_4$

Erythritol

Hydrolysis (pH 5)

phosphorylated polyol

routine electrophoresis in oxalate buffer where the linear phosphates usually have faster mobilities. The reaction was stopped by adding excess (0.1-0.3 g) solid sodium borohydride. This borohydride reduction reaction was left standing overnight to allow the complete reduction of the phosphorylated dialdehyde and periodate. The solution was then adjusted to pH 5 with glacial acetic acid and hydrolysed in a sealed tube at 110°for 48 hr. The hydrolysed substance was passed through a Dowex 50 H^+ (AG 50W-x4-400 mesh) column (1 x 7 cm). The effluent was evaporated to dryness with methanol (3x volume) (to remove boric acid) in a rotatory evaporator; then the residue was taken up in a minimal volume of water (1-3 ml) It was deionised with a mixed bed of Amberlite resin (Amberlite Monobed Resin MB-1, analytical grade, B.D. Houses Ltd., England). The solutions containing the sugar alcohols were concentrated and examined by paper chromatography using solvent systems 1 and 2 (Table 4). A sample of the sugar alcohol was further acetylated (see Section 2.3.3.1) and the polyol acetate was examined, with standards, by gas-liquid chromatography (see Section 2.3.3).

2.5 Biological and chemical materials

2.5.1 Commercially obtained

The ascomycete, *Neurospora crassa* (wild type, STA 4) was obtained from the Fungal Genetics Stock Centre (Dartmouth College, U.S.A.).

A sample of *Pseudomonas* SB2 phytase was a gift from Dr. D.J.

Cosgrove (Division of Plant Industry, CSIRO, Canberra, Australia).

Wheat bran and mung beans were obtained locally from Whiting and Chambers (Adelaide, South Australia).

Olympic wheat (harvested in 1966) was obtained from Dr. P.B. Nicholls and barley malt (5 days old) from Dr. D.B.H. Sparrow, both of the Waite Institute, University of Adelaide, South Australia.

Sigma Chemical Corp., St. Louis (U.S.A.) supplied corn (*Zea mays*), sodium phytate grade V, the calcium salt of glyceryl phosphoryl choline, phosphatidic acid, snake venom (the source of phospholipase), NAD, NADP, glucose-1-phosphate, glucose-6-phosphate, fructose-1,6 diphosphate, α and β-glycerophosphate, α-naphthyl phosphate, pyrophosphate, phenyl phosphate, ATP, ADP, AMP.

Tris buffer and sodium acetate, hexokinase, cytochrome *c* from horse heart; n-butanol; palmitic, stearic and oleic acids; egg lecithin, protamine sulphate and nigrosine were obtained from British Drug Houses (Poole, England).

Synthetic 1-O-palmitoyl glyceryl phosphoryl choline was from Koch-Light Conbrook (Bucks, England).

Calbiochem. Inc. U.S.A. supplied bovine serum albumin and synthetic 1,2 di-O-palmitoyl glyceryl phosphoryl choline and G.T. Gurr Ltd. (London, England) the fast blue B salt No. 16077 stain used in starch gel electrophoretic studies (see Section 2.3.1.1).

The methyl esters of fatty acids used as chromatographic standards were obtained from Supelco, Inc. (Bellafonte, Pa., U.S.A.).

Solutes for liquid scintillation fluids were from Packard Instrument Co.

Sephadex G-100 and Blue Dextran 2000 were from Pharmacia Fine Chemicals (Uppsala, Sweden).

Merck silica gel H was purchased from Lab. Supplies, Adelaide, South Australia and starch gel from Connaught Medical Res. Lab., Toronto, Canada.

Bacto-agar used in the preparation of culture media for *N. crassa* was from Difco Laboratories (Michigan, U.S.A.).

Whatman chromatography products were obtained from H. Reeve Angel and Co. (London, England).

Eastman chromatogram sheets of silica gel H were purchased from Eastman Organic Chemicals (N.Y., U.S.A.).

All other chemicals were of analytical reagent grade and were obtained either from Drug Houses of Australia or from May and Baker, Dagenham, England.

2.5.2 Preparation of synthetic and related compounds of lyso-lecithin

References to the methods used to prepare compounds for the study in Section 3.2.1.2 were as follows:-

1,2-di-O-acetyl glyceryl phosphoryl choline - Kogl *et al.* (1960); 1-O-palmitoyl glyceryl phosphoryl choline (using snake venom) - prepared from synthetic lecithin by the method of Hanahan, Kodbell

and Turner (1954);1-O-alkenyl glyceryl phosphoryl choline (using ox-heart extract) - Panghorn (1945) and Hartree and Mann (1960).

The determination of fatty acid ester groups in the phospholipids was that of Goddu *et al.* (1955) and Rapoport and Alonzo (1955), using methyl stearate as the standard.

3. EXPERIMENTAL RESULTS

3. EXPERIMENTAL RESULTS

3.1 Phytase from *Neurospora crassa*

This section deals with the production of phytase in *Neurospora crassa* (STA 4, wild type) and with its purification and properties.

3.1.1 Production of phytase

The effects of cultural conditions on the production of phytase were studied.

Stock cultures were stored on anhydrous silica-gel (Grivell and Jackson, 1969) since these remained viable for several years. This simple technique does not require frequent sub-culturing of the strain.

In the preparation of phytase from *Neurospora crassa* the suspension of conidia from 3 to 5 day old cultures grown on agar slopes was normally used. Optimum growth for the fungus on the slopes occurred with phosphate concentrations in the range of 1.25×10^{-3} M and 2.5×10^{-3} M KH_2PO_4.

In liquid cultures similar to that illustrated in Plate 2, growth of the mycelial mat occurred under vigorous aeration at 28-30^{o} in the basal medium (see Section 2.1.2, Materials and Methods) containing 0.01% w/v phytate instead of inorganic phosphate. This amount of phytate resulted in the maximum production of phytase (see Table 5) after three days of growth (Figure 11) in the medium either aerated by mechanical shaking or by sparging with sterile water.

PLATE 2

GROWTH OF *NEUROSPORA CRASSA* IN PHYTATE MEDIUM

[Photography by Mr. R.J.F. Jenkins]

Table 5. Effect of phytate concentration in the culture medium on phytase production by *Neurospora crassa* STA 4

Concentration of phytate ($Na_{12}\phi.34H_2O$) (g/250 ml basal medium)	Total protein (mg)	Total activity (units)
0.01	400	40
0.02	600	100
0.05	629	148
0.10	640	120
0.50	612	103

Total volume of homogenate was 250 ml.

Neurospora crassa STA 4 was grown in culture medium containing various amounts of phytate listed in this Table. The fungus was grown for three days, after which it was harvested and the protein concentration and enzyme assay determined as described in Methods (Section 2.4.1; 2.4.2).

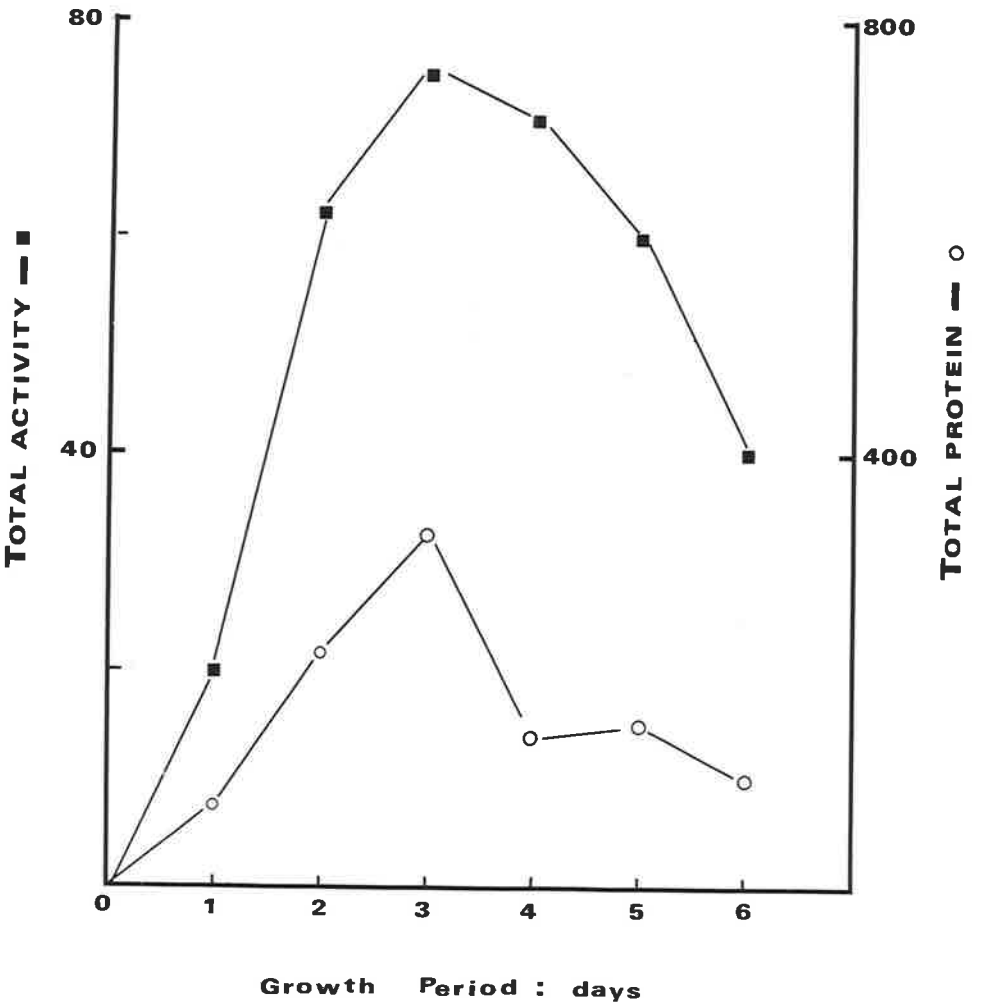
Figure 11.

Growth of *Neurospora crassa* in phytate medium for the production of phytase.

Cell homogenate prepared as described in Section 2.1.2 was made up to a total volume of 50 ml. Protein was determined as in Section 2.4.2 and phytase activity determined as described in Section 2.4.1. Protein and enzyme activity are for 50 ml homogenate as shown in Figure 11.

0 ——— 0 Total protein (mg/50 ml)

■ ——— ■ Enzyme activity (μmoles P_i/50 ml)


80
40
0
800
400
1
2
3
4
5
6
TOTAL ACTIVITY — ■
TOTAL PROTEIN — ○
Growth Period : days

3.1.2 Breakage of cells

Four methods of cell disruption were compared on the basis of the specific activity of the enzyme found in the 7,500 *g* supernatant fraction after centrifuging for 20 min (see Table 6). In addition the mycelia were examined with a light microscope for broken cells. Glass homogenisation proved to be the least satisfactory since microscopic examination of the homogenate showed that most of the cells were undamaged. Breakage in the French pressure cell (20,000 lb/in^2) gave a more efficient and better extraction of the enzyme than did glass homogenisation, ultrasonication (20 Kcycles/sec, 3 min) or extraction with liquid nitrogen in a mortar and pestle. The French pressure cell technique was adopted as the standard method for the preparation of cell extracts from *N. crassa*.

3.1.3 Distribution of enzyme

Table 7 shows the distribution of the phytase activity in cell-free extracts. Frozen mycelia (1 g in 2 ml of 0.02 M sodium acetate buffer, pH 5.0) were minced in a Waring blendor for 5 min. The preparation was then passed through a French pressure cell at 20,000 lb/in^2 at 3^o. The homogenate was centrifuged at various g values (Table 7). All the phytase activity originally present in the crude extract was found in the supernatant fraction. Whether the extracts were prepared by glass homogenisation, ultrasonication or liquid nitrogen, the same distribution of the enzyme was achieved as that with the French pressure cell. Thus the phytase is a soluble

Table 6. Comparison of 4 methods for extracting phytase from *Neurospora crassa*

Method	Enzyme activity	Specific activity
1. Glass homogeniser	0.014	0.017
2. Liquid nitrogen (mortar and pestle)	0.017	0.028
3. French pressure cell (20,000 lb/in^2)	0.035	0.075
4. Ultrasonication (3 min,20 Kcycles/sec)	0.021	0.051

Portions of *Neurospora crassa* (2 ml/gm wt) cells were disrupted by one of the methods listed in Table 6. After centrifuging at 7,500 *g* for 20 min all the cell-free extracts were diluted 2-fold with the sodium acetate buffer (pH 5) and assayed for phytase activity (Section 2,4.1). Protein determinations were made by the method of Lowry *et al.*, (1951).

Table 7. Distribution of phytase in cell-free extracts of *Neurospora*

Fraction	Total enzyme activity (units/ml)	Total protein (mg/ml)
1. Supernatant fraction left after centrifuging homogenate at 7,500 *g* for 15 min	0.2	1
2. Supernatant left after centrifuging fraction 1 at 20,000 *g* for 15 min	0.2	1
3. Supernatant fraction left after centrifuging fraction 2 at 100,000 *g* for 30 min	0.2	0.8

The enzyme activity in cell-free extracts was assayed in 1 ml. Details of the standard assay are as described in Section 2.4.1. Protein concentration was by the method of Lowry *et al.* (1951), Section 2.4.2.

intracellular enzyme rather than one tightly bound to cell-membranes. The method of differential centrifugation was not included in the purification procedure since the specific activity of the enzyme in the 100,000 *g* (30 min) supernatant fraction was similar to that of the crude extract.

3.1.4 Phytase purification procedures

The results of a 100-fold purification of the fungal phytase using the methods below are summarised in Table 8.

All enzymic operations were carried out in a cold room at 2° and centrifuging was done at 7,500 *g* for 20 min on a Servall SS3, unless otherwise stated.

(i) The washed mycelia suspended in 0.02 M sodium acetate buffer, pH 5.0, were minced as described earlier. The homogenate was then passed through a French pressure cell, then centrifuged to remove cell debris and the supernatant fraction was used as the crude extract (Fraction 1, Table 8).

(ii) Cold 2% (w/v) protamine sulphate (1 ml/10 ml enzyme fraction, v/v) was slowly added to Fraction 1 with stirring. After standing for 10 min, the small precipitate was removed by centrifuging and the resultant supernatant fraction which contained all the enzyme activity yielded Fraction 2 (Table 8).

(iii) Absolute ethanol at -15° was slowly added to the supernatant Fraction 2 (50% v/v), immersed below surface to avoid denaturation

Table 8. Partial purification of phytase

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Specific Activity	Purification
1. Crude extract: Supernatant fraction left after centrifuging homogenate at 7,500 *g* for 20 min	500	2250	117	0.05	1
2. Protamine sulphate treatment of 1. Supernatant fraction left after adding protamine sulphate	510	917	110	0.12	2.4
3. 50-70% v/v ethanol fraction of 2. Supernatant fraction after dialysis against cold distilled water	133	83	33	0.4	8.0
4. 50-100% $(NH_4)_2SO_4$ fraction of fraction 3. Supernatant fraction dialysed for 12 hr against water	100	10	25	2.5	50
5. Fraction 4 loaded onto Sephadex G-100 (5.6 x 30 cm) and enzyme eluted with 0.02 M sodium acetate buffer, pH 5.0. Pooled active enzyme fractions	25	5	25	5.0	100

Felts grown and harvested as in Section 2.2 were disrupted in a French pressure cell and fractionated as described in Results, Section 3.1.2.

The enzymic reaction mixture was as given in Section 2.4.1. Phytase activity expressed in International units was determined as given in Section 2.4.1. Protein was determined by the Folin method using bovine serum albumin as standard.

After 1 hr the precipitate was removed by centrifuging. Cold ethanol was again added to the supernatant fraction to a final 70% v/v saturation. The precipitate was centrifuged and dissolved in distilled water. The solution was dialysed against cold distilled water for 24 hr and centrifuged again. The supernatant fraction containing the enzyme (Fraction 3, Table 8) was used for the ammonium sulphate fractionation as described below.

(iv) Solid ammonium sulphate was added with stirring to Fraction 3 (0-50% saturation). The mixed fraction was allowed to stand for 2 hr. The supernatant fraction from this saturation (50%) was again made to 100% saturation with solid ammonium sulphate. (The pH of the solution remained at about 5). The second supernatant fraction contained the enzyme activity. It was dialysed against 2 changes of double distilled water to give Fraction 4 of Table 8.

(v) Fraction 4, concentrated by freeze-drying to 30 ml, was dialysed against 0.02 M sodium acetate buffer (pH 5) for 3 hr. About 15 ml of the enzyme was loaded each time onto a Sephadex G-100 column (5.6 x 30 cm) which had been previously washed and equilibrated as described in Section 2.3.2.1. The column was eluted with the same buffer. Fractions containing the active enzyme obtained as in Figure 12 were combined to give Fraction 5 (Table 8). Fraction 5 of such a preparation as presented in Table 8 had only a 22% recovery of the initial activity but a purification of 100-fold. A freeze-dried sample of the enzyme (Fraction 5) remained active for at least 6 months. Any further attempts at purification, such as alumina C α gel

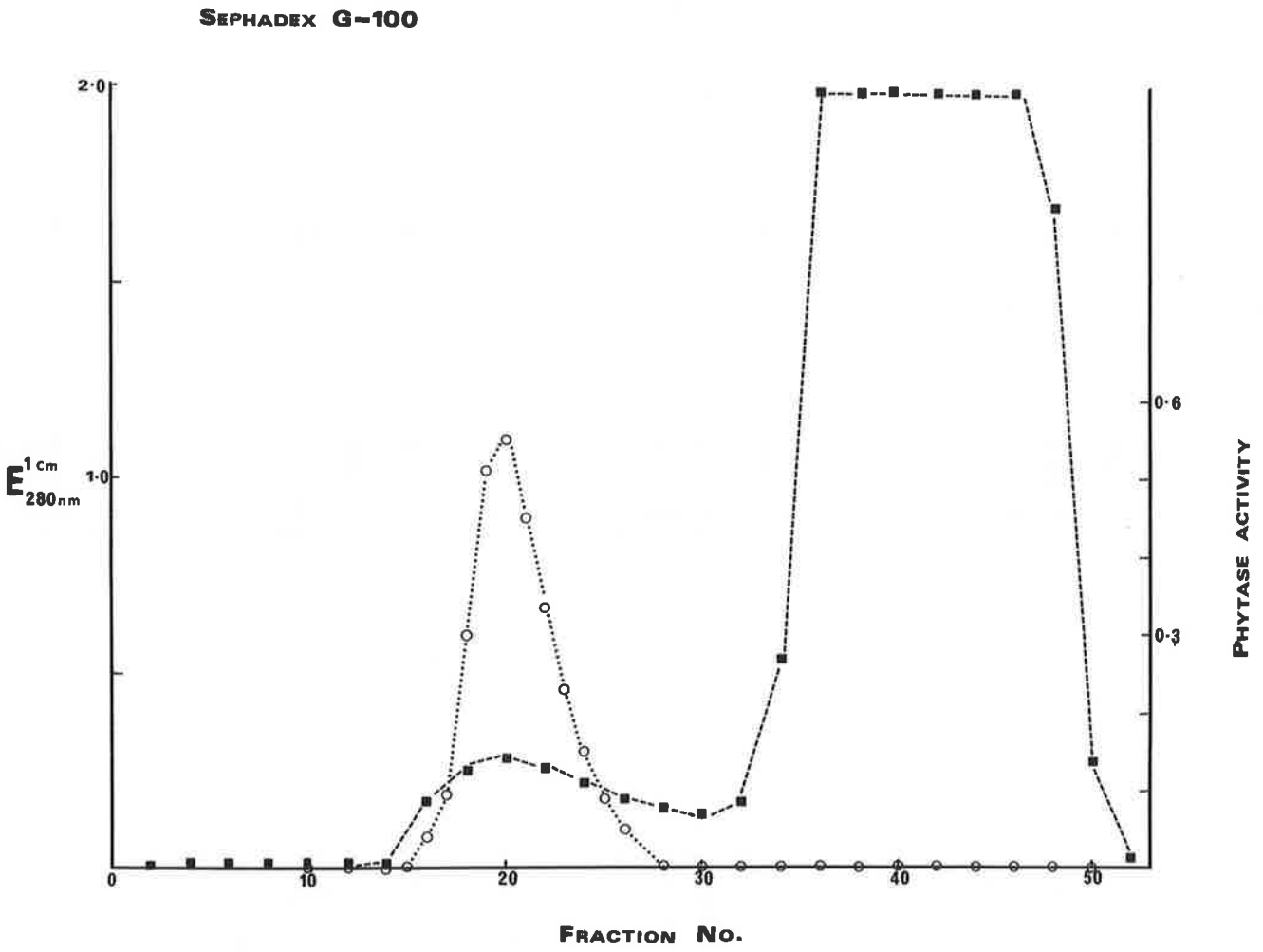
Figure 12.

Elution pattern for phytase from Sephadex G-100

Phytase fraction 4, Table 8 was loaded onto a Sephadex G-100 column (1.6 x 52 cm) which had been previously equilibrated with 0.02M sodium acetate buffer (pH 5.0). The enzyme eluted with the same buffer was used for phytase activity determinations (as described in Section 2.4.1). Empirical determinations of proteins were measured at 280 nm.

■ ——— ■ Protein reading at $E^{1cm}_{280\ nm}$

o ——— o Phytase activity.


SEPHADEX G-100
2·0
1·0
$E^{1\,cm}_{280\,nm}$
0·6
0·3
PHYTASE ACTIVITY
0
10
20
30
40
50
FRACTION NO.

absorption and ion-exchange chromatography, resulted in poor recovery of the enzyme.

3.1.5 Properties of phytase

Some properties of the partially purified phytase from *N. crassa* obtained as in Section 3.1.4 are described in this Section.

3.1.5.1 Starch-gel electrophoresis

Figure 13 compares the starch gel electrophoretic patterns of the crude enzyme (lane 1, Figure 13) with the partially purified (100-fold) enzyme in lane 2. Mobilities of phosphatase (α-naphthyl phosphate-substrate detection; see Section 2.3.1.1) bands relative to horse heart cytochrome c ($M_{cyto\ c}$) were:- crude enzyme lane 1, $M_{cyto\ c}$ 0.16 and 0.31; purified enzyme lane 2, $M_{cyto\ c}$ 0.27. The major band with $M_{cyto\ c}$ 0.31 in lane 1 and the single band $M_{cyto\ c}$ 0.27 (Figure 13) contained phytase activity. The bulk of the protein as stained by nigrosine (Section 2.3.1.1) was not associated with the enzymes. The crude enzyme gave protein bands at $M_{cyto\ c}$ 0.5 and 0.75; the purified enzyme showed a single band of $M_{cyto\ c}$ 0.63. The slight retardation of phosphatase and protein bands in the purified enzyme may be due to removal of impurities from the crude enzyme. Recovery of the phytase from the gel by a number of procedures was poor and thus starch gel was not used as a preparative method.

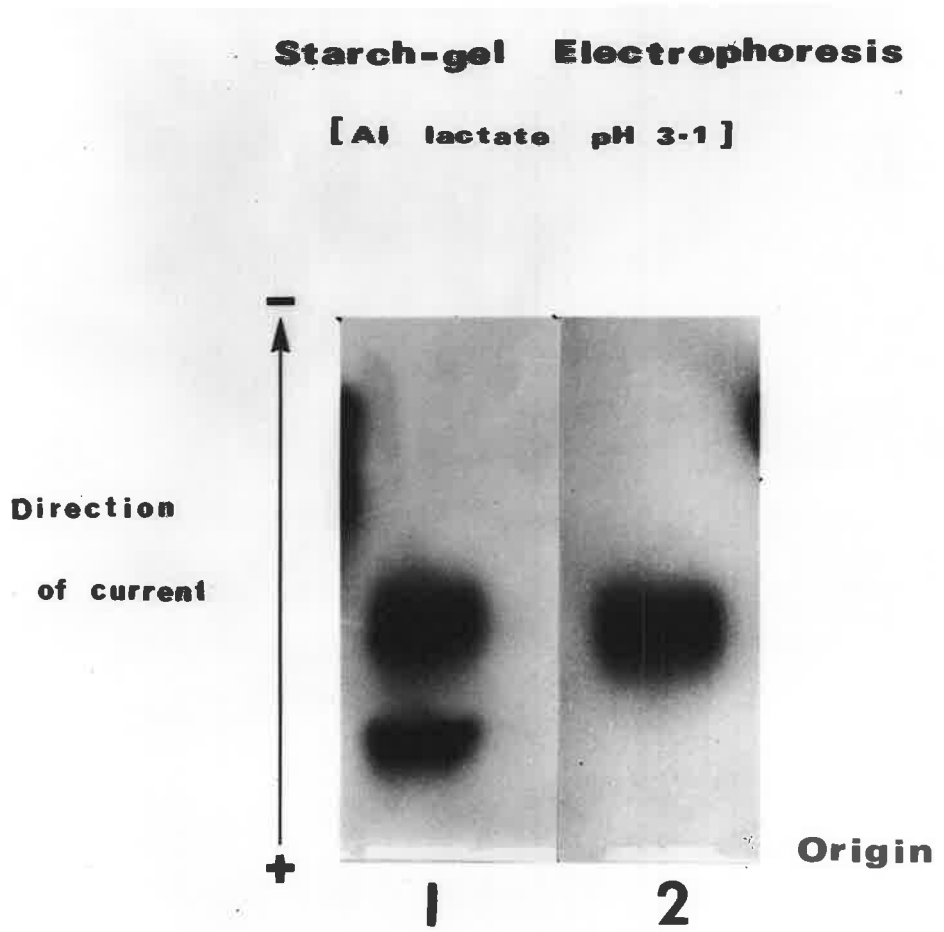
Figure 13.

Starch gel electrophoresis

The gel containing 12% starch w/v was prepared in 0.017 M aluminium lactate buffer (μ = 0.1, pH 3.1) and 2 M urea. Enzyme samples (0.1 ml) were contained in each slot. Electrophoresis was conducted around 5 volts/cm for 6 hr. Phosphatase activity was detected on a cut half of the gel with α-naphthyl phosphate and fast blue (as given in Materials and Methods).

Lane 1 = crude *N. crassa* enzyme (Fraction 1, Table 8)

Lane 2 = partially purified *Neurospora crassa* enzyme (Fraction 5, Table 8).


Starch-gel Electrophoresis
[Al lactate pH 3-1]
Direction
of current
−
+
Origin
1
2

3.1.5.2 Molecular weight determination

The apparent molecular weights of phytases from *Neurospora crassa*, *Pseudomonas* SB2 (see Section 2.5.1) and wheat bran were determined by gel-filtration using Sephadex G-100 following the method of Andrews (1965). The distribution coefficient (K_d) of each enzyme was calculated according to the formula given in Section 2.4.4 and that of the fungal phytase was found to be 0.27. This gave a molecular weight of 45,000 ± 2,000 and this result was compared to the bacterial and cereal phytases which showed a similar K_d value of 0.25 (Figure 14).

3.1.5.3 Effect of enzyme concentration and time of incubation

The effect of enzyme concentration on the production of phosphate is shown in Figure 15a.

The effect of time of incubation on the production of phosphate by *N. crassa* phytase is shown in Figure 15b. The enzymic dephosphorylation of phytate was linear up to 45 min with only a slight decline over the 45-60 min period.

3.1.5.4 Stability of the enzyme

The enzyme from *Neurospora* was more stable at -15^{o} than at 0^{o}. The partially purified Fraction 5 (Table 8) was less stable when diluted. One such preparation retained 40% of the initial activity after 6 months while the concentrated fraction, 70%

Figure 14.

Determination of approximate molecular weights of phytases from *Neurospora crassa*, *Pseudomonas* SB2, and wheat bran by gel filtration

Sephadex G-100 was calibrated as described in Section 2.3.2.1. Standard proteins of known molecular weights as well as blue Dextran and tritiated water were used. The distribution coefficient (K_d value) calculated as in Section 2.4.4 for the *Neurospora* enzyme was 0.27 which corresponded to a molecular weight of around 45,000. The K_d obtained for bran phytase Fractions F_1 and F_2 and the phytase from *Pseudomonas* SB2 was 0.25; giving a molecular weight of around 47,000.

The marker proteins used were:-

Proteins	Molecular weight	K_d	Symbol used
Bovine serum albumin	66,500	0.18	A
Hexokinase	45,000	0.27	B
Chymotrypsin	24,000	0.45	C
Cytochrome c (horse heart)	13,000	0.56	D
Neurospora crassa phytase		0.27	N □
Pseudomonas SB2 phytase		0.25	SB2 ○
Wheat bran phytase (F_1 and F_2)		0.25	W ■

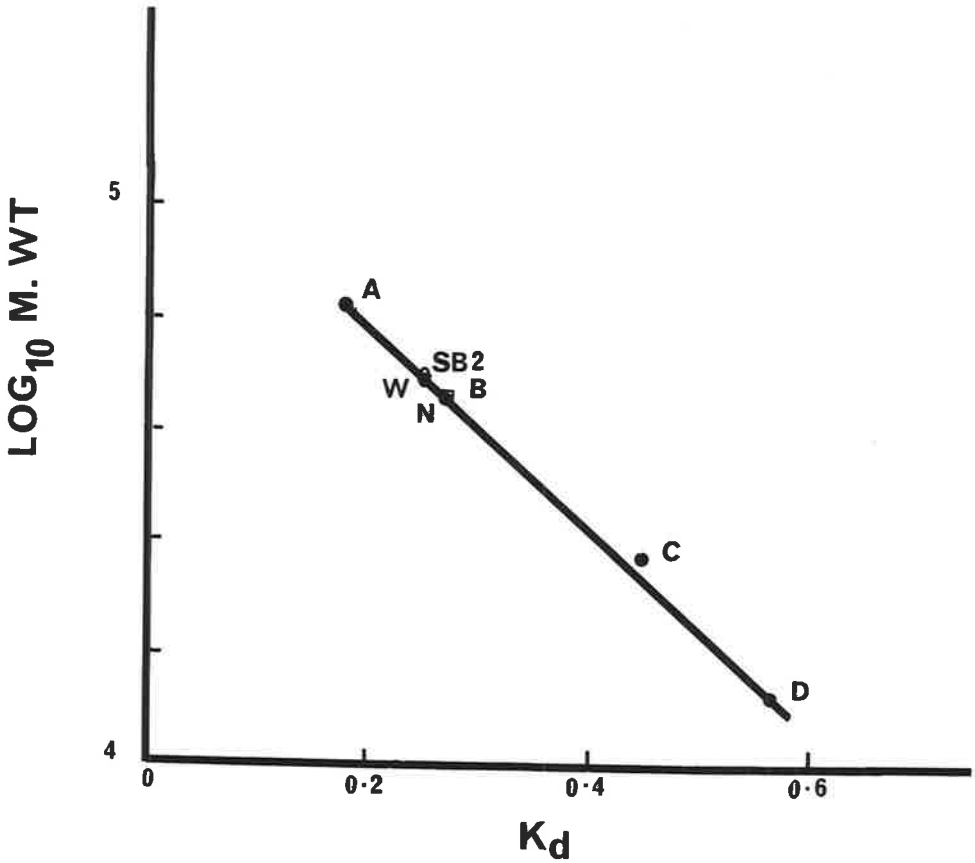

LOG10 M. WT
5
4
A
SB2
W
B
N
C
D
0
0·2
0·4
0·6
Kd

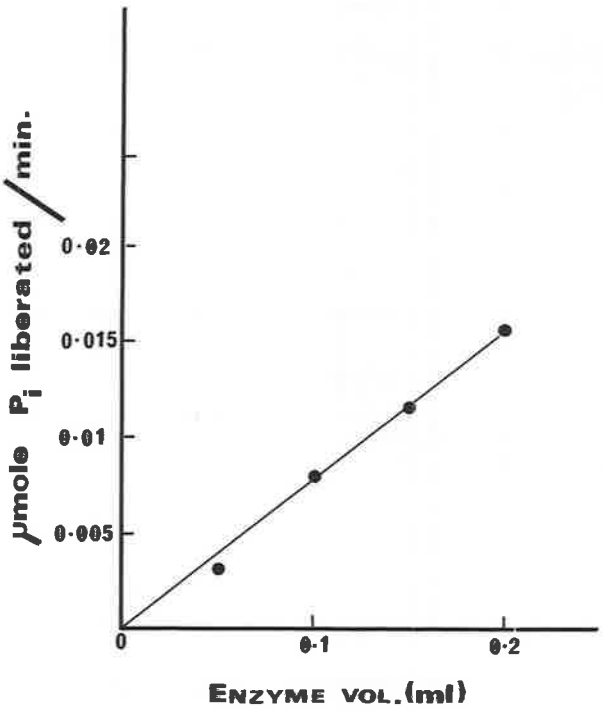
Figure 15.

(a) The effect of enzyme concentration on the production of P_i

Standard enzymic reactions as described in Section 2.4.1. were carried out but with various concentrations of enzyme (as given in Figure) at 37° and with phytate as the substrate. The liberation of inorganic phosphate was detected by the method previously given in Section 2.4.1.

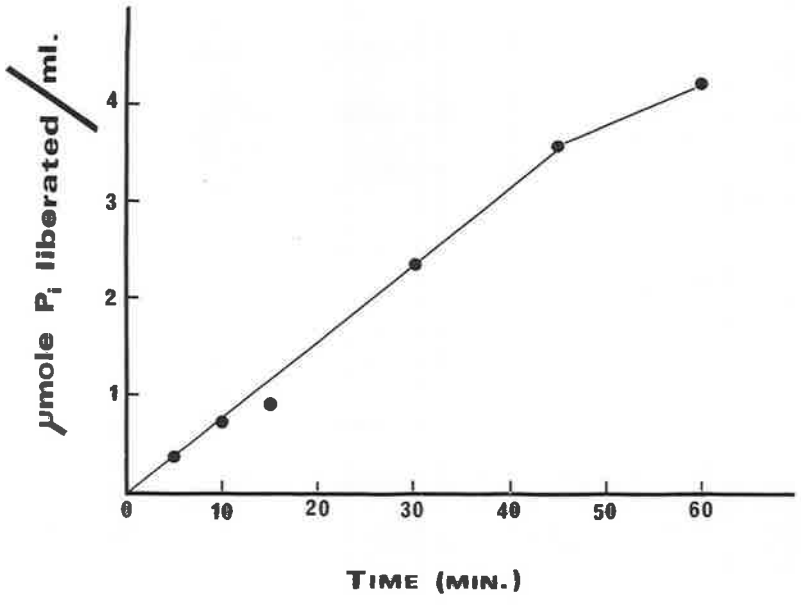
(b) The effect of incubation times on P_i production by *Neurospora* phytase

Fraction 5, Table 8 was incubated for time varying from 0 to 60 min with sodium phytate as the substrate at 37°. P_i released was detected by the ascorbic acid method as described in Methods (Section 2.4.1).


µmole P_i liberated /min.
0·02
0·015
0·01
0·005
0
0·1
0·2
ENZYME VOL.(ml)

Ia

b


µmole P_i liberated /ml.
4
3
2
1
0
10
20
30
40
50
60
TIME (MIN.)

over the same period.

3.1.5.5 Effect of temperature

The effect of temperature on phytase activity is shown in Figure 16a. The optimum temperature of the enzyme was 60°. All enzyme was inactivated within 20 min at 80°. The same loss of activity was recorded with two different substrates (other than phytate) when aliquots of the heated enzyme were assayed for residual phosphomonoesterase activity (Figure 16b).

3.1.5.6 Effect of pH

Figure 19a shows that optimal phosphatase activity for β-glycerophosphate was near pH 5.4 whereas Figure 17b shows that the optimal phytase activity was at pH 5.0.

3.1.5.7 Effect of metallic cations and various reagents

The effects of several monovalent and divalent cations on enzyme activity are shown in Table 9. Addition of copper, zinc or silver resulted in maximal inhibition, which may be due to the precipitation of phytate.

The chelating agents listed in Table 10 did not affect the *N. crassa* enzyme. Potassium cyanide and citrate at 2.5×10^{-3} M and 5×10^{-3} M respectively appeared to inhibit phytase activity, but the inhibition was not proportional to the concentration of the inhibitor used.

Figure 16.

Optimum temperature for phytase activity

(a) Phytase from *N. crassa* (Fraction 5, Table 8) was assayed in varying incubation temperatures as shown in Figure 16a. The enzyme activity was determined as in Section 2.4.1 (as given in Methods).

(b) Relative rates of hydrolysis of phosphoesters during the course of heat-inactivation of the *N. crassa* phytase

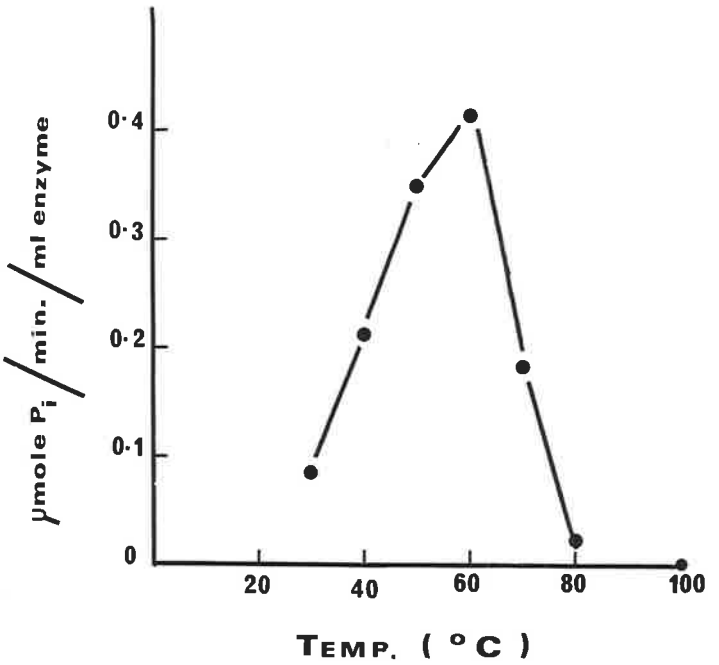
Samples of the purified enzyme in 0.05 M acetate buffer (pH 5) were heated for 10 min at the indicated temperatures shown in this Figure 16b and then cooled. Aliquots of the enzyme were removed and assayed for the liberation of inorganic phosphate from each of the substrates by the standard assay procedure given in Section 2.4.1. Each substrate concentration was 0.02 M.

● ——— ● phytate

▲ ——— ▲ β-glycerophosphate

○ ——— ○ glucose-6-phosphate

a


μmole P_i / min. / ml enzyme
0·4
0·3
0·2
0·1
0
20
40
60
80
100
TEMP. (°C)

b

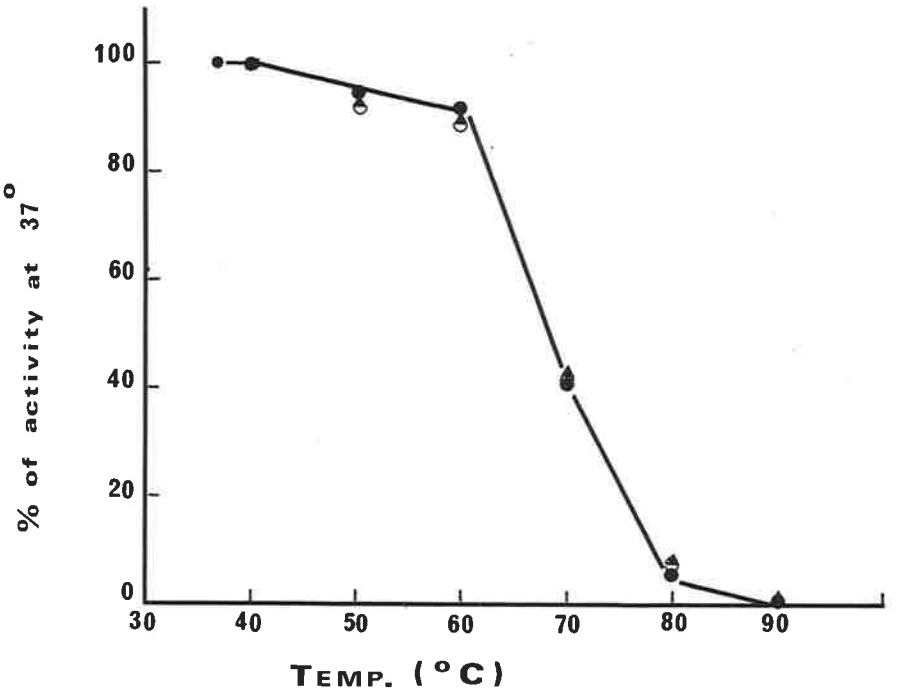

% of activity at 37°
100
80
60
40
20
0
30
40
50
60
70
80
90
TEMP. (°C)

Figure 17.

Optimum pH for *Neurospora crassa* phytase

Phytase was extracted from *Neurospora crassa* as described in Section 2.1.2.2. The enzyme fraction was each dialysed against 0.05 M sodium acetate buffer of pH values 4 to 6; Tris-maleate buffer pH 7 to 9 was used in the higher range. The P_i release was determined at various pH values as specified in Figure. The reaction mixture is given in Section 2.4.1. The substrate used was β-glycerophosphate in Figure 17(a) and phytate in Figure 17(b).

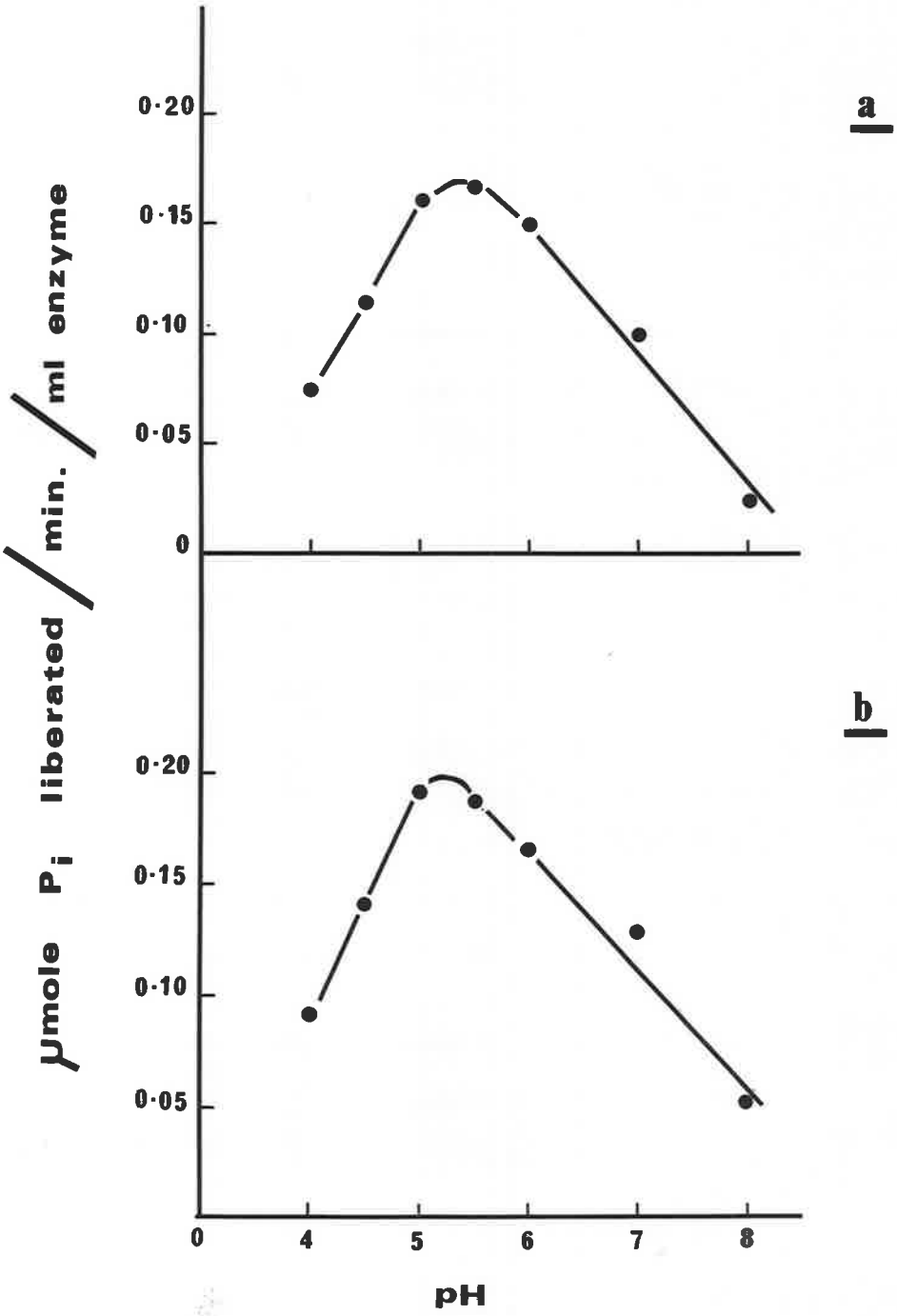

a
b
0·20
0·15
0·10
0·05
0
0·20
0·15
0·10
0·05
0
4
5
6
7
8
pH
μmole Pi liberated / min. / ml enzyme

Table 9. Effect of metallic ions on phytase from *Neurospora crassa*

Cation (Final concentration 2.5×10^{-2}M)	Relative rate of hydrolysis of phytase %
No addition	100
NaN_3	96
NaCl	100
$NaNO_3$	96
KCl	96
KNO_3	96
$CaCl_2$	105
$MgCl_2$	100
$MgSO_4$	100
$MnCl_2$	100
$CdCl_2$	100
$HgCl_2$	64
$ZnCl_2$	47
$CuSO_4$	40
$AgNO_3$	50
$FeCl_3$	80
$AlCl_3$	95
$NiSO_4$	95

The effect of various cations (listed in Table 9) on enzyme activity was determined with assayed procedures as described in Methods (Section 2.4.1). The cation was preincubated with the enzyme for 5 min before starting the reaction. The activity of the phytase without the cation was 1.00 International Unit which is equivalent to 100%.

Table 10. Effect of inhibitors and various compounds on phytase

Compound	Final concentration (M)	% Inhibition
Without addition	-	0
NaF	5×10^{-3}	51
KH_2PO_4	4×10^{-3}	68
(+) tartrate	1.8×10^{-3}	49
	3.6×10^{-3}	100
(-) tartrate	3.6×10^{-3}	0
	5.0×10^{-2}	0
meso tartrate	3.6×10^{-3}	0
	5.0×10^{-2}	0
sodium molybdate	2×10^{-3}	80
EDTA	2.5×10^{-3}	0
KCN	2.5×10^{-3}	4
oxalate	2.5×10^{-3}	2
citrate	5.0×10^{-3}	8
glutathione	2.5×10^{-3}	0
PCMB	2.5×10^{-3}	0
PCMB + glutathione	2.5×10^{-3}	0
iodoacetate	1.0×10^{-3}	5
iodobenzoate	1.0×10^{-3}	2

The effect of various inhibitors on enzyme activity was assayed using the standard techniques as described in Methods (Section 2.4.1). The compound was incubated for 5 min before starting the reaction. Enzyme activity was expressed as % inhibition of the activity of phytase without the addition of inhibitors. The activity of the phytase without the inhibitor was 0.7 International Unit.

The presence of sulphydryl reagents and alkylating compounds (Table 10) also had no effect on the enzyme.

3.1.5.8 Effect of inhibitors

The enzyme was inhibited by the common inhibitors of acid phosphatases, e.g. molybdate, (+) tartrate,, fluoride and ortho-phosphate. Thus the enzyme was extremely sensitive to molybdate, which at 2×10^{-3} M inhibited 80% of the initial activity of the enzyme (Table 10). Phosphate, the end product of phytate hydrolysis when added as KH_2PO_4, was inhibitory (68%) at 4×10^{-3} M; sodium fluoride at 5×10^{-3} M reduced the activity by half. While only (+) tartrate was inhibitory to the phytase activity, the (-) and meso forms had no effect, even at 5×10^{-2}M.

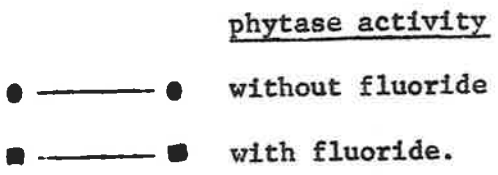
Further study of the effects of fluoride and (+) tartrate on *N. crassa* phytase are as shown in Figures 18 and 19. In each experiment the concentration of the substrate was varied in the presence or absence of 5×10^{-4} M fluoride and 1×10^{-3} M (+) tartrate. The data are presented by means of double reciprocal plots (lineweaver and Burk, 1934). In Figure 18 extrapolation of the linear portion of the graph which occurs at low substrate concentration gives the approximate K_i value with fluoride at 1.6×10^{-4} M. A non-competitive type of inhibition was observed. The plot with (+) tartrate gave similar results (Figure 19).

Figure 18.

Double reciprocal plots of the effect of fluoride on the activity of phytase from *Neurospora crassa*

Phytase activity with and without fluoride as an inhibitor was determined at various concentrations of phytate as shown in this figure.

The velocity of reaction with the inhibitor (5×10^{-4}M) against substrate concentration was plotted as the double reciprocal plot (Lineweaver and Burk, 1934).



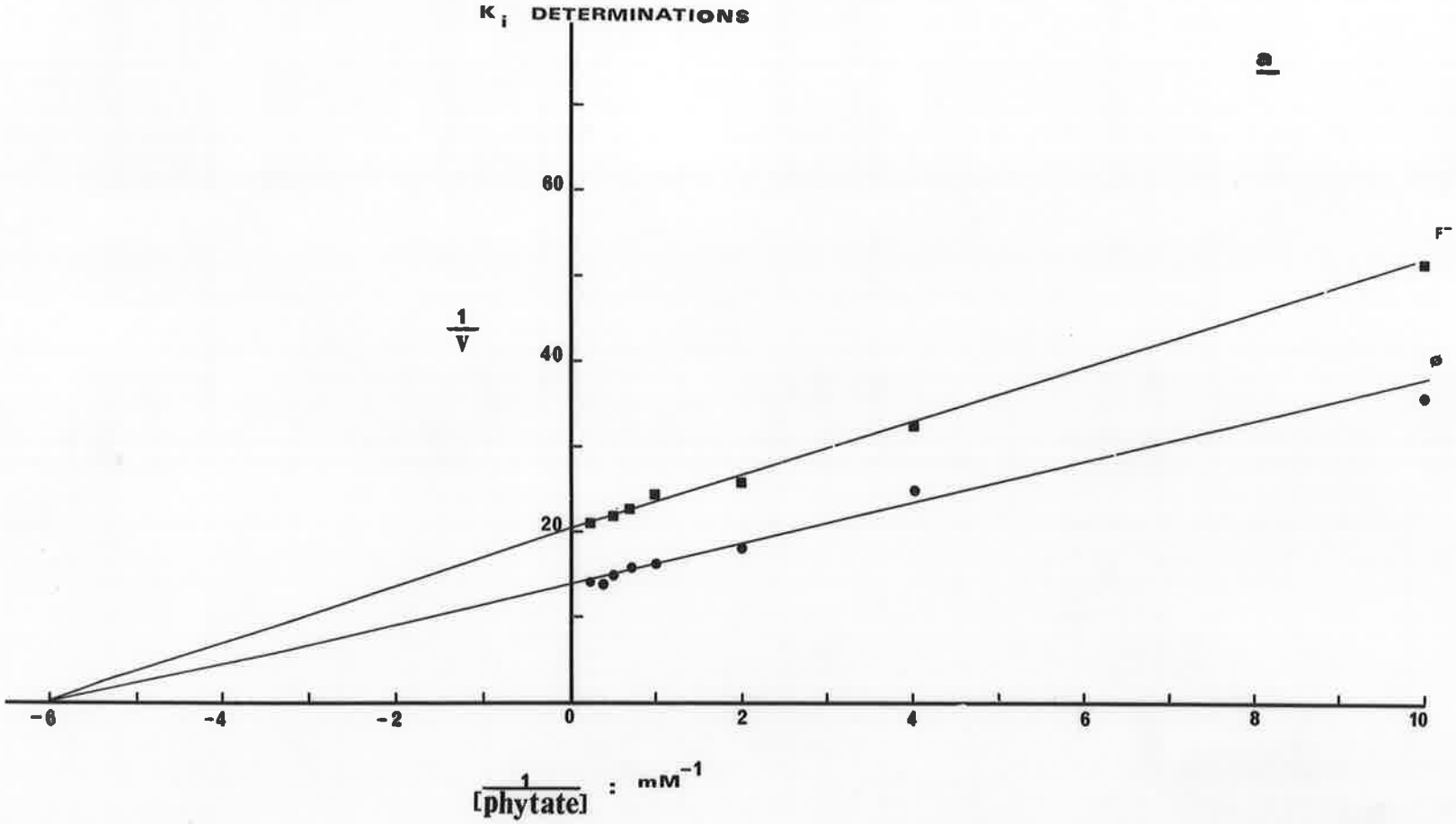

K_i DETERMINATIONS
a)
60
40
20
$\frac{1}{V}$
F⁻
-6
-4
-2
0
2
4
6
8
10
$\frac{1}{[\text{phytate}]}$: mM^{-1}

Figure 19.

Effect of (+) tartrate and phytate on the activity of phytase from *Neurospora crassa*

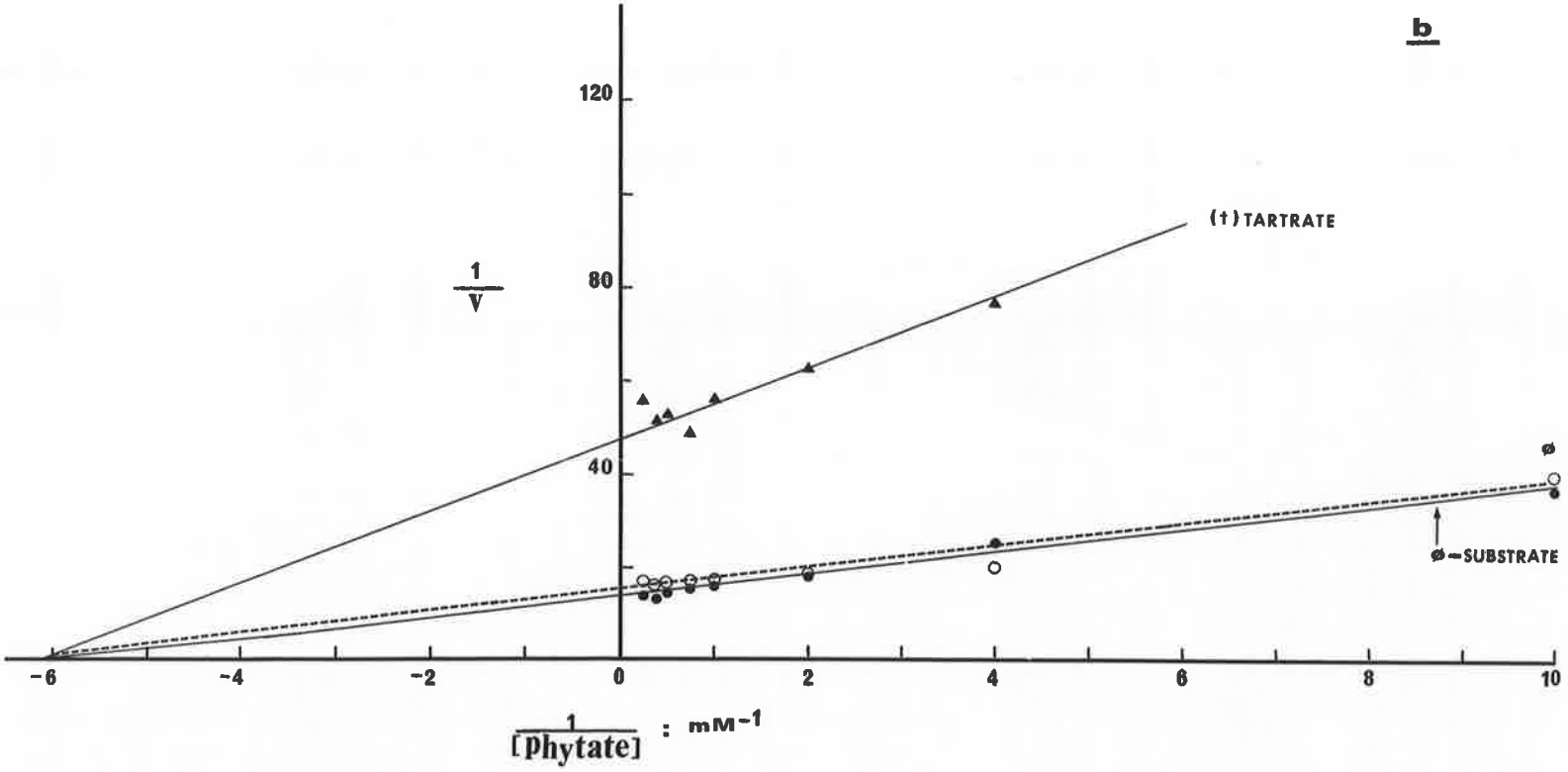
Phytase activity was determined as in Section 2.4.1 with and without the addition of (+) tartrate and excess phytate.

The velocity of reaction with (+) tartrate (1×10^{-3}M) and phytate (1×10^{-4}M) against substrate concentration was plotted as the double reciprocal plots (Lineweaver and Burk, 1934) as shown in this Figure.

● ——— ● phytase activity (as control)

o ------- o phytase activity with excess phytate

▲ ——— ▲ phytase activity with added (+) tartrate.


b
120
80
40
1/V
(†) TARTRATE
ø
ø – SUBSTRATE
-6
-4
-2
0
2
4
6
8
10
1/[Phytate] : mM⁻¹

3.1.5.9 Michaelis constants

The apparent K_m of the enzyme for its substrate, phytate, was calculated from the double reciprocal plot (Lineweaver and Burk, 1934) shown in Figure 18 and again in 19, when the reciprocal of the velocity of the dephosphorylation of phytate is plotted against the reciprocal of phytate concentration. It was estimated at 1.6×10^{-4} M.

When β-glycerophosphate was used as the substrate, the apparent K_m value was 4.4×10^{-3} M (Figure 20). At high concentrations of phytate and glycerophosphate the rate of reaction fell off sharply. This indicates substrate inhibition (Dixon and Webb, 1964).

3.1.5.10 Substrate specificity

The simple phosphomonoesters as listed in Table 11 were tested as substrates for the phytase under the conditions of the standard assay at pH 5. The specificity of the enzyme for the phosphate bonds in glucose-1-phosphate and NAD were not cleaved but ATP and ADP readily served as substrates. Pyrophosphate was hydrolysed more rapidly than inositol hexaphosphate.

3.1.5.11 Activity of phytase on mixed substrates

The activity towards mixed substrates (with simple phosphomonoesters as listed in Table 12) was measured with the enzyme preparation from *N. crassa*. If the phytase preparation consisted of many enzymes which are highly specific to one substrate then

Figure 20.

Michaelis constant for phytase from *Neurospora crassa* using β-glycerophosphate as the substrate

The activity of phytase from *Neurospora crassa* (Fraction 5, Table 8) was determined in the presence of varying amounts of β-glycerophosphate as the substrate. The reaction mixture was as described in Section 2.4.1 except that the varying concentrations of the substrate were as given in the Figure here. Inorganic phosphate was detected by the colorimetric method of Watanabe and Olsen (1960).

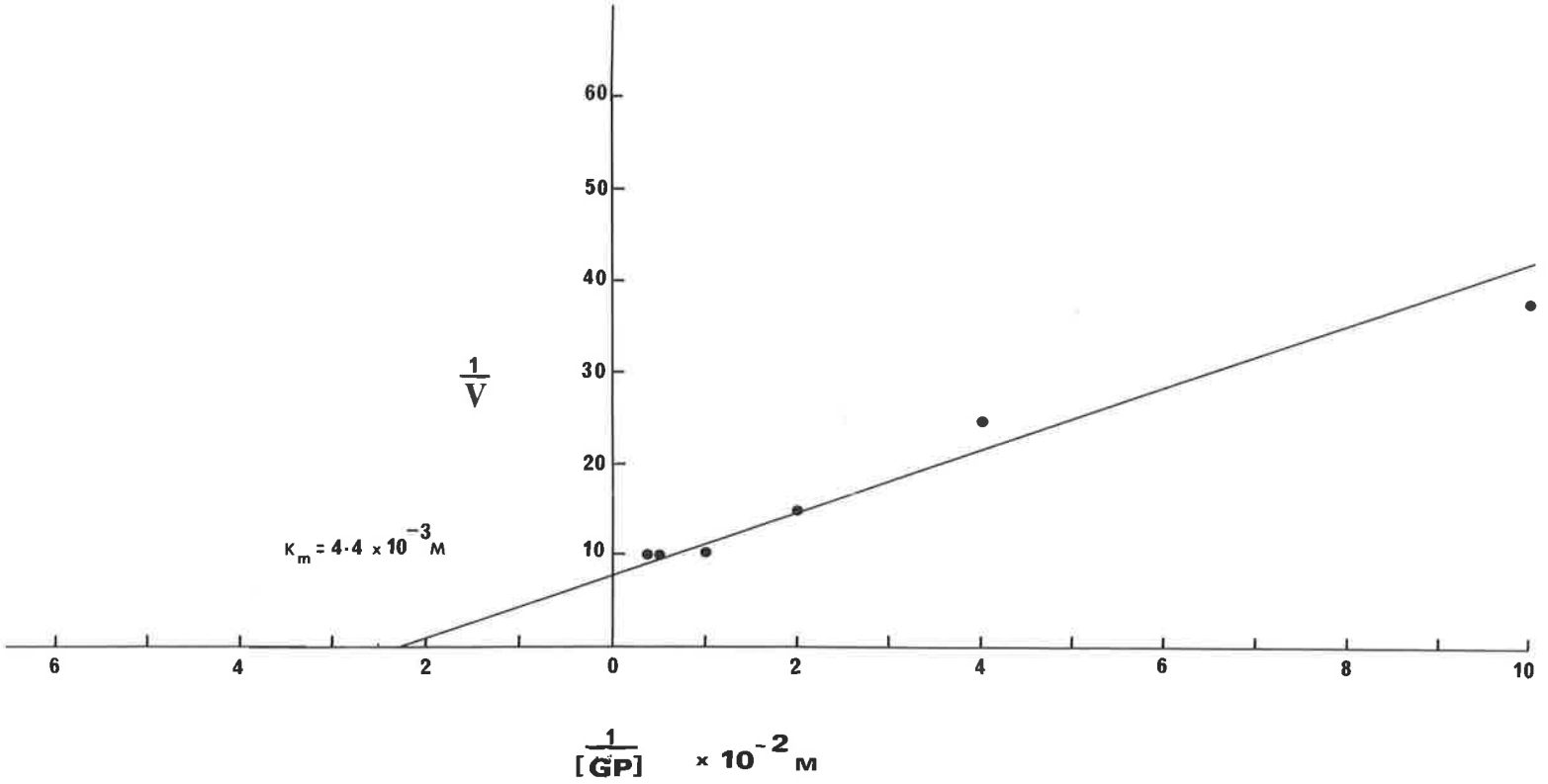

60
50
40
30
20
10
1/V
$K_m = 4{\cdot}4 \times 10^{-3}$ M
6
4
2
0
2
4
6
8
10
1/[GP] × 10^{-2} M

Table 11. Substrate specificity of phytase

Substrate (1×10^{-2} M)	Relative activity (%)
Inositol hexaphosphate	100
NAD	0
NADP	94
ADP	250
ATP	370
Glucose-1-phosphate	0
Glucose-6-phosphate	90
Fructose 1,6 diphosphate	140
α-glycerophosphate	70
β-glycerophosphate	80
α-naphthyl phosphate	110
Phenyl phosphate	90
Inorganic pyrophosphate	200

The enzyme assay was as describedin the text, Section 2.4.1, except that the type of substrate used was as listed in this Table. All substrates were adjusted to pH 5.0. The amount of phosphorus liberated from phytate (0.15) International Unit was considered as 100%; other values are indicated as percentages of this value.

Table 12. Activity of phytase toward mixed substrates

Substrate	Activity of enzyme to a single and a mixed substrate
Inositol hexaphosphate	0.20
β-glycerophosphate	0.08
Glucose-6-phosphate	0.085
Inositol hexaphosphate + β-glycero-phosphate	0.10
Inositol hexaphosphate + glucose-6-phosphate	0.15
β-glycerophosphate + glucose-6-phosphate	0.12

All substrates were 0.01 M. Where 2 substrates were used together each substrate was 0.01 M. The liberated P_i was colorimetrically detected using the ascorbic method of Olsen and Watanabe (1965). Activity of the enzyme was in μmole P_i/min/ml enzyme.

the enzyme activity towards the mixed substrate should be equal to the sum of each activity tested with each individual substrate. Table 12 shows that the additivity of the enzymic activity did not exist with any of the mixed substrate test. Each phosphatase activity was not independent, but correlated with each other, indicating that each substrate competed with the other for the enzyme.

3.1.6 Products of the enzymic hydrolysis of *myo*-inositol hexaphosphate

The intermediates in the hydrolysis of *myo*-inositol hexaphosphate or phytate by the enzyme are described in this Section.

Figure 21 shows the stepwise dephosphorylation of *myo*-inositol hexaphosphate by the *N. crassa* enzyme after 15, 30, and 50% of the organic phosphorus had been converted into inorganic phosphorus (lanes 1,2 and 3 respectively). When 50% of the organic phytate phosphorus was degraded the following compounds in the order of their highest mobilities were separated:- hexa-, penta-, tetra-, tri-, di- and mono-phosphates. The non-migratory spot (at the origin of lanes 1, 2 and 3 in Figure 21 is a sugar contaminant in the enzyme extract. Such a large scale enzymic (using Fraction 5, Table 8) preparation of the polyphosphate intermediates was used for structural studies. It was observed that the time course curve for the enzymic hydrolysis of *myo*-inositol hexaphosphate or phytate decreased in rate with time for the large scale preparation. This is due to denaturation of the enzyme and also to inorganic phosphate (phytase inhibitor) accumulation in the reaction mixture. However, reaction rate could be speeded up

Figure 21.

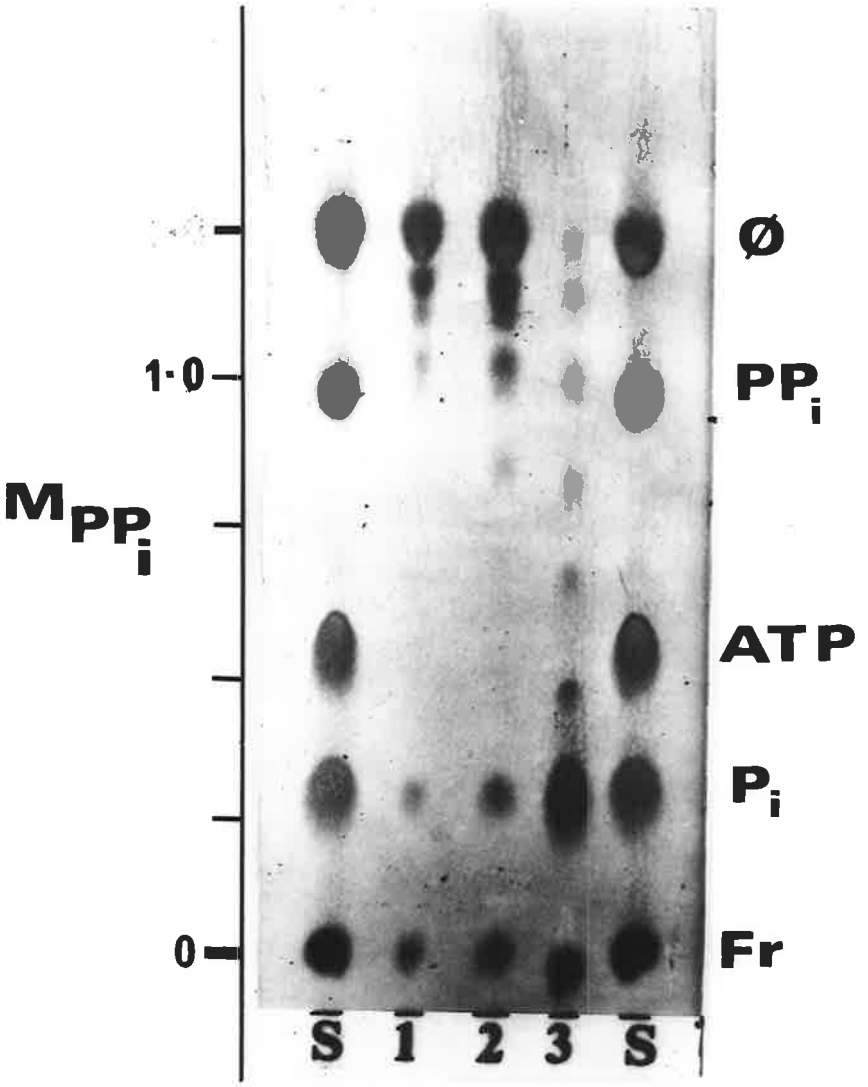
Stepwise dephosphorylation of *myo*-inositol hexaphosphate by phytase from *Neurospora crassa*.

Enzymic hydrolysis of *myo*-inositol hexaphosphate by crude phytase from *Neurospora crassa* was allowed to proceed until 15%, 30% and about 50% of the organic phosphorus was liberated as inorganic phosphorus. An aliquot (0.02 ml) of the reaction mixture at each stage of dephosphorylation was spotted onto Whatman No. 3 cellulose paper and examined by high voltage electrophoresis (Tate, 1968, Section 2.3.1.2).

Lane S = standard with ϕ(phytate); PP_i (inorganic pyrophosphate); ATP (adenosine triphosphate); P_i (inorganic phosphate) and Fr (fructose).

Lane 1,2,3 = represents 15%, 30% and 50% of phytate degradation respectively.

M_{PP_i} = mobility of compound with respect to inorganic pyrophosphate (see Section 2.4.5).


Ø
PPi
ATP
Pi
Fr
1·0
0
MPPi
S
1
2
3
S

if fresh enzyme was added; this increase in reaction rate then, is proportional to the amount of enzyme added and was not a multiple of the initial rate.

When the individual components were resolved on ion-exchange (Dowex Cl^- 400 mesh, Section 2.3.2.3) resin they gave an organic phosphorus profile as illustrated in Figure 22. The *myo*-inositol penta-, tetra-, tri-, di- and mono-phosphates were characterised by their electrophoretic mobilities in 0.1 M oxalate buffer (pH 1.5) and in 0.1 M citrate (pH 5) and chromatographic values (Table 13) with authentic standards. The individual polyphosphate ester was used for the determination of the phosphorus to inositol ratio and the molecular rotation at pH 1 and 11 respectively (Table 14) and for the study of structural assignment as presented in Section 3.1.6.1 below.

3.1.6.1 Intermediates in the *N. crassa* phytase route

Table 14 also lists the structures present in the electrophoretically homogeneous fractions which were numbered as indicated in the electrophoretogram, Figure 22. The evidence for the assignments of the polyphosphate fractions obtained in the degradation of *myo*-inositol hexaphosphate by the *N. crassa* phytase is in the following paragraphs:-

(a) *myo*-inositol pentaphosphates

The major pentaphosphate structure was deduced and shown to

Figure 22.

Paper electrophoretic separation (pH 1.5) of the polyphosphates from a partial dephosphorylation of *myo*-inositol hexaphosphate by the *N. crassa* phytase

Electrophoretic conditions:- 0.1 M oxalate buffer, pH 1.5

1 hr, 1,500 volt.

(a) Electrophoretogram of a partial dephosphorylation of *myo*-inositol hexaphosphate by the phytase from *Neurospora crassa* (50% degradation).

(b) Organic phosphorus profile of (a).

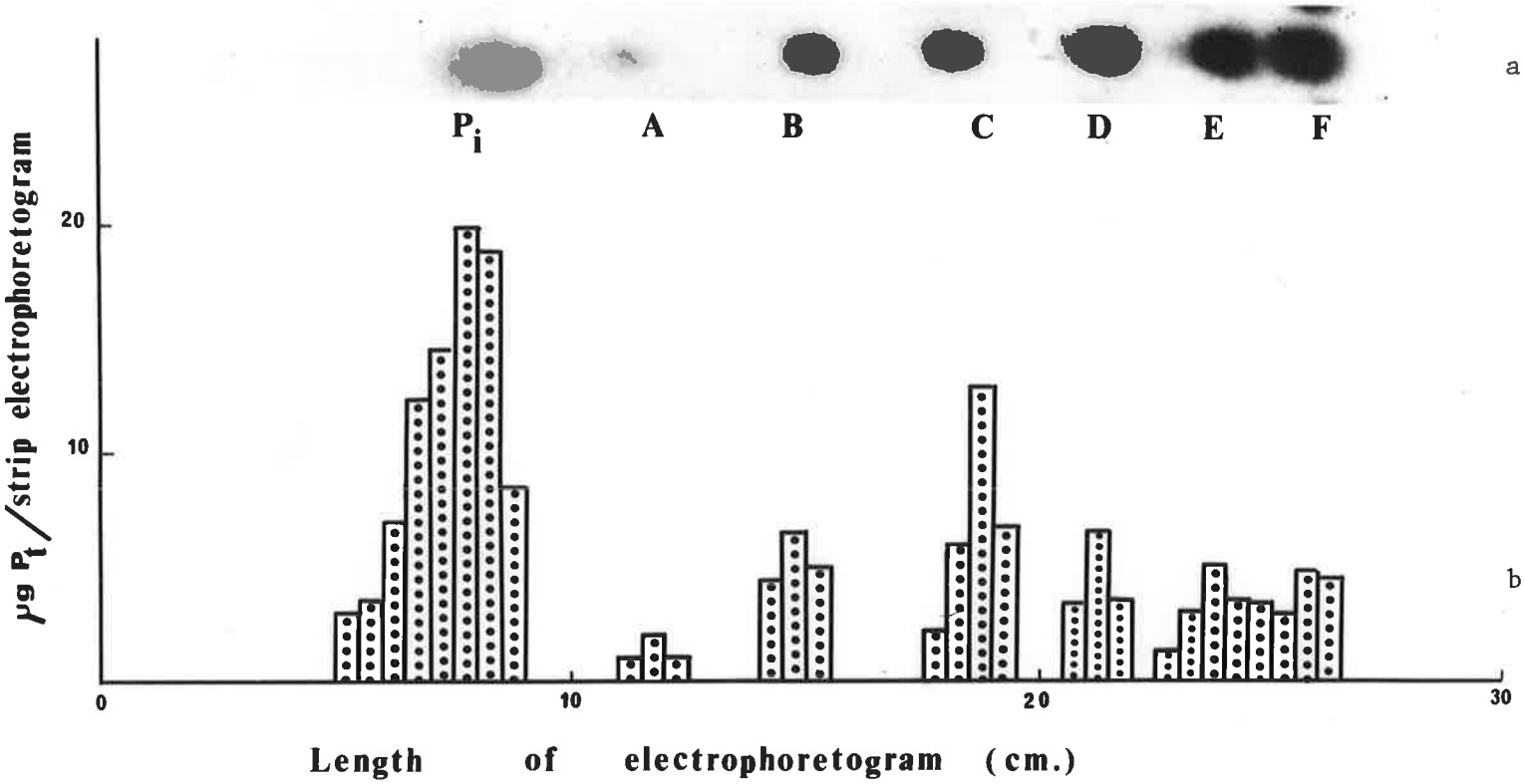

a
P_i
A
B
C
D
E
F
b
$\mu g\ P_i$ /strip electrophoretogram
20
10
0
10
20
30
Length of electrophoretogram (cm.)

Table 13.

Mobilities of reference compounds and *myo*-inositol polyphosphates obtained in the degradation of *myo*-inositol hexaphosphate by the *Neurospora crassa* phytase.

The polyphosphates were examined by electrophoresis in buffer systems: (a) 0.1M oxalate, pH 1.5 (Seiffert and Agranoff, 1965) and (b) in 0.1M citrate, pH 5.0 using the apparatus described by Tate (1968).

The mobilities (M_{PP_i}) of the compounds in buffers (a) and (b) were calculated relative to inorganic pyrophosphate using fructose as the non-migrating marker.

The compounds were again examined by paper chromatography using isopropanol/ammonia/water (v/v, 60/10/30) as the solvent system. The mobilities (R_{PP_i}) of the compounds were again calculated with reference to inorganic pyrophosphate (PP_i).

All compounds were detected with the phosphomolybdate dip reagent (Harrap, 1960).

The numbers represent the phosphate groups on the inositol (I) molecule, i.e. IP_6 = inositol hexaphosphate; IP_5 = inositol pentaphosphate, etc. The starred IP_5*and IP_4* are the isomeric compounds detected on the electrophoretogram (see Figure 21).

Table 13. Mobilities of reference compounds and *myo*-inositol polyphosphates obtained in the partial degradation of *myo*-inositol hexaphosphate by *Neurospora crassa* phytase

Compound	Buffer (a) 0.1M oxalate (pH 1.5)	Buffer (b) 0.1M citrate (pH 5.0)	Solvent Isopropanol/ ammonia/water (v/v, 60/10/30) ≈ 13 hr
	M_{PP_i} ± 0.02		R_{PP_i}
PP_i	1.00	1.00	1.00
ϕ	1.27	1.06	0.30
P_i	0.31	0.73	1.54
Polyphosphates in *N. crassa* hydrolysate			
IP_6	1.27	1.06	0.30
IP_5	1.19	1.03	0.39
IP_5*	1.14	1.03	0.44
IP_4	1.04	1.00	0.53
IP_4*	0.99	0.99	0.53
IP_3	0.86	0.89	0.67
IP_2	0.71	0.74	0.98
IP_1	0.48	0.46	1.20
P_i	0.31	0.73	1.54

Table 14. Analysis of the phosphate-containing fractions obtained by ion-exchange separation of a partial digest of phytic acid by the *Neurospora crassa* phytase

Fraction (as indicated in Figure 22)	Phosphorus:inositol ratio	Molecular rotation $[M]_D$ pH 1	Molecular rotation $[M]_D$ pH 11	Structure
P_i	-	-	-	
A	1	-	-	I
B	2	-90 ± 30	-62 ± 30	II
C	3	-24 ± 8	-80 ± 8	III
D	4	-36 ± 4	-30 ± 4	IV
E	5	-55 ± 8	-47 ± 3	Va
F	6	-	-	ϕ

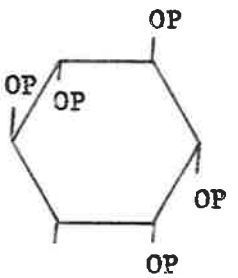
Analysis of the phosphate-containing fractions obtained by ion-exchange chromatography of a partial digest (50%) of phytic acid by *Neurospora* phytase.

Each polyphosphate fraction separated by anion-exchange chromatography was used for the determination of its phosphorus to inositol ratio (Section 2.4.10.1), the calculation of the molecular rotation using the optical activity at pH 1 and at pH 11 respectively as in Section 2.4.10.1.

The fraction was lettered as indicated in the organic phosphorus profile as shown in Figure 22(b); and the structure was as given in the text.

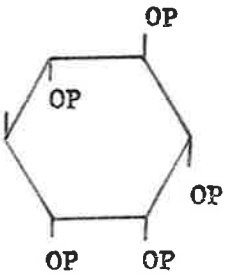
be of the structure Va by Johnson and Tate (1969). The phosphorus to inositol ratio (5:1), optical activity, chromatographic (in solvent system 1, Table 4, Section 2.3.5) and electrophoretic mobilities (Table 13) when taken in conjunction with the structure of the triphosphate produced by further degradation, characterise the major pentaphosphate as D-*myo*-inositol 1,2,4,5,6-pentaphosphate (Structure Va)

Va.



D-*myo*-inositol 1,2,4,5,6-pentaphosphate

Vb.



L-*myo*-inositol 1,2,3,4,5-pentaphosphate

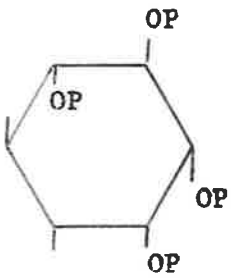
The minor pentaphosphate detected at 15 and 30% P_i release (Figure 21) was characterised only by its electrophoretic mobility which suggests it is either L-*myo*-inositol 1,2,3,4,5-pentaphosphate as from wheat bran phytase or its enantiomorph.

(b) *Myo*-inositol tetraphosphates

Upon enzymic hydrolysis (using the *N. crassa* phytase) the major pentaphosphate (Structure Va, above) gave rise to an optically active major tetraphosphate IVa (Table 14). This tetraphosphate fraction (M_{PP_i} = 1.04) yielded a new isomer (M_{PP_i} = 0.99) under acid conditions (1N HCl/100°/15 min) which induced *cis* migration. The inositol:phosphorus ratio, electrophoretic mobility, isomerisation

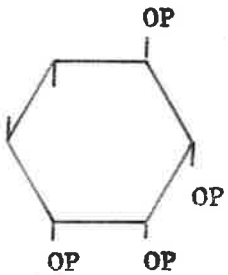
behaviour and optical activity of this fraction are consistent with structure IVa. The attempted periodate oxidation of IVa was not successful, but the similarity in properties to the major tetraphosphate from bran (Johnson and Tate, 1969*b*) together with the structure of the triphosphate produced by further degradation characterise it as D-*myo*-inositol 1,2,5,6-tetraphosphate IVa.

IVa.



D-*myo*-inositol 1,2,5,6-tetraphosphate

IVb.



L-*myo*-inositol 1,2,3,4-tetraphosphate

In some electrophoretograms a spot with the mobility of L-*myo*-inositol 1,2,3,4 tetraphosphate IVb was observed as a minor product, but no further characterization has been attempted.

(c) *Myo*-inositol triphosphate

The inositol triphosphate fraction (Figure 22, Table 13) was shown to be a triphosphate by its 3:1 phosphorus to inositol ratio. It was subjected to periodate-oxidation-borohydride-reduction-hydrolysis reaction as described in Section 2.4.10.5. Paper chromatography (as illustrated in Figure 23) of the product obtained from this reaction demonstrated that major products were arabitol and some *myo*-inositol. The latter is probably due to incomplete oxidation. Gas chromatography (Figure 24) of the polyol acetates confirmed the presence of arabitol which was purified by fractional sublimation and characterised by its infrared spectrum and melting point 74°. A mixed melting point with authentic D arabitol acetate (melting point

Figure 23.

Paper chromatography of arabitol obtained from the periodate-oxidation-borohydride-reduction and dephosphorylation reaction of the *myo*-inositol triphosphate from *Neurospora crassa* hydrolysis

The paper was run in the ascending manner using the solvent system acetone/water, v/v, 4/1 for 4 hr.

The polysaccharides were developed by the silver nitrate method of Anet and Reynolds (1954).

Gly, Glu, Myo = glycerol, glucose, *myo*-inositol

IP_3N.c [O] = *myo*-inositol triphosphate from *N. crassa*

Arab = arabitol

Arab + Ery = arabitol + erythritol.

PAPER CHROMATOGRAPHY

(ACETONE/WATER, 4/1)

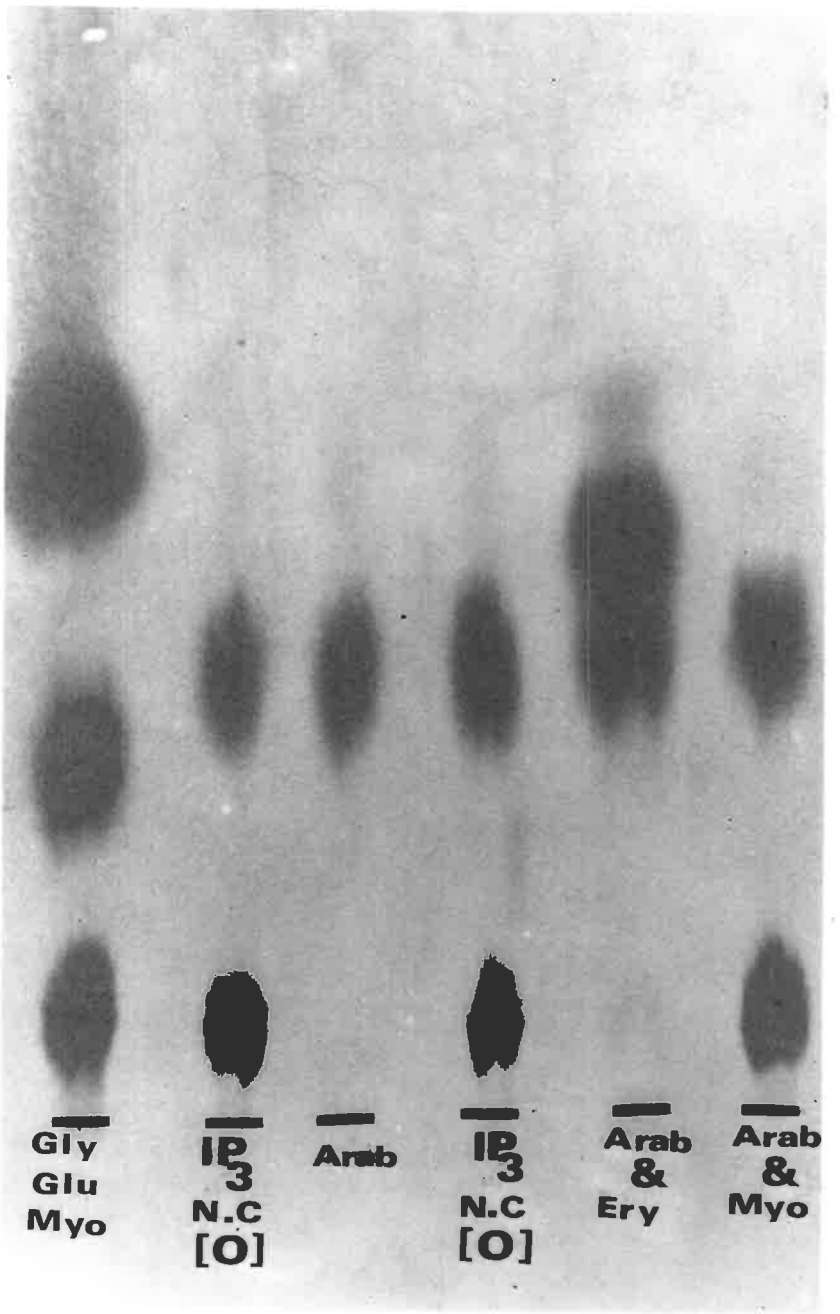


Figure 24.

Gas-liquid chromatography of sugar alcohol acetates

The standard technique used was as described in Section 2.3.3.1.

Trace (S) Standard compounds

(a) obtained from *N. crassa* and bran Fraction F_1 hydrolysates

(b) obtained from wheat bran Fraction F_2 hydrolysate

Standard acetates are as follows:-

(1) Glycerol

(2) Threitol

(3) Erythritol

(4) Ribitol

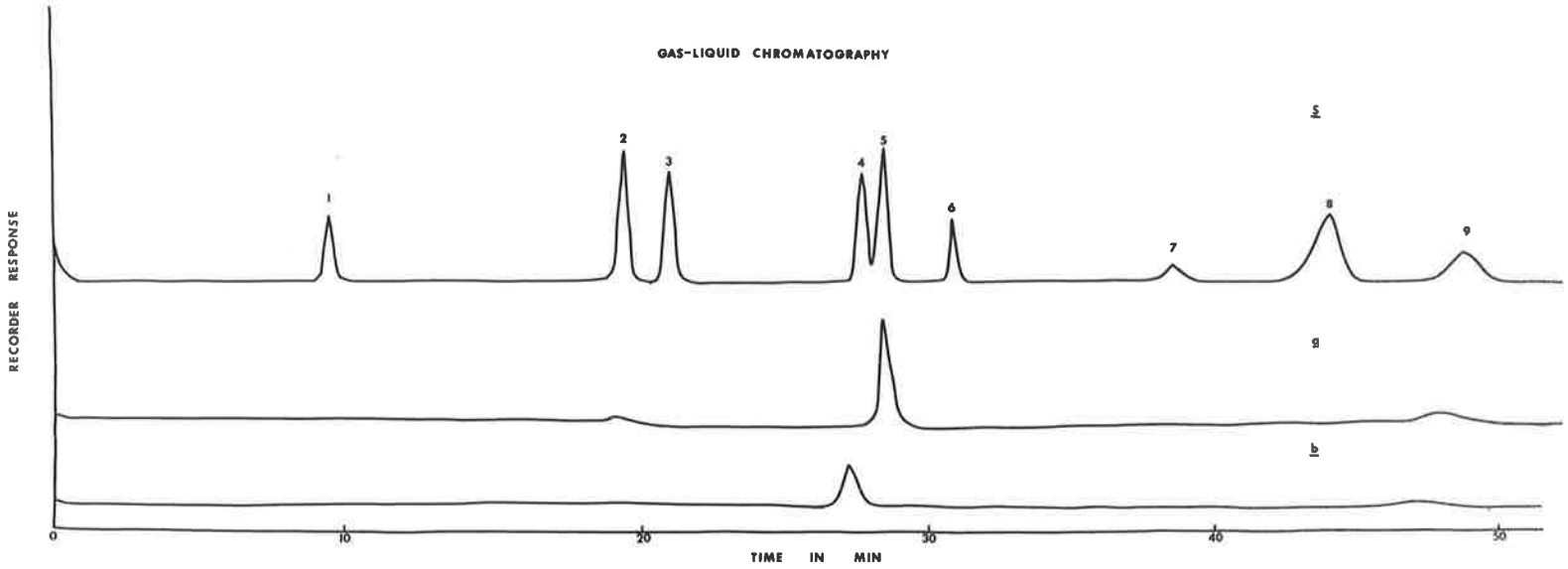
(5) Arabitol

(6) Xylitol

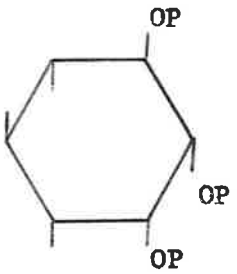
(7) Altritol

(8) Sorbitol

(9) *Myo*-inositol


GAS-LIQUID CHROMATOGRAPHY
RECORDER RESPONSE
1
2
3
4
5
6
7
8
9
5
8
1
0
10
20
30
40
50
TIME IN MIN

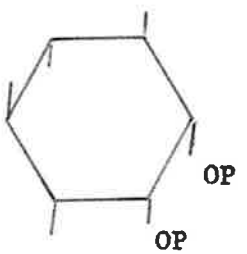
74°) was undepressed, but a mixed melting point with authentic L arabitol acetate was elevated to 95° which is identical with the mixed melting point of the authentic DL racemate. Thus the original polyol must be D arabitol, which can only be derived from Structure III.

III. 

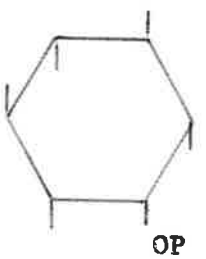
D-*myo*-inositol 1,2,6 triphosphate

(d) *Myo*-inositol diphosphate

Periodate oxidation of the diphosphate component yielded only erythritol; no other polyol was found. Under alkaline conditions it gave *myo*-inositol 1 and 2 monophosphates which were successfully separated by paper chromatography (see Table 15). The levorotatory sign of its molecular rotations both in acid and alkaline medium (Table 14) and its formation from III define its structure as D-*myo*-inositol 1,2 diphosphate (Structure II)

II 

Myo-inositol 1,2 diphosphate

I 

Myo-inositol 2 monophosphate

Table 15. Mobilities of *myo*-inositol di-phosphates

Compound	Electrophoretic mobility (M_{PP_i}) 0.1M oxalate pH 1.5, 1 hr at 1500 volt	Chromatographic mobility (R_{PP_i}) Isopropanol/ ammonia/water v/v 60/10/30, 15 hr	Polyol from *myo*-inositol diphosphate after the periodate-oxidation-borohydride and hydrolysis sequence
PP_i	1.00 ± 0.02	1.00	
P_i	0.31	1.53	
ϕ	1.27	0.38	
Myo-inositol diphosphates			
1,2* diphosphate	0.70	1.24	erythritol
1,4 diphosphate	0.70	1.06	carbon dioxide
1,6 diphosphate	0.75	1.04	threitol
1,3 diphosphate	0.70	1.07	ribitol
2,4 diphosphate	0.70	1.24	arabitol

* Numbers denote the carbon position on the inositol ring which is esterified to a phosphate.

The electrophoretic and chromatographic techniques used for the determination of the mobilities of the compounds are of standard procedures and are as given in Sections 2.3.1.2 and 2.3.5 respectively.

The mobilities of the compounds in the respective buffer or solvent system were calculated with reference to inorganic pyrophosphate.

All phosphate containing compounds were developed by the phosphomolybdate reagent of Harrap (1960).

(e) *Myo*-inositol monophosphate

The monophosphate component in the fungal hydrolysate was found to be a mixture of the 1 and 2 monophosphates but predominantly the latter. Further enzymic hydrolysis of this monophosphate resulted in free *myo*-inositol which was detected on paper chromatograms in the solvent systems 4 and 5 (Table 4, Section 2.3.5).

3.1.6.2 Comparative study of the degradation pathways of *myo*-inositol hexaphosphate

Figure 25 compares the electrophoretic phytate degradation patterns (at 35% P_i release) of a very active phytase from *Pseudomonas* SB2 (supplied by Dr. D.J. Cosgrove, see Materials and Methods) with the pattern from the *Neurospora crassa* phytase. The similarity in mobilities is in accord with the similarity in structures proposed by Cosgrove (1970) for the intermediates produced by the *Pseudomonas* enzyme.

Figure 26 shows the markedly different phytate degradation patterns of the *N. crassa* enzyme and the crude wheat bran phytase. The intermediates of the bran phytase route will be detailed in Section 3.2.4.

3.2 Phytases from higher plants

Phytases extracted from wheat bran were fractionated and partially purified. Some enzymic properties are presented.

Figure 25.

Electrophoretogram of the enzymic degradation of phytic acid compared between the phytases from *Neurospora crassa* and *Pseudomonas* SB2

The reaction mixture (1 ml) contained 0.25 M sodium acetate buffer (pH 5.0), 0.02 M sodium phytate (pH 5.0), the various phytases (each at 0.2 ml) and water. The mixture was incubated at 37° until the percentage of total phosphorus liberated was at 35%.

Electrophoretic conditions:- 0.1M oxalate buffer, pH 1.5
1 hr at 1,500 volts

Phosphorus compounds were detected by the phosphomolybdate reagent (Harrap, 1960).

M_{PP_i} = Electrophoretic mobility with respect to PP_i

PP_i = Inorganic pyrophosphate

ϕ = Phytic acid (*myo*-inositol hexaphosphate)

ATP = Adenosine triphosphate

P_i = Inorganic phosphate

Fr = Fructose

Lane S = Standard compounds

Bran IP_5 = *Myo*-inositol pentaphosphate

Lane SB2 = Degradation pattern by *Pseudomonas* SB2 enzyme

Lane NC = Degradation pattern by *Neurospora crassa* phytase

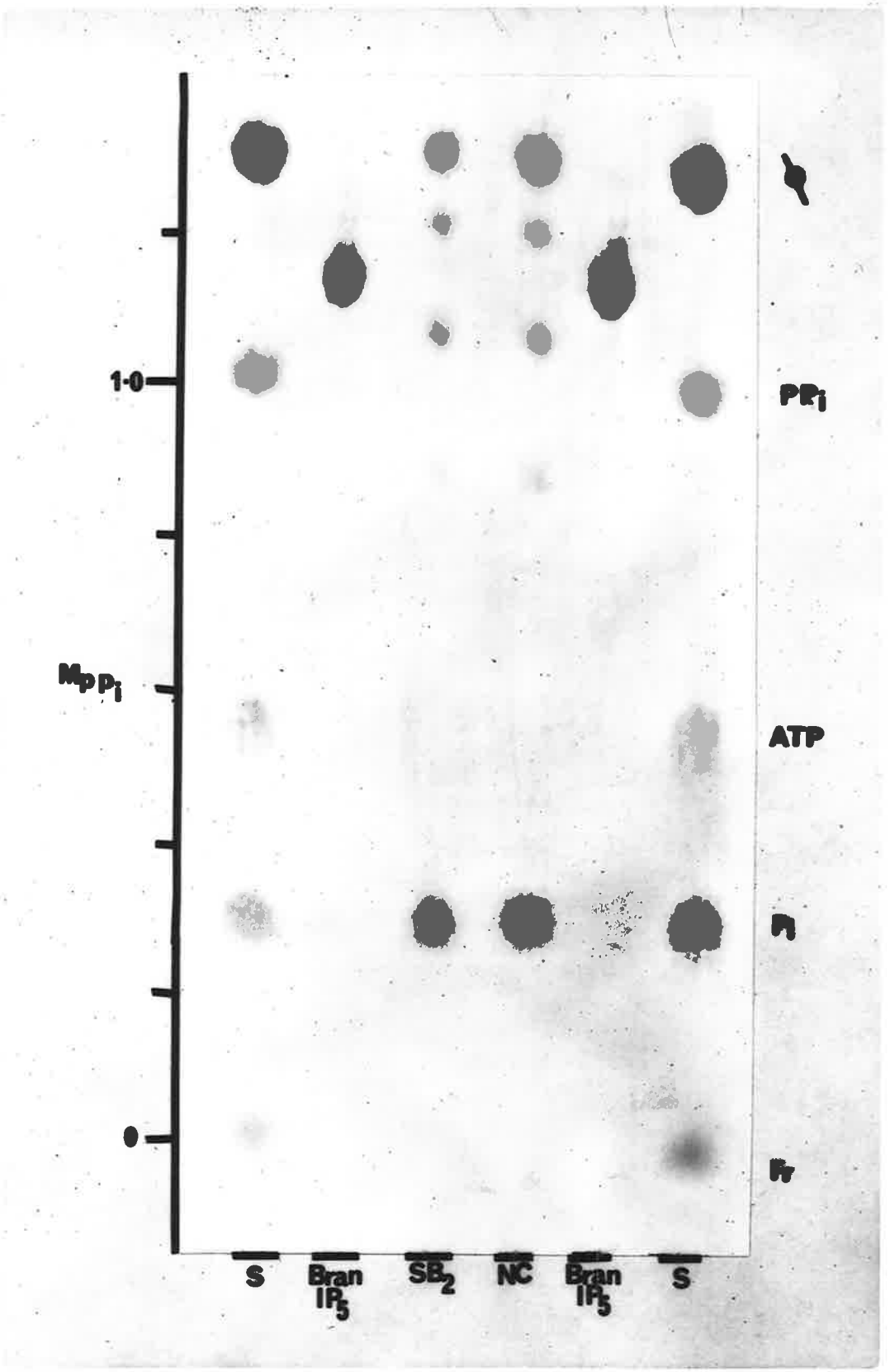

1·0
0
Mp
p_i
PP_i
ATP
Pi
Pr
S
Bran IP_5
SB_2
NC
Bran IP_5
S

Figure 26.

Electrophoretogram of the enzymic degradation of phytic acid by *Neurospora* and bran phytases

The reaction mixture was as given for Figure 25 and an aliquot (0.01 ml) was examined when 20-30% of the phytate phosphorus was liberated as inorganic phosphate (P_i).

Electrophoretic conditions:- 0.1 M oxalate buffer, pH 1.5
1 hr at 1,500 volts

All phosphate compounds were developed with the phosphomolybdate reagent (see Section 2.3.1.3).

The scale in the Figure 26 denotes the mobility of the compound with respect to inorganic pyrophosphate (PP_i) using fructose (Fr) as the non-migratory marker.

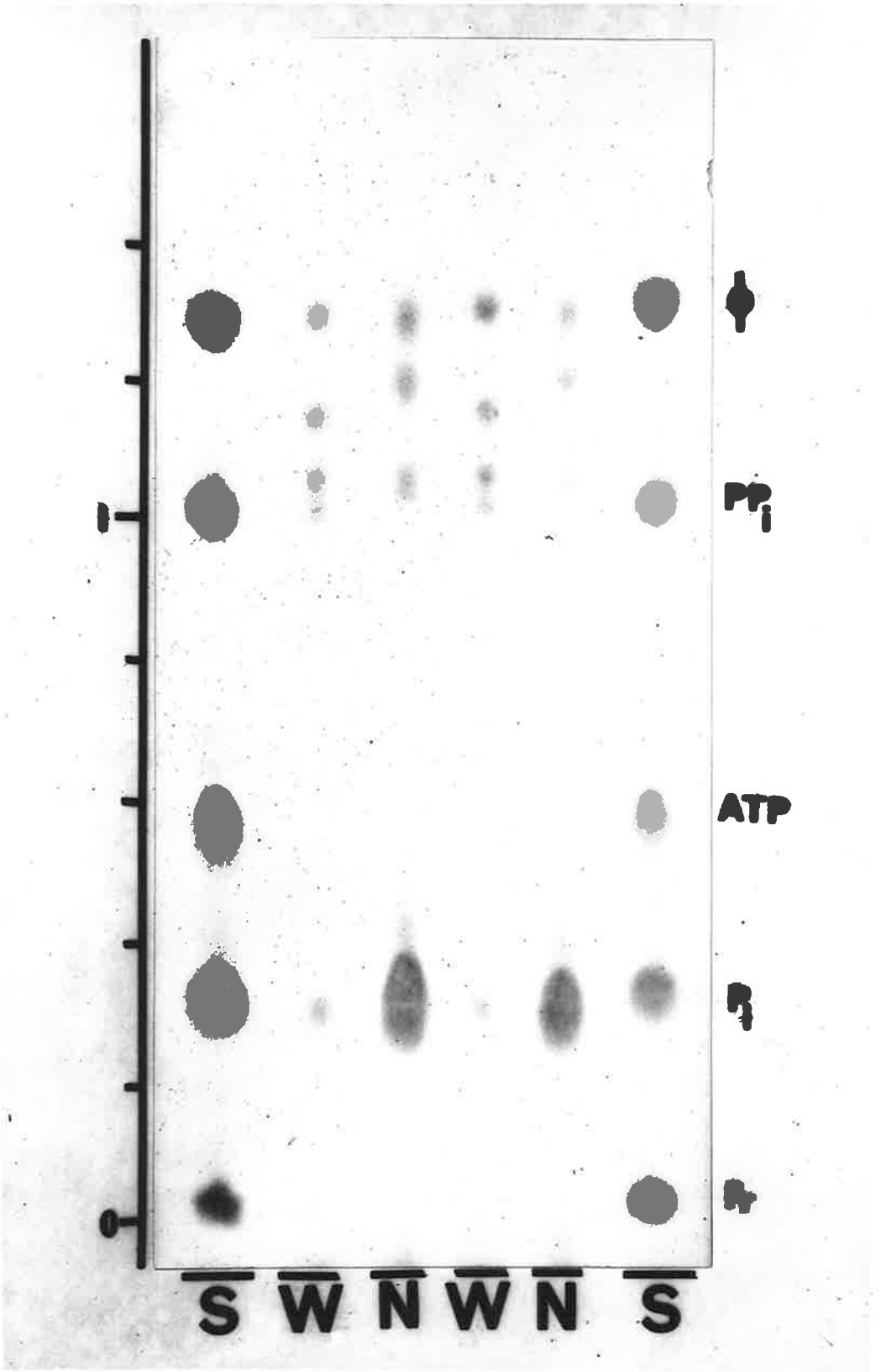
ATP = adenosine triphosphate

ϕ = phytic acid or *myo*-inositol hexaphosphate

Lane S = standard compounds

Lane W = degradation pathway of ϕ using wheat bran phytase

Lane N = degradation pathway of ϕ using *Neurospora crassa* phytase


ATP
S W N W N S

3.2.1 Butanol treatment

The crude aqueous extract from wheat bran prepared as described in Section 2.2.2.1 was treated with water-saturated n-butanol (see Section 2.2.2.2). Figure 27 shows the sequential butanol extractions (n times) of the crude enzyme which resulted in a successive decrease in enzymic activity. The activity was restored by combining the exhaustively extracted enzyme with the concentrated butanol extract (Figure 27). Plate 3 shows that at least seven iodine staining components of the concentrated butanol extract are separable by thin layer chromatography (see Section 2.3.4). Only two components were detected by the phospholipid reagent of Vaskovsky and Kostetsey (1968). These phospholipids (R_f 0.5 and 0.16) corresponded to standard lecithin (R_f = 0.5) and lysolecithin (R_f = 0.16) respectively. The phospholipid (R_f 0.16) when isolated by preparative thin layer chromatography restored enzyme activity whereas all other lipids were inactive. Since the increase in activity could have resulted from a degradation of either the added phospholipid or the substrate (*myo*-inositol hexaphosphate) an assessment was made of this possibility. Thus enzyme activity was determined with graded amounts of phospholipid without the substrate, in the reaction mixture. No increase in enzyme activity was recorded. A similar assessment of the phytase activity with only the substrate showed that much of the initial *myo*-inositol hexaphosphate remained at each incubation time indicating that enzyme activity was not enhanced due simply to the complete hydrolysis of *myo*-inositol hexaphosphate.

Figure 27.

Phytase activation by lysolecithin

Aliquots of the enzyme were assayed for phytase activity after 'N' sequential extractions with water-saturated n-butanol as shown in Figure 27.

The reaction mixture (total 1 ml) contained 0.02 M sodium phytate (pH 5.0), 0.25 M sodium acetate buffer (pH 5.0), enzyme solution and water.

■ ——— ■ phytase activity before 'N' (number) extractions

..... phytase activity restored with the addition of lysolecithin (15-20 µg P_t/ml) after the 10th extraction

● ——— ● phytase activity after the 4th extraction

▲ ——— ▲ " " " 8th "

● ——— ● " " " 10th "

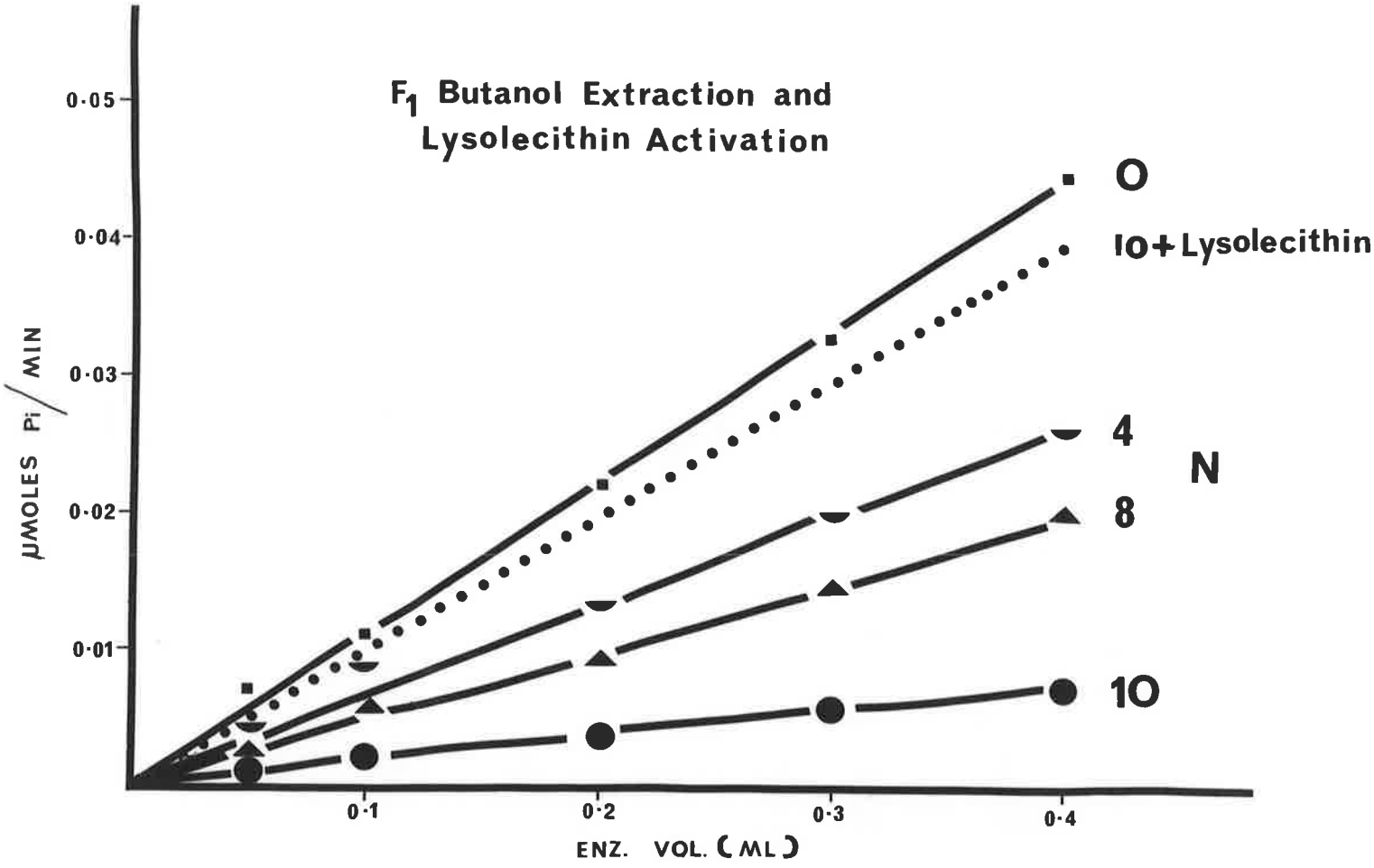

F1 Butanol Extraction and
Lysolecithin Activation
0·05
0·04
0·03
0·02
0·01
μMOLES Pi / MIN
0·1
0·2
0·3
0·4
ENZ. VOL. (ML)
O
10+Lysolecithin
4
N
8
10

Plate 3.

Thin-layer chromatography of the butanol extract

Activated silica gel H plates were run in solvent system $CHCl_3/MeOH/H_2O$ (v/v, 65/30/ 5).

Lipids were developed by iodine vapour as shown in Plate and also by the phospholipid spray of Vaskovsky and Kostetsey (1968).

Lane 1 = Lysolecithin extracted from wheat flour

Lane 2 = Phospholipid activating factor in the butanol extract

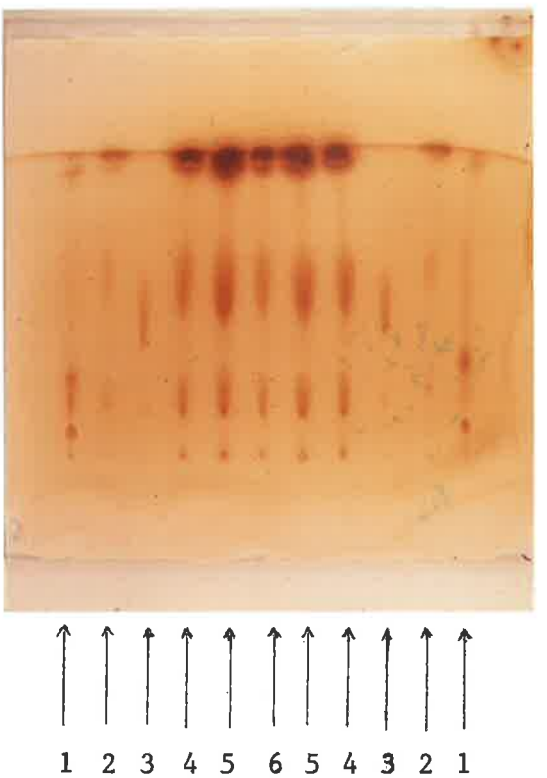
Lane 3 = Standard lecithin

Lane 4,5,6 = Standard egg lecithin (faster moving component) and its impurity lysolecithin (slower moving spot) at various concentrations respectively.

The darker colour observed with the iodine spray is due to unsaturation in the carbon chain.

PLATE 3

THIN LAYER CHROMATOGRAPHY OF THE BUTANOL EXTRACT



3.2.1.1 Identification of factors in the butanol extract

The two phospholipids described in Section 3.2.1 which had mobilities similar to those of lecithin and lysolecithin respectively were subjected to mild de-O-acylation. This was carried out in sodium methoxide solution for 15 min. The reaction was followed on thin-layer chromatography and the product formed was characterised by its chromatographic and electrophoretic properties. Both the phospholipids gave glyceryl phosphoryl choline (GPC) (see Table 16). With the aid of their chromatographic and electrophoretic mobilities of authentic standards, choline and a mixture of α and β glycerophosphate were identified in acid hydrolysis (1N HCl/100°/15 min) of glyceryl phosphoryl choline, Table 16. The fatty acids obtained from the mild de-O-acylation and characterised by gas-liquid chromatography (see Section 2.3.3.2) were predominantly C 16:0, C 18:1 and C 18:2 straight chain fatty acids. From the data obtained it was deduced that the slowest moving phospholipid on thin-layer chromatography and the enzyme activator was lysolecithin. The effect of this compound in restoring phytase activity is shown in Figure 27.

3.2.1.2 Lipids and phospholipids as activators

Table 17 shows a series of lysolecithin preparations, synthetic and naturally occurring, and related compounds which were tested for activity with the butanol treated phytase. The phosphorus content of the test solution was adjusted to the phosphorus

Table 16. Mobilities of some compounds

Compound	Electrophoretic mobility (M_{PP_i}) 0.1M oxalate, pH 1.5 1 hr, 1500 volt	Chromatographic mobility (R_{PP_i}) isopropanol/ammonia/water v/v, 60/10/30 11 hr
PP_i	1.00	1.00
ϕ	1.27	0.30
ATP	0.54	1.05
P_i	0.31	1.3
α-glycerophosphate	0.55	1.97
β-glycerophosphate	0.54	2.13
Glyceryl phosphoryl choline	0.00	2.54
Phosphoryl choline	-0.049	
Phosphoryl ethanolamine	-0.106	1.87
Choline	-1.96	2.80
Serine	0.091	2.50

The compounds listed in this table are as given under Abbreviations.

The standard techniques of high voltage electrophoresis (Section 2.3.1) and paper chormatography (Section 2.3.5) were employed using Whatman No. 3 paper for electrophoretic runs and prewashed Whatman No. 1 paper in the latter method.

Phosphorus compounds were detected by the phosphomolybdate dip method of Harrap (1960), heated for 15 min at 65° and then irradiated under U.V. light.

Choline was developed using the Dragendorff reagent (Wagner *et al.*, 1961) and amino acids by dipping in 0.3% w/v ninhydrin in acetone and heating at 100° for 2 min.

The mobilities of all compounds were calculated with reference to inorganic pyrophosphate (PP_i) as given in Section 2.4.5.

Table 17. Effect of lipids and phospholipids on the activity of the butanol-treated enzyme (10 extractions)

Enzyme reaction mixtures	activity (%)
Crude	100
Butanol-extracted (10x) enzyme	50
Synthetic 1-O-acetyl glyceryl phosphoryl choline	50
Synthetic 1,2 di-O-acetyl glyceryl phosphoryl choline	51
Synthetic lecithin (1,2 di-O-palmitoyl glyceryl phosphoryl choline)	51
Egg lecithin	57*
1-O-palmitoyl glyceryl phosphoryl choline (snake venom)	70
1-O-palmitoyl glyceryl phosphoryl choline (commercial)	70
Egg lysolecithin	75
Wheat endosperm lysolecithin	85
Phosphatidic acid	50
Oleic acid	50
Stearic acid	50
Palmitic acid	50
1-O-alkenyl glyceryl phosphoryl choline (ox-heart)	50
Glyceryl phosphoryl choline	50
Phosphatidyl ethanolamine	50

*A small amount of lysolecithin was detected by thin-layer chromatography in this sample.

The reaction mixture contained in a total of 1 ml, 0.25 M sodium acetate buffer, pH 5.0, 0.02 M sodium phytate, pH 5.0, 0.2 ml of enzyme solution (4-5 mg protein/ml) and water, with or without the lipid (15-20 µg P_t). Conditions of incubation and assay are given in Section 2.4.1. Phytase activity was expressed as a percentage of the original activity of the bran phytase which had not been subjected to butanol extraction. 100% activity is equivalent to 0.053 International Unit.

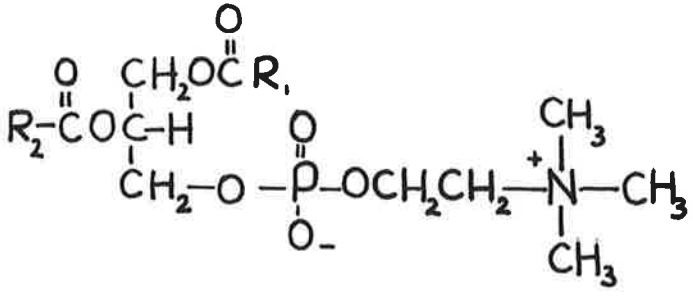
content of the original butanol extract. Of the compounds tested the only significant activation was by lysolecithin preparations. Major changes such as shortening the chain length by reducing the number of carbon atoms to C 2 as in acetyl lysolecithin or substitution of an enol ether for the ester linkage failed to restore enzyme activity. Similarly, total acylation or total de-O-acylation of the carbon residues as in lecithin and glyceryl phosphoryl choline (see structures as shown in Plate 4) also failed to restore enzyme activity. Because of the ease of acyl migration it is not possible to be certain that activity is due only to 1-O-acyl lysolecithins although the activity of the snake venom preparations supports this supposition.

The fact that phytase activity was not reduced to zero by successive exhaustive butanol extractions (see Figure 27) suggested a possible additional enzyme which might not require this cofactor. The substrate degradation patterns on electrophoretogram for both the crude phytase and the butanol treated phytase were indistinguishable suggesting that either the butanol extraction was incomplete or the hypothetical second enzyme had the same degradation pattern as the lysolecithin activated enzyme or was obscured by it.

Figure 28 shows the presence of two phytases F_1 and F_2 in the elution patterns for the crude enzyme from a DE-11 cellulose column (1.8 x 18 cm, Section 2.3.2.2). Butanol extractions revealed that only Fraction F_1 contained the phospholipid requiring enzyme and that the butanol extracted F_1 had a substrate degradation pattern which was indistinguishable from the crude enzyme. However, as shown in

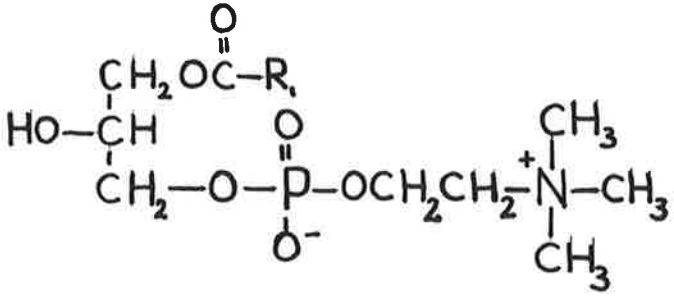
PLATE 4

STRUCTURES OF LECITHIN, LYSOLECITHIN AND GLYCERYL PHOSPHORYL CHOLINE

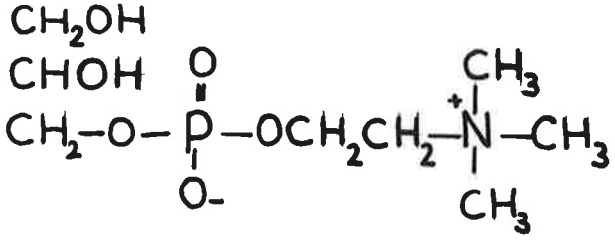


Phosphatidyl choline

Lecithin



Lysolecithin



Glycerylphosphoryl choline

Figure 28.

DE-11 cellulose chromatography of wheat bran phytase

Method used is as described in Section 2.3.2.2. Protein content in the fractions were determined at $E_{280\ nm}$. The reaction mixture was incubated at 37^{o} and contained in a total of 1 ml, 0.02 M sodium phytate (pH 5.0) as substrate, 0.25 M sodium acetate buffer (pH 5.0) and enzyme solution (0.2-0.5 ml) and water. Phytase activity was assayed as given in Section 2.4.1.

●------● Extinction coefficient $E_{280\ nm}$

■———■ Phytase activity in µmole P_i/min/fraction

▼- - - ▼ M Tris-HCl buffer (molarity), pH 7.3

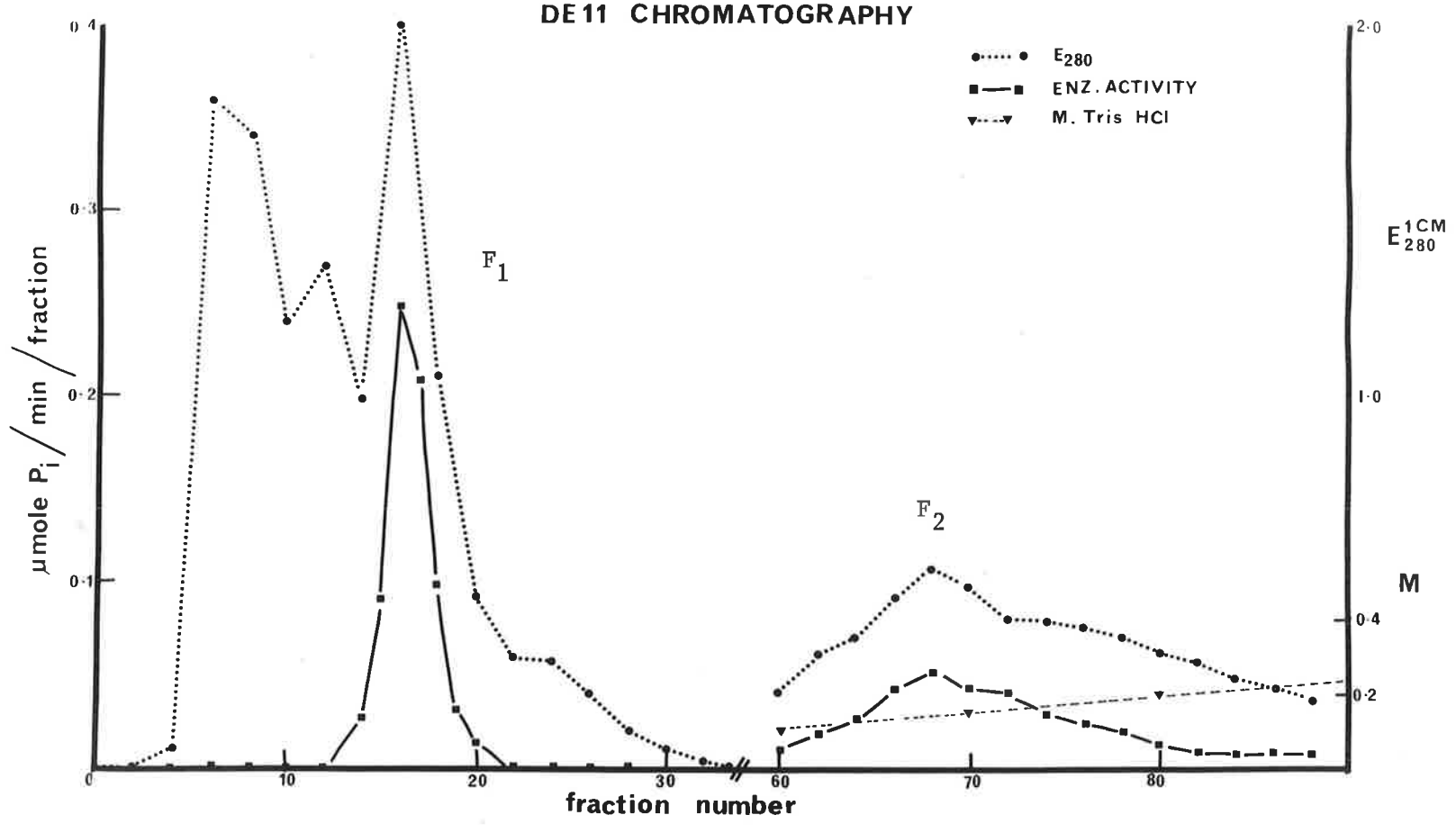

DE 11 CHROMATOGRAPHY
E280
ENZ. ACTIVITY
M. Tris HCl
F1
F2
μmole Pi / min / fraction
E 1CM 280
M
fraction number
0·4
0·3
0·2
0·1
2·0
1·0
0·4
0·2
0
10
20
30
60
70
80

Figure 29, phytase Fraction F_2 has a markedly different pattern which was masked by the presence of the more active F_1 enzyme in the crude enzyme preparations. A detailed discussion of the F_2 substrate degradation pattern and the structure of the intermediates will be given in Section 3.2.4.1.

3.2.1.3 Lipids and thermal stability

It has been reported that lecithin and lysolecithin confer thermal stability to the bovine ATPase (Tanaka, 1969). Figure 30 shows that a 60% loss in enzyme activity was recorded when the butanol treated enzyme was heated at 60° for 10 min and that the butanol treated enzyme is much less stable to temperature than the crude enzyme. However, in contrast to the ATPase (Tanaka, 1969) addition of lysolecithin, lecithin or the butanol extract failed to restore thermal stability to the butanol treated phytase.

3.2.2 Extraction, fractionation and purification of wheat bran phytase fractions F_1 and F_2

The following sequence was developed for the partial purification of phytase:-

(i) The crude enzyme extract was obtained from pre-soaked (>6 hr) wheat bran (after a selection of presoaking times for the maximum production of enzyme + protein; see Figure 31) (30 g/100 ml distilled water) following the osmotic shock method of Nagai and Funahashi (1962). This and all enzymic operations were conducted near 2°. The bran,

Figure 29.

Electrophoretogram of phytic acid degradation by bran phytase Fractions F_1 and F_2

The incubation mixture contained in a total of 1 ml, 0.02 M phytic acid (pH 5.0), 0.25 M sodium acetate buffer (pH 5.0), enzyme solution (0.2 ml) and water. The mixture was incubated at 37° until the inorganic phosphate liberated from ϕ was 35% of the total organic phosphorus. 0.02 ml of the reaction mixture spotted on Whatman 3 MM paper was examined in oxalate buffer (Seiffert and Agranoff, (1965) on high voltage electrophoresis of the type described by Tate (1968).

Electrophoretic conditions:- 0.1 M oxalate buffer, pH 1.5
1 hr, 1,500 volt.

Phosphate compounds were developed by the phosphomolybdate reagent of Harrap (1960).

Lane 1 = bran phytase F_1

Lane 2 = bran phytase F_2

Lane S = standard compounds with ϕ *myo*-inositol hexaphosphate, PP_i inorganic pyrophosphate, ATP adenosine triphosphate, P_i inorganic phosphate and Fr fructose

M_{PP_i} = mobility of the compound from the non-migratory marker, fructose with reference to PP_i (Section 2.4.5).

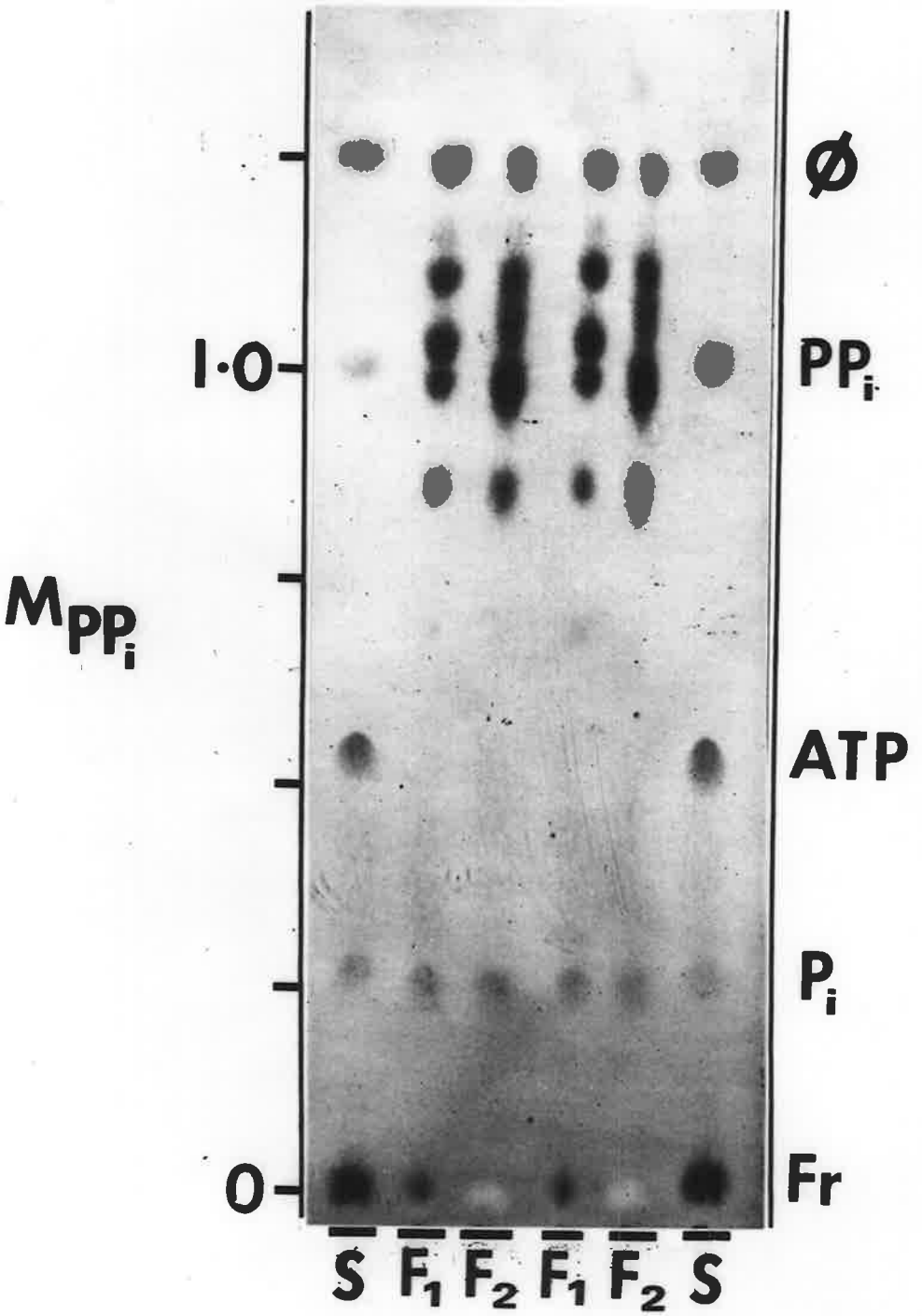

Ø
PPi
1·0
MPPi
ATP
Pi
Fr
0
S
F1
F2
F1
F2
S

Figure 30.

Lipids and thermal stability

The enzyme solution (4-5 mg protein/ml) was heated at 60°. At times specified as in Figure 30, aliquots of the enzyme solution were removed and assayed for phytase activity with or without the addition of lipid.

The reaction mixture (incubated at 37° for 30 min) contained in a total of 1 ml, 0.02 M sodium phytate (pH 5.0) as substrate, 0.25 M sodium acetate buffer (pH 5.0) and 0.2 ml enzyme and water.

●——● untreated enzyme

■——■ enzyme subjected to butanol treatment

O——O extracted enzyme plus lysolecithin

▲——▲ extracted enzyme plus lecithin

Readdition of the combined and concentrated butanol extracts to the treated enzyme heated at 60° did not detectably alter the enzyme activity (similar to ■——■).

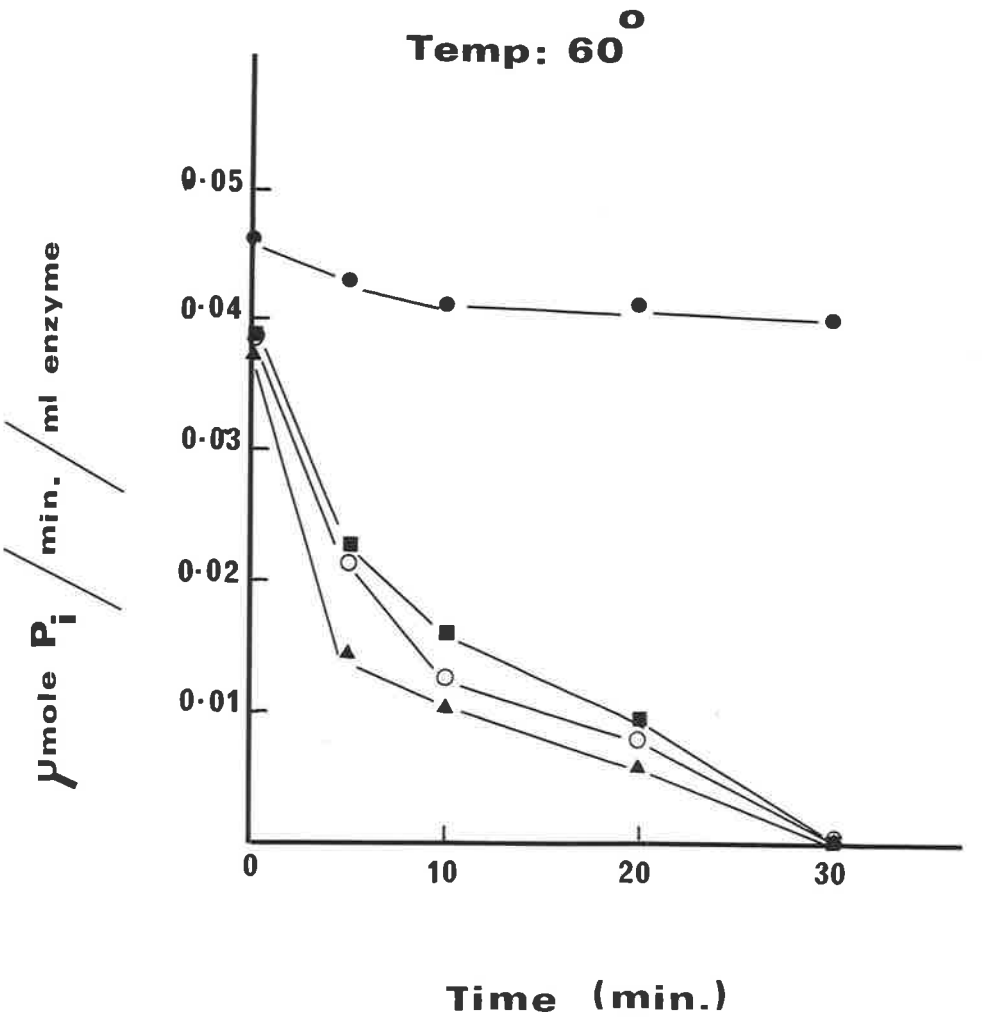
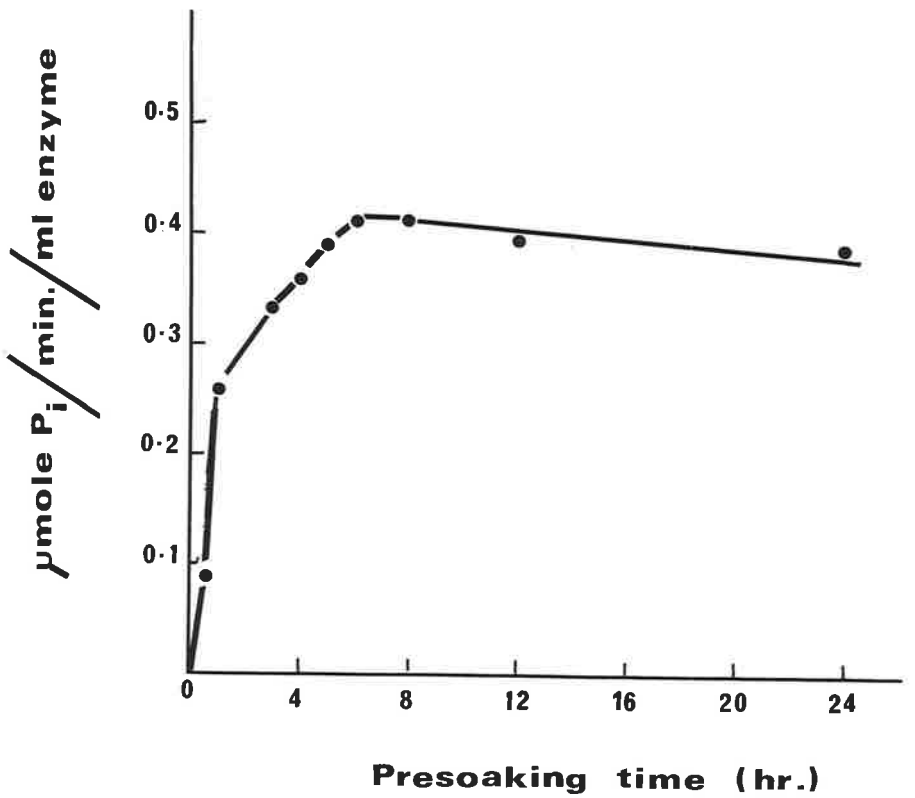

Temp: 60°
0·05
0·04
0·03
0·02
0·01
μmole P_i / min. / ml enzyme
0
10
20
30
Time (min.)

Figure 31.

Effect of pre-soaking on the extraction of bran phytase

Wheat bran (15 g/50 ml) was soaked in double distilled water for varying periods as shown in Figure 31. The extracted enzyme solution (Section 2.2.2) was made up to 40 ml. The activity of phytase was determined by the release of inorganic phosphorus from 0.01 M phytate as described in Methods (Section 2.4.1).

EFFECT OF PRESOAKING ON THE EXTRACTION OF PHYTASE FROM WHEAT BRAN



after an overnight soaking in water, was squeezed through double layers of muslin cloth. The yellow supernatant fraction left after centrifuging at 7,500 *g* for 20 min (Fraction 1, Table 18) was fractionated with ammonium sulphate.

(ii) Solid ammonium sulphate to a 50% saturation was slowly stirred into the crude enzyme Fraction (1). The suspension was allowed to stand for 2 hr before centrifuging at 7,500 *g* for 20 min and the residue was discarded. Solid ammonium sulphate was again added to completely saturate the 50% supernatant fraction and the mixture was allowed to stand for another 2 hr. The precipitate obtained after the second centrifuging dissolved in a minimal volume of cold distilled water was dialysed overnight against two volumes of cold distilled water. After dialysis the insoluble material was centrifuged and discarded, while the supernatant fraction (Fraction 2, Table 18) was used for gel chromatography.

(iii) The dialysed fraction was freeze-dried and dissolved in 15 ml water, then redialysed against 0.02 M sodium acetate buffer (pH 5) for 3 hr. It was then loaded onto a Sephadex G-100 column (1.6 x 52 cm) which had been previously equilibrated with the same buffer and calibrated (see Section 2.3.2.1). The enzyme eluted with 0.02 M sodium acetate buffer (pH 5) as a single peak (Figure 32) was collected in 3 to 4 ml fractions per tube. The fractions were then bulked to give Fraction 3 (Table 18).

(iv) Fraction 3 was dialysed against cold distilled water before

Table 18. Partial purification of bran phytase Fractions F_1 and F_2

Fraction	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity	Purification
1. Crude aqueous extract: Supernatant fraction left after centrifuging at 7,500 *g* for 20 min	76	1140	785	0.7	1
2. 50-100% $(NH_4)_2SO_4$ precipitate of (1). Active enzyme fraction dialysed overnight at 4° against double distilled water (2)	20	240	373	1.6	2
3. (2) passed through a Sephadex G-100 column (1.6 x 52 cm). Active enzyme fractions collected and dialysed as for step 4, below	28	10	330	33	47
4. (3) passed through a DEAE-(DE-11) column (1.8 x 18 cm).					
a) Fraction F_1 eluted in a stepwise manner with 0.02M Tris-HCl buffer, pH 7.3	40	1	200	200	285
b) Fraction F_2 eluted by a linear gradient of 0.02M to 0.5M Tris-HCl buffer, pH 7.3	50	0.5	70	140	200

Phytase was extracted from commercial wheat bran which had been presoaked as described in Section 2.2.2.1. The enzymes were partially purified with full details as given in Results, Section 3.2.2.

Phytase activity was determined as in Section 2.4.1. Protein was assayed by the Folin method (Section 2.4.2).

Figure 32.

Gel chromatography for phytase from wheat bran

15 ml of dialysed enzyme Fraction 2 (Table 18) containing active phytase was loaded onto a Sephadex G-100 column (1.6 x 52 cm) which had been previously equilibrated with 0.02 M sodium acetate buffer, pH 5.0 as given in Section 2.3.2.1. The enzyme was eluted with the same buffer from the column.

The concentration of protein in the collected fractions was determined by optical density measurements at 280 nm. Phytase activity was measured as described in Section 2.4.1.

●——● $E_{280\ nm}$ (optical extinction at 280 nm)

■.....■ Phytase activity expressed in μmole P_i/30 min/fraction.

2
1
100
50
E^{1CM}_{280}
μmole P_i liberated/30 min/Fraction
0
10
20
30
FRACTION Nº

loading onto the DEAE-(DE-11) cellulose (Section 2.3.2.2) column which had been previously washed with water. The column was washed first with distilled water before eluting with 0.02 M Tris-HCl buffer (pH 7.3). Finally a linear gradient of buffer between 0.02 M and 0.5 M Tris-HCl buffer (pH 7.3) was used. The eluate was collected in 5 ml fractions. An enzyme fraction (F_1) was eluted by the stepwise method whereas a second enzyme fraction (F_2) was eluted around 0.1 M (see Figure 28). The respective fractions were collected and each was dialysed overnight against cold distilled water to give Fractions 4 (a) and 4 (b) (Table 18) respectively.

The data from such a preparative fractionation are presented in Table 18. This purification procedure resulted in a 285-fold and 200-fold over the crude phytase extract for enzyme Fractions F_1 and F_2.

Other procedures which were attempted but did not result in further purification, included acetone extractions at -15° and methanol precipitation at 2°. Gel chromatography on Sephadex G-100 revealed only one homogeneous peak of enzyme activity as shown in Figure 32. This was resolved into enzyme Fractions F_1 and F_2 by anion-exchange chromatography (Figure 28), indicating the charge differences of the fractions. Good recovery of the phytase activity and protein from the DEAE column was only obtained after irreversible adsorption sites had been covered with bovine serum albumin (see Section 2.3.2.2 in Materials and Methods).

3.2.3 Properties of the enzymes

3.2.3.1 Presence of other enzymes

The bran phytase Fraction F_1 (or Fraction 4(a), Table 18) contained 30% of adenosine triphosphatase (ATPase), 40% of pyrophosphatase (PPase) and 50% of β-glycerophosphatase (β-GPase) as compared to their initial activities (100%) in the crude extract. Similarly PPase, β-GPase and ATPase were detected in the enzyme Fraction F_2 (or Fraction 4(b), Table 18). Both F_1 and F_2 were not active with glucose-1-phosphate whereas the crude extract (Fraction 1, Table 18) had weak glucose-1-phosphatase activity. In contrast to F_1 hydrolysis of glucose-6-phosphate was not detected in the F_2 fraction. The crude extract had a glucose-6-phosphatase activity (G-6-Pase) of 0.38 μmole P_i liberated/min/mg protein as compared to Fraction F_1 (Fraction 4(b), Table 18), which had only 0.1 μmole P_i/min/mg protein. Thus the G-6-Pase activity had been reduced to less than 26% of the original level. Although the phosphatases had been reduced considerably in the purification steps they were not eliminated completely in the Fractions 4(a) and 4(b), Table 18, respectively.

3.2.3.2 Starch gel electrophoresis

Bran phytase Fractions F_1 (285-fold) and F_2 (200-fold) (see Table 18, Section 3.2.2) contained other phosphatase activity as outlined in Section 3.2.3.1. Starch gel electrophoretic studies of the Fractions F_1 and F_2 were undertaken to establish whether phytase was a single protein capable of hydrolysing

Figure 33.

Starch gel electrophoresis

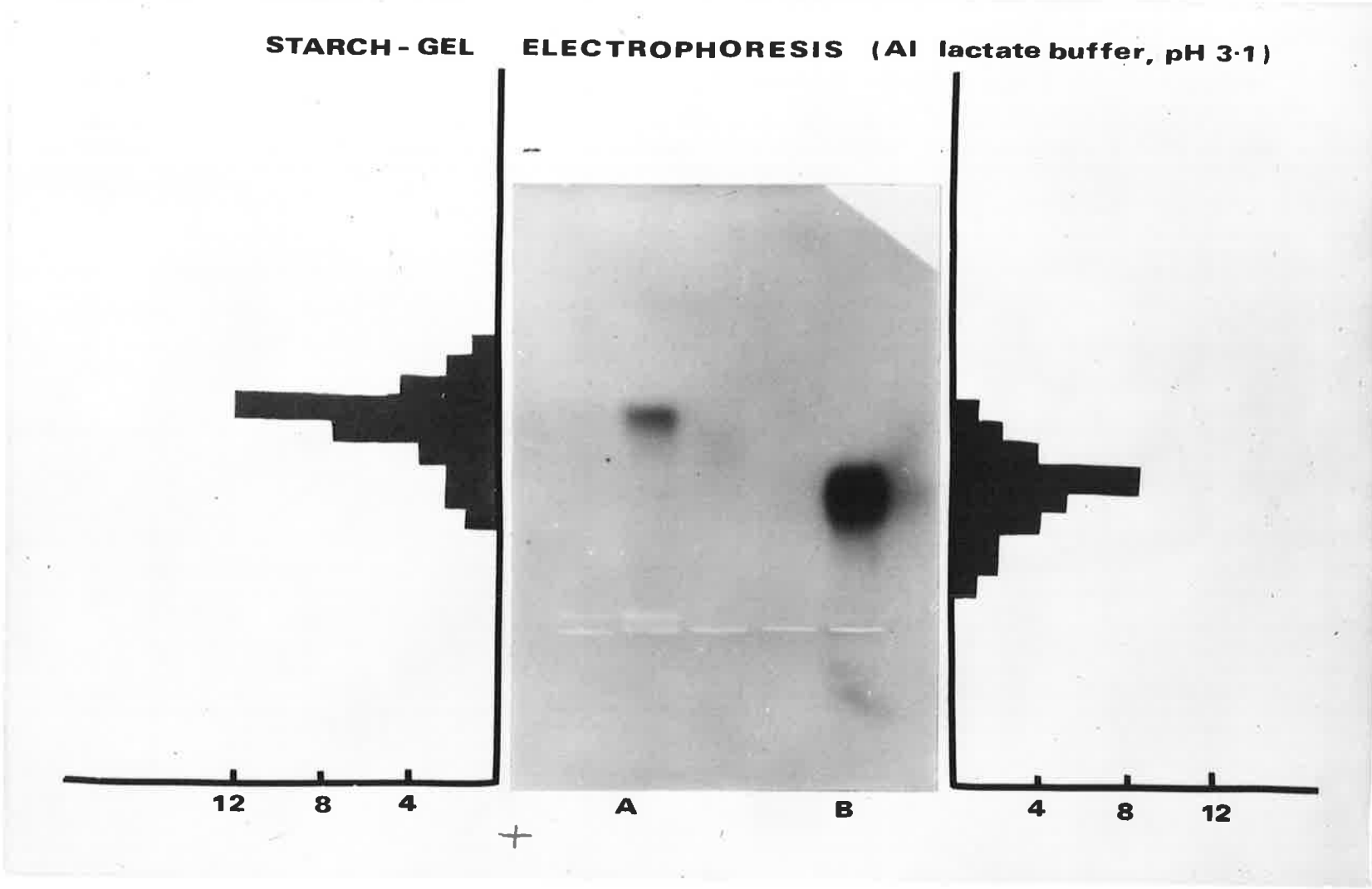
The gel containing 12% w/v starch was prepared in 0.017 M aluminium lactate buffer (ionic strength 0.1, pH 3.1) and 2 M urea. Samples of the enzyme Fractions F_1 and F_2 (Fractions 4 (a) and 4 (b), respectively of Table 18) were contained in slots prepared in the gel. Electrophoresis was around 3-4 volt/cm for 6 hr.

Half the gel was stained for phosphatase activity with buffered α-naphthyl phosphate and the coupling agent, fast blue (see Section 2.3.1.1). Protein was stained with 0.015% nigrosine for 12 hr. Excess stain was washed out using a mixture of acetic acid/methanol/water (v/v, 1/5/1).

The scale in the figure indicates the μgm P_i liberated from incubated sections of the gel (0.5 x 1 cm) with 0.0001 M sodium phytate as the substrate.

Lane A = Enzyme Fraction F_2 (Fraction 4 (b), Table 18)

Lane B = Enzyme Fraction F_1 (Fraction 4 (a), Table 18).


STARCH - GEL ELECTROPHORESIS (Al lactate buffer, pH 3·1)
−
12
8
4
A
B
4
8
12
+

simple phosphomonoesters or a mixture of enzymes. No stain specific for phytase has been found. However, dissected sections of the gel, when tested for phytase activity, showed that enzyme activity corresponded with the α-naphthyl phosphatase activity (Figure 33). The mobility of the phosphatase pattern of Fraction F_1 and F_2 relative to horse heart cytochrome *c* on starch gel, using aluminium lactate buffer, pH 3.1 (see Section 2.3.1.1) were 0.47 and 0.58 respectively. The mobilities of the proteins in F_1 and F_2 enzymes stained with 0.015% nigrosine (see Section 2.3.1.1) again with respect to horse heart cytochrome *c* were 0.61, 0.74 for Fraction F_1 and 0.46, 0.54 and 0.76 for the F_2 fraction respectively, which confirms the presence of much inactive protein.

Further resolution by starch gel electrophoresis using 0.41 M histidine-citrate buffer (pH 7), showed that F_1 and F_2 contained 2-3 bands of phosphatases. These species moved towards the positive electrode. This confirmed that F_1 and F_2, i.e. Fractions 4(a) and 4(b), Table 18, were still relatively impure.

3.2.3.3 Apparent molecular weight determination

The apparent molecular weights of the partially purified Fractions F_1 and F_2, Table 18, were determined by a Sephadex column technique (see Section 2.3.2.1). The distribution coefficients of the F_1 and F_2 enzyme were compared with those of reference proteins of known molecular weights and with phytases from microorganisms (see Section 3.1.5.2). This was shown in Figure 14.

Bran phytase F_1 had a K_d value of 0.25 (see Figure 14), which was indistinguishable from the K_d of Fraction F_2. This was equivalent to a molecular weight of 47,000 ± 2,000 indicating that the enzymes were relatively small proteins. When enzyme Fractions F_1 and F_2 were combined together and re-examined on the Sephadex G-100 column (as described before) only one peak of enzyme activity (equivalent to one distribution coefficient value) was obtained. Again a similar K_d value of 0.25 was recorded for the combined fractions as for the individual fraction either F_1 or F_2.

3.2.3.4 Stability

The ammonium sulphate fraction (Fraction 2, Table 18) was more stable than the partially purified F_1 preparation (Fraction 4(a), Table 18). The former preparation could be stored at -15° for a period of 9 months without loss in activity. The partially purified enzyme F_1, however, retained only 40% of the initial activity after 6 months at -15°. F_1 had an optimum temperature at 50° (Figure 34). A loss of about 10% in activity was recorded after heating the enzyme for 10 min at 60°. All enzyme activity was destroyed within 10 min above 80°.

The partially purified enzyme F_2 (Fraction 4(b), Table 18) was unstable at dilute concentrations. The addition of a surface activator, e.g. Triton X-100, did not restore activity. However, preparations could be concentrated by freeze-drying and stored at -15° for 6 months without substantial loss in phytase activity. The enzyme was stable at 37° but was completely inactivated after 2 min

Figure 34.

Optimum temperature for phytase Fraction F_1

Phytase Fraction F_1 was incubated for various temperatures with a reaction mixture of composition as given in the text (Section 2.4.1). The enzyme activity assay was as described in the same Section.

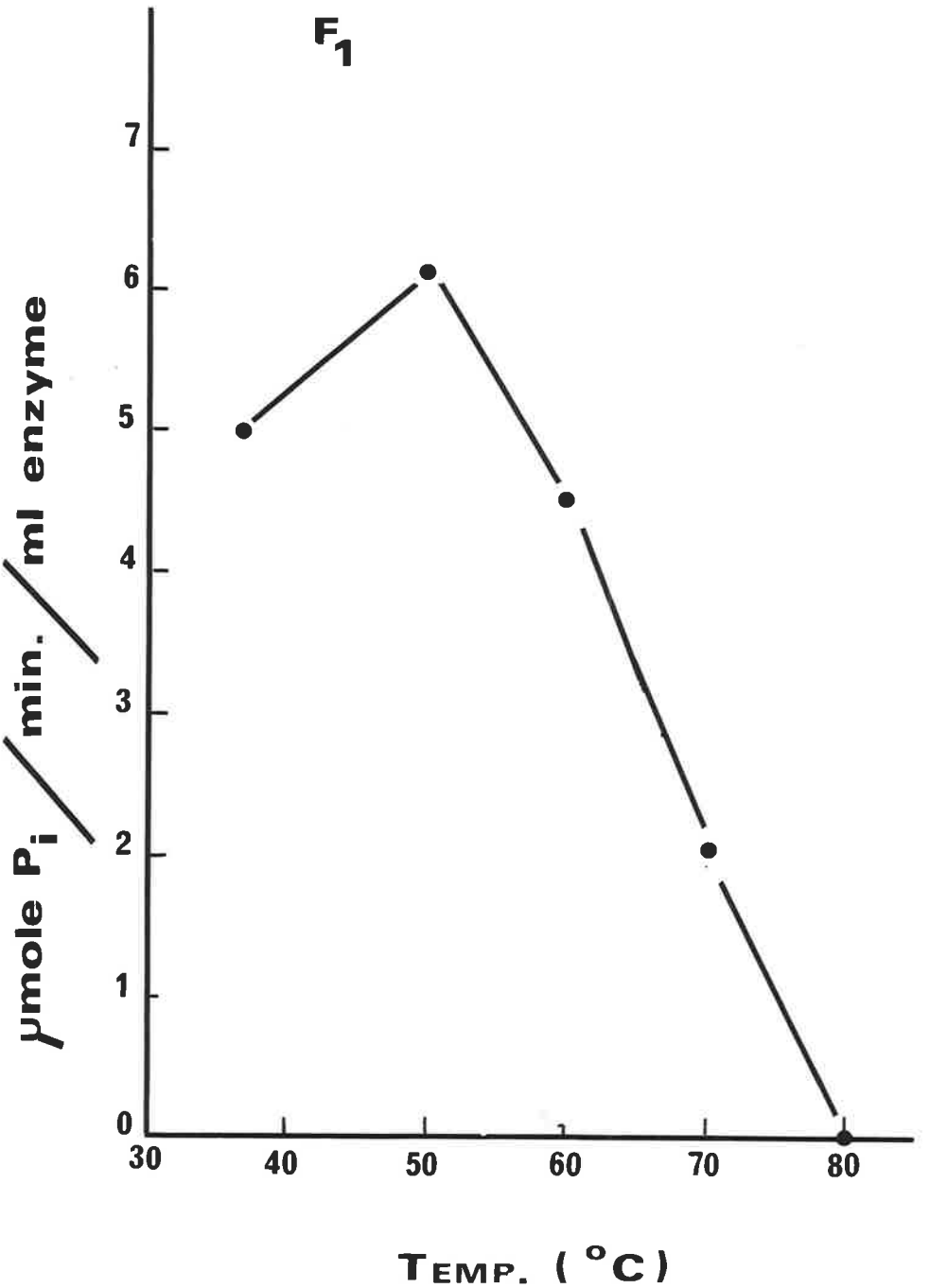
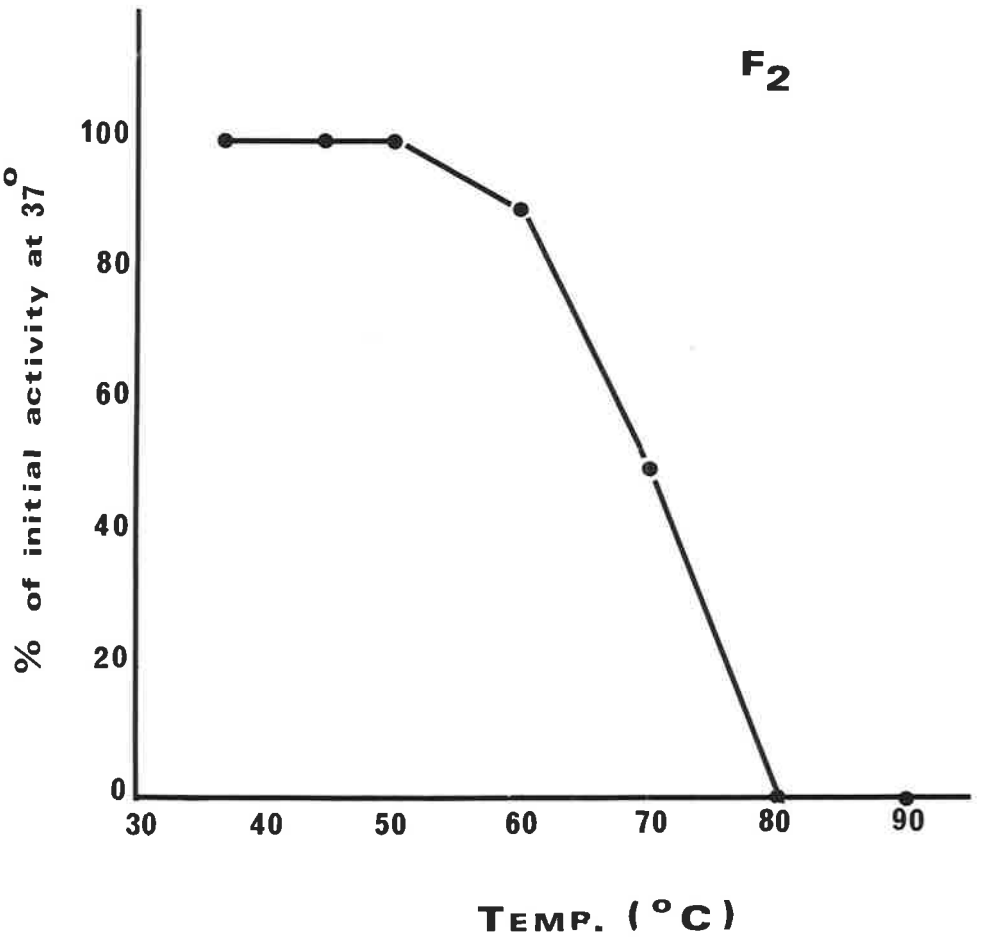

F1
7
6
5
4
3
2
1
0
μmole Pi / min. / ml enzyme
30
40
50
60
70
80
TEMP. (°C)

Figure 35.

Effect of various temperatures on the activity of phytase F_2

Aliquots of enzyme Fraction F_2 were heated for 10 min, at various temperatures as listed in Figure 35. Samples were then removed and normal phytase activity was carried out at 37° as described in Section 2.4.1. The residual phytase activity was expressed as a percentage of the activity at 37°.


F2
% of initial activity at 37°
100
80
60
40
20
0
30
40
50
60
70
80
90
TEMP. (°C)

at 80° (Figure 35). The optimum temperature for enzyme activity of Fraction F_2 was around 50°, similar to that for phytase Fraction F_1.

3.2.3.5 Effect of time of incubation

The effect of varying incubation times on the enzymic hydrolysis of phytic acid (*myo*-inositol hexaphosphate) for Fractions F_1 and F_2 is shown in Figures 36 (a) and 36 (b) respectively. Dephosphorylation of phytate was linear with time for the first 60 min for both Fractions F_1 and F_2. These linear relationships demonstrate that the enzyme fractions were stable in the reaction mixture at 37°. As shown in Figure 36 (a), Fraction F_1 showed a change in P_i release which was linear from 60 to 120 min. This suggests the possible utilisation of a second substrate, e.g. *myo*-inositol pentaphosphate.

3.2.3.6 Effect of pH

The effect of pH on the activity of phytase Fractions F_1 and F_2 is shown in Figure 37. A broad pH profile with a maximum activity at 7.0 was obtained for F_2 using phytate as the substrate. Variation in activity over the pH range of 4 to 8 was greater for phytase F_1 which showed a sharp optimum at pH 5.0.

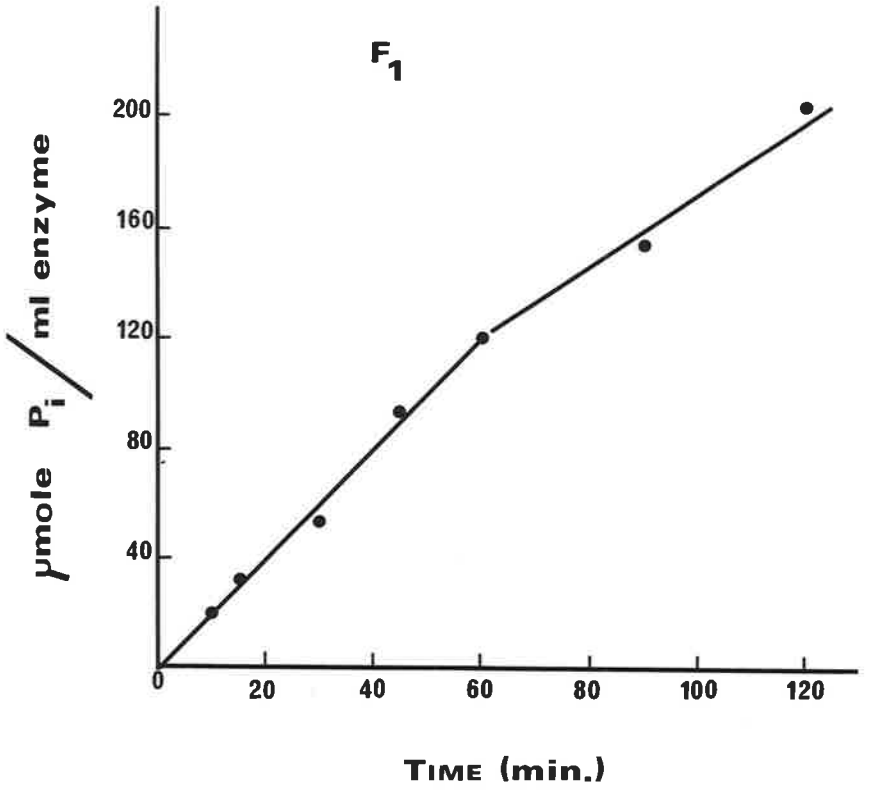
3.2.3.7 Effect of cations and various reagents

The effects of several mono- and divalent cations on the enzyme activities is shown in Table 19. There were no

Figure 36.

Effect of incubation times on the production of P_1 of bran phytase Fractions F_1 and F_2

Phytase Fraction F_1 and F_2 respectively were incubated for various times shown in Figure 36a and b with phytate as the substrate (Section 2.4.1). The liberation of inorganic phosphate was determined using the ascorbic method of Watanabe and Olsen, 1965.


a
F1
μmole Pi / ml enzyme
200
160
120
80
40
0
20
40
60
80
100
120
TIME (min.)

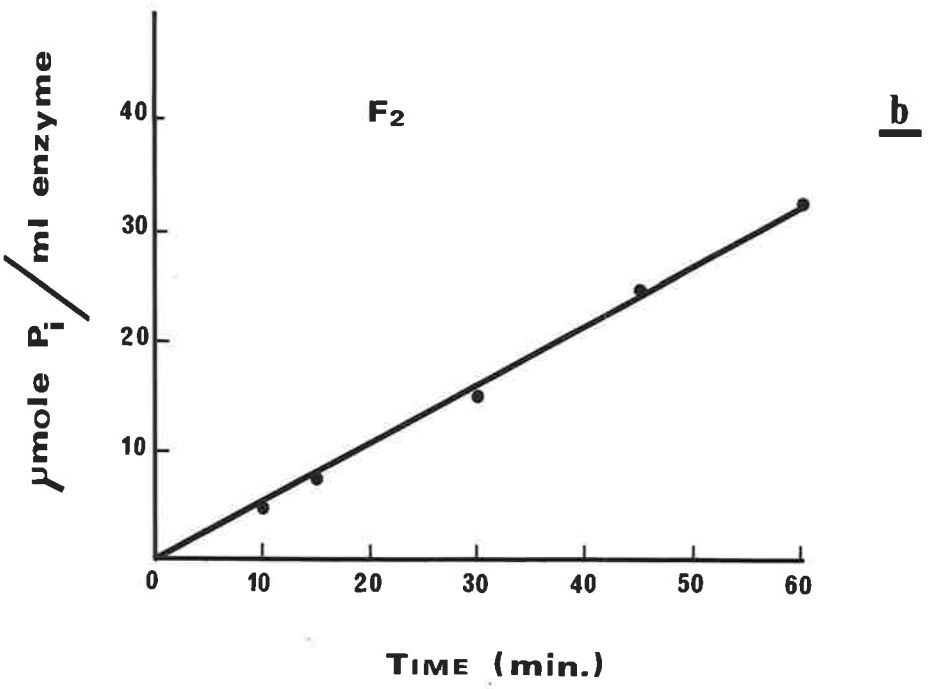

b
F2
μmole Pi / ml enzyme
40
30
20
10
0
10
20
30
40
50
60
TIME (min.)

Figure 37.

Influence of pH on phytase activity.

Phytase activity at various pH values (as indicated in Figure 37) was determined with sodium phytate as the substrate. 0.05 M sodium acetate buffer pH 4 to 6 and Tris-HCl pH 7 to 8 were used. Details of the assay are described in the text.

●——● Phytase Fraction F_1

▲——▲ Phytase Fraction F_2

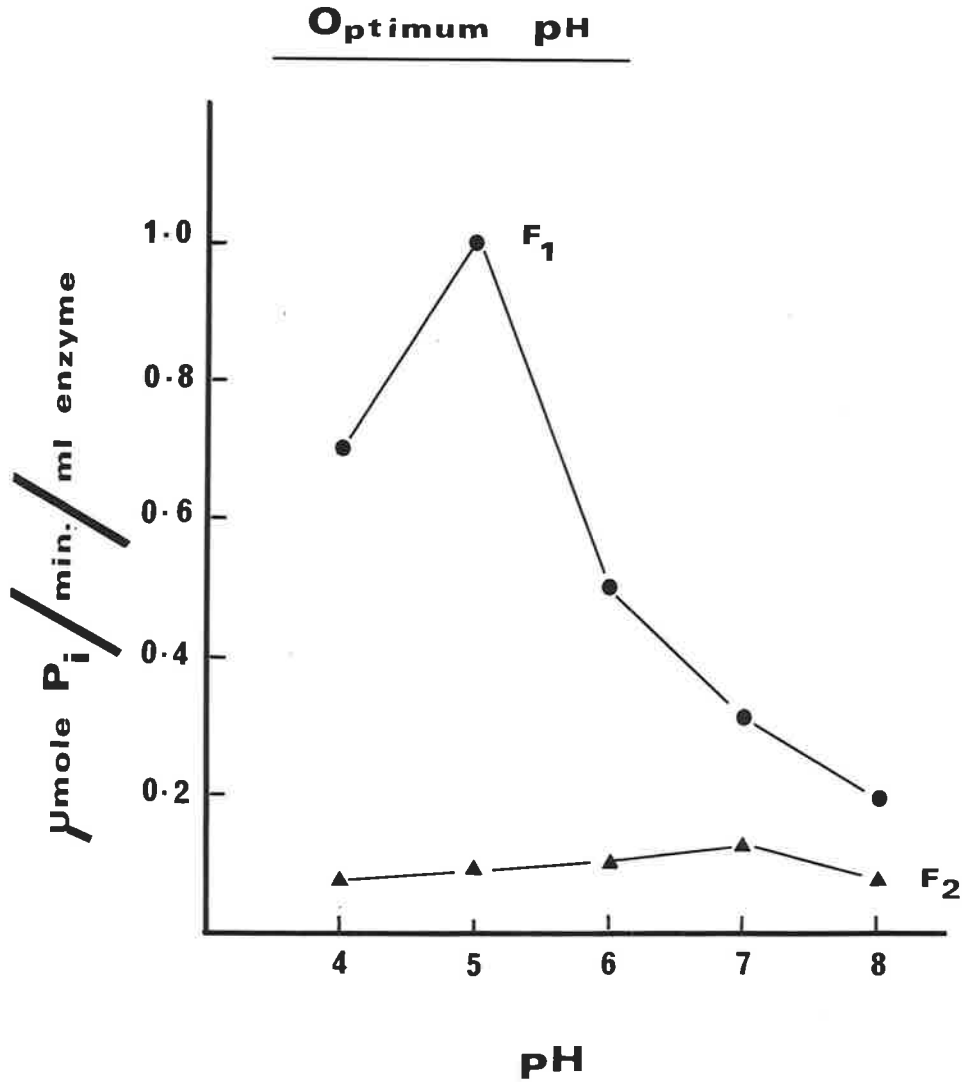

Optimum pH
1·0
0·8
0·6
0·4
0·2
μmole Pi / min. / ml enzyme
F1
F2
4
5
6
7
8
pH

Table 19. Cations and phytase activity

Cation (2.5×10^{-3}M)	Percentage activity of enzyme Fractions F_1	F_2
No addition	100	100
NaCl	100	100
$NaNO_3$	100	100
NaN_3	100	100
KCl	100	100
KNO_3	100	100
$CaCl_2$	110	105
$MgCl_2$	100	100
$MgSO_4$	100	100
$MnCl_2$	100	100
$CdCl_2$	100	90
$HgCl_2$	60	31
$ZnCl_2$	48	42
$CuSO_4$	45	40
$AgNO_3$	66	40
$FeCl_3$	80	75
$AlCl_3$	90	90
$NiSO_4$	90	90

The enzyme was incubated with the cation for 5 min before normal reaction time was taken. The rate of enzymic hydrolysis was expressed as percentage of the activity of phytase without the addition of the cation, taken as 100%, which was equivalent to 5 µmole P_i/min/ml enzyme and 0.4 µmole P_i/min/ml enzyme for phytase Fractions F_1 & F_2 respectively.

significant increases in phytase activity when magnesium ions were used. Addition of heavy divalent ions resulted in various degrees of inhibition, e.g. copper and zinc reduced enzyme activity by half.

Chelating agents such as Na, EDTA, KCN, oxalate, citrate and 2,2' dipyridyl were without effect and this further supports the evidence (Nagai and Funahashi, 1962) that metallic cations are not necessary for phytase activity.

The activity of the partially purified Fractions F_1 and F_2 were not affected by adding a sulphydryl inhibitor such as p-chloromercuri-benzoate. Glutathione, mercaptoethanol, cysteine and cystine and alkylating agents (see Table 20) were also without effect. It is therefore unlikely that sulphydryl groups are involved at the active site of the phytase.

3.2.3.8 Effect of phosphatase inhibitors

The effects of various phosphatase inhibitors are given in Table 21 for F_1 and F_2 respectively. F_1 was inhibited by phosphate whereas F_2 was not affected by phosphate even up to a concentration of 10×10^{-3} M (Table 21). On a molar basis fluoride was a more effective inhibitor of both Fractions F_1 and F_2 than was (+) tartrate. The effects of fluoride and (+) tartrate on phytase Fraction F_1 are presented in Figure 38 (a). When plotted as the double reciprocal plots (Lineweaver and Burk, 1934) the effects of fluoride and (+) tartrate on the activity of F_1 were non-competitive inhibitory (Figure 38 (b)). Similarly it was observed that the F_2

Table 20. Effect of inhibitors and various reagents on the activity of enzyme Fractions F_1 and F_2

Compound	Final concentration (M)	Percentage phytase activity F_1	Percentage phytase activity F_2
No addition	-	100	100
Fluoride	0.5×10^{-3}	61	61
	1.0×10^{-3}	20	20
(+) tartrate	0.5×10^{-3}	100	96
	1.0×10^{-3}	76	85
Oxalate	0.5×10^{-3}	95	95
Citrate		98	96
2,2 dipyridyl		100	100
EDTA		100	100
KCN		96	100
PCMB	2.5×10^{-3}	100	101
Glutathione		100	100
Glutathione + PCMB		96	98
L-cystine		101	96
Mercaptoethanol		96	96
Iodoacetamide	0.5×10^{-3}	100	100
Iodoacetate		100	100
Iodosobenzoate		100	100

The enzyme was preincubated with the buffered compound at pH 5.0 for 5 min before normal reaction time was started. Phytase activity with or without the compound was assayed as in Section 2.4.1. Normal enzymic activity was taken as 100%; all other activity was expressed as a percentage of this activity.

Table 21. Effect of phosphate on the activity of enzyme Fractions F_1 and F_2

Phosphate concentration (final concentration in M)	Percentage activity*	
	F_1	F_2
Without phosphate	100	100
0.4×10^{-3}	83	100
1.0×10^{-3}	68	100
2.0×10^{-3}	50	100
5.0×10^{-3}	25	100
10.0×10^{-3}	0	100

*Expressed as percentage of the rate of reaction in a control incubation which was 1.4 µmole P_i liberated/min/assay for F_1 and 0.5 µmole P_i liberated/min/assay for F_2.

Phytase activity was determined as described in Section 2.4.1. The reaction mixture was as given in Section 2.4.1 but with or without phosphate added as shown in this Table.

Figure 38.

Effect of inhibitors on the activity of enzyme Fraction F_1

(a) The enzyme was incubated with the buffered inhibitor at various concentrations of substrate at pH 5.0 as shown in this Figure. Phytase activity was determined as described in Section 2.4.1 and the rate of dephosphorylation of the substrate *myo*-inositol hexaphosphate (phytic acid) was plotted against concentrations of substrate.

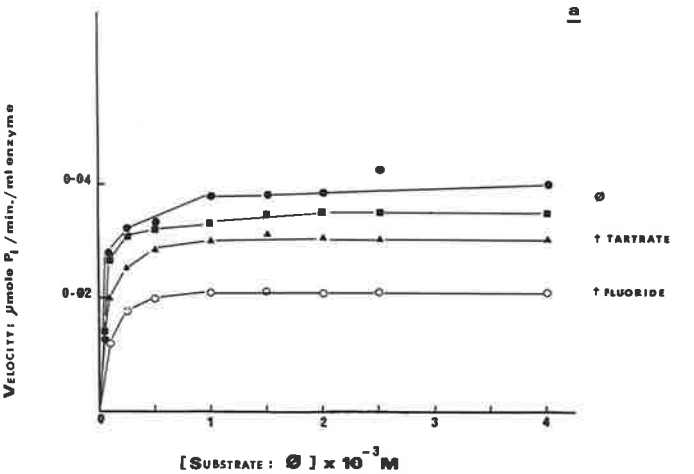
(b) The results from Figure 38(a) were plotted as the double reciprocal plots of the type described by Lineweaver and Burk, 1934.

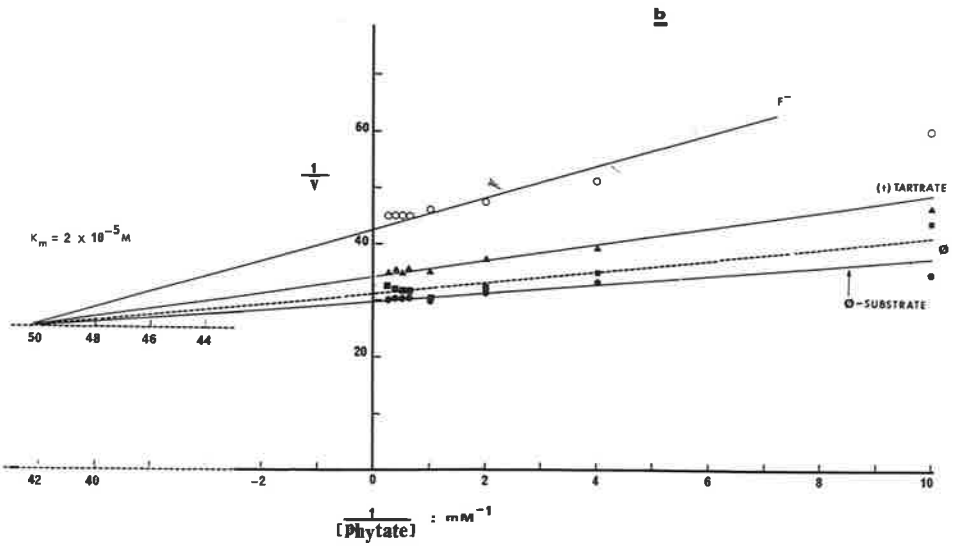
●——● without inhibitor

■------■ with 1×10^{-3} M excess phytate

○——○ with 5×10^{-4} M fluoride

▲——▲ with 1×10^{-3} M (+) tartrate


a
VELOCITY: μmole P_i / min./ml enzyme
0·04
0·02
0
1
2
3
4
Ø
† TARTRATE
† FLUORIDE
[SUBSTRATE: Ø] x 10^{-3} M


b
$\frac{1}{V}$
$K_m = 2 \times 10^{-5}$ M
60
40
20
50
48
46
44
42
40
−2
0
2
4
6
8
10
F^-
(†) TARTRATE
Ø
Ø-SUBSTRATE
$\frac{1}{[Phytate]}$: mM^{-1}

enzyme also showed non-competitive inhibition with these substances: fluoride and (+) tartrate.

3.2.3.9 Michaelis constants

The results in Figure 38 (a) of the dephosphorylation of *myo*-inositol hexaphosphate by Fraction F_1 when the concentration of the substrate was varied are given as a double reciprocal plot in Figure 38 (b). By extrapolating the straight-line portion of the curve representing the lower concentrations of phytate the apparent K_m value of the enzyme F_1 for phytate (see Section 2.4.3) is 2×10^{-5} M.

Figure 39 shows a similar double reciprocal plot (Lineweaver and Burk, 1934) of the enzymic hydrolysis of phytate by phytase Fraction F_2. At higher concentrations of the substrate the rate of reaction was reduced which suggests a substrate inhibition (Dixon and Webb, 1964). The apparent K_m value calculated and as described in Section 2.4.3 is 1.82×10^{-4} M.

3.2.3.10 Substrate specificity

The partially purified Fractions F_1 and F_2 were allowed to react with the phosphoesters (as listed in Table 22) as substrates. F_1 and F_2 showed a potent inorganic pyrophosphatase activity in contrast to the lack of activity with NAD. The fractions did not contain glucose-1-phosphatase activity. The slightly enhanced activity which was observed with ATP (Table 22) may result

Figure 39.

Michaelis constant of bran phytase F_2

Double reciprocal plots (Lineweaver and Burk, 1934) of the rate of dephosphorylation of phytate versus various concentrations of sodium phytate are as shown in this Figure. Details of enzyme assay are given in Section 2.4.1 .

Figure 39 shows the Lineweaver and Burk (1934) plot for enzyme Fraction F_2.

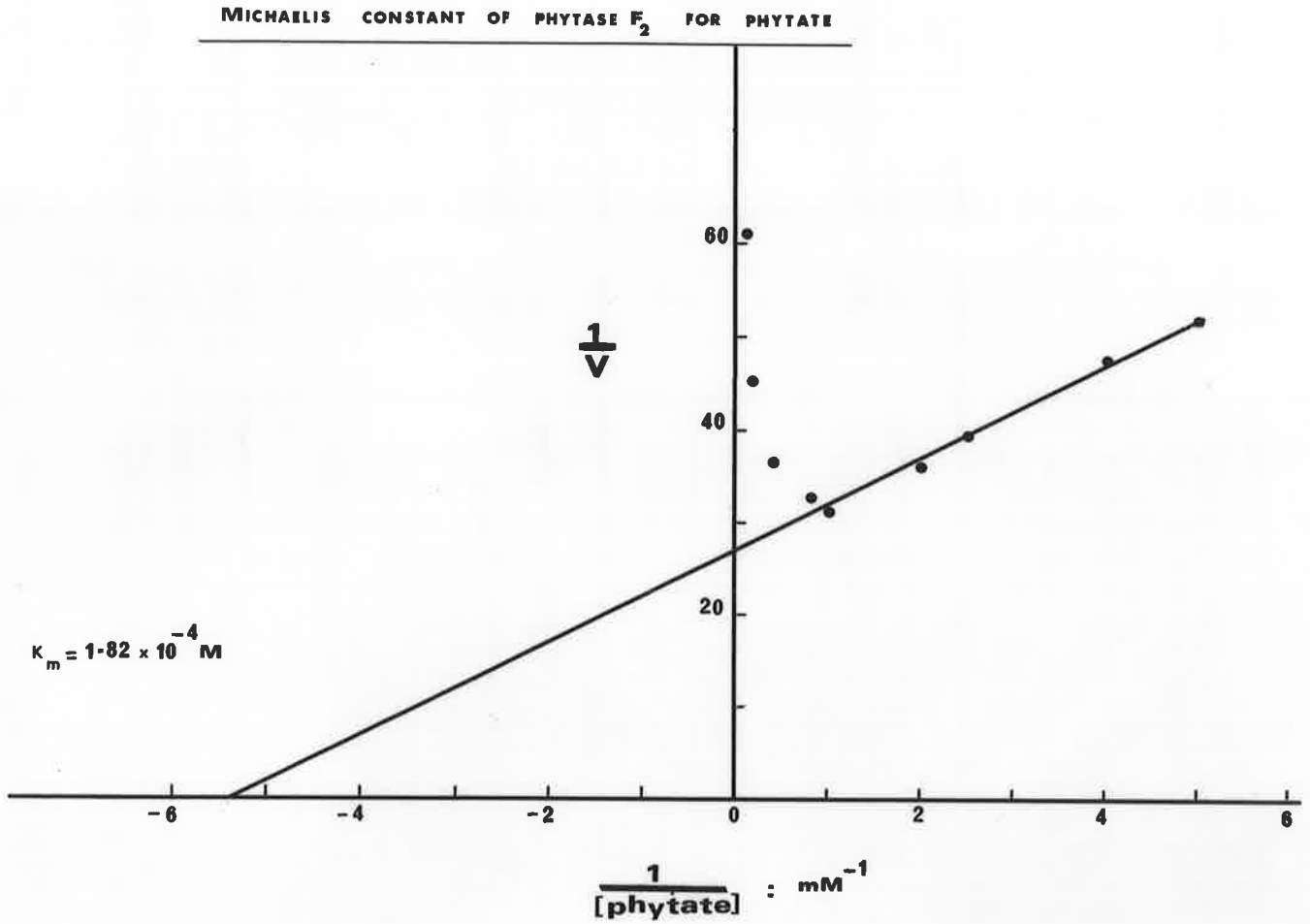

MICHAELIS CONSTANT OF PHYTASE F2 FOR PHYTATE
60
40
20
1/V
Km = 1·82 x 10^-4 M
-6
-4
-2
0
2
4
6
1/[phytate] : mM^-1

Table 22. Substrate specificity study

Substrate concentration (1×10^{-2} M)	Percentage activity of phytase	
	F_1	F_2
myo-inositol hexaphosphate	100	100
α-glycerophosphate	80	70
β-glycerophosphate	60	100
inorganic pyrophosphate	350	200
phenyl phosphate	100	80
glucose-1-phosphate	0	0
glucose-6-phosphate	25	50
adenosine triphosphate	160	200
NAD	0	0
α-naphthyl phosphate	80	90

The release of inorganic phosphate from various compounds as listed in this Table was determined with the reaction mixture as given in Section 2.4.1. The activity with inositol hexaphosphate as the substrate was given as 100% which was 2 μmole P_i/min/ml enzyme for Fraction F_1 and 0.4 μmole P_i/min/ml enzyme for Fraction F_2. All activity was expressed as a percentage of the activity with inositol hexaphosphate.

from nucleotide enzymes catalysing the hydrolysis of ATP to ADP, AMP and P_i.

3.2.4 Products of the enzymic hydrolysis of *myo*-inositol hexaphosphate

The *myo*-inositol polyphosphate intermediates in the F_1 and F_2 hydrolysates are studied in this Section.

The electrophoretic and paper chromatographic mobilities relative to inorganic pyrophosphate of the detectable inositol phosphate intermediates of enzyme Fractions F_1 and F_2 are listed in Table 23. In this table each observable spot is given a name in which the first two symbols (F_1 or F_2) denote the enzyme fraction; the second two symbols (P_n) denote the degree of phosphorylation of the inositol ring and the last letter is used to differentiate isomeric polyphosphates in decreasing order of mobility. It should be noted that the pentaphosphate F_2P_5C and the tetraphosphate F_1P_4A are unique to each pathway and the basis for the electrophoretic assignments of degradation pathway, and that the existence of three isomeric pentaphosphates such as F_2P_5A, F_2P_5B and F_2P_5C are peculiar to the F_2 route. The electrophoretogram (see Section 2.3.1.2) representing a 10-20% conversion of phytate phosphorus to inorganic phosphorus of the F_1 and F_2 enzymes is as shown in Figure 40. The same figure shows the partial degradation of *myo*-inositol hexaphosphate of the crude phytase (Fraction 1, Table 18) and that of *Neurospora crassa* (see Section 3.1.6). The pattern for F_1 could not be easily

Table 23. Electrophoretic and chromatographic mobilities of inositol polyphosphates produced by enzyme Fractions F_1 and F_2 (P_i release = 50% substrate P_t)

Substance	Electrophoretic mobility (M_{PP_i}) 0.1M oxalate, pH 1.5, 1 hr 1500 volt	Chromatographic mobility (R_{PP_i}) isopropanol/ammonia/water v/v, 60/10/30, 17 hr
PP_i	1.00	1.00
F_1P_6	1.27	0.20
F_1P_5	1.14	0.74
F_1P_4A	1.04	0.77
F_1P_4B	0.99	0.77
F_1P_3	0.86	1.01
F_1P_2	0.71	1.55
F_1P_1	0.48	1.86
F_2P_6	1.27	0.20
F_2P_5A	1.33	0.50
F_2P_5B	1.14	0.74
F_2P_5C	1.08	0.52
F_2P_4	0.99	0.77
F_2P_3	0.86	0.82
F_2P_2	0.71	1.55
F_2P_1	0.48	1.86
P_i	0.30	1.70

The electrophoretic mobilities (M_{PP_i}) using 0.1 M oxalate buffer at pH 1.5 and paper chromatographic mobilities (R_{PP_i}) using isopropanol/ammonia/water, v/v 60/10/30 were calculated with reference to inorganic pyrophosphate.

The first two symbols (F_1 or F_2) denote the enzyme fraction, the second two symbols (P_n) denote the degree of phosphorylation of the inositol ring and the last letter is used to differentiate isomeric polyphosphates in decreasing order of electrophoretic mobility.

The retardation in R_{PP_i} of $F_{1,2}P_6$ may be due to overloading of the compound under examination.

Figure 40.

Electrophoretogram of the enzymic degradation of phytic acid

The incubation mixture contained in a total of 1 ml, 0.02 M sodium phytate (pH 5.0), 0.25 M sodium acetate buffer, pH 5.0, enzyme solution (0.2-0.5 ml) and water. The mixture was incubated at 37° until the percentage of total phosphorus liberated as inorganic phosphorus was between 10 and 20%. 0.02 ml of the reaction mixture was examined by high voltage paper electrophoresis (Tate, 1968).

Electrophoretic conditions:- 0.1 M oxalate buffer, pH 1.5
1 hr, 1,500 volts

Phosphate compounds were developed by the phosphomolybdate reagent of Harrap, 1960.

Lane 1 = crude phytase from bran

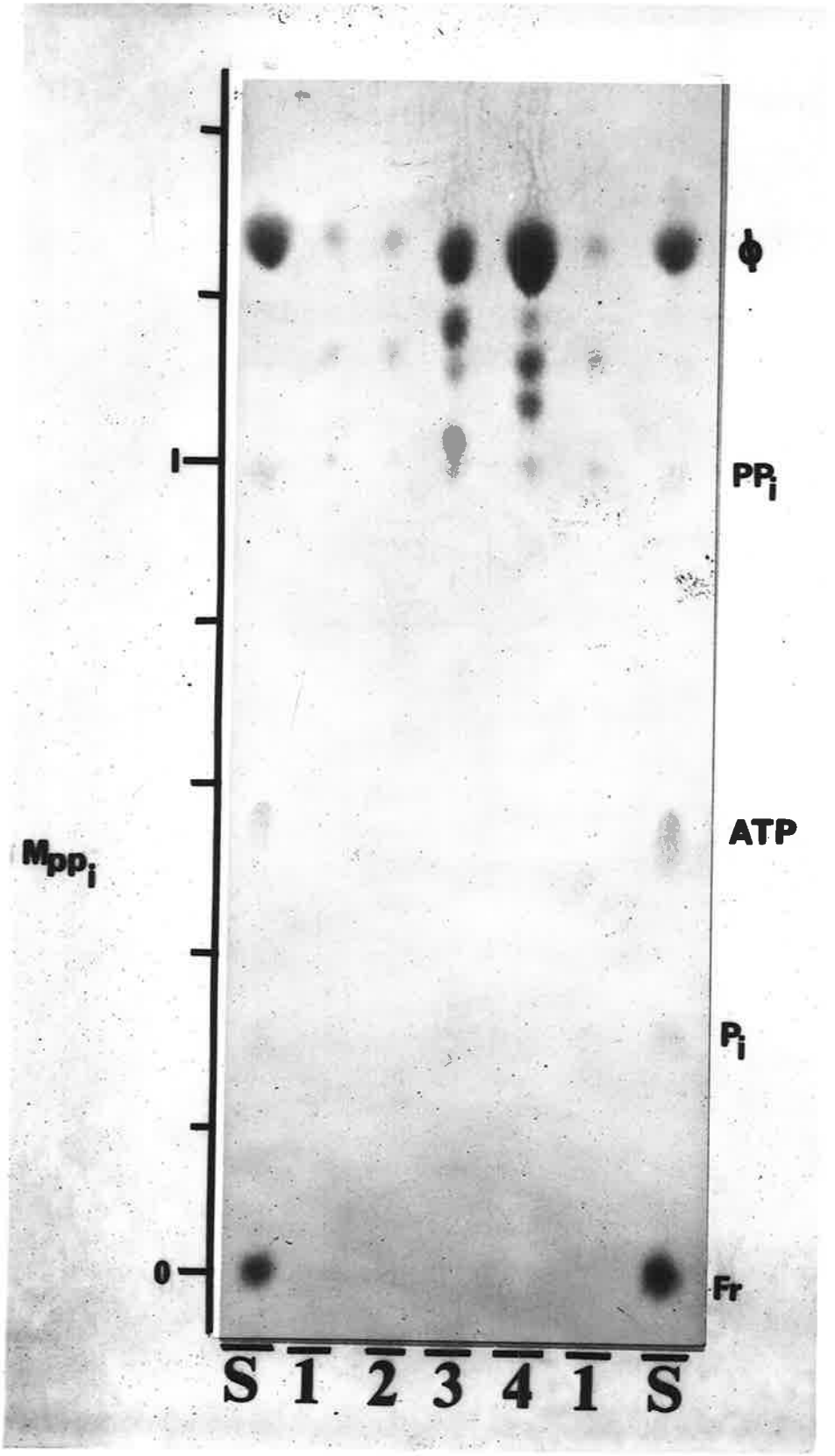
Lane 2 = bran phytase Fraction F_1

Lane 3 = *Neurospora crassa* phytase

Lane 4 = bran phytase Fraction F_2

Lane S = standard compounds with ϕ, *myo*-inositol hexaphosphate; PP_1, inorganic pyrophosphate; ATP, adenosine triphosphate; P_i, inorganic phosphate and Fr, fructose.

M_{PP_i} = mobility of compound with reference to PP_i.


Mpp$_i$
I
0
ϕ
PP$_i$
ATP
P$_i$
Fr
S 1 2 3 4 1 S

differentiated from the pattern of the crude enzyme (also see Section 3.2.1.2). The markedly different pattern of F_2 was obscured in the crude enzyme pattern but the products formed by this enzyme account for some of the observations made by Tomlinson and Ballou, (1962) and are presented in Section 3.2.4.1. The pattern for the fungal (*Neurospora crassa*) phytase has already been discussed in Section 3.1.6.1. The *Neurospora crassa* data are presented in Figure 40 as a third example of a phytase degradation pattern which is discernibly different at the pentaphosphate stage.

3.2.4.1 Structural assignments for the intermediates in the F_2 hydrolysate

Table 24 lists the electrophoretic mobilities, structural assignments and molecular rotations at pH 1 and pH 11 of the *myo*-inositol polyphosphates isolated from the action of phytase Fraction F_2 on *myo*-inositol hexaphosphate. Electrophoresis in 0.1 M oxalate buffer (pH 1.5, see Section 2.3.1.2) gave a quantitative determination of the eight separable organic phosphates including the initial substrate, *myo*-inositol hexaphosphate. By the combined techniques of ion-exchange chromatography (see Section 2.3.2.3) and high voltage paper electrophoresis (Section 2.3.2.1) it was possible to isolate sufficient of the electrophoretically homogeneous compounds to assign their structural configuration as follows:-

(a) *Myo*-inositol pentaphosphates

Three pentaphosphates F_2P_5A, F_2P_5B and F_2P_5C (Table 24)

Table 24. Products of the hydrolysis of *myo*-inositol hexaphosphate by bran phytase Fraction F_2

Compound	Phosphorus: Inositol ratio	Structures of main component	M_{PP_i} 0.1M oxalate buffer pH 1.5	Molecular rotation $[M]_D$ pH 1	pH 11
F_2P_1A*	1	I	0.48	-	-
F_2P_1B	1	I	0.48	-	-
F_2P_2	2	II	0.71	-15±1	-15±1
F_2P_3	3	IIIb	0.86	-14±14	0±15
F_2P_4	4	IVb	0.99	+30±15	-70±17
F_2P_5C	5	Vc	1.06	0±13	0±19
F_2P_5B	5	Vb	1.14	+9±4	+27±5
F_2P_5A	5	Va	1.23	+18±36	+15±30

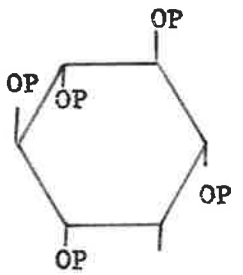
* This nomenclature was explained in Section 3.2.4.

Myo-inositol polyphosphate products obtained in the F_2 hydrolysate were used to determine for their phosphorus to inositol ratio (Section 2.4.10.1); electrophoretic mobility (Section 2.4.5) and their molecular rotation in acid and alkaline medium (Section 2.4.10.2).

The symbols used in the naming of the compounds are as given in Section 3.2.4. The structures of the main components are given in the text.

were separated from the F_2 hydrolysates. F_2P_5A was identified by its mobility with moving paper electrophoresis (Tate, 1968), its isomerisation behaviour under *cis* migration conditions, its lack of optical activity, and finally its enzymic hydrolysis pattern indicated it as the somewhat unexpected *myo*-inositol pentaphosphate (Structure Va, below).

Va.



Myo-inositol 1,3,4,5,6 pentaphosphate

The enzymic hydrolysis study of F_2P_5A which led to the assignment of *myo*-inositol 1,3,4,5,6-pentaphosphate is as follows:- Hydrolysis of known pentaphosphate would give tetraphosphates of higher or lower electrophoretic mobilities. F_2P_5A was subjected to a 30-40% enzymic (Fraction 1, Table 18) degradation and its pattern was compared with that of a similar enzymic hydrolysis of chicken-blood pentaphosphate (*myo*-inositol 1,3,4,5,6-pentaphosphate; Johnson and Tate, 1969*a*) as well as that of *Neurospora crassa* pentaphosphate (D-*myo*-inositol 1,2,4,5,6-pentaphosphate). Figure 41 shows that the products obtained in the biodegradation of fraction F_2P_5A correspond to those of the chicken-blood pentaphosphate (D-*myo*-inositol 1,3,4,5,6-pentaphosphate) and not those of the D-*myo*-inositol 1,2,4,5,6-pentaphosphate pattern. A tetraphosphate of electrophoretic mobility (M_{PP_i} = 1.04) was the major component formed from F_2P_5A although it contained less than 20%

Figure 41.

Enzymic hydrolysis of *myo*-inositol pentaphosphate

Myo-inositol pentaphosphates were subjected to about 40% degradation by bran phytase (F_1) in a buffered condition as given in Section 2.4.1. The enzymic products were examined by high voltage paper electrophoresis (Tate, 1968, Section 2.3.1) and developed using the phosphomolybdate reagent (Harrap, 1960).

Lane 1 = chicken-blood *myo*-inositol 1,3,4,5,6-pentaphosphate

Lane 2 = F_2P_5A (pentaphosphate isolated from F_2 hydrolysate)

Lane 3 = D,L *myo*-inositol 1,2,4,5,6-pentaphosphate

Lane S = standard compounds using:-

ϕ = *myo*-inositol hexaphosphate (phytic acid)

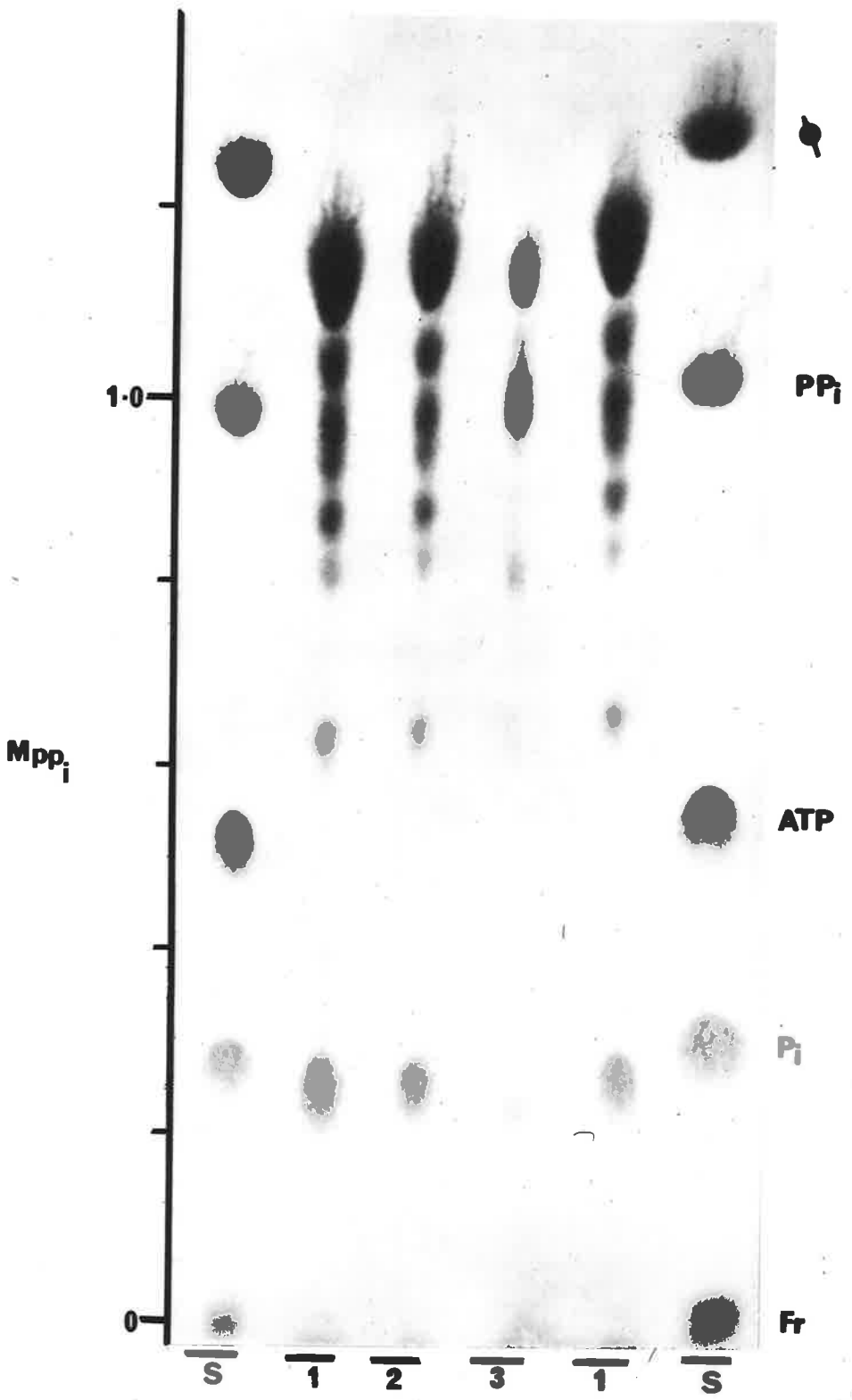
PP_i = inorganic pyrophosphate

ATP = adenosine triphosphate

P_i = inorganic phosphate

Fr = fructose, a non-migratory marker

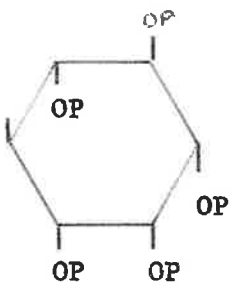
Scale M_{PP_i} = mobility of compounds with reference to PP_i.


1·0
0
Mpp$_i$
PP$_i$
ATP
P$_i$
Fr
S
1
2
3
1
S

of the total phosphorus of the initial substrate. That F_2P_5A is of the structure D-*myo*-inositol 1,3,4,5,6-pentaphosphate is confirmed by moving paper electrophoresis (see Tate, 1968) and was shown to have a similar M_{PP_i} as that of an authentic D-*myo*-inositol 1,3,4,5,6-pentaphosphate sample.

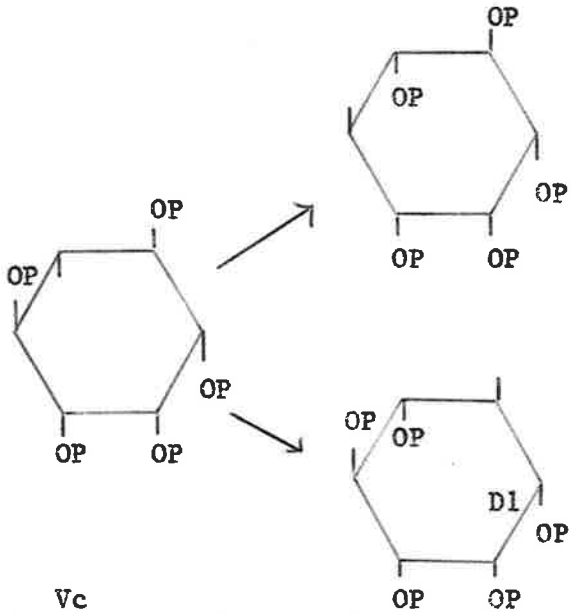
F_2P_5B was the major pentaphosphate component obtained in the crude phytase hydrolysate (see electrophoretogram, Figure 40). The phosphorus to inositol ratio, optical activity and electrophoretic mobility are listed in Table 24 and are in agreement with the properties of L-*myo*-inositol 1,2,3,4,5 pentaphosphate as outlined by Johnson and Tate (1969*b*).

Vb.



L-*myo*-inositol 1,2,3,4,5-pentaphosphate

Compound F_2P_5C (Table 24) was shown to be pentaphosphate Vc by its 5:1 phosphorus to inositol ratio, its electrophoretic mobility and its isomerisation under conditions of *trans* migration to yield pentaphosphates of greater mobility as shown below. These data coupled with its negligible optical activity characterise it as *myo*-inositol 1,2,3,4,6-pentaphosphate Vc.



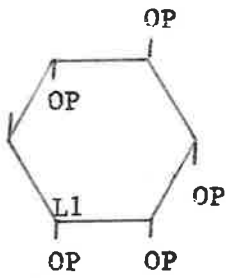
Myo -inositol 1,2,3,5,6-pentaphosphate

M_{PP_i} = 1.12

Vc

Myo-inositol 1,2,3,4,6-pentaphosphate

M_{PP_i} = 1.06



D & L-*myo*-inositol 1,2,3,4,5-pentaphosphate

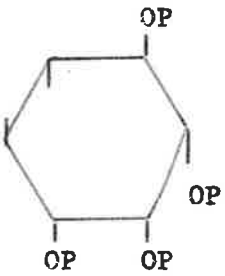
M_{PP_i} = 1.14

(b) *Myo*-inositol tetraphosphate F_2P_4

Only one tetraphosphate component was detected among the F_2 products. Since this tetraphosphate F_2P_4 did not undergo *cis* migration, was optically active (see Table 24), and was obtained by the enzymic (F_2) hydrolysis of either F_2P_5B or F_2P_5C, the major component could only be L-*myo*-inositol 1,2,3,4-tetraphosphate (Structure IVb). Additional evidence in favour of Structure IVb was obtained when it was found that enzyme Fraction F_2 further degraded it to the symmetrical *myo*-inositol 1,2,3-triphosphate (Structure IIIb) as discussed in the next Section.

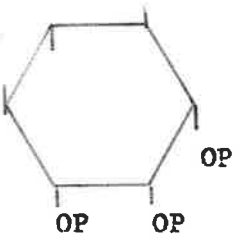
Although Johnson and Tate (1969*a*) were able to oxidise a tetraphosphate with a *cis* glycol, the attempted periodate oxidation with the tetraphosphate from bran and that from *N. crassa* which have *trans* glycols was unsuccessful. Oxidation would only occur when P_i was released to the triphosphate ester, indicating that hydrolysis was also occurring.

IVb.



F_2 →

IIIb.



L-*myo*-inositol 1,2,3,4-tetraphosphate

Myo-inositol 1,2,3-triphosphate

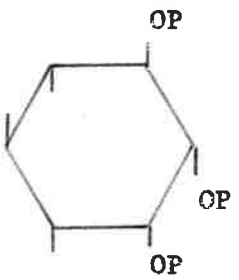
(c) *Myo*-inositol triphosphate F_2P_3

The triphosphate fraction among the F_2 enzyme products had a lower paper chromatographic mobility than the triphosphates IP_3 and F_1P_3 from the *N. crassa* hydrolysate (Section 3.1.6.1) and the F_1 pathway (Table 23) respectively. Periodate oxidation of the F_2P_3 triphosphate (M_{PP_i} = 0.86) yielded a compound of a higher electrophoretic mobility (M_{PP_i} = 0.93) in the oxalate system (Section 2.3.1). The periodate oxidised triphosphate was reduced with the borohydride and dephosphorylated at pH 5 (Section 2.4.10.5) to yield ribitol which was detected by paper chromatography (Solvents 4 and 5, Table 4). That the polyol was ribitol was confirmed by gas chromatography of the

acetate as shown in Figure 24. *Myo*-inositol 1,2,3-triphosphate (Structure IIIb) is the only inositol triphosphate which can yield ribitol from the periodate reaction sequence (Section 2.4.10.5, also see below). These properties coupled with the inositol to phosphorus ratio (1:3) and lack of optical rotation characterise the major component of the triphosphate F_2P_3 as *myo*-inositol 1,2,3-triphosphate (Structure IIIb). D-*myo*-inositol 1,2,6-triphosphate (Structure IIIa) gives arabitol by the periodate reaction sequence as previously discussed and is also shown below:-

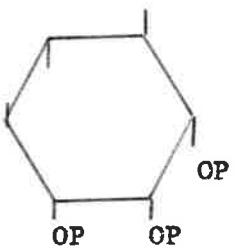
Myo-inositol triphosphates

IIIa.



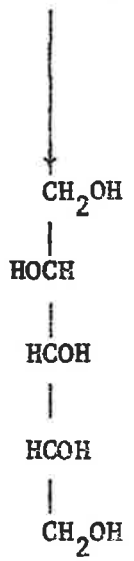
D-*myo*-inositol 1,2,6-triphosphate

IIIb.

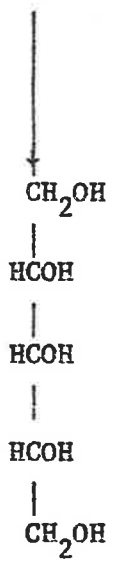


Myo-inositol 1,2,3-triphosphate

(periodate-oxidation-)
(borohydride-reduction)
(and dephosphorylation)
(sequence)



D arabitol

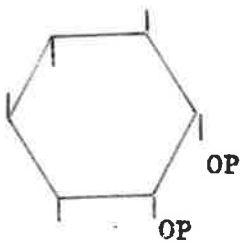


ribitol

(d) *Myo*-inositol diphosphates F_2P_2AB

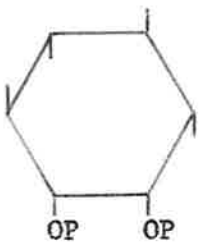
The periodate-oxidation-borohydride-reduction and hydrolysis sequence of the diphosphate fraction gave erythritol as its sugar alcohol. Upon partial alkaline hydrolysis of the diphosphate compound, *myo*-inositol 1 and 2 monophosphates were obtained. These were identified by their electrophoretic and chromatographic mobilities which were consistent with authentic *myo*-inositol 1- and 2-monophosphate (Table 25). The magnitude and sign of the molecular rotations in acid and in alkaline (Table 24) medium suggested it is a mixture of the enantiomorphs having Structures IIa and IIb in which D-*myo*-inositol 1,2-diphosphate (Structure IIa) predominated.

IIa.



D-*myo*-inositol 1,2-diphosphate

IIb.



L-*myo*-inositol 1,2-diphosphate

(e) *Myo*-inositol monophosphate

Myo-inositol 2-monophosphate (Structure Ia) was the only detectable monophosphate obtained by prolonged paper chromatography (using solvent 3, Table 4, Section 2.3.5) and electrophoresis in 0.1 M citrate buffer (pH 5). Further treatment of this monophosphate with enzyme Fraction F_2 yielded free *myo*-inositol which was detected by paper chromatography (see Section 2.3.5) using the silver nitrate

developing method of Anet and Reynolds (1954). The *myo*-inositol 2-monophosphate fraction which appeared in the F_2 route could be distinguished in mobility patterns from that of the isomeric *myo*-inositol 1-monophosphate as shown in Table 25.

(a) (b)

Myo-inositol 2-monophosphate *Myo*-inositol 1-monophosphate

[1]Structure I.

3.2.4.2 Products of the F_1 hydrolysate

The *myo*-inositol polyphosphate intermediates formed in a partial dephosphorylation of *myo*-inositol hexaphosphate by bran phytase have been investigated by Tomlinson and Ballou (1962); Cosgrove (1963*b*, 1969); Johnson and Tate (1969*b*) (see Section 1.3.2). Table 23 lists the electrophoretic mobilities and chromatographic properties of inositol polyphosphates produced by enzyme Fraction F_1 which are similar to the available products isolated by Johnson and Tate (1969*b*) with a crude bran enzyme. Thus the degradation route is electrophoretically and chromatographically indistinguishable from the pathway originally deduced by Tomlinson and Ballou (1962) and was not examined further.

Table 25. Mobilities of *myo*-inositol 1- and 2-monophosphates

Compound	Buffers			Solvent		
	0.1 M oxalate pH 1.5 1 hr	0.1 M citrate pH 5 1 hr	0.1 M formic acid pH 1 2.5 hr	Isopropanol/ammonia/water (v/v) 60/10/10 9-10 hr	80/10/10 48 hr	70/10/20 48 hr
		$[M_{PP_i}]$			$[R_{PP_i}]$	
PP_i	1.00	1.00	1.00	1.00	1.00	1.00
P_i	0.31	0.73	0.56	1.6	2.14	1.58
IP_i (2P)*	0.48	0.73	0.65	1.89	2.18	1.97
IP_1 (1P)*	0.48	0.48	0.58	1.68	1.77	1.39
NP_1 a	0.48	0.73	0.65	1.89	2.18	1.97
NP_1 b	0.48	0.48	0.58	1.68	1.77	1.39
F_2P_1	0.48	0.73	0.65	1.89	2.18	1.97

* Numbers indicate position on the inositol ring which is esterified to a phosphate group.

NP_1 inositol monophosphates isolated from *N. crassa* hydrolysate in which a is the major component.

F_2P_1 inositol monophosphate from F_2 hydrolysate.

The electrophoretic and chromatographic mobilities of the inositol monophosphates in the listed buffer and solvent systems are expressed with reference to inorganic pyrophosphate. The normal standard technique of the runs is as described in Section 2.3.1 and Section 2.3.5.

All phosphate compounds were developed with the phosphomolybdate reagent (Harrap, 1960).

4. DISCUSSION

4. DISCUSSION

4.1 Presence of more than one phytase in wheat bran

As a consequence of a study of the enzymic degradation pathways of phytic acid it has been suggested (Johnson and Tate, 1969*b*; Cosgrove, 1970; Theodoru, 1971) that several phytases may coexist in the one organism. Results presented in Figure 28 show that wheat bran phytase may be separated into two distinct fractions, F_1 and F_2 by chromatography on DEAE-cellulose.

4.1.1 Separation of F_1 and F_2

In attempting to follow the purification procedure of Nagai and Funahashi (1962) for the bran phytase, two main problems were encountered. First, the methanol-precipitation step invariably led to a large loss in phytase activity and second, the ion-exchange chromatography step resulted in almost complete adsorption of the enzyme by the DEAE-cellulose.

Although no variations of the methanol procedure were successful in retaining the enzyme activity, an important practical advance was made when it was found that good recoveries of enzyme activity and protein from the DEAE-cellulose column were obtained after saturating the cellulose with bovine serum albumin.

It has previously been suggested that phytase is a mixture of enzymes and that the isolation of the iso-enzymes is difficult (Preece *et al.*, 1966, see Section 1.4.2). Although Courtois and Perez (1949)

separated a 'phyto-phosphatase' from a phosphomonoesterase in plant extracts they were unable to achieve further purification. That Nagai and Funahashi (1962) did not observe a second peak of phytase activity in eluants of their DEAE-cellulose column, was probably due to the fact that the ionic strengths of their elution buffers were too low to elute Fraction F_2. Thus phytase Fraction F_1 was eluted at 0.02 M Tris-HCl buffer at pH 7.3; the second enzyme Fraction F_2 with 0.1 M buffer.

In a study of rice bran phytase, Ikawa *et al.* (1964) observed a multiplicity of peaks on elution from a DEAE-cellulose column. However, they did not investigate the phytate degradation patterns of these various fractions. Davies (1968) also obtained several enzyme fractions during the acetone precipitations of phytase from peanut, suggesting that there were iso-enzymes present. Nagai and Funahashi (1962) also found that even their most active preparations were still heterogeneous when examined by physical methods.

An essential feature of the present study has been an examination of the phytate degradation pathway during the fractionation procedures. Thus the data in Figure 29 clearly show the different degradation patterns for F_1 and F_2.

Since the crude phytase activity was eluted as a single peak from the Sephadex G-100 column (Step 3, Table 18) it is clear that the bran phytase proteins present in F_1 and F_2 are relatively small molecules and cannot be resolved by molecular size differences. On Sephadex G-100 both F_1 and F_2 gave K_d values equivalent to a molecular weight of 47,000 ± 2,000 (Figure 14). This is close to the molecular weight

of 55,000 of a non-specific acid phosphatase from wheat germ (Verjee, 1969).

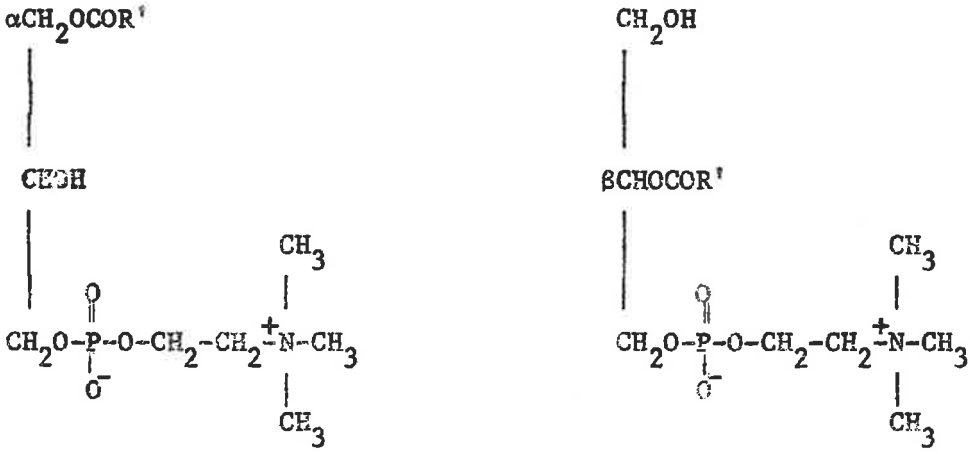
Figure 33 shows that on starch gels at pH 3.1 phytase Fraction F_1 differed in electrophoretic mobility from that of F_2. However, the apparently homogeneous phosphatase bands of enzyme Fractions F_1 and F_2 were resolved into 2-3 bands at pH 7.0 indicating that both fractions are still relatively impure preparations.

4.1.2 Enzyme Fraction F_1 and its lipid activator

Figure 28 shows that the bulk of the wheat bran phytase activity is associated with Fraction F_1 and its substrate degradation pattern is in accord with that reported by Tomlinson and Ballou (1962). Because of the large losses of enzyme activity observed in this work during the methanol precipitation step described by Nagai and Funahashi (1962) the possible loss of a methanol soluble activator or stabilising factor was considered. Substitution of butanol for methanol as a milder extractant (Morton, 1950) indicated the presence of a lipid activator as shown in Figure 27 with a substantially complete restoration of activity when the pooled butanol extracts were concentrated and recombined with the deactivated enzyme.

Preparative thin layer chromatography of the concentrated butanol extract showed that the lipid activator was associated with one or two phospholipids which were present. The mobility on thin layers of silica gel and the isolation of glyceryl phosphoryl choline from the de-O-acylation of this fraction suggested that the lipid activator

was lysolecithin. This was confirmed (Table 17) with several samples of lysolecithin both synthetic and naturally occurring and a remarkable specificity for the lysolecithin molecule was observed. Apart from minor changes in the degree of unsaturation and chain length of the acyl group all other alterations to the molecule failed to restore activity to the deactivated enzyme. Although lipid activators are known to affect many particulate enzymes, e.g. Na^{+}-K^{+}-Mg^{2+}-activated adenosine triphosphatase and K^{+}-Mg^{2+}-activated p-nitro-phenyl phosphatase (Tanaka, 1969) this appears to be the first example of a soluble enzyme which contains an endogenous lipid activator which has been successfully characterised. As shown in Table 17 substitutions of various phospholipids and lipids as possible activators of F_1 phytase has indicated the minimum requirements in the molecular structure for effective activation. Minor alterations in the degree of unsaturation in the chain and small variations in the chain length (C_{16}-C_{18}) of the lysolecithin successfully restore the activity to the deactivated enzyme F_1. All other modifications to the lysolecithin structure which were examined failed to reactivate the enzyme. Because of the ease of acyl migration in lysolecithins (see Van Deneen, 1968) there can be no certainty as to whether there is any specificity for 1-O-acyl (see formula a, below) or the 2-O-acyl (formula b) lysolecithins.



(a) (b)

4.1.3 Comparison of properties of F_1 and F_2

Figure 37 shows that phytase F_1 exhibits a relatively sharp pH optimum at pH 5.0, whereas F_2 had a broad profile with the optimum at 7.0. The existence of two pH optima in phytase preparations from higher plants has been noted on several occasions. Thus lettuce seed phytase (Mayer, 1958, Section 1.4.1) recorded two pH optima at 5 and 7. Similarly the enzyme from cotton (Thomas *et al.*, 1946) showed twin optima at pH 4 and 5.

Although the phytase Fraction F_2 has an alkaline pH, it is different from the general alkaline phosphomonoesterase classified by Folley and Kay (1936). The latter enzyme is known to have a requirement for metallic ions. As considered in Section 1.4.3, some alkaline phytases are known to be activated by magnesium ions (Patwardhan, 1937; Pileggi, 1959), but neither of the wheat bran phytase Fractions F_1 and F_2 has a magnesium requirement. The absence of a detectable effect by either EDTA or KCN on the F_1 and F_2 enzyme activities also

indicates that there is no requirement for polyvalent cations. This conclusion is in accord with the results obtained by Nagai and Funahashi (1962).

Phytase Fraction F_1 has a K_m value of 2×10^{-5} M whereas F_2 has a K_m of 1.82×10^{-4} M. A possible important distinction is that end product inhibition was observed with F_1 but not with the F_2 enzyme (see Table 21).

As shown in Figure 40 one of the most obvious differences between F_1 and F_2 is their production of different *myo*-inositol pentaphosphates from the same substrate, *myo*-inositol hexaphosphate. Although the pH optimum of phytase Fraction F_2 is at pH 7.0, its broad pH profile means that it still contributes significantly to the stepwise enzymic degradation of *myo*-inositol hexaphosphate at pH 5.0. Figure 42shows that at pH 5.0 Fraction F_2 produces two additional *myo*-inositol 1,3,4,5,6-pentaphosphate (Structure Va) and *myo*-inositol 1,2,3,4,6-pentaphosphate (Structure Vc) as well as L-*myo*-inositol 1,2,3,4,5-pentaphosphate (Vb) which is the main pentaphosphate from the F_1 enzyme. Further characterisation and identification of the lower inositol esters in the F_2 pathway include:- L-*myo*-inositol 1,2,3,4-tetraphosphate (IV), *myo*-inositol 1,2,3-triphosphate (III), D and L-*myo*-inositol 1,2-diphosphates (IIa, IIb) and *myo*-inositol 2-monophosphate.

Figure 40 shows the intermediates in the F_1route are electrophoretically indistinguishable from those produced by the crude wheat enzyme and hence probably correspond to the pathway reported by

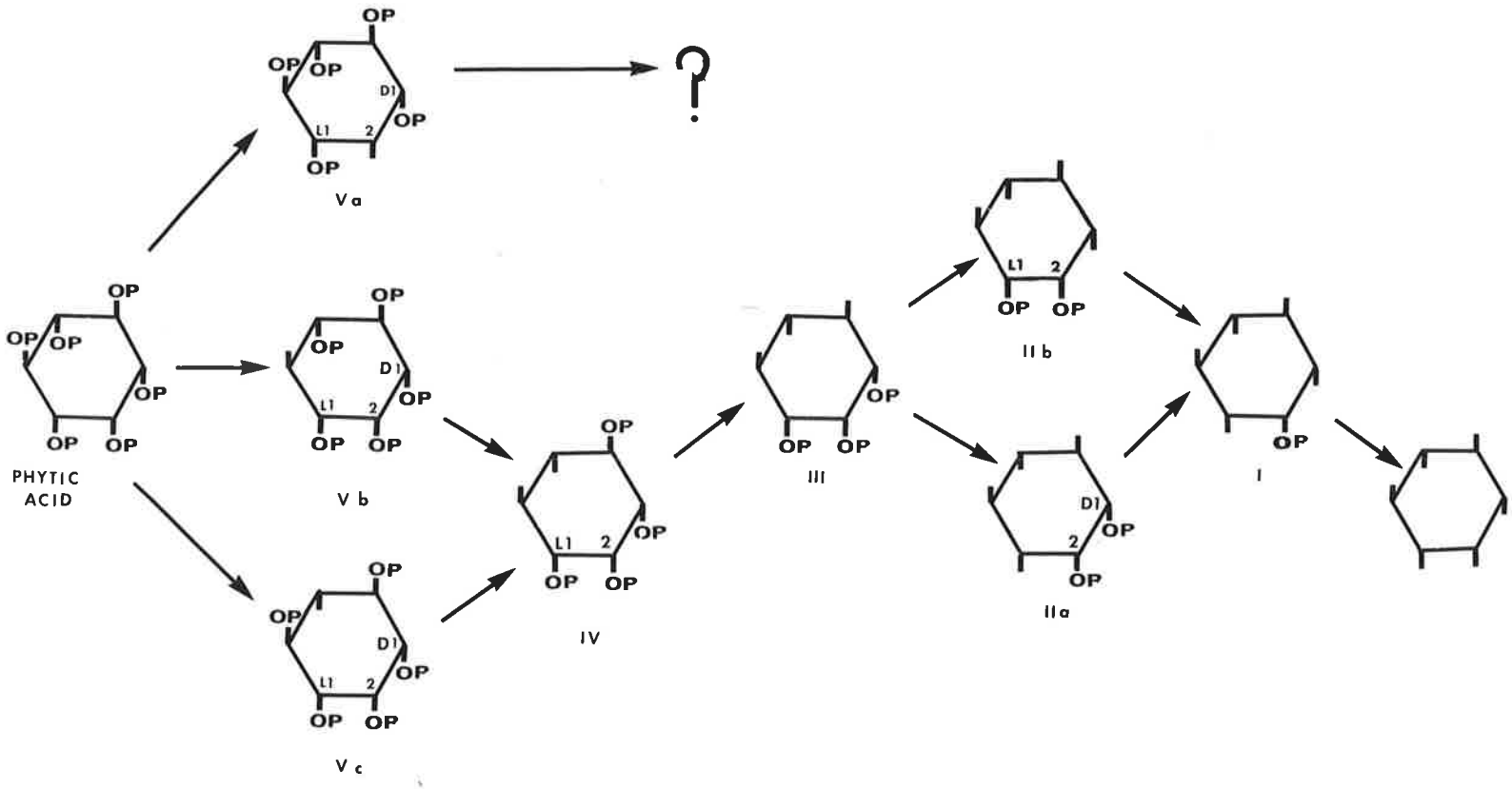
Figure 42.

Proposed scheme for the dephosphorylation of phytic acid by bran phytase Fraction F_2

The naming of the inositol polyphosphate intermediates in this scheme follows the IUPAC-IUB nomenclature (European J. Biochem. 5, 1968, 1). The relevant D 1 or L 1 positions are marked.

OP = OPO_3H_2

Dephosphorylation Of Phytic Acid By Bran Phytase Fraction F2



Tomlinson and Ballou (1962) (Figure 4). Because of the apparent similarity in these pathways the F_1 intermediates were not given in detail.

L-*myo*-inositol 1,2,3,4,5-pentaphosphate appears to be the major pentaphosphate present in phytate hydrolysates from F_1 and F_2 phytases. This same pentaphosphate (though not fully characterised by Tomlinson and Ballou, 1962) was postulated to be the initial intermediate of their bran phytase enzyme and has been isolated by Cosgrove (1963, 1969) and its optical properties were recorded by Johnson and Tate (1969*b*) and again by Cosgrove (1969). The detection of *myo*-inositol 1,3,4,5,6-pentaphosphate in enzyme F_2 hydrolysates seemed to be an unusual result at first sight since it is known that the phosphate group at the position 2 of the inositol ring is very resistant to enzymic and chemical hydrolysis (Iselin, 1949; Fleury *et al.*, 1954; Pizer and Ballou, 1959; Tomlinson and Ballou, 1962). However, the recent studies (Tate unpublished) on the conformational inversion (see Section 1.2.3) indicates that considerable doubt exists as to the exact conformation of the phytate molecule in solution and also in association with the enzyme. The identity of the *myo*-inositol 1,3,4,5,6-pentaphosphate with an authentic sample of chicken blood pentaphosphate of the same structure (see Johnson and Tate, 1969*b*) was established by the M_{PP_i} procedure (Tate, 1968) and also by comparing the enzyme degradation patterns (see Figure 41). The enzymic removal of the axial 2 phosphate does occur albeit slowly is apparent in the final stage of the phytate degradation to *myo*-inositol, as in the following reaction:-

myo-inositol 2-monophosphate $\xrightarrow{\text{phytase}}$ *myo*-inositol+inorganic phosphate

The pathway beyond *myo*-inositol 1,3,4,5,6-pentaphosphate (see Figure 42)was not characterised but is presumed to lead to *myo*-inositol, possibly via D or L *myo*-inositol 1-monophosphate which was noted as a minor product by Tomlinson and Ballou (1962) and also in this work.

The presence of the third pentaphosphate, *myo*-inositol 1,2,3,4,6-pentaphosphate in the F_2 hydrolysate is of interest (Section 1,3.1) since it has hitherto only been separated from an acid hydrolysis mixture of phytic acid (Tate, 1968; Cosgrove, 1963, 1969). Preliminary work has indicated that this pentaphosphate appears to be concentrated in the germ area of both germinated and ungerminated wheat and barley seeds. It has been detected in the hypocotyl region and also in the green and etiolated foliage leaves of 5 day old seedlings. Its presence is probably due to the action of phytase Fraction F_2 on *myo*-inositol hexaphosphate.

The enzymic hydrolysis of L-*myo*-inositol 1,2,3,4,5- and *myo*-inositol 1,2,3,4,6-pentaphosphates both yield the same L-*myo*-inositol 1,2,3,4-tetraphosphate in the F_2 degradative scheme. It is interesting to note that this tetraphosphate was postulated to be one of the intermediates in the bran phytase pathway (Tomlinson and Ballou, 1962) and was characterised as a minor product in the crude bran phytase route by Johnson and Tate (1969*b*) and Cosgrove (1969). Davies (1968) characterised one of the tetraphosphate intermediates from peanut phytase as D- or L-*myo*-inositol 1,2,3,4-tetraphosphate. In contrast, the major tetraphosphate fraction in F_1 is D-*myo*-inositol 1,2,5,6-

tetraphosphate (similar to the fungal component, Section 3.1.6.1).

The triphosphate in the F_2 route is the symmetrical *myo*-inositol 1,2,3-triphosphate whereas that in F_1 is D-*myo*-inositol 1,2,6-triphosphate. Both triphosphates have similar electrophoretic mobilities. However, as shown by Tomlinson and Ballou (1962) they may be distinguished by the periodate-oxidation-borohydride-reduction and dephosphorylation sequence (Section 2.4.10.5). *Myo*-inositol 1,2,3-triphosphate gives ribitol whereas D-*myo*-inositol 1,2,6-triphosphate yields D arabitol (see Section 3.2.4.1 (c)). The latter triphosphate was found in bran (Tomlinson and Ballou, 1962) and peanut hydrolysates (Davies, 1968). Cosgrove (1970) separated D-*myo*-inositol 1,2,6- and 1,2,5-triphosphates in a partial digest of *myo*-inositol hexaphosphate by the phytase from *Pseudomonas* SB2.

From the triphosphate stage the diphosphate fraction formed by the F_2 phytase is mainly D-*myo*-inositol 1,2 -diphosphate and appears to be common to all phytase degradative routes so far examined. Penultimately the enzymic hydrolysis of *myo*-inositol 1,2-diphosphate gives *myo*-inositol 2-monophosphate and finally *myo*-inositol.

A combination of the action of the phytase fractions (F_1 and F_2) would account for most aspects of the degradative scheme proposed originally by Tomlinson and Ballou (1962) (Figure 4, Section 1.3.2).

4.2 A phytase from *Neurospora crassa*

A phytase from *Neurospora crassa* (N.c. phytase) purified a 100-fold was used for a comparison of its properties with those of

bran phytase (Fractions F_1 and F_2) and with those from other sources. In addition its properties were compared with the reported properties for a non-specific acid phosphomonoesterase from the same fungus (Kuo and Blumenthal, 1961).

At pH 5.0, the N.c. phytase catalyses a stepwise hydrolysis of *myo*-inositol hexaphosphate to inositol and inorganic phosphate. This observation is consistent with the reported action of phytase from other sources (Section 1.4.3). But as reported by Johnson and Tate (1969b) the structure of *myo*-inositol pentaphosphate in the fungal hydrolysate differs from that of the major bran phytase *myo*-inositol pentaphosphate L-*myo*-inositol 1,2,3,4,5-pentaphosphate. In the present study it has also been found to differ from the major pentaphosphate, *myo*-inositol 1,2,3,4,6- and *myo*-inositol 1,3,4,5,6-pentaphosphates of the F_2 enzyme. The initial hydrolysis product of phytic acid by N.c. phytase is D-*myo*-inositol 1,2,4,5,6-pentaphosphate (Structure Va, Figure 43) together with a small amount of L-*myo*-inositol 1,2,3,4,5-pentaphosphate (Structure Vb,Figure 43). Subsequent hydrolysis proceeds in both cases to yield D-*myo*-inositol 1,2,5,6-tetraphosphate (IV), D-*myo*-inositol 1,2,6-triphosphate (III), D-*myo*-inositol 1,2-diphosphate (II) and *myo*-inositol 2-monophosphate (Ia) and finally to *myo*-inositol as shown in Figure 43. A comparison of these intermediates (in the fungal degradation route) with those of a phytase from *Pseudomonas* (Cosgrove, 1970) (see Figure 5) shows that the pathway for degrading phytic acid is similar in both microbial enzymes.

A key feature of the present study has involved the character-

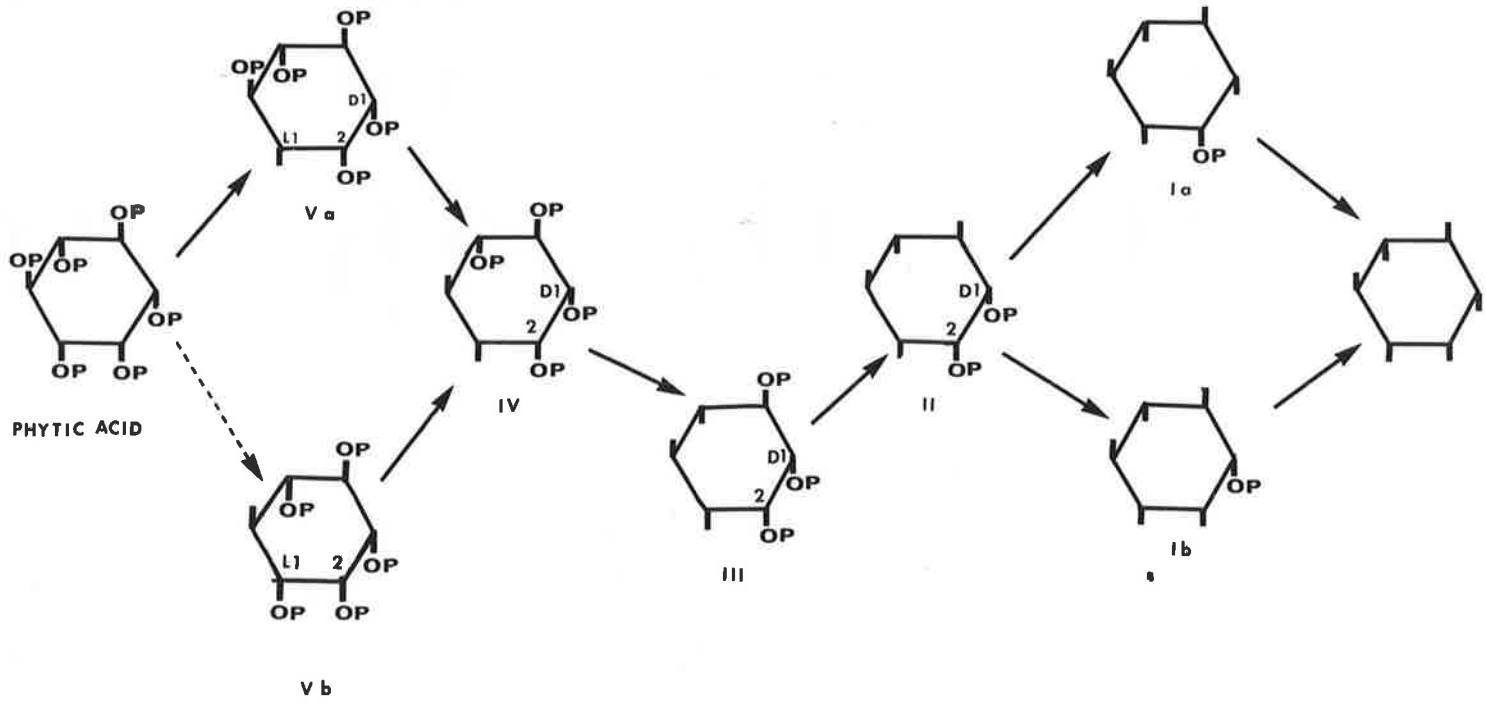
Figure 43.

Proposed scheme for the dephosphorylation of phytic acid by the *Neurospora crassa* phytase

The currently accepted IUPAC-IUB nomenclature (European J. Biochem. 5, 1968, 1) is used in this scheme. Details of structures are given in the text.

OP = OPO_3H_2

Dephosphorylation Of Phytic Acid By *Neurospora* Phytase



isation of the *myo*-inositol triphosphate in the degradation pathway of phytase from N.c. phytase as D-*myo*-inositol 1,2,6-triphosphate (Structure III, Figure 43). This was established by the isolation of D arabitol from the periodate-oxidation-borohydride-reduction and dephosphorylation sequence (Section 2.4.10.5). Cosgrove (1970) has postulated a similar structure for the bacterial triphosphate but did not isolate the D arabitol from the periodate degradation sequence. Johnson and Tate (1969*b*) isolated arabitol but obtained insufficient quantity for the determination of its absolute configuration. The characterisation of D-*myo*-inositol 1,2,6-triphosphate as a component in the bran phytase hydrolysis mixture by Tomlinson and Ballou (1962) has been the corner-stone on which all configurational assignments for asymmetric inositol phosphates from phytic acid have been made (Tomlinson and Ballou, 1962; Johnson and Tate, 1969*b*). Once the structure of the triphosphate as D-*myo*-inositol 1,2,6-triphosphate had been established the configurational assignments for the asymmetric pentaphosphate, tetraphosphate and diphosphate follow as outlined by the latter workers (Johnson and Tate, 1969*b*) and are shown in Figure 43.

The preferential positional attack on the L 1 position of *myo*-inositol hexaphosphate by the phytases from micro-organisms (*N.crassa* and *Pseudomonas*) is in contrast to the F_1 and F_2 phytases from higher plants, which attack the D 4, D 2 amd D 5 positions as shown below. The different *myo*-inositol pentaphosphates thus formed are shown in Figure 44.

Figure 44.

Proposed phytase routes

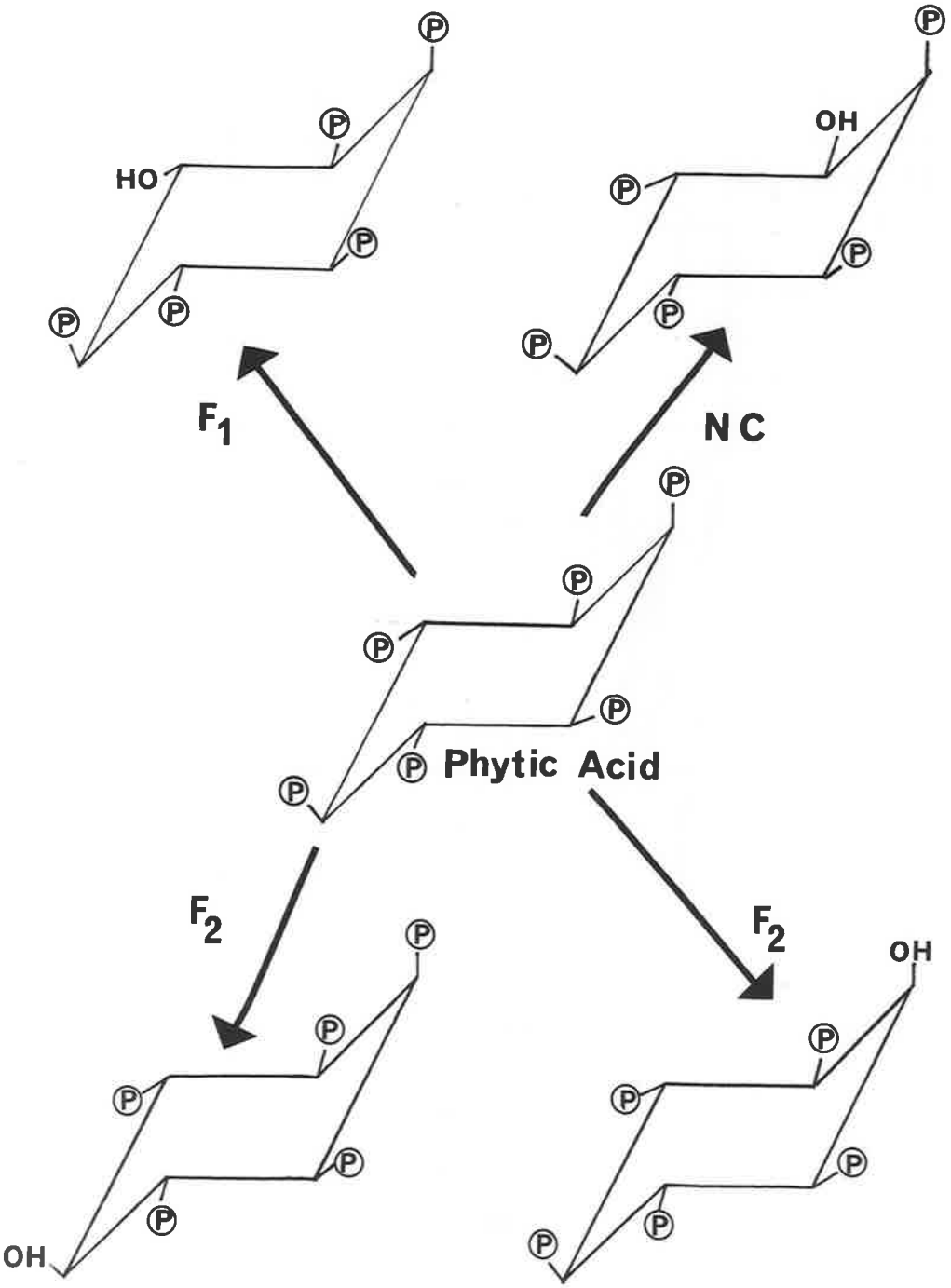
Figure 44 shows that dephosphorylation of *myo*-inositol hexaphosphate initiates either at the L 1, D 2, D 4 or D 5 position of the molecule.

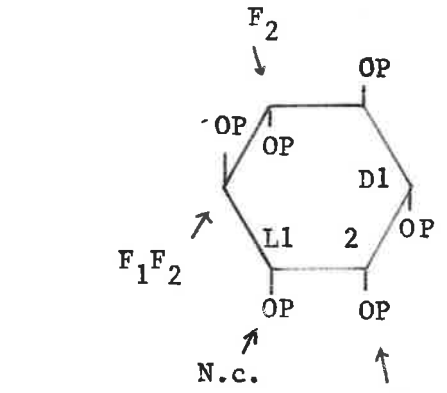
F_1 = Action of wheat bran phytase F_1

F_2 = Action of wheat bran phytase F_2

NC = Action of phytase from *Neurospora crassa*

PHYTASE ROUTES





Pseudomonas SB2

With the exception of the position of attack on the phytic acid molecule the N.c. phytase exhibits properties which are similar to those of bran phytase F_1 and with those of cereal phytases reported previously (Nagai and Funahashi, 1962, 1963; Peers, 1953; Chang, 1967; Davies, 1968; see Section 1.4.3). Both the N.c. phytase and bran F_1 exhibit a similar pH optimum at pH 5 of most plant phytases (see Table 1, Section 1.4.3). Bran phytase F_2 has an optimum activity at pH 7.0. The optimum at pH 5 of the fungal and F_1 enzymes differs from (a) the *E. coli* enzyme which has twin pH optima at pH 2.2 and 4.5 (Courtois and Manet, 1952); (b) the alkaline phytase from *Phaseolus aureus*, pH 7.5 (Mandal and Biswas, 1970) and (c) the rat phytase, pH 7.9 (Pileggi, 1959) and the enzyme from *Bacillus subtilis*, pH 7.5 (Powar and Jagannathan, 1967). As considered in Section 1.4.3.1 it appears that most phytases exhibit a pH range between 4 and 8 with very few enzymes well below pH 4 or above pH 8. It has been suggested that some phytase enzymes are inactivated at low pH values (Hill and Tyler, 1954;

Anderson, 1965).

Studies of the effects of cations (Table 9) on N.c. phytase show that like the wheat bran phytase Fractions F_1 and F_2 it was not affected by cations; unlike the Ca^{++} and Mg^{++} requirement of the phytase from *Bacillus subtilis* (Powar and Jagannathan, 1967) and some cereals (Peers, 1953; Chang 1967). Peers (1953) reported a 35-fold increase in activity of phytase preparation on adding magnesium ions at 0.01 M. In contrast, 0.05 M magnesium competitively inhibited the activity of a phytase from *Pseudomonas* (Irving and Cosgrove, 1971*a*). The activation caused by some metallic ions, e.g. calcium ions on the *B. subtilis* (Powar and Jagannathan, 1967) and corn phytases (Chang, 1967) may be a consequence of the removal of phosphate as calcium phosphate from the site of reaction which then proceeds as shown below:-

$$\text{Inositol hexaphosphate} \xrightarrow{\text{phytase}} \text{inositol pentaphosphate} + P_i$$

$$Ca^{++} \downarrow \text{calcium phosphate}$$

Phytase activity was determined by following the utilization of phytate.

The apparent Michaelis constants for phytate for N.c. phytase was 1.6×10^{-4} M and for wheat bran enzyme F_2, 1.82×10^{-4} M were consistent with values obtained for phytase from other sources (Table 2, Section 1.4.3), whereas that for F_1 was much lower (2×10^{-5} M). Low Michaelis constants, a characteristic feature of phytase, suggest that the enzyme has a strong affinity for its substrate. At higher concen-

trations of phytate, substrate inhibition was observed. This may be explained by the fact that phytate is a protein precipitant and around pH 5-6, the phytic acid-protein association is most stable (Posternak, 1965).

The N.c. phytase hydrolysed ATP, ADP, inorganic pyrophosphate in addition to phytic acid and to a lesser extent it hydrolysed glucose-6-phosphate, α- and β-glycerophosphate and NADP. Similarly both enzyme Fractions F_1 and F_2 exhibited a broad substrate specificity (Section 3.2.3.10). The enzyme isolated from wheat bran by Nagai and Funahashi (1962) and from peanut by Davies (1968) showed similar effects. Further studies with phytase preparations would be necessary to determine the factors controlling the specificity of the enzymes. However, it seems likely that in view of the broad substrate specificity shown by the phytases, including the most highly purified one from wheat bran (1,500-fold) reported by the Japanese workers (1962), they are non-specific enzymes. The enzyme from *B. subtilis* (Powar and Jagannathan, 1967) which exhibits a high degree of specificity towards phytic acid is an exceptional case.

4.3 Comparison of the properties of a phytase with those of a non-specific acid phosphomonoesterase from *Neurospora crassa*

Kuo and Blumenthal (1961) extracted a non-specific acid phosphomonoesterase from *Neurospora crassa* which had some properties in common with the fungal phytase. Both enzymes have similar pH optima and K_m values (with β-glycerophosphate as the substrate). Neither enzyme requires a co-factor or a cation for activity. Fluoride and

(+) tartrate inhibit both enzymes. The N.c. phytase has pyrophosphatase activity but no phosphotransferase activity which distinguishes it from the phosphomonoesterase.

Phytase activity may be a result of a number of closely related phosphomonoesterases. This is further supported by the broad substrate specificity of the enzyme. However, enzyme assays using combinations of two substrates would be expected to give additive activities, if separate enzymes were responsible for the activity. The results were not additive since the amount of P_i formed corresponded closely with the amount expected from a single enzyme acting simultaneously on two substrates. At no time was a non-specific phosphatase separated from phytase.

Attempts were made to detect more than one phosphatase by heating phytase preparations (at various temperatures) and then measuring residual activity with different substrates. Although small differences in the amount of inactivation were observed at a given temperature there was no consistent indication of a loss of activity against any one substrate at a rate different from others over a wide range of temperatures. Thus, in the absence of evidence for the presence of more than one phosphomonoesterase, the N.c. enzyme is probably a single phytase responsible for all the phosphomonoesterase properties.

4.4 General conclusion

At least three distinct modes of attack by phytase are shown in wheat bran (F_1 and F_2) and yet a fourth in *Neurospora crassa*,

Figure 44. If the formation of isomeric inositol polyphosphates is indicative of the number of phytases in Fraction F_2 then F_2 consists of at least three iso-enzymes. This is supported by starch-gel electrophoresis which revealed 2 to 3 bands of phosphatase activity at pH 7.0, although these were represented as one band at pH 3.1. Further studies with the enzyme preparation would be required to determine the factors responsible for the separation of these bands on starch gel. Hence, it is possible to suggest that phytase degradation of phytic acid is initiated by forming one of these pentaphosphates:- *myo*-inositol 1,3,4,5,6-pentaphosphate; *myo*-inositol 1,2,3,4,6-pentaphosphate; L-*myo*-inositol 1,2,3,4,5-pentaphosphate (these are peculiar to F_1 and F_2) and D-*myo*-inositol 1,2,4,5,6-pentaphosphate (found mainly in *N. crassa* and *Pseudomonas*).

However, other explanations may well apply. It is possible that the initial orientation of the substrate to the enzyme determines the locus for dephosphorylation of *myo*-inositol hexaphosphate at either the L 1, D 2, 4 and 5 position of the molecule (see Figure 44). Some of the requirements for the active centre of phytases have been discussed by Irving and Cosgrove (1971*b*).

4.5 Physiological importance of phytases

The metabolic function of phytases (or of acid phosphatases) particularly in micro-organisms is rather obscure. In examining the properties of phytases both from *Neurospora crassa* and wheat bran it is difficult to relate the properties of the partially purified enzymes

to a specific function in the intact organisms. At present the function of the fungal phytase and Fractions F_1 and F_2 can only be speculated upon.

Thus phytase is a widely distributed enzyme in the plant kingdom (see Section 1.4.1). The enzyme plays an important role in supplying inorganic phosphate (from phytic acid) for plant tissues (Stumpf, 1952).

Despite the striking correlation between phytase activity and the decrease in phytate phosphorus with the concomitant increase in phosphate level during germination, supplying P_i may not be the sole function of phytases (see Section 1.4.4). The broad substrate specificity of the enzymes may reflect the non-specificity of phytases *in vivo*. However, the substrate specificity of the phosphatases might be made more selective *in vivo* by the presence of an inhibitor which could act selectively in preventing the hydrolysis of some biochemical substrates at the expense of others. The effects of phosphate on some phytases (e.g. on F_1 and *N. crassa* enzymes, but not on F_2) suggest that the level of P_i in the tissues may be maintained through the control of the activity of these enzymes. The level of P_i may in turn control biochemical processes sensitive to P_i such as those in the Embden-Meyerhoff pathway.

Phosphatase enzymes are known to be directly involved in controlling the intra-cellular metabolism of carbohydrates (Kiesow and Doge, 1961) and to catalyse transfer reactions (Atkinson and Morton, 1960; Morton, 1965; Roberts, 1967). Therefore phytases may be

involved in such reactions rather than in the simple hydrolytic cleavage of phosphoesters. However, present preliminary experiments suggest that transphosphorylation reactions are not associated with phytase preparations.

Mitchell (1959) suggested that a phosphatase enzyme from *E. coli* was involved in regulating the permeability properties of the cell since it is located in the 'periplasm' bounded by the cell membrane and the outer cell wall. Although the localisation of the enzyme was not determined, Kuo and Blumenthal (1961) and Rogers and Reithel (1960) also assumed such a function for their phosphatases. This role might also be extrapolated to the phytases.

The most interesting aspect of the enzymic study of phytases is the specific requirement of lysolecithin for activation of the main enzyme Fraction F_1 from wheat bran. Lysolecithin is now known to be a major phosphorus constituent of cereal grain endosperm (Nakamura *et al.*, 1958; Wren and Merryfield, 1970) and is second only to phytic acid as a store of phosphorus. The water miscible lysolecithin is known to form strong complexes with starch (Nakamura *et al.*, 1958; Wren and Merryfield, 1970) and the results presented herein suggest that it has a strong affinity for the F_1 enzyme. If the interaction is extrapolated to storage proteins of wheat endosperm in general, lysolecithin may be important as a modifier of the rheological properties of dough.

APPENDIX

APPENDIX: FORTRAN LISTING OF THE PROGRAMME

```
      PROGRAM W135   (INPUT, TAPE60=INPUT,OUTPUT,TAPE61=OUTPUT)
C     FIT OF HYPERBOLA.  WILKINSON,BIOCHEM.J.(1961)80,324. WI 135
      DIMENSION S(50),V(50),DH(50),H(50),TITLE( 9),IMT(10)
      READ(60,121) IMT
  121 FORMAT(10A4)
      WRITE(61,131)
  131 FORMAT(1H1)
      TKM=8H      KM
      TVK=8HINIT.SL.
      TVH=8H    VMAX
   99 READ(60,101)TITLE,NM,N
  101 FORMAT(9A8,214)
      IF(N)26,26,25
   25 READ (60,IMT)   (S(I),V(I),I=1,N)
      DF=N-2
      IF (DF) 1,2,3
    1 WRITE(61,103)TITLE
      WRITE (61,102)
  102 FORMAT (1x18H INSUFFICIENT DATA)
      GO TO 99
    2 DIV=1./(V(2)*S(1)-V(1)*S(2))
      VM=V(1)*V(2)*(S(1)-S(2))*DIV
      KM=S(1)*S(2)*(V(1)-V(2))*DIV
      WRITE(61,103) TITLE,KM,VM
  103 FORMAT(/1X9A8//1X3HKM=F10.4,8H,  VMAX=F10.4,19H, NO D.F. FOR ERROR
     $)
      GO TO 99
    3 IF (NM)1001,1002,1003
 1003 DO 1004 I=1,N
      S(I)=1/S(I)
 1004 CONTINUE
      IF(NM.GT.100)1001,1002
 1001 READ(60,IMT)EVM,EKM
      JJ=0
      GO TO 5
 1002 A=0
      B=0
      G=0
      D=0
      E=0
      DO 4 I=1,N
      X=V(I)*V(I)
      Y=X/S(I)
      A=A+V(I)*X
      B=B+X*X
      G=G+V(I)*Y
      D=D+X*Y
      E=E+Y*Y
    4 CONTINUE
```

```
      JJ=0
      DIV=A*E-G*D
      IF(DIV.NE.0) 13,14
   14 PRINT 110
  110 FORMAT(10X*DIVISOR ZERO IN CALCULATION OF INITIAL ESTIMATES*)
      GO TO 99
   13 DIV=1.0/DIV
      EKM=(B*G-A*D)*DIV
      EVM=(B*E-D*D)*DIV
      PRINT 111,EKM,EVM
  111 FORMAT(10X*INITIAL ESTIMATES*/10X*KM =*E11.4,* VMAX =*E11.4/)
    5 RSS=0
      JJ=JJ+1
      DO 6 I=1,N
      SAD=1./(S(I)+EKM)
      H(I)=EVM*S(I)*SAD
      DH(I)=-H(I)*SAD
      RSS=RSS+(V(I)-H(I))**2
    6 CONTINUE
      IF(JJ.GT.2) 8,7
    8 IF (RSS.EQ.0) 9,10
   10 IF ((RSSP-RSS)/RSS-1.E-6) 9,9,7
    7 IF (JJ.GT.10) 9,11
   11 A=B=G=D=E=0
      DO 12 I=1,N
      A=A+H(I)*H(I)
      B=B+DH(I)*DH(I)
      G=G+H(I)*DH(I)
      D=D+V(I)*H(I)
      E=E+V(I)*DH(I)
   12 CONTINUE
      DIV=1./(A*B-G*G)
      BI=(B*D-G*E)
      EVM=BI*EVM*DIV
      EKM=EKM+(A*E-G*D)/BI
      SVM=EVM*SQRTF(B*DIV)
      SKM=(SQRTF(A/DIV))/BI
      RSSP=RSS
      GO TO 5
    9 SD=SQRTF(RSS/DF)
      SEKM=SKM*SD
      SEVM=SVM*SD
      CVK=-G/SQRTF(A*B)
      RVK=EVM/EKM
      CVVM=SEVM/EVM
      CVKM=SEKM/EKM
      SEVK=RVK*SQRTF(CVVM*CVVM+CVKM*CVKM-2.0*CVK*CVVM*CVKM)
      CVVK=SEVK/RVK
      WRITE (61,104) TITLE,SD,JJ
  104 FORMAT(/1X9A8//1X5HS.D.=E11.4,6H,AFTER16,11H ITERATIONS)
      WRITE (61,105)
```

```
  105 FORMAT (29X8HESTIMATE9X14HSTANDARD ERROR4X18HCOEFF OF VARIATION/)
      WRITE (61,106) TKM,EKM,SEKM,CVKM
      WRITE (61,106) TVM,EVM,SEVM,CVVM
      WRITE (61,106) TVK,RVK,SEVK,CVVK
  106 FORMAT (15XA8,3XF16.8,3XF16.8,3XF16.8)
CALCULATE FITTED VALUES AND RESIDUALS
      DO 27 I=1,N
      DH(I)=V(I)-H(I)
   27 CONTINUE
      WRITE (61,108) (DH(I),I=1,N)
  107 FORMAT(14H FITTED VALUES11(1XF10.5))
      WRITE (61,107) (H(I),I=I,N)
  108 FORMAT(14H    RESIDUALS 11(1XF10.5))
      GO TO 99
   26 WRITE(61,999)
  999 FORMAT(1H-,22HJOB EXECUTION COMPLETE)
      END
```

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