STUDIES ON PHYTIC ACID

AND ITS

INOSITOL PHOSPHATE DERIVATIVES

A Thesis

Presented to

The Faculty of Graduate Studies

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Hershel William Kaufman

Department of Oral Biology

August 3, 1967

DC

STUDIES ON PHYTIC ACID AND ITS INOSITOL PHOSPHATE DERIVATIVES

ABSTRACT

The factors involved in the hydrolysis of phytate and its inositol phosphate derivatives and the properties of these compounds particularly as they may relate to dental caries and nutrition were examined. The inositol phosphates studied were phytic acid (IHP), inositol monophosphate (IMP), inositol diphosphate (IDP), inositol triphosphate (ITriP), inositol tetraphosphate (ITetraP), and inositol pentaphosphate (IP'P). IDP, ITriP, ITetraP, and IP'P were prepared from an acid hydrolysis of phytic acid and were identified from their P/I ratios. IMP was purchased. To determine the effect of pH on adsorption and elution of phytate from a column containing Dowex-1-Cl anion exchange resin and by analogy to teeth and soils, phytate at pH 2.1, 5.6 and 8.6 was put onto the column and eluted with LiCl under a variety of conditions. Similar experiments were also carried out using IMP at pH 2.4 and 8.6 for comparison. Contrary to what one might have expected, phytate was adsorbed more strongly to the resin at low than at high pH. With IMP, pH had the reverse effect. This effect with phytate was attributed to

the large number of polar OH groups on phytate which would favour physical adsorption. The sum of the physical and chemical adsorptive forces must be greater at low than at high pH. Because IMP does not contain a large number of polar OH groups, physical adsorptive forces would be weak, and the sum of the physical and chemical adsorptive forces would be greater at high than at low pH. These results may explain why, with decrease in pH, the adsorption of phytate to soils is increased (Anderson and Arlidge, 1962) and why phytate may have adsorbed to enamel at the low pH levels used in the experiments of Jenkins et al (1959). To compare sodium and calcium binding by phytic acid and its inositol phosphate derivatives, differences between their titration curves with NaOH and $Ca(OH)_2$ were observed. The effect of pH on the solubility of their calcium salts was determined by noting during titration with $Ca(OH)_2$, the pH at which a precipitate first formed. The curves for IMP with NaOH and for all 6 inositol phosphates with $Ca(OH)_2$ were diphasic and similar. Between 50 - 100% neutralization of each inositol phosphate, the NaOH curves showed progressively larger deviations from the $Ca(OH)_2$ curves as the phosphorus/myoinositol (P/I) ratio increased from 2 to 6, probably due to the possibility that some of the hydrogens on these molecules (other than IMP)

are bound between oxygens on adjacent phosphates and that, whereas Ca^{++} can displace these hydrogens easily, Na^{+} cannot. pK_2 values determined with $Ca(OH)_2$ were similar and lower than those for organic phosphates without a ring structure. Those values determined with NaOH were higher than those with $Ca(OH)_2$. Because Na^{+} does not appear able to displace all of the hydrogens on the inositol phosphates other than IMP, the pK_2's determined with NaOH probably only reflect the second ionizable hydrogens of those phosphate groups not involved in hydrogen bonding. pK_1 values are meaningless for these compounds since the pH titration curves for IMP, ITetraP, and IHP in the 0-50% neutralization region were lower for higher concentrations of inositol phosphate, indicating that these compounds behave more like strong acids than weak ones in this region. The pH at which a precipitate formed with $Ca(OH)_2$ decreased as the P/I ratio increased, indicating that calcium inositol phosphate salts with higher P/I ratios are less soluble than those with lower ratios. Because phytate can be hydrolyzed sequentially by phytases, and little is known about the factors that may be involved during such hydrolysis, the effect of pH, enzyme, substrate, and P_i concentrations on the hydrolysis of the inositol phosphates by acid and alkaline phosphatases was examined. The

pH optima for alkaline phosphatase decreased with decreasing substrate concentration whereas those for acid phosphatase did not. This was explained in terms of a scheme proposed by Krupka and Laidler (1960) and also in terms of relative charges on enzymes and substrates involved. Km and Vmax values were calculated from Lineweaver-Burk plots of the reciprocal of reaction velocity as a function of the reciprocal of substrate concentration. Km data indicated that whereas molecules with P/I ratios between 3 and 6 would all be hydrolyzed at similar rates with both enzymes, IMP and IDP would be hydrolyzed at slower rates with acid phosphatase, suggesting that phosphate groups linked by hydrogen bonds (in the higher intermediates) were resistant to hydrolysis with alkaline phosphatase. Vmax data suggested that the active sites on both enzymes are more accessible to IDP and ITriP than to the other inositol phosphates. Although P_i inhibited hydrolysis of all inositol phosphates with both enzymes, alkaline phosphatase was more susceptible to inhibition than acid phosphatase and with both enzymes, hydrolysis of ITriP and ITetraP was least affected. The Km, Vmax, and P_i inhibition data taken together suggest that IDP, ITriP, and ITetraP would yield more P_i upon enzymic hydrolysis than the other inositol phosphates and that, since IMP is quite

sensitive to P_i inhibition and is slowly hydrolyzed, complete depletion of organic P would be resisted in a sequential hydrolysis of phytate. Gel filtration was used to study the variables that might be involved in the interaction between P_i and alkaline phosphatase. Low levels of P_i were eluted from Bio-Gel P-4 with distilled water much sooner than one might have expected because of its small size indicating that the negative P_i molecules cannot enter the pores of the gel which might be negatively charged unless this charge is reduced, either by increasing P_i concentration or eluting with NaCl. Na phytate showed similar but less marked effects because of its higher charge. Alkaline phosphatase and P_i eluted with distilled water exhibited repulsion as evidenced by the P_i being eluted in a larger volume than when enzyme was absent. This repulsion was due to charge repulsion and was therefore eluainated with NaCl in the eluent. A sample containing alkaline phosphatase and P_i at a certain pH was put onto a Bio-Gel P-4 column equilibrated with P_i at the same concentration and pH as that in the sample, and eluted with the equilibrating P_i solution. The eluate was monitored for P_i and enzyme and if the pH was above the isoelectric point of the protein, a dip occurred in the P_i concentration in that portion of the eluate associated with the enzyme, indic-

ative of repulsion. This effect was increased by increasing the pH, enzyme or P_i concentrations (which would increase negative charge on enzyme, P_i, or both) and was eliminated in the presence of NaCl or below the isoelectric point of alkaline phosphatase. ^{32}P added to alkaline phosphatase and passed through a Bio-Gel P-4 column with distilled water separated from the P_i which was associated with the alkaline phosphatase used in this thesis, indicating that this residual P_i was not exchangeable. Dialysis of alkaline phosphatase and ^{32}P resulted in some ^{32}P binding to the enzyme. These findings are consistent with the hypothesis of Schwartz (1963) that two sites exist on alkaline phosphatase for P_i binding, one involving electrostatic bonds and the other covalent bonds. The findings in this thesis that relate to dental caries, the intestinal absorption of calcium and other biological phenomena were discussed.

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. I. Kleinberg, who supervised this project, for his unselfish contribution of time, advice and encouragement. Drs. C. Dowse, E. T. Pritchard, I. Hamilton, and C. Dawes have been of assistance at various times with both technical assistance and advice.

The author also would like to thank Dr. F. Kaminsky, Mr. J. Levinson, and Mr. H. Sawchuk for their technical assistance during portions of these studies and Mr. D. Summerfield for art work and photography.

A special vote of thanks goes to the author's wife, Louise, for the long hours spent recopying rough drafts of this thesis.

Finally, I wish to thank the Associate Committee on Dental Research of the National Research Council of Canada for awarding me Dental Research Fellowships during my studies and for supporting the research through grants made to Dr. Kleinberg.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE

both in the absence of inositol (blank) and in the presence of 0.5 μ Moles of inositol.

II·4 Change in % transmittance as a function of wave length for a solution of a) active inositol oxygenase b) inactive inositol oxygenase c) the inactive yellow component eluted in the void volume from G-100 sephadex.

II·5 The elution pattern obtained on gel filtration on a P-4 Bio-Gel column of alkaline phosphatase. Each fraction eluted was monitored at 280 mμ and analyzed for P_i.

II·6 The elution pattern obtained on gel filtration on a P-4 Bio-Gel column of acid phosphatase. Each eluted fraction was monitored at 280 mμ.

II·7 Inositol standard curve: change in optical density at 660 mμ as a function of inositol concentration following incubation of known inositol standards with inositol oxygenase and analysis for the glucuronic acid formed in the enzyme reaction with $FeCl_3$-orcinol reagent.

II·8 Inositol standard curve: Change in optical density at 440 mμ as a function of inositol concentration following reaction of known inositol standards with $K_2Cr_2O_7$ reagent.

II·9 Chloride standard curve: The number of millivolts is plotted as a function of the negative log of the chloride

concentration.

III·1 The elution pattern obtained when phytate, pH 2.1, 5.6 or 8.6 is eluted from a Dowex-1-Cl anion exchange resin using a stepwise LiCl gradient containing 2 ml of LiCl in each step. Eluted fractions were assayed for organic P.

III·2 The elution pattern obtained when IMP, pH 2.4 and 8.6 is eluted from a Dowex-1-Cl anion exchange resin using a stepwise LiCl gradient containing 2 ml of LiCl in each step. Eluted fractions were assayed for organic P.

III·3 The elution pattern obtained when IMP, pH 2.4 and 8.6 is eluted from a Dowex-1-Cl anion exchange resin using a stepwise LiCl gradient (reduced a factor of 5X from that in Fig. III·4) containing 2 ml of LiCl in each step. Eluted fractions were assayed for organic P.

III·4 The elution pattern obtained when IMP, pH 2.4 and 8.6 is eluted from a Dowex-1-Cl anion exchange resin using a stepwise LiCl gradient (reduced a factor of 15X from that in Fig. III·4) containing 2 ml of LiCl in each step. Eluted fractions were assayed for organic P.

III·5 The elution pattern obtained when phytic acid, pH 2.1, was eluted from a Dowex-1-Cl anion exchange column using either 0.50, 0.55, 0.60, 0.65, 0.70, or 0.75 M LiCl as eluent. Fractions eluted were monitored for organic P.

optical density of acid phosphatase at 700 mμ are plotted as a function of pH.

V·11 Isoelectric point of alkaline phosphatase: Changes in optical density of alkaline phosphatase at 700 mμ are plotted as a function of pH.

V·12 Lineweaver-Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of the substrate concentration) for acid phosphatase at pH 5.0 with IMP both in the absence of P_i and in the presence of either 0.84×10^{-4} M P_i or 1.7×10^{-4} M P_i.

V·13 Lineweaver-Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of the substrate concentration) for alkaline phosphatase with IMP at pH 9.0 both in the absence of P_i and in the presence of either 0.84×10^{-4} M P_i or 1.7×10^{-4} M P_i.

V·14 Lineweaver-Burk plots (reciprocal of the initial rate of hydrolysis against the receiprocal of the substrate concentration) for acid phosphatase at pH 5.0 with phytate both in the absence of P_i and in the presence of 1.7×10^{-4} M P_i.

V·15 Lineweaver-Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of the substrate concentration) for alkaline phosphatase at pH 9.0 with

of elution of alkaline phosphatase is also shown to mark the void volume.

VI·3 The elution pattern obtained on gel filtration on a Bio-Gel P-4 column (equilibrated with distilled water) of different concentrations of P_i mixed with a fixed concentration of alkaline phosphatase using distilled water as eluent. Fractions eluted were assayed for P_i and monitored at 280 mμ for enzyme.

VI·4 The elution pattern obtained on gel filtration on a Bio-Gel P-4 column (equilibrated with 0.1 M NaCl) of different concentrations of P_i mixed with a fixed concentration of alkaline phosphatase using 0.1 M NaCl as eluent. Fractions eluted were assayed for P_i and monitored at 280 mμ for enzyme.

VI·5 Effect of pH on the elution characteristics of P_i and alkaline phosphatase on Bio-Gel P-4 equilibrated with 3 μg P_i/ml or 3 μg P_i/ml in 0.1 M NaCl using either 3 μg P_i/ml or 3 μg P_i/ml in 0.1 M NaCl as eluent. Fractions eluted were monitored for both enzyme and P_i.

VI·6 Effect of pH on the elution characteristics of P_i and alkaline phosphatase on Bio-Gel P-4 equilibrated with 3 μg P_i/ml or 3 μg P_i/ml in 0.1 M NaCl using either 3 μg P_i/ml or 3 μg P_i/ml in 0.1 M NaCl as eluent. Fractions eluted

were monitored for both enzyme and P_i.

VI·7 Effect of different enzyme concentrations on the elution characteristics of P_i and alkaline phosphatase on Bio-Gel P-4 equilibrated with 3 µg P_i/ml at pH 7.0. Samples were eluted with 3 µg P_i/ml, pH 7.0 and fractions monitored for enzyme and P_i.

VI·8 Effect of increasing P_i concentration on the elution characteristics of P_i and alkaline phosphatase on Bio-Gel P-4 equilibrated with a solution containing P_i in the same concentration as the sample, at pH 7.0. A constant enzyme concentration was used, and samples, eluted with the equilibrating P_i solution, were monitored for P_i and enzyme.

VI·9 Elution of ^{32}P and alkaline phosphatase from a Bio-Gel P-4 column equilibrated with 3 µg P_i/ml, pH 7.0 when the ^{32}P was added to the 1 ml of eluent (a) following the enzyme and (b) containing the enzyme, using the equilibrating solution as eluent. Fractions eluted were monitored for enzyme, P_i, and ^{32}P.

VI·10 Elution of ^{32}P from a Bio-Gel P-4 column equilibrated with 3 µg P_i/ml, pH 7.0 when the ^{32}P was added to the 1 ml of eluent (a) immediately preceeding the enzyme and (b) 2 ml ahead of the enzyme, using the equilibrating solution

as eluent. Fractions eluted were monitored for enzyme, P_i, and ^{32}P.

VI·11 Elution of ^{32}P and alkaline phosphatase from a Bio-Gel P-4 column equilibrated with distilled water. ^{32}P was added to alkaline phosphatase which contained a small amount of bound P_i and this sample was eluted with distilled water. Eluate fractions were monitored for enzyme, P_i, and ^{32}P.

VI·12 Elution of ^{32}P and alkaline phosphatase from a Bio-Gel P-4 column equilibrated with distilled water. ^{32}P was added to alkaline phosphatase containing, along with a small amount of bound P_i, 25 μg P_i/ml, and this sample was eluted with distilled water. Eluate fractions were monitored for enzyme, P_i, and ^{32}P.

LIST OF TABLES

Definition of Symbols Used in This Thesis

Inositol monophosphate	IMP
Inositol diphosphate	IDP
Inositol triphosphate	ITriP
Inositol tetraphosphate	ITetraP
Inositol pentaphosphate	IPP
Inositol hexaphosphate (phytic acid)	IHP
Inositol pentaphosphate +Inositol hexaphosphate	Inositol pentaphosphate' or IP'P
Glycerophosphate	GP
Inorganic phosphate	P_i
Optical density	O.D.
Phosphorus/myoinositol ratio	P/I ratio
Change in the reciprocal of the reaction velocity per unit change in inhibitor concentration	$\Delta \frac{1}{v} / \Delta I$
Void volume of a gel filtration column	Vo
Elution volume of a solute from a gel filtration column	Ve

CHAPTER I

INTRODUCTION

Phytic acid, the hexaphosphate of mesoinositol (Fig. II.1), is a compound which, in its salt forms, is ubiquitous in plants and is also found in animal tissues.

It has been suggested that the removal of phytate from cereals and sugars in the refining process (Jenkins et al, 1959), may be responsible for the elimination of the protective effect of the unrefined forms of these foodstuffs against dental caries in animals (McClure, 1963), and in humans (Osborn and Noriskin, 1937; Osborn, Noriskin and Staz, 1937).

Because of its ability to bind multivalent cations, phytate has also been implicated as a dietary constituent which can prevent the absorption in the intestine of a number of nutritionally essential elements such as calcium, magnesium, zinc, and iron (McCance and Widdowson, 1942a; McCance, Edgecombe and Widdowson, 1943; Likuski and Forbes, 1965).

The rachitogenic property of oatmeal has been attributed to the presence of phytate in the oatmeal which, by binding calcium in the diet and forming poorly soluble calcium phytate, may, under certain circumstances, reduce the intestinal absorption of calcium and its availability for bone formation (Harrison and Mellanby, 1939).

Although these effects might suggest that phytate is a

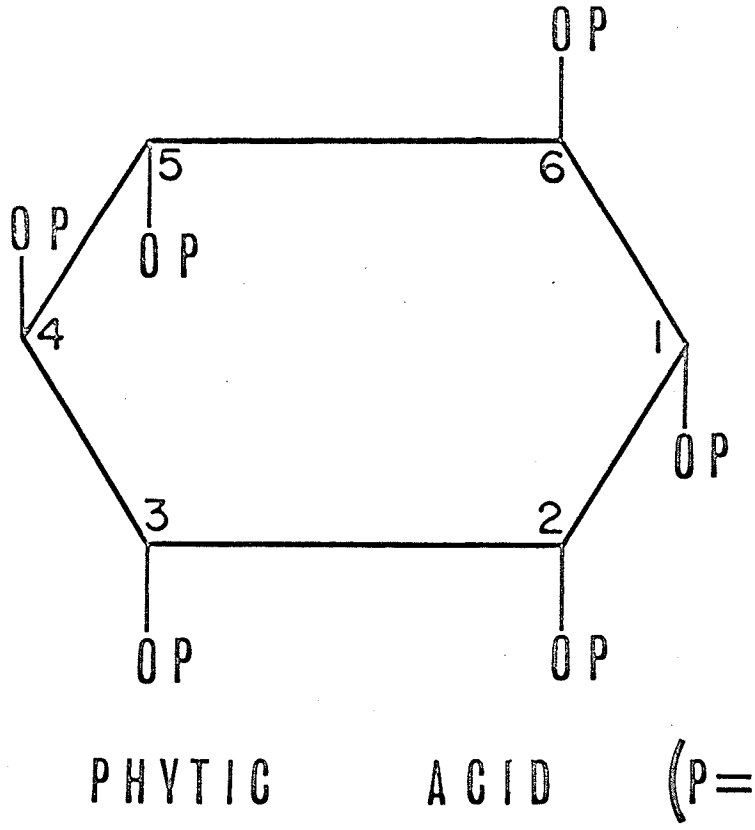


FIGURE I·1

Schematic drawing of the Phytic acid molecule, inositol hexaphosphate (IHP), which consists of a 6-carbon ring with a phosphate group attached to each carbon atom. The possibility exists for hydrogen bonding between oxygen atoms on adjacent phosphate groups such as those attached to carbons 1 and 2, and 4 and 5. The phosphate groups can each be replaced by OH groups and if all 6 phosphates are replaced, the resulting structure is myoinositol (also referred to as mesoinositol).

harmful dietary constituent, several studies have indicated that this compound can be beneficial in the treatment and prevention of a number of pathological conditions. For example, phytate has been used with some success in humans for the treatment of hypercalcaemia (Stapleton, MacDonald and Lightwood, 1956) and hypercalciuria (Vagelos and Henneman, 1957).

Studies concerned with the effect of phytate on the nutrition of zinc and iron have produced conflicting results. For example, addition of sodium phytate to the diets of growing chicks (Maddaiah, Kurnick and Reid, 1964; also turkey poults; Vohra and Kratzer, 1966; and rats; Likuski and Forbes, 1965; Byrd and Matrone, 1965; Oberleas, Muhrer and O'Dell, 1966) reduced the uptake of zinc and, as a result, the growth of these animals. Formation of an insoluble zinc-phytate complex in the intestine and, as a result, decreased absorption of the zinc, has been suggested as the explanation. In other studies with chicks (Nielson, Sunde and Hoekstra, 1966), phytic acid, when fed in a diet consisting of hydrolyzates of egg white and casein and in amounts comparable to those found in soy protein, showed little or no effect on the availability of zinc for normal leg development in these animals. As explanation, it was suggested that soy protein may contain a

factor that nullifies the effect of phytic acid. With respect to iron, McCance et al (1943) showed that when phytate was added to the diet of humans, absorption of iron in the intestine was reduced. However, in rats, phytate had no effect on iron absorption (Cowan et al, 1966).

Until more is known about the properties of phytate and its metabolism in relation to these conditions, the inclusion of phytate in human diets, should this compound prove to be an effective preventive agent against dental caries, is likely to be controversial.

Essential to this problem, would appear to be the necessity for obtaining information which would make it possible to determine whether altering the conditions of phytate administration (e.g. its salt form, pH, use of hydrolyzed products of the phytate, etc.) would eliminate any harmful, and retain any beneficial effects that it might have.

During its metabolism, phytate is usually hydrolyzed, either partially or completely, by phytases, producing various inositol phosphate intermediates, inositol, and Pi. Therefore, before determination of the best conditions for phytate administration can be determined, it is necessary to obtain basic information on the properties of phytate and its inositol phosphate derivatives, and also on the factors influencing the hy-

drolytic action of phytase on these substances. In this thesis an attempt has been made to obtain some of this information.

The studies that have been reviewed below in this introduction are those that relate to (1) the role of phytate in the intestinal absorption of calcium and in dental caries, and (2) the factors involved in the sequential hydrolysis of phytate and the properties of the inositol phosphate intermediates formed during such hydrolysis.

The Role of Phytate in the Intestinal Absorption of Calcium

The implication of phytate as the rachitogenic agent in oatmeal and in other cereals resulted from a number of nutritional studies carried out mostly between 1921 and 1942.

In 1921, Mellanby observed that the rickets produced in puppies fed a diet deficient in vitamin D was more severe if the diet also contained oatmeal. Initial boiling of the oatmeal with dilute HCl resulted in the disappearance of the rachitogenic effect of the oatmeal which could be reduced by increasing the level of vitamin D or calcium (as calcium carbonate or phosphate) in the diet (Mellanby, 1925).

The antirachitic action of vitamin D was also shown by Pileggi, DeLuca and Steenbock (1955) in rats fed cereal diets in which the Ca/P ratio of the diet was high. Since the Ca/P

ratio of the diets used by Mellanby was low, this indicates that the action of vitamin D is independent of the dietary Ca/P ratio.

In 1939, Harrison and Mellanby isolated phytate from both oatmeal and commercial phytin (a CaMg salt of phytic acid) and showed that feeding puppies either the acid or the sodium form of this compound reproduced the rachitogenic effect of the oatmeal. They concluded that phytate exerted its rachitogenic effect by binding the calcium in the diet, thus reducing the availability of calcium in the intestine for absorption. That this effect of phytate may be more complex is suggested by their finding that replacing phytic acid and sodium phytate with phytin, not only resulted in the elimination of the rachitogenic effect of phytate, but actually reduced the rickets over that in the control animals.

The possibility that, in humans, phytate in bread prepared from wheat flour might reduce the availability of calcium from the diet was examined by McCance and Widdowson (1942a).

Bread made from flour of both high and low extraction rates (the less material removed from the original flour, the higher the extraction rate) was fed to a small number of healthy men and women and their calcium balances determined.

When the subjects were fed bread prepared from flour of high extraction rate (brown bread), the subjects rapidly went into negative calcium balance, whereas when fed bread prepared from flour of low extraction rate (white bread), little or no shift in balance was observed. Addition of sodium phytate to white bread reproduced the effects with brown bread. Since the addition of calcium to the diet improved calcium absorption in the presence of brown bread, McCance and Widdowson recommended that calcium, as $CaCO_3$, should be added to wheat flour.

McCance and Widdowson also concluded from their experiments that some of the phytate was hydrolyzed in the small intestine, and that the availability of the phytate phosphorus for absorption was less than from other phosphates present in the experimental diet. In spite of this reduced availability of phytate P from brown bread, the data of McCance and Widdowson showed that the subjects went into positive phosphorus balance.

In another series of experiments, McCance and Widdowson (1942b) showed that enzymic hydrolysis of the phytate in brown bread prior to feeding did not completely remove the effect of brown bread on the calcium balance. They suggested, as explanation, that the inorganic phosphate produced interfered with calcium absorption, and that hydrolysis of the phytate

in brown bread is not a satisfactory procedure for improving calcium absorption.

That the negative calcium balance observed by McCance and Widdowson is a temporary condition was shown by Walker, Fox and Irving (1948), who carried out their experiments for much longer times. They found that their subjects also went into negative calcium balance almost immediately, but returned to normal calcium balance after three to five weeks. They suggested as explanation that calcium phytate was hydrolyzed within the digestive tract, since any dietary phytase that might have been present would probably have been destroyed by cooking.

Should calcium phytate be completely hydrolyzed in the gut, then inorganic phosphate would be released and, in the presence of calcium, would form calcium phosphate. For the explanation of Walker et al (1948) to be correct, the calcium phosphate formed would have to be more soluble than calcium phytate.

The in vitro experiments by McCance and Widdowson (1942a) support this possibility since they showed that calcium phytate precipitates at a pH of approximately 3, a pH at which calcium phosphate is very soluble. That calcium phytate may precipitate more readily than calcium phosphate in the small

intestine, is also suggested from experiments carried out by Hoff-Jorgenson (1944) in which he simulated conditions likely to prevail in the duodenum (substances entering the duodenum from the stomach would be subjected to a change in pH from approximately 2 in the stomach to approximately 5 to 7 in the duodenum). In his experiments, raising the pH of solutions of either calcium phytate or calcium phosphate from about 3.5 to about 5.0, produced immediate precipitation of calcium phytate and very slow precipitation of calcium phosphate.

Whereas these studies indicate that calcium phytate is less soluble than calcium phosphate under experimental conditions involving precipitation, other experiments, those in which their solubilities are compared by dissolving, indicate a reverse relationship.

Hoff-Jorgenson (1944) showed, in this type of exper iment, that between pH 4 and 7, calcium phytate is more soluble than calcium phosphate. In support of this, Jenkins et al (1959) showed that, when sodium phytate was added to a saturated solution of calcium phosphate at pH 4.76 or 5.52, no precipitation of calcium occurred.

From these studies, it appears as if the effect of unhydrolyzed phytate on the solubility of calcium in the presence of phosphate is not simple and, since phytate can undergo

hydrolysis, the effect of the products produced during such hydrolysis would be expected to be considerably more complex.

The Effect of Phytate on Hypercalcaemia in Humans

Idiopathic hypercalcaemia of infants has been a serious problem in Britain (Rhaney and Mitchell, 1956; Morgan et al, 1956; Stapleton et al, 1956). The cause is obscure but the symptoms resemble those of hypervitaminosis D. The high level of vitamin D in baby foods has been cited as a possible cause. Evidence has also been presented suggesting that a defect in cholesterol metabolism may be the principal cause (Forfar et al, 1956).

Treatment of patients, suffering from hypercalcaemia, with phytate added as "all bran" (breakfast cereal) to their diets was ineffective in lowering serum calcium (Bonham Carter et al, 1955).

However, a diet containing low-calcium milk and low-calcium cereal was effective in reducing serum calcium in patients with the same condition (MacDonald and Stapleton, 1955; Stapleton et al, 1956). These workers suggested that the high phytate and inorganic phosphate content in the cereal may increase the loss of calcium in the faeces by binding calcium in the intestinal secretions.

The Effect of Phytate on Hypercalciuria in Humans

During the acute, but not during the chronic stages of poliomyelitis a severe hypercalciuria may occur which can produce kidney calcification and cessation of kidney function. To carry patients through this acute stage, Vagelos and Henneman (1957) treated nine patients orally with sodium phytate. Five of the nine patients showed a marked reduction in 24 hour urinary calcium excretion while the results on four patients were inconclusive. These workers suggested that the calcium liberated during the acute stage was diverted into the gastrointestinal tract by phytate and that the mechanism for such an effect is unkown.

Hypercalciuria also occurs in patients with vitamin D poisoning and sarcoid, conditions in which the hypercalciuria has been shown to be due to excessive absorption of calcium from the gastrointestinal tract (Henneman, Caroll and Albright, 1956).

Treating these patients with sodium phytate (9g/day orally) to block absorption of calcium from the intestine, decreased urinary levels of calcium and magnesium. Since the calcium balance was hardly altered, these workers suggested that sodium phytate blocked the absorption of dietary calcium (therefore, more in faeces- less in urine) and did not alter the reabsorp-

tion of calcium secreted into the intestinal tract through the bile and other intestinal juices.

The Role of Phytate in Dental Caries

The incidence of dental caries in the South African Bantu, whose diet is primarily unrefined carbohydrate, is low when compared to that for both the Bantu and European on so-called modern diets (Osborn and Noriskin, 1937; Staz, 1938). To determine whether unrefined dietary carbohydrate (cereals and sugars) contained a factor protective against this disease, Osborn et al (1937) carried out experiments which showed that decalcification of human teeth in vitro occurred more readily in mixtures of saliva and refined carbohydrate than when the crude forms of the same carbohydrates were used. These in vitro observations could not be explained on the basis of formation of acid by the oral micro flora in these incubation mixtures.

These results were confirmed and extended by Jenkins et al (1959) who showed that when calcium phosphate was incubated with saliva in the presence of white and brown flour, the amount of calcium dissolving in the presence of brown flour was about 2/3 of that dissolving in the presence of white flour. The fall in pH was the same with both flours. Acetic acid extracts of the brown flour reproduced the effects of

the brown flour. Sodium phytate prepared from wheat bran also reproduced these effects.

Removal of phytate from brown flour with $FeCl_3$ showed that other substances in the brown flour extract, possibly phosphates other than phytate, were also protective.

An interesting finding was that teeth, exposed to phytate and subsequently washed repeatedly with distilled water, showed protection against dissolution with acid buffer when compared to their controls. On the other hand, teeth exposed to brown flour extracts did not show the retention of protection observed with phytate.

These workers suggested that phytate adsorbs to the surface of the teeth and forms either a protective layer or a less soluble surface complex.

These findings were essentially repeated and confirmed by Andlaw (1960a,b), Bibby and Andlaw (1961), and by Grenby (1967a,b,c).

In more recent experiments, Jenkins and Smales (1966) showed that aqueous extracts of wheat bran, wheat germ, oat hulls, pecan hulls, and cocoa also reduced the solubility of tricalcium phosphate in incubated saliva, and as in the earlier experiments of Jenkins et al (1959), had little inhibitory effect on acid production. They concluded that any anti-

caries effect exerted by these materials is more likely to be brought about by water extractable substances diffusing into the saliva and affecting the solubility of teeth than by inhibition of bacterial acid production.

The effect of phytate on animal caries has been tested by McClure (1960, 1963, 1965) and by Dawes and Shaw (1965) on rats.

McClure showed that phytate, as well as several other phosphates (orthophosphates, metaphosphates, β-glycerophosphate) markedly reduced dental caries in rats. This effect of phytate was later confirmed by Dawes and Shaw (1965).

When rats were fed either Na_2HPO_4 or sodium phytate by stomach tube (McClure and Muller, 1959; McClure, 1965 respectively), rather than in the diet, a significant reduction in caries was not observed. These results indicate that these compounds exert their effect locally in rats, rather than systemically. Hydrolysis of phytate would provide inorganic phosphate which may alter the critical pH in the plaque and that for the dissolution of the underlying enamel (Fosdick and Starke, 1939).

To answer this question, the same lack of information, as pointed out in the earlier section in relation to the role of phytate in the intestinal absorption of calcium and other

dietary elements, would apply.

Studies Related to the Hydrolysis and Properties of Phytate and its Inositol Phosphate Intermediates

Phytate can be sequentially hydrolyzed with acid or base (Kerr and Kfoury, 1962) or with enzymes (Tomlinson and Ballou, 1962) to produce IPP, ITetraP, ITriP, IDP, IMP, inositol and P_i. The amounts of the various intermediates produced depend upon the time of hydrolysis.

Tomlinson and Ballou (1962) have presented a scheme for the enzymic hydrolysis of phytic acid which is shown in Fig. IV.6 and seems to indicate a resistance of adjacent phosphate groups to hydrolysis which is supported by data in this thesis (Chapter IV and V).

Enzymic Hydrolysis of Phytic Acid

Enzymes which catalyze the sequential hydrolysis of phytic acid are usually referred to as phytases and are found in some microorganisms and in most plant and animal tissues.

The phytases isolated from microorganisms and plant tissues seem to have lower pH optima than those from animal cells and tissues.

A phytase isolated from colibacilli had a pH optimum of 2.2 (Courtois and Manet, 1952), and one from Aerobacter aerogenes showed a pH optimum between 4 and 5 (Greaves, Anderson

and Webley, 1967).

In studies comparing the pH of optimum phytase activity in a crude extract of Manitoba flour (100% extraction rate) to that of a purified preparation, Peers (1953) found that in both, phytase activity was optimal at pH 5.15.

The blood plasmas and erythrocytes of various lower vertebrates (birds, reptiles, batrachians, fish) have been found to contain phytases of optimum pH 5.7 to 7.2 (Rapoport et al, 1941).

Rat organs (intestinal mucosa, kidney, brain, spleen, liver, and plasma) examined by Pillegi (1959), all showed phytase activity. The pH optimum for intestinal enzyme was 7.3, a figure lower than that for glycerophosphate.

Summarizing the studies in plant phytases, Posternak (1965e) concluded that since all phytases studied also split monoesters like the glycerophosphates, the question as to whether this is owing to a lack of specificity or whether the preparations are contaminated with phosphomonoesterases remains unsolved.

Pillegi (1959) concluded after studying rat intestinal phytase, that it could be a form of alkaline phosphatase.

Studies Related to the Properties and Metabolism of Inositol Phosphates

Most studies on inositol phosphates have been concerned with phytic acid (the most commonly formed ester - see above). and little is known about its lower inositol phosphate derivatives.

These lower derivatives are most commonly found free in plant tissue (Anderson, 1915, Pøsternak, 1965c) and in soils (Bower, 1945); in the latter, these compounds probably arise from bacterial and plant residues present in the soil.

In some cases, these inositol phosphates may be products of hydrolysis of phytic acid (Saio, 1964), while in others, they may be intermediates in the synthesis of phytic acid (Asada and Kasai, 1959; Saio, 1964).

In soils, availability of phosphate from phytate for plant growth is poor, and this has been attributed in part to the formation of insoluble salts (Whiting and Heck, 1926; Conrad, 1939; Rogers, Pearson and Pierre, 1940; Anderson and Arlidge, 1962). Since the solubility of calcium and magnesium salts of inositol phosphates with lower P/I ratios is greater than that for the inositol phosphates with higher P/I ratios (Posternak, 1965d), the availability of phosphate from the compounds with fewer phosphate groups would appear to be better than that from phytic acid.

That the formation of insoluble calcium phytates is not

the whole answer, is suggested by a finding that phytate is less available from acid than from alkaline soils (Goring and Bartholomew, 1950; Anderson and Arlidge, 1962).

In animal tissues inositol-1-phosphate is present as an intermediate in the synthesis of inositol from glucose-6-phosphate (Eisenberg, Jr. and Bolden, 1965). Mono-, di-, and triphosphates of inositol are also found as constituents of the phosphoinositide component of phospholipids in animal tissues (Hawthorne, 1960, 1964).

The phosphoinositides play an important role in the transport of substances through cellular membranes and in membrane contraction (Hawthorne, 1964).

A variety of substances (e.g. sugars, amino acids, ions) can be transported actively across cell membranes and recent evidence suggests that a protein-phospholipid complex is responsible (Hokin and Hokin, 1961; Nikaido, 1962; Tria and Barnabei, 1963). It is also suggested that each substrate can combine with the protein part of this complex, and that a conformational change is induced in the protein through a change in the lipid (e.g. phosphorylation or dephosphorylation of phosphatidic acid or phosphoinositide) allowing transport of the substrate and its removal once transport is accomplished.

One of the sources of energy for the active transport is the compound ATP, produced during the glycolytic breakdown of glucose. Inositides have been implicated as playing a role in coupling the energy in the ATP molecule via the membrane ATPase system to membrane transport processes.

A protein fraction, similar to actomyosin of skeletal muscle, required for ATP-induced contraction of mitochondrial membranes and probably plasma membranes, has been separated by Ohnishi and Ohnishi (1962a,b). Much of the contractile effect of this protein was due to phosphatidyl inositol which adheres to the protein (Vignais, Vignais and Lehninger, 1963). This contractile function would have an effect on the permeability of membranes for transport.

For example, Neifakh and Kazakova (1963) suggested that the actomyosin-like protein may affect regulation of release from the mitochondria of glycolysis-stimulating factors. The membrane, which is contracted in the presence of high mitochondrial ATP, would swell when ATP is low, releasing factors which stimulate glycolysis, increasing the ATP level.

It is important to note that inositol, a functional isomer of glucose and classified as a vitamin within the B group (Woolley, 1941), is a constituent of the phosphoinositides.

Inositol has been shown to cure alopecia (baldness) in

mice (Woolley, 1941), although dietary inositol was ineffective in the absence of pantothenic acid in the diet, presumably due to a reduction in inositol synthesis by intestinal organisms (Woolley, 1942).

It also prevented the type of fatty liver caused by biotin deficiency in rats (Gavin and McHenry, 1941).

Inositol is required for the growth and survival by a number of yeasts and fungi (Posternak, 1965a; Matile, 1966), and bacteria (Posternak, 1965b; Tsukagoshi, Lembach and Charalampous, 1966), and for every cultured human cell examined (Eagle et al, 1957).

Bacterial and animal cells, both normal and malignant, and viruses are protected by inositol from damage due to partial dessication from X-ray irradiation, drying or aging (Webb, 1960a,b; Webb, 1961; Webb, Bather and Hodges, 1963; Webb, 1963; Webb and Dumasia, 1964; Webb, Cook and Bather, 1964; Bather, Sebastian and Webb, 1964). Dessication above 60% relative humidity resulted in the removal of bound water from the nucleotides of DNA and RNA and possibly from enzymes, causing disorganization of the helix and cell death, possibly through a lack of ability to synthesize protein. It is suggested that inositol, because of its structure, acts by replacing this bound water, thus maintaining the integrity of

the nucleotides and allowing protein synthesis to continue.

Purpose of This Thesis

Phytic acid and the compounds formed during its partial and complete hydrolysis are obviously involved in a wide variety of biological processes. Little is known of the properties of phytate and of the intermediates formed during its hydrolysis as they relate to these processes. In this thesis, an attempt has been made to investigate a few of these properties.

Because experiments by Jenkins et al (1959) have suggested that phytate can reduce the solubility of enamel possibly by forming an adsorbed layer on the tooth surface, the characteristics of the phytate molecule which might account for this adsorption have been examined.

Because phytate may be hydrolyzed in the oral cavity and in the gastrointestinal tract should phytate be present in the diet, the factors affecting this hydrolysis and the properties of the phytate molecule as well as of its inositol phosphate intermediates involved in such hydrolysis have been examined in some detail.

Plan of Thesis

In Chapter I, the role of phytate in the intestinal absorption of calcium, in dental caries, and in other biological phenomena has been reviewed.

Chapter II is concerned with the general preparative and analytical methods used in this thesis. The preparation of phytic acid and the inositol phosphate intermediates and the enzymic method for the quantitative analysis of inositol used in the identification of the intermediates are described in detail.

In Chapter III, anion exchange chromatography was used to study the effect of pH on the properties of the phytate molecule which affect its adsorption to and elution from a Dowex-1-Cl resin.

To compare sodium and calcium binding by phytic acid and its inositol phosphate derivatives and the solubility of their calcium salts, in Chapter IV, IMP, IDP, ITriP, ITetraP, IP'P, and phytic acid were each titrated with $Ca(OH)_2$ and compared to similar titration with NaOH. Differences in their titration curves gave a measure of calcium and sodium binding, and the pH at which a precipitate appeared during titration with $Ca(OH)_2$ gave a measure of the relation between pH and the solubility of their calcium salts.

In the experiments in Chapter V, phytate and each of its inositol phosphate derivatives were hydrolyzed with acid and alkaline phosphatases. pH optima for each enzyme-substrate combination and the effect of substrate concentration on their

pH optima were determined. Km values for each enzyme-substrate combination and the influence of P_i on the rate of hydrolysis of each substrate by either acid or alkaline phosphatase were also determined.

In Chapter VI a gel filtration procedure was used to examine some of the variables (pH, enzyme concentration, P_i concentration) that might affect the repulsion between P_i and alkaline phosphatase.

A discussion on the relation of the findings in this thesis to dental caries, the intestinal absorption of calcium, and other biological phenomena is presented in Chapter VII, followed in Chapter VIII by a summary of the thesis.

CHAPTER II

METHODS

Introduction

The methods presented in this chapter have been classified into those that are preparative and those that are analytical.

1) Preparative Procedures

In order to carry out the experiments in this thesis, it was necessary to prepare several inositol phosphate intermediates from phytic acid and the enzyme inositol oxygenase from male rat kidney. Other chemicals and enzymes were purchased and in some cases (phytic acid and the enzymes, acid and alkaline phosphatase), additional purification was carried out. Those not requiring such treatment that were purchased, were almost all reagent grade.

i) Purification of Phytic Acid

100 gm sodium phytate, purchased from Nutritional Biochemicals (Cleveland, Ohio), was dissolved in 200 ml distilled water and the pH adjusted to pH 1.0 with concentrated HCl.

0.26 M ferric chloride was added, resulting in the formation of a white precipitate of ferric phytate. The ferric chloride was added in excess as indicated by the yellow color of the solution and the precipitate that was formed was cent-

rifuged for 20 minutes at 1600 x g (International Centrifuge - Universal Model UV). The supernatant was discarded. The precipitate was then washed three times using approximately 1.5 litres of distilled water for each washing.

The precipitate was then suspended in distilled water and the pH raised to between 11.0 and 12.0 with NaOH. A flocculent precipitate of ferric hydroxide formed and was sedimented by centrifugation at 1600 x g for 20 minutes. The supernatant was decanted and the precipitate washed once with NaOH. The washings and supernatant were combined and the precipitation process repeated twice.

The sodium phytate solution was then put through a Dowex cation exchange resin (Bio·Rad 50W -x8, 100-200 mesh, H^+ form; purchased from Calbiochem, Los Angeles) and the phytate converted to phytic acid.

Analysis for inorganic and total P showed that this phytic acid solution, which was 0.011 M, contained an inorganic phosphate concentration of 0.002 M. This concentration of P_i was approximately 3% of the total P concentration.

The percentage of the total P present as P_i was reduced to 0.1% of the total by the following procedure.

An aliquot, usually 20 ml, of the stock 0.011 M solution of phytic acid was put onto a glass column (1.0 x 30 cm) con-

taining Dowex anion exchange resin (Bio.Rad AG 1 -x8, 200-400 mesh, Cl^- form; purchased from Calbiochem, Los Angeles). Elution, first with 50 ml of 0.025 M LiCl, eluted P_i; elution, with 40 ml of 1.0 M LiCl, eluted the phytate.

The lithium phytate fraction was lypophilized, and the LiCl in the fraction removed by washing the residue 6 times with 5 ml volumes of absolute methanol. Residual methanol was removed by evaporation with a stream of air. The lithium phytate was then dissolved in 10 ml of distilled water.

The lithium phytate was converted to phytic acid by putting the lithium phytate solution onto a glass column (1.0 x 30 cm) containing a Dowex cation exchange resin (50 -x8, H^+ form) and eluting with 20 ml of distilled water. The eluate was lypophilized to concentrate the phytic acid and to remove residual HCl. The residue was dissolved and lypophilized 6 times to ensure complete removal of HCl.

Attempts to reduce the P_i level below 0.1% of the total P by repeating this portion of the purification procedure (anion exchange chromatography with LiCl) were not successful.

ii) <u>Preparation of the Inositol Phosphates Produced when Phytic Acid is Hydrolyzed</u>

Separation of phytic acid and each of its inositol phosphate derivatives from an acid or enzyme hydrolyzate of phytic

acid is usually accomplished by anion exchange chromatography, using chloride in progressively increasing concentrations as eluent (Tomlinson and Ballou, 1962; Saio, 1964).

In this study, phytic acid was hydrolyzed by heating aliquots of the stock phytic acid in sealed tubes under vacuum, and the inositol phosphates produced were isolated by anion exchange chromatography. This was done as follows:

5 ml of the stock phytic acid was added to each of 6, 20 ml pyrex test tubes. The tubes were sealed under vacuum and the contents partially hydrolyzed by heating for three hours at 120^{o}C in a hot air oven (Arnold, 1956). The contents were then put onto a Dowex anion exchange resin (1 -x8) as in section (i), and the inositol phosphate intermediates successively eluted stepwise with concentrations of LiCl between 0 and 1.0 M (Fig. II.1).

Because phytate is eluted in multiple peaks, a large step in the LiCl gradient was used (i.e. elution with 0.5 M LiCl was followed immediately by 1.0 M LiCl) to elute the final fraction. This peculiar elution pattern of phytate can be attributed to adsorption effects and this property of the phytate molecule is examined in Chapter III.

Fractions labelled II, III, IV, and V in Fig. II.1 were each taken to dryness and LiCl removed as described in section

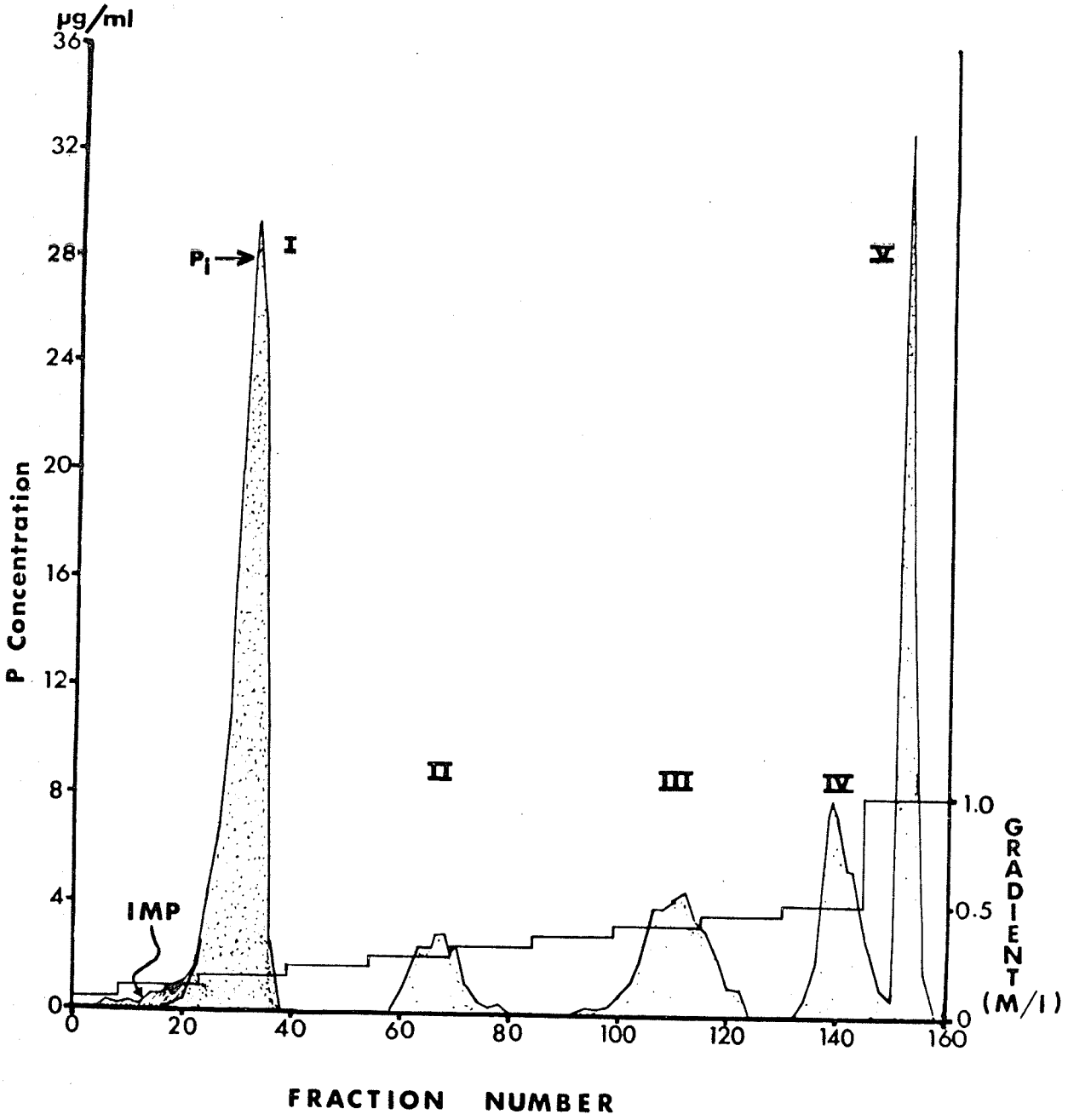


FIGURE II·1

The elution pattern obtained on ion exchange separation on a Dowex-1-Cl column of the phosphate products from a partial acidic dephosphorylation of phytic acid. For each step in the gradient, 20 ml of LiCl was used as eluent and approximately 1 1/3 ml fractions collected and analyzed for phosphorus.

(i) for phytic acid. The final residue was dissolved in 3 ml of distilled water.

To identify the inositol phosphate in each of these fractions, aliquots were removed from each and analyzed for phosphorus and inositol so that their phosphorus/inositol (P/I) ratios could be calculated. The inositol was determined by two procedures; in one series of separations and analyses, an enzyme method using inositol oxygenase was used (see below), while in a second series, as a check, inositol was analyzed by the non-specific dichromate oxidation procedure of Johnson (1949).

To prepare aliquots from each fraction for analysis by the inositol oxygenase method (always carried out at least in duplicate), the following procedure was followed. A 100 µl aliquot was transferred from each hydrolyzate, along with 100 µl of concentrated HCl, to a 10 x 75 mm pyrex tube, the open end of which had been previously drawn out to facilitate sealing. The tube was sealed under vacuum and with heat, and the contents hydrolyzed completely by heating at 120°C for 24 hours.

Following hydrolysis, the contents of each tube were taken to dryness, and twice redissolved in 100 µl of distilled water and again taken to dryness. The residue was then dis-

solved in 100 µl of distilled water and duplicate 5 µl aliquots were removed for P_i analysis.

75 µl of the remainder was passed through a micro-column (0.4 x 4 cm) containing a Dowex anion exchange resin (1 -x8, Cl^- form) to separate P_i from inositol, the latter not being adsorbed.

The eluate containing the inositol was taken to dryness, the dried sample redissolved with water, and the solution again taken to dryness. The dried sample was redissolved in 75 µl of distilled water and three 20 µl aliquots then removed from each sample and analyzed for inositol.

Because the dichromate oxidation procedure was carried out at a more macro level, a 1 ml aliquot was transferred from each hydrolyzate, along with 1 ml of concentrated HCl, to a 30 ml pyrex test tube. The tube was sealed under vacuum and the contents hydrolyzed completely by heating at 120°C for 24 hours.

Following removal of HCl by desiccation, the dried sample was redissolved in 900 µl of distilled water, and duplicate 400 µl aliquots then removed and analyzed for inositol. Inositol, purchased from Matheson, Coleman, and Bell, was used as standard in both inositol procedures.

The P/I ratios for fractions II, III, IV, and V, deter-

mined by the two procedures, are shown in Table II.1.

In view of the difficulty in separating IPP from IHP (cf. Tomlinson and Ballou, 1962), it was decided to utilize this mixture (labelled hereafter as IP'P) in our study, since, in the experiments involving the inositol phosphates, we were interested mainly in comparing the characteristics of these compounds as a series in which the P/I ratio progressively varied.

IMP was purchased from Calboichem, Los Angeles, because sufficient quantities could be obtained more easily this way than by the above hydrolysis. Analysis for P_i showed that no P_i was present; IMP from other sources of supply contained large quantities of P_i. IMP on a one-dimensional paper chromatogram ran as a single spot (Bandurski and Axelrod, 1951). When analyzed for P_i and inositol by the procedures used to identify the inositol phosphates in the above phytic acid hydrolyzate (only the inositol oxygenase method was used), IMP showed a P/I ratio of 1.0(5)/1.

The P/I ratio for phytic acid (inositol being determined by the dichromate oxidation procedure) was 6.0(0)/1.

iii) Preparation of Inositol Oxygenase for the Enzymic Analysis of Myoinositol with this Enzyme

Inositol has been analyzed by microbiological and enzymic procedures (Beadle, 1944; Wiss, 1950; Weissbach, 1958; Charalampous, 1959; Garcia-Bunuel, and Garcia-Bunuel, 1964), gas

TABLE II·1

Identification of the Various Inositol Phosphates by their Phosphorus/Myoinositol Ratios

These materials were prepared by anion exchange chromotography of an acid hydrolyzate of phytic acid. Inositol was analyzed by two methods, the inositol oxygenase procedure and the dichromate oxidation procedure.

FRACTION NUMBER	INOSITOL OXYGENASE ANALYSIS	DICHROMATE OXIDATION ANALYSIS	COMPOUND
	PHOSPHORUS/ MYOINOSITOL RATIO	PHOSPHORUS/ MYOINOSITOL RATIO	
II	2.1(9)	2.3(2)	INOSITOL DIPHOSPHATE
III	3.0(0)	3.2(8)	INOSITOL TRIPHOSPHATE
IV	3.8(2)	3.7(8)	INOSITOL TETRAPHOSPHATE
V	5.4(5)	5.4(5)	INOSITOL PENTAPHOSPHATE + INOSITOL HEXAPHOSPHATE

chromatography (Roberts, Johnston and Fuhr, 1965), and by chemical methods involving periodate oxidation of the inositol (Platt and Glock, 1943).

All of the above methods suffer from disadvantages. In the present study, the inositol oxygenase procedure of Charalampous was selected and was modified so that it could be used reasonably conveniently, considering that a stable preparation of this enzyme cannot be prepared.

The principle of the method is the enzymic conversion of inositol to glucuronic acid catalyzed by inositol oxygenase, and the colorimetric measurement of a green complex formed when glucuronic acid is subsequently reacted with orcinol-$FeCl_3$.

In this section, the procedure used to prepare inositol oxygenase is described; the use of this enzyme in the analysis for inositol is described in the analytical section below.

As carried out by Charalampous, the procedure for preparing the enzyme was complex, requiring a minimum of about 14 hours to complete. Once prepared, the enzyme retained full activity for a maximum of ten days under the best of conditions (freezing at -20^{o} under nitrogen).

We confirmed the observation of Charalampous that the enzyme was unstable, and therefore decided to scale the

analytical portion of the method to a more micro level (factor of 20) so that less enzyme would be required per analysis, and more analyses could be done during the period that the enzyme was stable.

By introducing gel filtration into the preparative procedure, this procedure was simplified, and only three hours were required to obtain a satisfactory enzyme preparation.

Each time fresh enzyme was prepared, one male adult rat was decapitated, the kidneys rapidly removed and immediately embedded in cracked ice and transferred to a cold room at 4°C, where all subsequent purification procedures were carried out. All fatty tissue was stripped from both kidneys and each kidney was cut into small pieces with a sharp scissors prior to homogenization in a medium prepared by mixing 4.5 ml of 1.4 M KCl and 4.5 ml of 0.1 M phosphate buffer, pH 7.2.

Homogenization was carried out in a 30 ml pyrex homogenizing flask (with diagonal fluting) using a Virtis "45" Macro Homogenizer with a macro shaft and two macro stainless steel blades rotated at 3/4 speed for 15 seconds (obtained from Virtis Co. Inc., Gardiner, N.Y.).

The homogenate, which was pink in color, was then centrifuged at 90,300 x g for 30 minutes and at 0°C in a Spinco Model L ultracentrifuge using rotor #40. Material at the

surface of the supernatant was removed by filtering the supernatant through Whatman #31 filter paper.

Approximately 2 gm of dry sephadex (G-25, 60-100 mesh; a dextran polymer that can be purchased from Pharmacia, Uppsala, Sweden) was added to concentrate the filtrate approximately four times. The sephadex was removed by filtering the kidney extract through nylon mesh and using centrifugation (400 x g) to remove residual filtrate (Fig. II.2).

The filtrate was red and slightly murky and was then passed through a 1.0 x 60 cm column containing G-100 sephadex (100-200 mesh; also purchased from Pharmacia, Uppsala, Sweden), using 0.05 M phosphate buffer, pH 7.2 as the eluent. Because different characteristics of a protein molecule can be observed by monitoring its absorption at 230 and 280 mμ, the fractions eluted from the column were monitored at both wavelengths, rather than only at 280 mμ as is common. Optical density readings were made on a Beckman DU spectrophotometer in which the cell compartment was altered to enable the rapid reading of many micro-samples (approximately 50 μl).

For determination of inositol oxygenase activity, four, 25 μl aliquots were removed from each eluate fraction and treated as follows. Two of the aliquots were added to tubes containing 20 μl of 0.025 M inositol and 5 μl of 1.0 M phos-

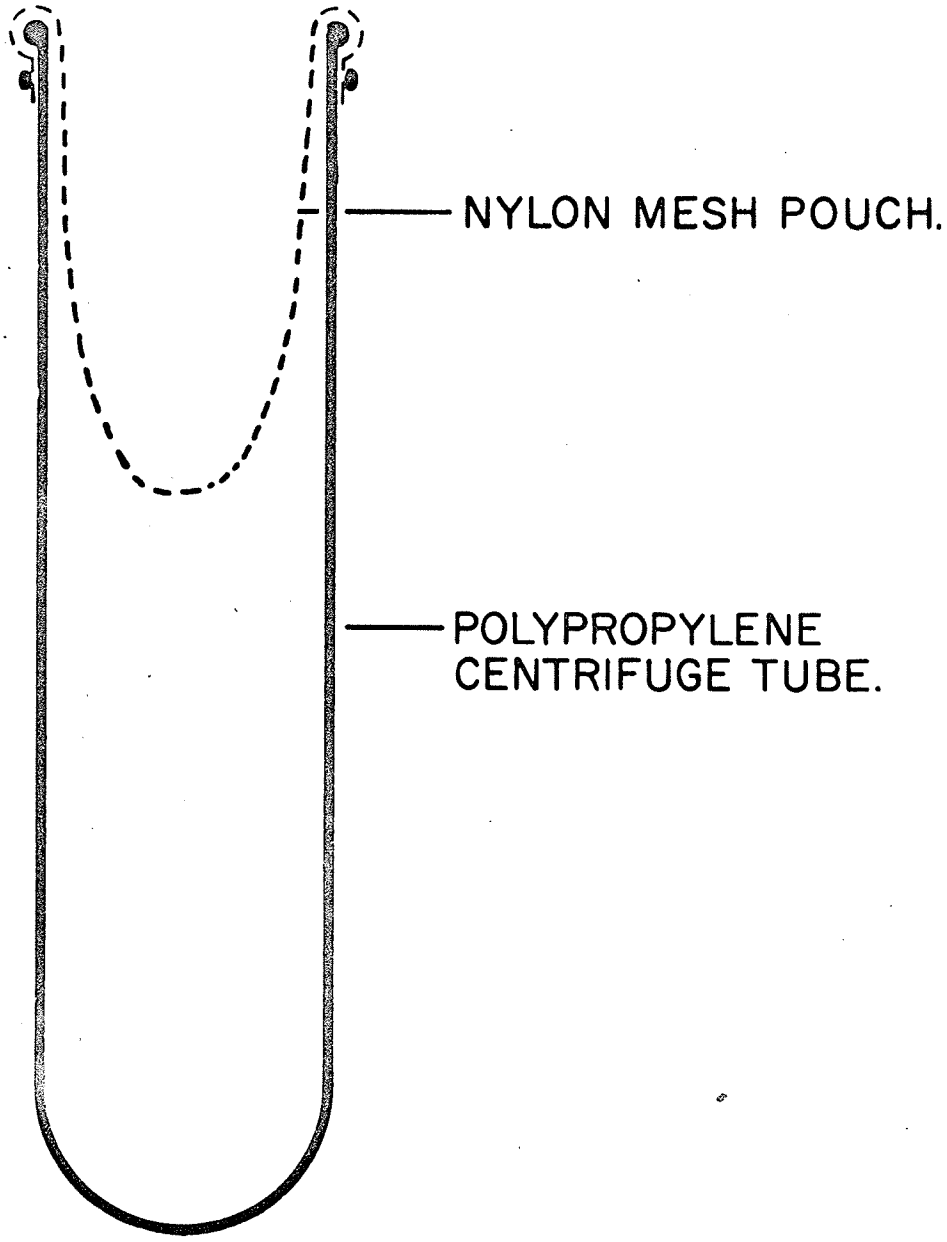


FIGURE II·2

Centrifuge tube adapted with nylon mesh to facilitate sample concentration with G-25 sephadex.

phate buffer, pH 7.2, while the other two were added to tubes containing 20 ul of distilled water and the same volume of buffer. Each mixture was then gassed with O_2, incubated at 35°C for 40 minutes, heated in a boiling water bath for two minutes, and then assayed for glucuronic acid by the procedure described below.

The results are shown in Fig. II.3. The eluate between 9.0 and 13.5 ml was yellow, that between 13.5 and 28.0 ml was reddish-orange, and that between 28.0 and 50 ml was colorless.

Only the reddish-orange component showed activity, as indicated in Fig. II.3 by a difference in the 660 mμ O.D. between the blank and the inositol-containing tubes. The reddish-orange color of the active fraction enabled easy isolation of this component from the G-100 sephadex column in subsequent preparations, since monitoring in a spectrophotometer was unnecessary.

The fractions showing maximum activity (containing maximum reddish-orange color; between eluate volume 16.5 ml and eluate volume 21.5 ml) were pooled and this was the enzyme preparation used in the analytical portion of the inositol oxygenase method for the estimation of inositol.

Other porosities of gel gave poorer separations; G-25, G-50, and G-75 were tested. Rechromatography on G-100 of the

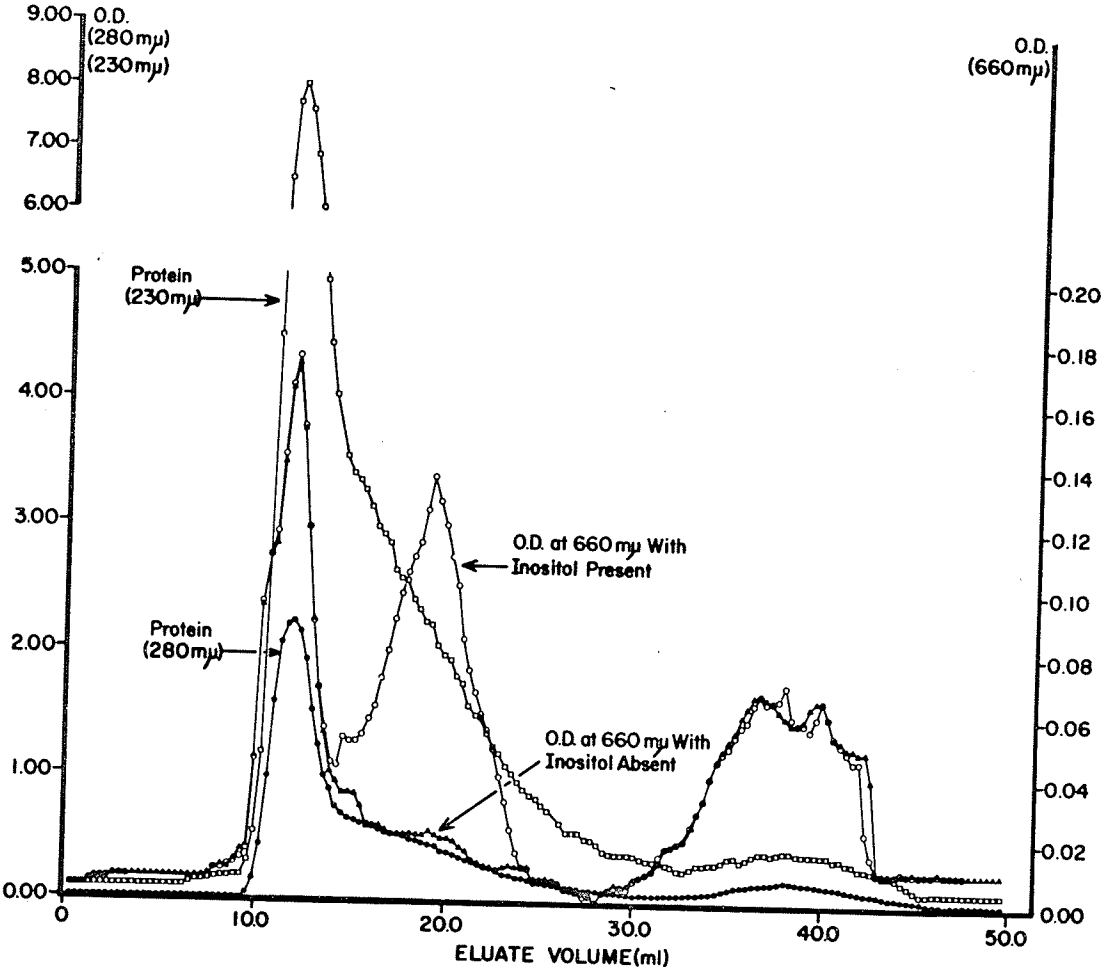


FIGURE II.3

The elution pattern obtained on gel filtration on a G-100 sephadex column of a crude kidney extract of inositol oxygenase. Each fraction eluted was monitored at 230 and 280 mμ and analyzed for inositol oxygenase activity both in the absence of inositol (blank) and in the presence of 0.5 μ Moles of inositol.

active peak, obtained by initial chromatography on the same resin, resulted in removal of some material from the active fraction, but did not provide a more stable enzyme preparation. This was omitted subsequently because it increased the time of preparation.

Contrary to the findings of Charalampous, whose preparation retained activity for 3 to 4 days with glutathione added prior to freezing at -20°, our enzyme preparation immediately lost 100% of its activity on freezing at -20°C, even with glutathione or mercaptoethanol added. On standing exposed to air for 12 hours, as was observed by Charalampous, most of the activity was lost.

When stored under nitrogen at 4°C, the best conditions for storing our enzyme, the enzyme retained 100% of its activity for 4 days. At the end of one week, 50% of the original activity remained. The inositol oxygenase preparation of Charalampous (1959), stored under his best conditions (frozen at -20°C under N_2), retained full activity for 10 days and 50% activity after two weeks.

Although our enzyme preparation was not stable for quite as long as that of Charalampous, the fact that we could prepare enzyme in a much shorter time and much more easily, far outweighed the disadvantage of the enzyme retaining activity

for this shorter time. In other words, preparation of fresh enzyme was not a major undertaking.

To determine whether any spectrophotometric change occurred, in the visible region of the spectrum in the enzyme preparation with age, and to uncover possible reasons for differences in stability between our preparation and that of Charalampous, a fresh aliquot and one from the same preparation that had been left standing exposed to air for one week were scanned between 800 and 320 mμ in a Beckman DB recording spectrophotometer.

Because the enzyme preparation obtained by Charalampous was yellow in color, and a yellow fraction was also obtained in the present study (which incidentally contained most of the protein and was inactive), a similar scan was also done on this yellow fraction.

The spectrum for the active inositol oxygenase fraction (Fig. II.4a) showed characteristics indicative of a heme protein, since it showed absorption in the Soret band region (approximately 400-452 mμ; Soret, 1878; Neilands, 1958).

A comparison of this spectrum with that of oxyhemoglobin shows absorption maxima at 350, 415, 541.5 and 576 mμ (Sidwell et al, 1938), whereas that of our enzyme preparation shows peaks at 345, 415, 545, and 565 mμ.

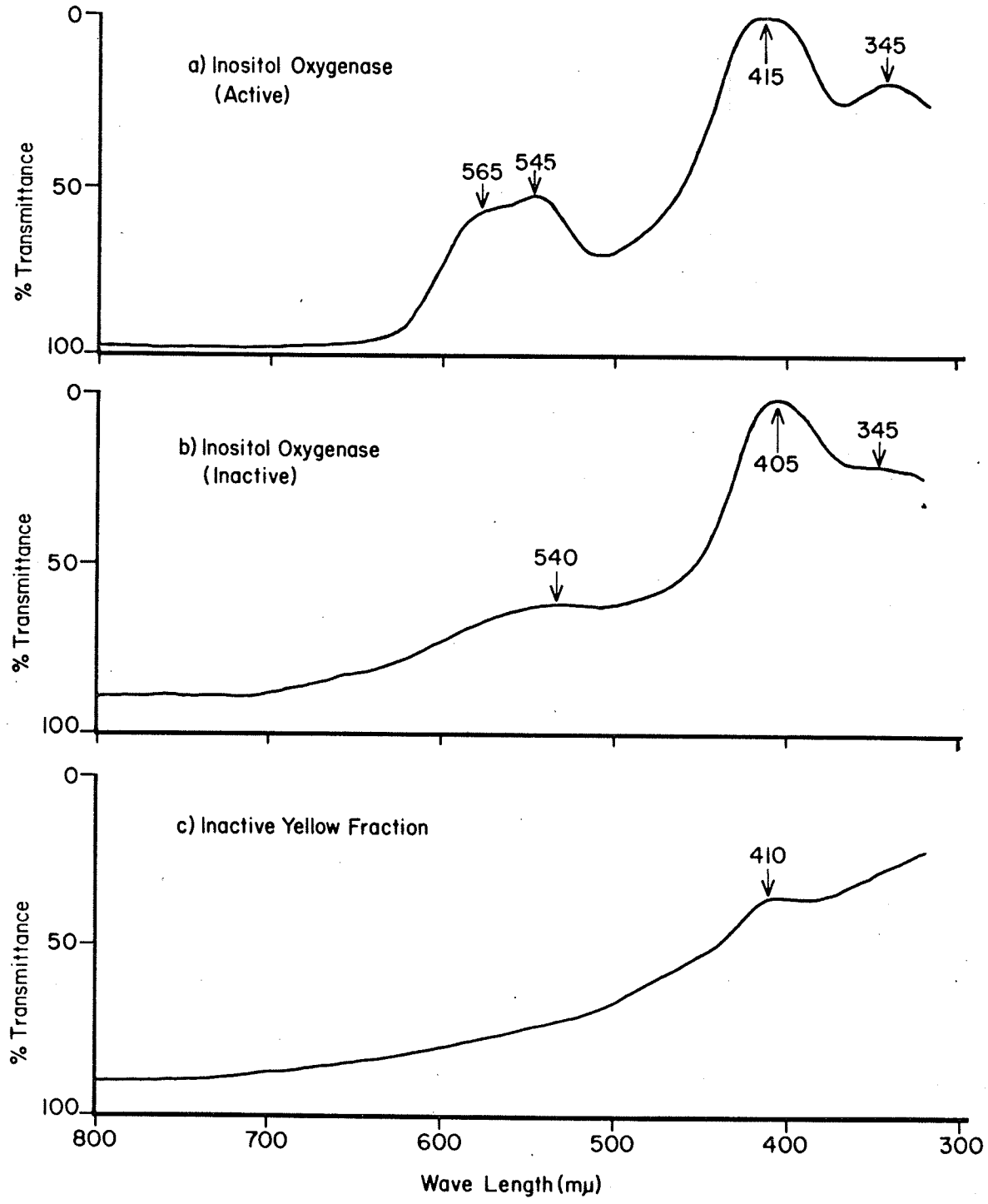


FIGURE II·4

Change in % transmittance as a function of wave length for a solution of a) active inositol oxygenase b) inactive inositol oxygenase c) the inactive yellow component eluted in the void volume from G-100 sephadex.

Our method of preparing inositol oxygenase could allow contamination with hemoglobin. To avoid this, one would have to perfuse the kidneys prior to removal as was done by Strittmatter and Ball (1954) with rat liver. These workers isolated nuclear, mitochondrial, microsomal, and supernatant fractions. Their supernatant was free of hemoglobin and was consequently yellow in color, rather than reddish like our supernatant. A spectrum of their reduced supernatant showed a Soret peak at 405 mμ and a broad low peak at 550 mμ which they suggested was due to small amounts of various hemochromogen substances.

The spectrum of our preparation may therefore be due to a mixture of heme proteins, one of which is probably hemoglobin and the other inositol oxygenase.

The spectrum of the yellow material eluted in the void space of the G-100 column (Fig. II.4c) is similar to that of the supernatant fraction of Strittmatter and Ball (1954).

Hemoglobin, having a molecular weight of 67,000, would not be expected to be eluted in the void space of G-100 sephadex and would therefore separate from some of the larger compounds, but would be eluted later along with the inositol oxygenase fraction.

Because there was a remote possibility that the inositol oxygenase activity of our preparation was due to the hemo-

globin, hemoglobin alone was tested for activity. An experiment was also carried out to determine whether, when added to a preparation of inositol oxygenase, hemoglobin had any effect on its activity. Both tests proved negative.

The spectrum for the inositol oxygenase preparation after aging showed a disappearance of the 565 mμ peak and some dimmunition of the 545 mμ peak. As a result, only one peak remained in this region of the spectrum (at 540 mμ). A slight shift in the Soret band, to 405 mμ from 415 mμ, was observed (Fig. II.4b). Although not visible to the eye, these spectral changes seem to indicate a shift towards a more yellow color and therefore to a more stable but inactive compound.

According to Charalampous (1959), inactivation of inositol oxygenase is probably due to oxidation, probably of SH groups, of the reduced enzyme, and the changes seen in the spectrum support this contention as they seem to be characteristic of the oxidation of oxidoreductases such as the cytochromes (Horie and Morrison, 1963; Azoulay and Couchoud-Beaumont, 1965).

Examination of the spectrum for the inositol oxygenase preparation of Charalampous (1959) showed only one peak, in the visible part of the spectrum, at 415 mμ. A scan of our enzyme preparation also showed a peak at 415 mμ, but the iron

peak at 345 mμ, and the peaks at 545 and 565 mμ present in our spectrum were absent in his. These findings are in agreement with the fact that Charalampous has very little hemin iron in his preparation; these peaks in the visible region of the spectrum are characteristic of the relationship between the iron and the porphyrin (Friedli, 1924) and, of the type of side chains on the porphyrin (Slater, 1961). A loss of hemin iron would alter this relationship, thus altering the spectrum.

Because its divalent iron is protected against oxidation, hemoglobin has the ability to bind oxygen without becoming denatured (Keilin, 1960).

Inositol oxygenase apparently does not possess any protective mechanism against oxidation as is evidenced by its lability in oxygen.

The iron-porphyrin and SH groups of hemoglobin can be oxidized independently (Mirsky and Anson, 1936), and, if this were true of inositol oxygenase, the removal of hemin iron by Charalampous with NH_4SO_4 during his preparative procedure, would leave a protein with fewer groups available for oxidation, and therefore, a more stable enzyme. This does not preclude the possibility that Charalampous may have removed a contaminating heme protein during NH_4SO_4 fractionation.

Drying hemoglobin results in the formation of a hemo-

chromogen due to the removal of the water molecule that is often bound to iron and its replacement by a nitrogenous group from the globin portion of the hemoglobin molecule (Keilin, 1960). The binding of a molecule of water with iron in our enzyme could explain the immediate denaturation of our enzyme preparation on freezing, whereas the preparation of Charalampaus, because it has the iron removed and probably the water with it, does not denature upon freezing.

From the above, it would appear that the preparation of a stable enzyme is not likely to be easy.

iv) Preparation of Alkaline Phosphatase

The alkaline phosphatase used in these studies was a purified preparation from calf intestine and was purchased from Mann Research Laboratories (New York 6, N.Y.).

Intestinal alkaline phosphatase was chosen rather than the more pure alkaline phosphatase from E.coli so that studies with this enzyme could be more readily applied to the intestinal metabolism of phytic acid. Because of this and because of the reported heterogeneity of intestinal alkaline phosphatase (Behal and Center, 1965), the only further purification that was carried out was the removal of as much of the P_i present as possible.

To remove P_i from this preparation, 25 mg of enzyme was

dissolved in 5 ml of distilled water, and 3 ml of this solution was passed through a 1.0 x 60 cm column containing Bio-Gel P-4 (50-150 mesh, a copolymer of acrylamide and methylenebisacrylamide purchased from Calbiochem, Los Angeles) using distilled water as eluent. This treatment removed most, but not all, of the P_i, as well as a small 280 mμ absorbing fraction (Fig. II.5). A small amount of phosphate remained with the enzyme fraction and could not be removed by a number of procedures. Some experiments on the nature of this association of P_i and enzyme have been described in Chapter VI below.

The most active portion of the enzyme peak (between eluate volume 12 ml and eluate volume 15 ml, Fig. II.5) was the enzyme preparation used in the experiments in this thesis.

v) Preparation of Acid Phosphatase

The acid phosphatase utilized in this study was a preparation from wheat germ and was obtained from Mann Research Laboratories (New York 6, N.Y.).

Because we were interested in the role of dietary phytate present in unrefined carbohydrate in dental caries and nutrition, wheat germ acid phosphatase was chosen for the studies in this thesis.

To further purify the acid phosphatase, 50 mg was dissolved in 10 ml of distilled water and an insoluble component

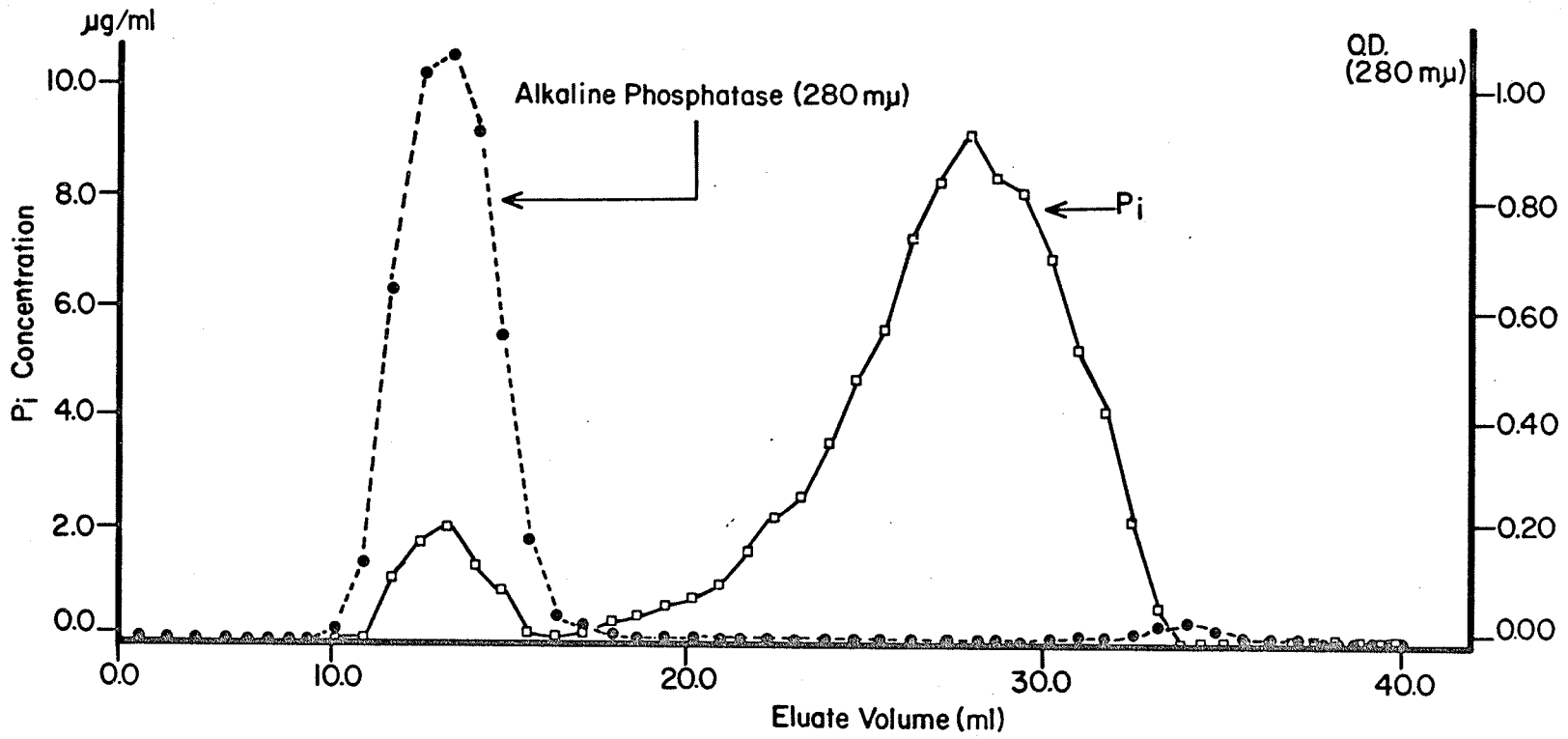


FIGURE II·5

The elution pattern obtained on gel filtration on a P-4 Bio-Gel column of alkaline phosphatase. Each fraction eluted was monitored at 280 mµ and analyzed for P_i.

was removed by filtering through Whatman #31 filter paper.

The filtrate was then put onto a 1.0 x 60 cm column containing Bio-Gel P-4 and eluted with distilled water. A 280 mμ absorbing fraction of inactive material was eluted at a Ve/Vo ratio greater than 1 (where Ve is the elution volume of the solute, and Vo is the void volume; Flodin, 1962) and was discarded (Fig. II.6).

The most active portion of the enzyme peak (between eluate volume 12 ml and eluate volume 15 ml, Fig. II.6) was the enzyme preparation used in the experiments in this thesis.

2) Analytical Procedures

i) Inorganic and Organic Phosphate

Inorganic phosphate was analyzed by a micro-adaptation (developed in this laboratory) of the method of Kuttner and Cohen (1927). For experiments in this thesis, the method of Kuttner and Cohen was scaled down by a factor of 10 in order to analyze 0 to 0.5 μg of P_i.

In this method, phosphate forms a colorless complex with ammonium molybdate which is then reduced with stannous chloride. The reduced phosphomolybdate complex is blue in color.

In the experiments in this thesis, this reaction was carried out in 10 x 75 mm pyrex test tubes, and the blue color measured in a Klett-Summerson colorimeter (adapted to hold

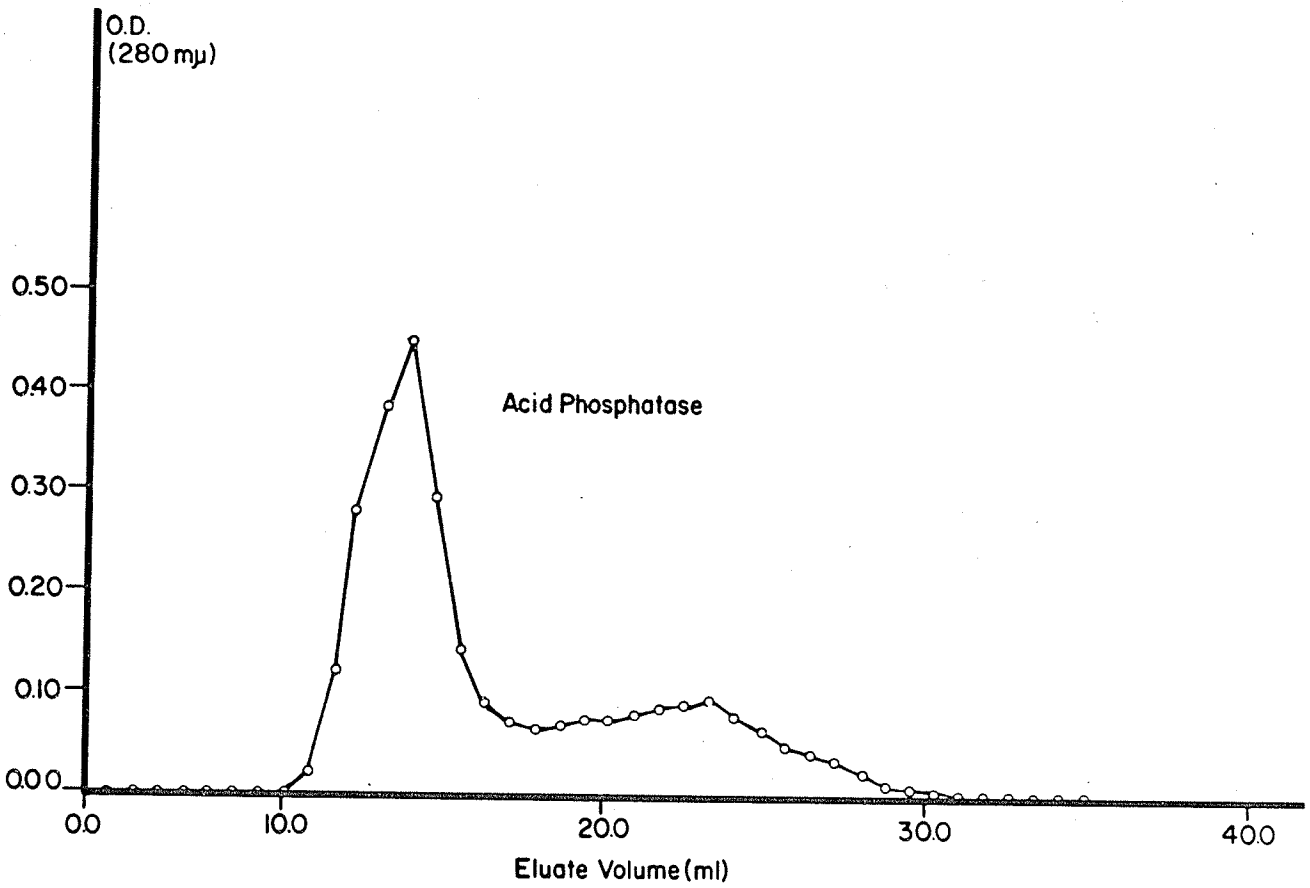


FIGURE II·6

The elution pattern obtained on gel filtration on a P-4 Bio-Gel column of acid phosphatase. Each eluted fraction was monitored at 280 mμ.

10 x 75 mm test tubes) with a number 69 filter.

The precision of the method is very high, having a coefficient of variation of ±0.8% when the P_i was 0.2 µg and ±0.3% when it was 0.4 µg.

Organic phosphates were analyzed by the same method as for P_i after samples were wet ashed with H_2SO_4 and H_2O_2. The details are as follows:

Aqueous aliquots (5.0 to 100 µl) of the organic phosphate were transferred to 5 x 50 mm Kimax test tubes, 2 µl of concentrated H_2SO_4 then added, and the contents heated on a digestion shelf (Precision Scientific Co., Chicago) at 140°C for approximately one hour to take the samples to dryness. The temperature was then raised until refluxing of the H_2SO_4 occurred in the bottom portion of the tube. This was done for approximately 1½ hours. The samples were allowed to cool, 5 µl of H_2O_2 was added, and heating and refluxing continued for at least one hour.

Samples were then cooled and diluted with distilled water. P_i analyses were carried out on an aliquot.

ii) $\underline{^{32}P}$

Phosphorus-32 orthophosphate was used in experiments designed to determine P_i binding to alkaline phosphatase. For these experiments, ^{32}P (specific activity = 100 curies/mgP;

purchased from Radiochemical Center, Amersham, Buckinghamshire, England) was diluted in distilled water so that when an aliquot was added to one ml of enzyme solution, this solution contained approximately 0.4 μ curies (8.88×10^5dpm). The enzyme solution was passed through chromatographic columns (see Chapter VI) and the amount of radioactivity in samples of eluate determined by plating the samples onto aluminum planchets, drying them with an infrared heat lamp (General Electric, 250W, 115-125V), and counting with a gas flow counter (Nuclear Chicago, low background, Model 181B).

iii) Inositol Analysis

a) Inositol Oxygenase Procedure

In this method, enzyme, as prepared above, is used to convert inositol to glucuronic acid, which forms a chromophore with a $FeCl_3$-orcinol reagent, which is measured spectrophotometrically. The details are as follows:

Reagents and Materials

1. 0.025 M myoinositol was prepared as a stock solution from which aliquots were removed to prepare 0.125, 0.250, and 0.375 μ M inositol standards.

2. 1.0 M phosphate buffer, pH 7.2.

3. Inositol oxygenase- This enzyme was prepared from male rat kidney, and its preparation is described in the prepara-

tive section of this Chapter.

4. $FeCl_3$ stock solution- 90 mg $FeCl_3.6H_2O$ was dissolved in 100 ml of concentrated HCl.

5. Orcinol-$FeCl_3$ reagent- This reagent was prepared by dissolving 0.04 g orcinol in 10 ml of the $FeCl_3$ stock solution just prior to use.

Using inositol standards and the inositol oxygenase obtained from the G-100 sephadex column, a standard curve was obtained by the following procedure.

Eight 5 x 50 mm Kimax tubes were set up in 4 groups containing 2 tubes in each group. 5 µl of phosphate buffer, 25 µl of inositol oxygenase, and either 0, 5, 10, or 15 µl of 0.025 M inositol were transferred to each tube. The final volume in each tube was made up to 50 µl with distilled water. The enzyme was always added last, and the tubes were held in an ice bath while these mixtures were prepared.

Each tube was gassed with O_2 for 15 seconds, covered with a glass bead, and incubated in a water bath at 35°C for 40 minutes. Each tube was then immersed in a boiling water bath for 2 minutes to stop the reaction. 150 µl distilled water was added, the contents mixed well, and centrifuged at 1100 xg for 15 minutes in a Clinical Centrifuge (International Equipment Co., Boston). 50 µl of supernatant was removed

from each tube and pipetted into another 5 x 50 mm Kimax tube. 100 µl orcinol-$FeCl_3$ reagent was added, the contents mixed, and each tube covered with a glass bead and placed in a boiling water bath for 20 minutes. Optical density was read on a Beckman DU spectrophotometer at 660 mµ; a typical standard curve is shown in Fig. II.7.

Since, above 0.250 µM inositol, the curve deviated from linearity, unkowns were kept between 0 and 0.250 µM.

b) Dichromate Oxidation Procedure

Johnson (1949) developed a procedure for estimation of the organic material in a non-volatile compound.

The principle of the method involves the oxidation of an organic compound by dichromate in sulfuric acid, and the extent of this oxidation is determined by measuring colorimetrically, at 440 mµ, the amount of dichromate not reduced by the compound.

Although this method had not previously been used for the analysis of inositol, it was found satisfactory providing one modification is made.

To a 25 x 150 mm test tube, an aliquot of the sample to be analyzed was added, along with sufficient distilled water so that the total volume was 400 µl. Corresponding blanks and standards were prepared in similar tubes and having the same

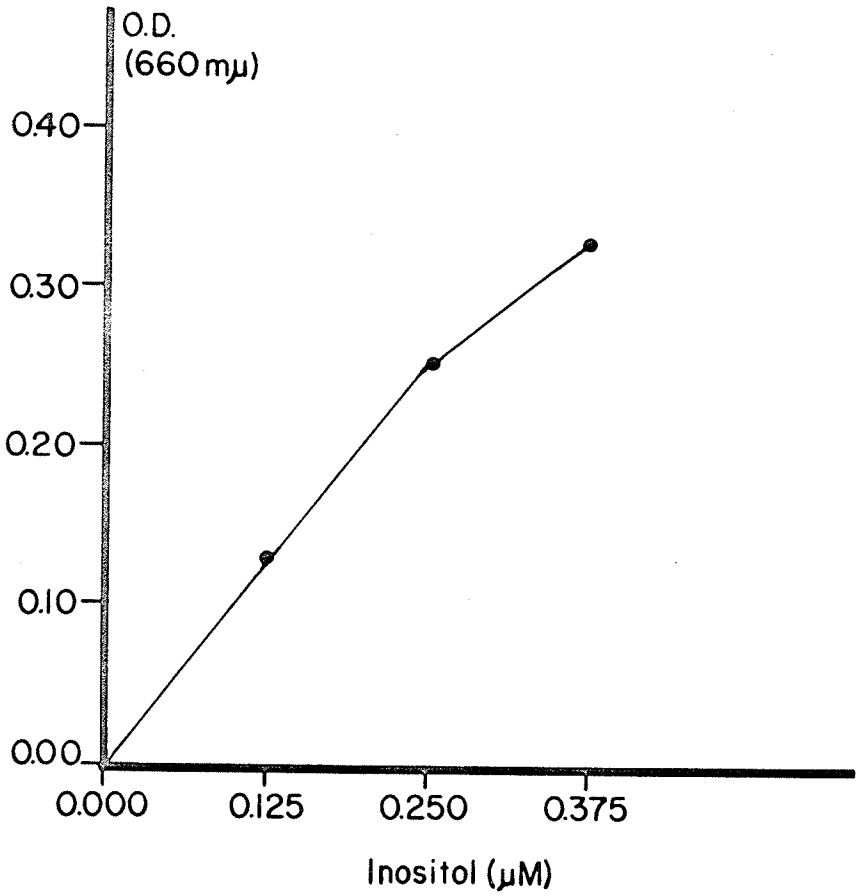


FIGURE II·7

Inositol standard curve: Change in optical density at 660 mμ as a function of inositol concentration following incubation of known inositol standards with inositol oxygenase and analysis for the glucuronic acid formed in the enzyme reaction with $FeCl_3$-orcinol reagent.

volume.

1 ml of the dichromate-H_2SO_4 reagent was added to each tube, the contents well mixed, heated in a boiling water bath for 20 minutes, and cooled immediately with ice water.

Following boiling, 1 ml of distilled water was added to each tube, rather than 10 ml as used in Johnson's procedure. This was to avoid diluting the color too much.

To one of the blanks, about 10 mg of Na_2SO_3 was added, and this reduced blank was used to set the spectrophotometer at zero absorbance at 440 mμ. The remaining tubes are then read against this blank.

A typical standard curve, obtained when 0.55, 1.1, and 2.2 μM of inositol were oxidized by this method, is shown in Fig. II.8.

iv) pH Measurements

All pH measurements were made on a Beckman Model G pH meter using an electrode assembly consisting of a glass electrode and a KCl bridge connected to a calomel electrode. The electrode assembly was sufficiently small in size so that it could be directly inserted into 10 x 75 mm test tubes.

v) Na Analysis

Na analyses were carried out on a Perkin-Elmer 330 Atomic Absorption Spectrophotometer at 589 mμ as described in

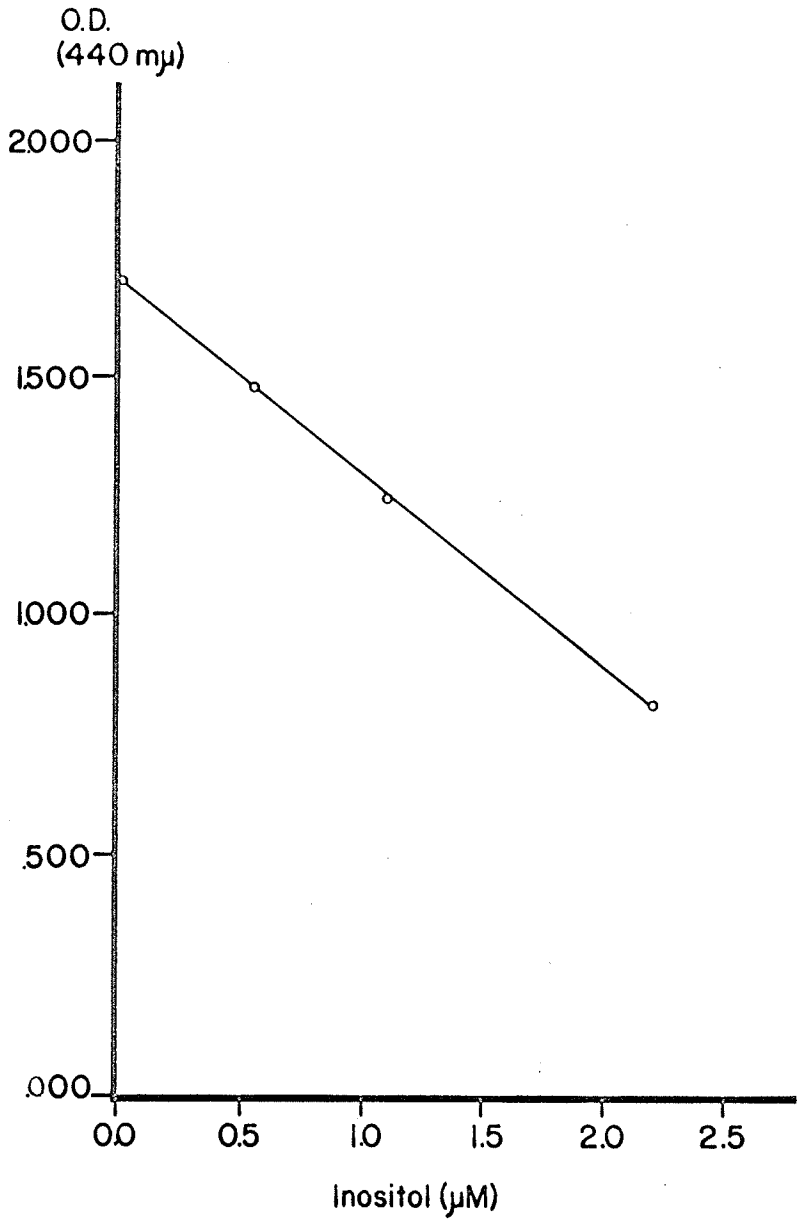


FIGURE II·8

Inositol standard curve: Change in optical density at 440 mμ as a function of inositol concentration following reaction of known inositol standards with $K_2Cr_2O_7$ reagent.

the Perkin-Elmer manual (Analytical Methods for Atomic Absorption Spectrophotometry) except that standards and samples were diluted in 100 ppm KCl.

vi) Chloride Analyses

Chloride was measured electrometrically, using the following electrode arrangement: $Ag|AgCl\ KCl|Hg_2Cl_2|Hg_2Cl_2(S)|Hg$. Changes in potential for known chloride standards and unkown samples were measured in millivolts on a Beckman Model G pH meter. The chloride concentrations of the unkowns were determined from a standard curve as shown in Fig. II.9.

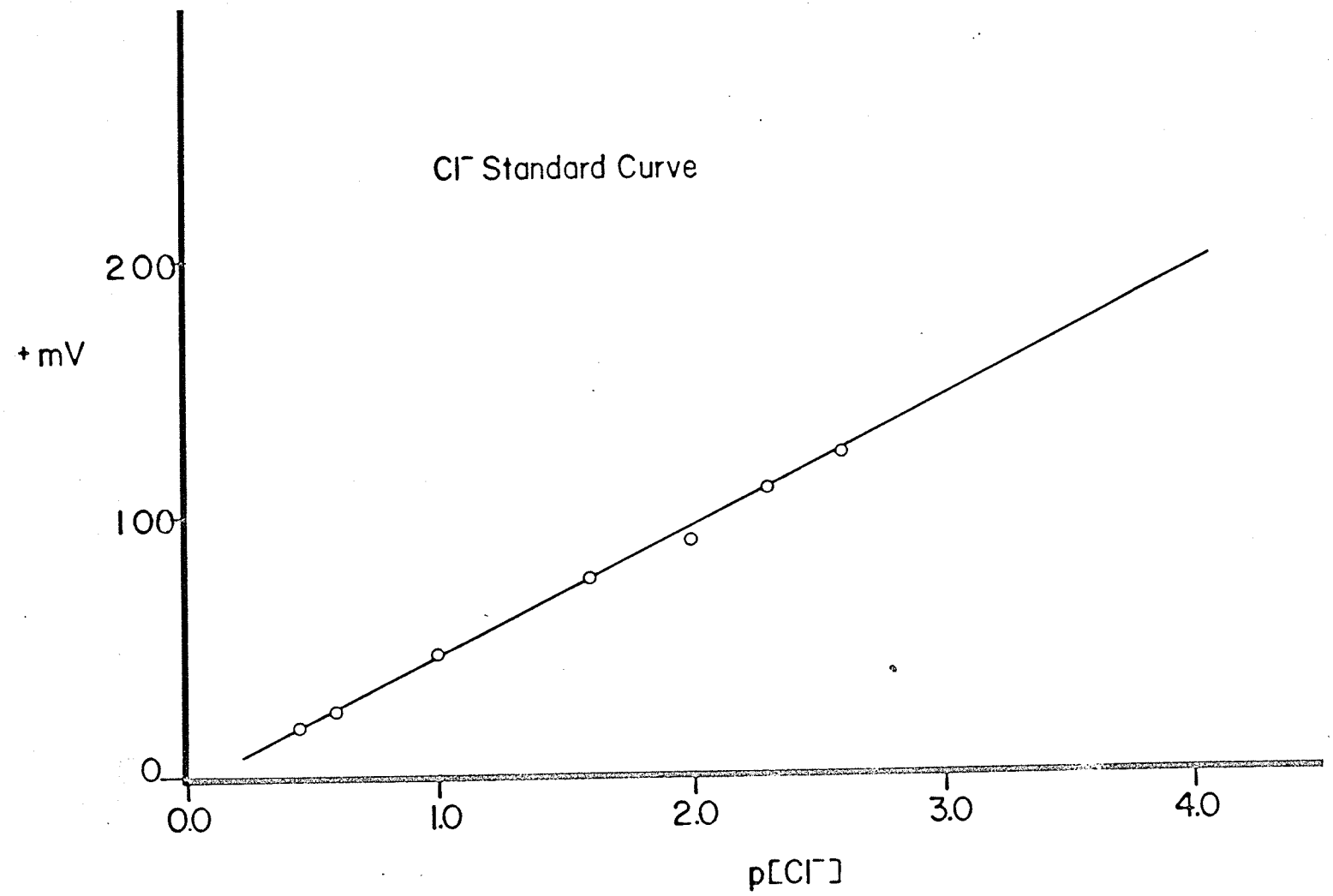


FIGURE II·9

Chloride standard curve: The number of millivolts is plotted as a function of the negative log of the chloride concentration.

CHAPTER III

THE USE OF AN ANION EXCHANGE RESIN TO STUDY THE EFFECT OF pH ON THE ADSORPTION CHARACTERISTICS OF PHYTIC ACID

Introduction

During experiments to determine the conditions for preparing inositol phosphate derivatives from an acid hydrolyzate of phytic acid by anion exchange chromatography (see Chapter II), the concentration of LiCl required to elute phytate was higher if the phytate applied to the column was in its acidic form rather than in its salt forms.

These observations agree with those observed earlier by Tomlinson and Ballou (1962) and by Saio (1964) but not commented upon by either investigator. The former showed that when a hydrolyzate of lithium phytate was applied to a Dowex-1-Cl column, elution of phytate from the resin did not occur until the chloride concentration was increased to 0.38 M. On the other hand, Saio showed that when a hydrolyzate of phytic acid (i.e. the acid rather than the salt form of phytate) was applied to the resin, elution of phytate did not occur until a chloride concentration of 0.6 M was reached.

Adsorption of phytate to the inorganic material in soil is also greater at low than at neutral or high pH, which may

be responsible for the low availability in acidic soils of phytate P for plant nutrition (Goring and Bartholomew, 1950; Anderson and Arlidge, 1962).

Jenkins et al (1959) found that teeth exposed to phytate were less soluble in acetic acid buffer than teeth not exposed to phytate. Subsequent washing of the teeth did not remove this protective effect, strongly indicating that some phytate was still adsorbed to the surfaces of the teeth. Although not actually studied, this adsorption may have been favored by the acid pH.

Although the adsorption of phytate to anion exchange resins may not be identical to its adsorption to the inorganic material in soils and to tooth surfaces, the properties of the phytate molecule responsible for its adsorption in the different systems is probably the same.

Because the active group on the resin (the quaternary ammonium) does not change appreciably with pH whereas, the inorganic material in soils (e.g. silicates) and the calcium phosphate in teeth do, in this chapter, the effect of pH on the adsorption properties of phytate was studied on the Dowex-1-Cl resin. The more complicated problem of adsorption to tooth surfaces, although probably important in the determination of the role of phytate in dental caries, has not been

dealt with in this thesis, since this requires and awaits more knowledge of the changes in calcium phosphate which occur with change in pH.

The elution of orthophosphate from Dowex-1-Cl is also dependent upon the pH, but this phosphate is eluted with a higher chloride concentration when the pH is high than when it is low (Hoff, 1963). This is opposite to that observed with phytate. This difference may be related to the difference in the number of phosphate residues in P_i and in phytate, since in a study by Beukenkamp, Rieman III and Lindenbaum (1954) on the behaviour of condensed phosphates on Dowex-1-Cl, P_i behaved like that observed by Hoff, triorthophosphate like that for phytate, and pyrophosphate in an intermediate fashion.

For comparative purposes, the elution characteristics of the monophosphate of inositol, IMP, were studied in a manner similar to but less extensive than that for phytate.

In the present study, the effect of pH on the adsorption of phytate and IMP to Dowex-1-Cl was examined by determining the concentration of LiCl required to displace adsorbed phytate and IMP under a variety of conditions.

Experiments were also carried out to determine the changes that occur as phytate and IMP are adsorbed to the resin.

Methods

The Dowex-1-Cl column used for the experiments in this chapter was prepared by packing a glass chromatographic column (4 x 125 mm) with this resin and washing the resin with distilled water prior to and following the application of a 250 µl aliquot of an 0.0054 M solution of either phytic acid or IMP. The phytic acid was put on the column at each of three pH levels, viz. 2.1, 5.6 and 8.6, and IMP, because of its less complicated ionization with change in pH, was studied only at pH 2.4 and 8.6. The amount of sample was the same in all the experiments (i.e. 1.35 µM). Phytate, pH 5.6 and 8.6 and IMP, pH 8.6 were prepared by adding appropriate amounts of NaOH to a given solution of phytic acid or IMP respectively.

In one series of experiments, following the putting of a sample onto the column and washing with water, the phytate or IMP was eluted with a stepwise LiCl gradient. In another series of experiments, to examine other aspects of the elution characteristics of phytate, a number of phytate samples were each eluted at any one of the above three pH levels and any one of a series of LiCl concentrations (0.30 to 0.75 M). This type of experiment could not be carried out with IMP since it did not show the irregular adsorption characteristics of phytate. In a third series of experiments to determine the changes

that occur as either phytate or IMP is adsorbed to the resin, the pH and Na^+ and Cl^- concentrations of the eluate prior to and after the application of phytate or IMP were monitored.

1) Stepwise Elution of Phytate and IMP with LiCl

To determine the concentration of LiCl required to displace adsorbed phytate, elution was carried out in a stepwise fashion with solutions of LiCl between 0.0 and 0.7 M, and the eluate monitored for phytate by measuring the amount of organic P present (see Chapter II).

The elution characteristics of IMP at pH 2.4 and 8.6 were determined by the same procedures as those used for phytic acid except that stepwise elution was carried out with solutions of LiCl between 0.0 and 0.2 M, the range in which IMP is eluted.

2) Elution of Phytate with Individual LiCl Concentrations

Because the amount of phytate remaining on the column progressively decreases with stepwise elution with LiCl, the effect of each step in the gradient on the elution of phytate is difficult to assess. Therefore, in this series of experiments, instead of stepwise elution of a phytate sample, elution was carried out with individual concentrations of LiCl solution. The eluate was monitored for total P as previously.

3) Changes in pH, Na^+ and Cl^- Associated with Putting

Phytate and IMP onto the Dowex-1-Cl Column

The changes occurring when phytate and IMP are adsorbed to the resin were determined by the following procedure. Samples of phytate at each of pH 2.1, 5.6 and 8.6 and IMP at either pH 2.4 or 8.6 were put onto the column and eluted with distilled water. The pH and Na^+ and Cl^- concentrations of the eluates prior to and after the application of samples were measured. The portion of the eluate that deviated from the base-line pH (which is the pH of the water eluate) was collected and back-titrated, either with NaOH or HCl, to the base-line level. This enabled the number of equivalents of acid or base associated with the adsorption of phytate or IMP to be determined.

Results

1) Stepwise Elution of Phytate and IMP with LiCl

i) Phytate

When phytate was eluted stepwise with LiCl, the elution pattern was irregular at each of the three pH levels examined, and occurred at lower LiCl concentrations when the phytate was at pH 8.6 than at 5.6 and in turn than at pH 2.1 (Fig. III ·1).

ii) IMP

Whether in its acidic or in its basic form, IMP was eluted

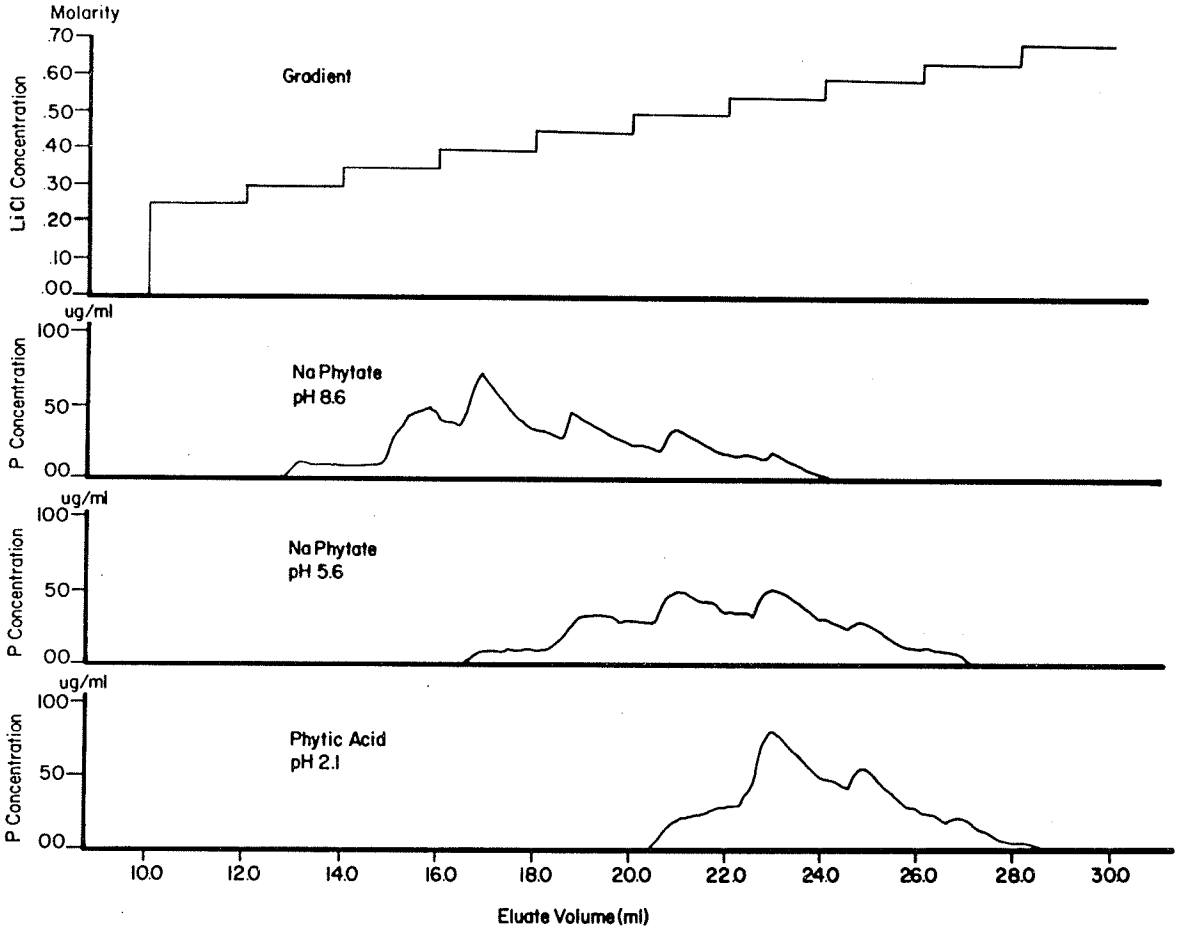


FIGURE III·1

The elution pattern obtained when phytate, pH 2.1, 5.6 or 8.6 is eluted from a Dowex-1-Cl anion exchange resin using a stepwise LiCl gradient containing 2 ml of LiCl in each step. Eluted fractions were assayed for organic P.

as a single symmetrical peak. When the step in the LiCl gradient was 0.05 M, elution of the two forms occurred at the same LiCl concentration (Fig. III·2) which was lower than that needed to elute any of the forms of phytate.

When the step was 0.01 M, both forms of IMP were eluted as single peaks, but the acidic form was eluted with a lower LiCl concentration than the basic form (Fig. III·3).

When the step was 0.0033 M, the results were similar to those observed with an 0.01 M stepwise gradient except that the elution peaks had a broader modal portion and were eluted with a lower LiCl concentration (Fig. III·4).

The effect of pH on the elution of the acidic and basic forms of IMP was opposite to that for phytate. Of interest, even reducing the step in the gradient by a factor of 15 did not result in the irregular pattern observed for phytate.

2) Elution of Phytate with Individual LiCl Concentrations

The results for the experiments in which a constant amount of phytate was eluted with individual concentrations of LiCl are shown in Figs. III·5, III·6, and III·7. Even at high LiCl concentrations, the elution peaks were not symmetrical.

When the maximum phytate concentration reached in the eluate (referred to here as the phytate response) is plotted in Fig. III·8 as a function of the LiCl concentration, a straight

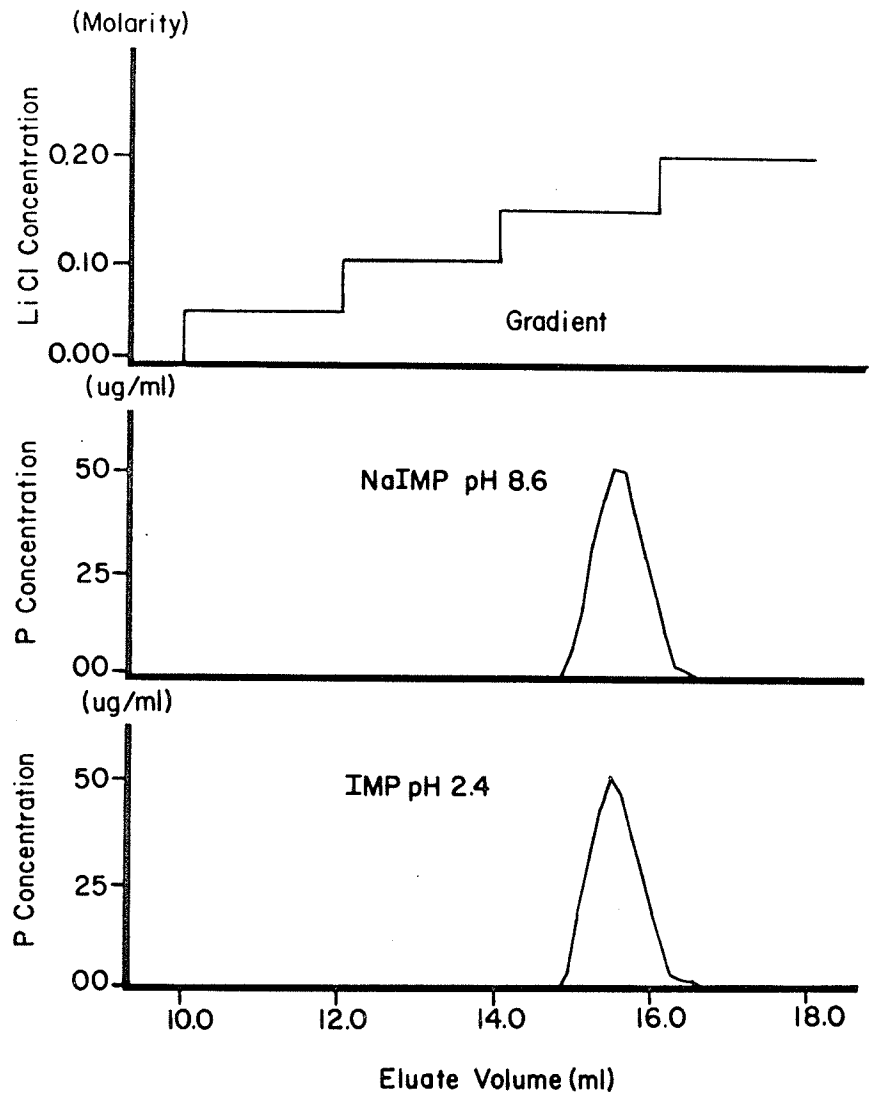


FIGURE III·2

The elution pattern obtained when IMP, pH 2.4 and 8.6 is eluted from a Dowex-1-Cl anion exchange resin using a stepwise LiCl gradient containing 2 ml of LiCl in each step. Eluted fractions were assayed for organic P.

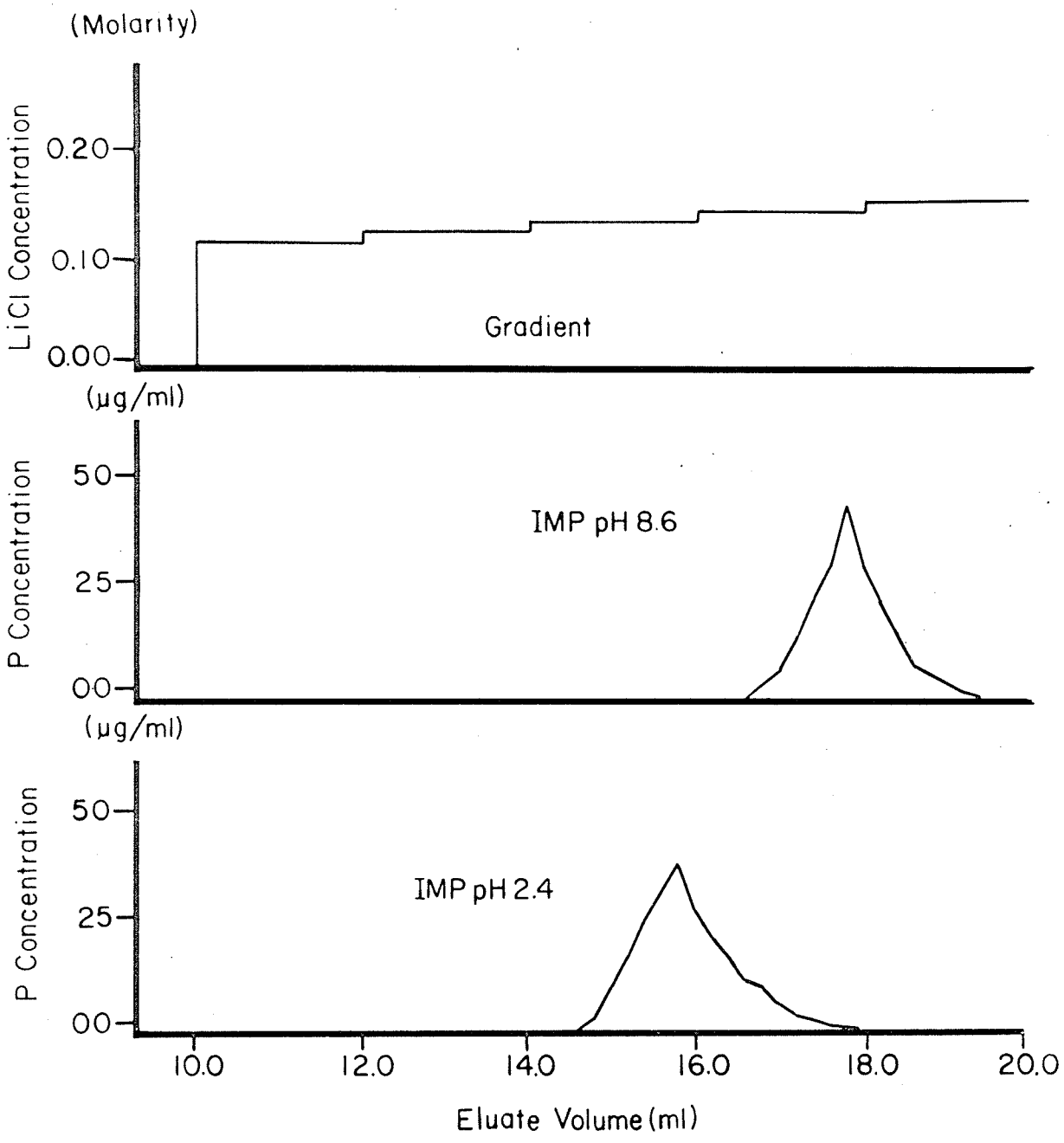


FIGURE III·3

The elution pattern obtained when IMP, pH 2.4 and 8.6 is eluted from a Dowex-1-Cl anion exchange resin using a stepwise LiCl gradient (reduced a factor of 5X from that in Figure III·4) containing 2 ml of LiCl in each step. Eluted fractions were assayed for organic P.

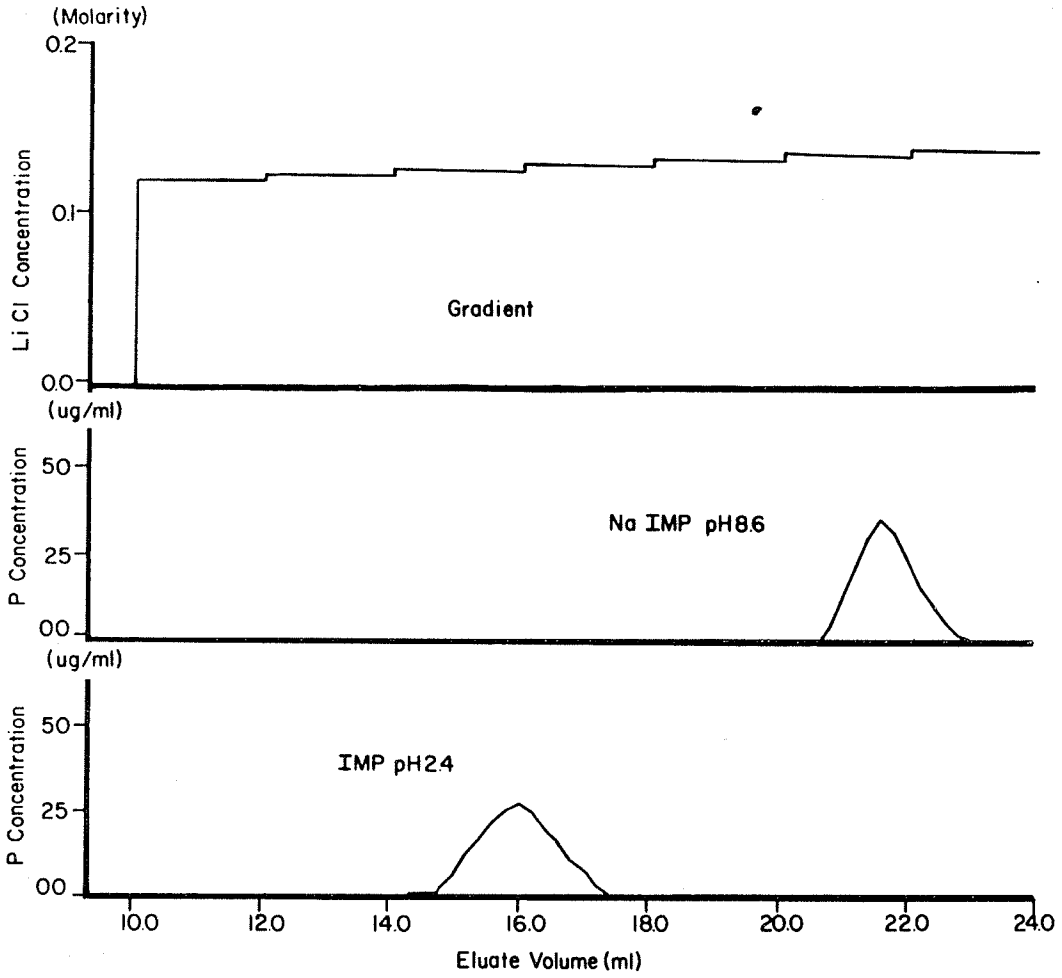


FIGURE III·4

The elution pattern obtained when IMP, pH 2.4 and 8.6 is eluted from a Dowex-1-Cl anion exchange resin using a stepwise LiCl gradient (reduced a factor of 15X from that in Figure III·4) containing 2 ml of LiCl in each step. Eluted fractions were assayed for organic P.

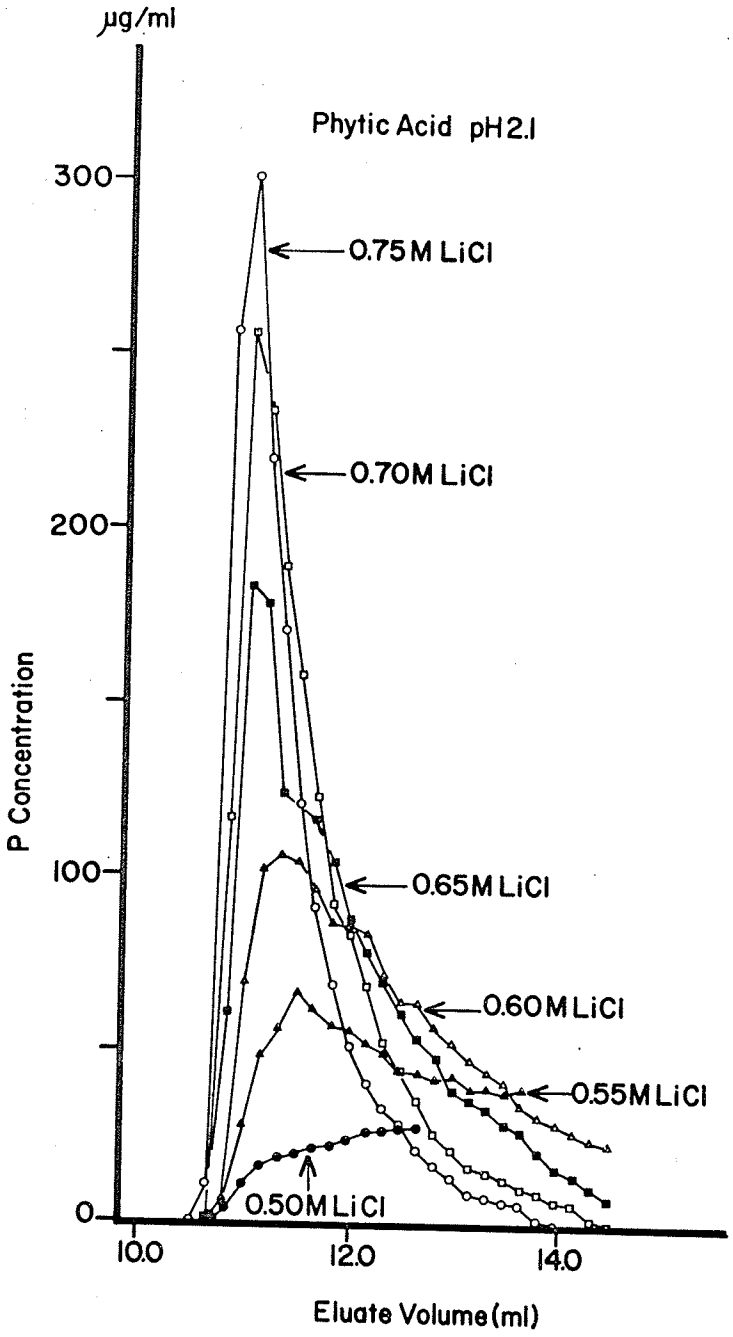


FIGURE III.5

The elution pattern obtained when phytic acid, pH 2.1, was eluted from a Dowex-1-Cl anion exchange column using either 0.50, 0.55, 0.60, 0.65, 0.70, or 0.75 M LiCl as eluent. Fractions eluted were monitored for organic P.

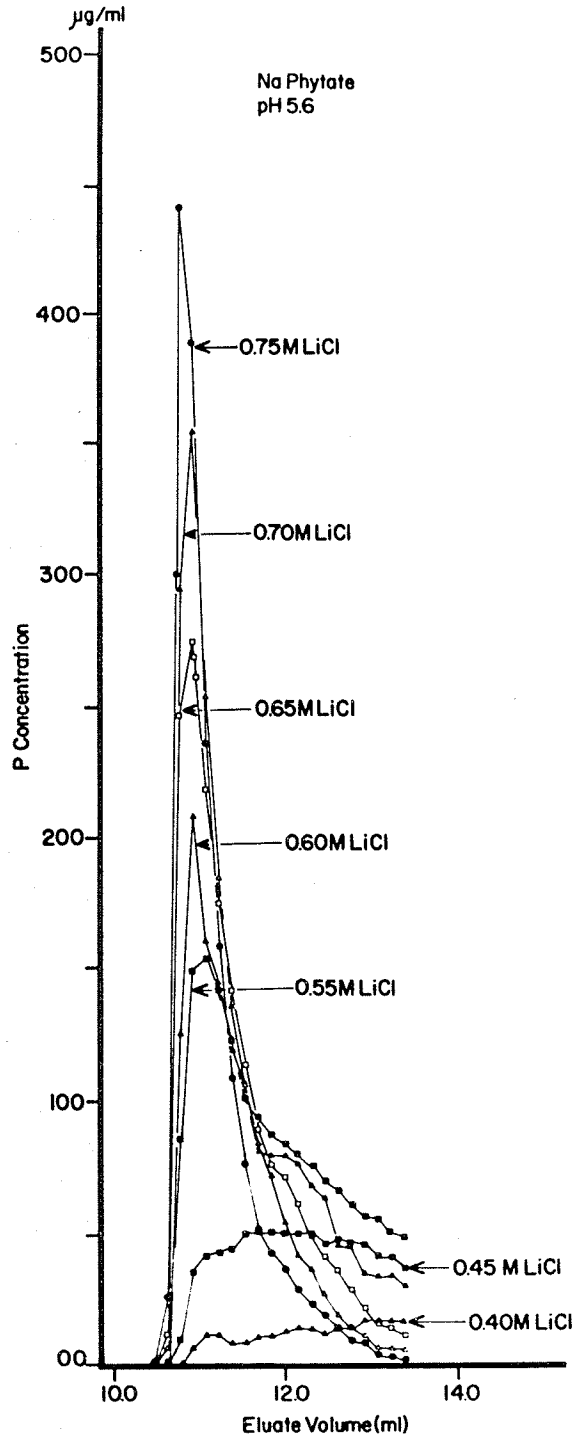


FIGURE III·6

The elution pattern obtained when Na phytate, pH 5.6, was eluted from a Dowex-1-Cl anion exchange column using either 0.45, 0.55, 0.60, 0.65, 0.70, or 0.75 M LiCl as eluent. Fractions eluted were monitored for organic P.

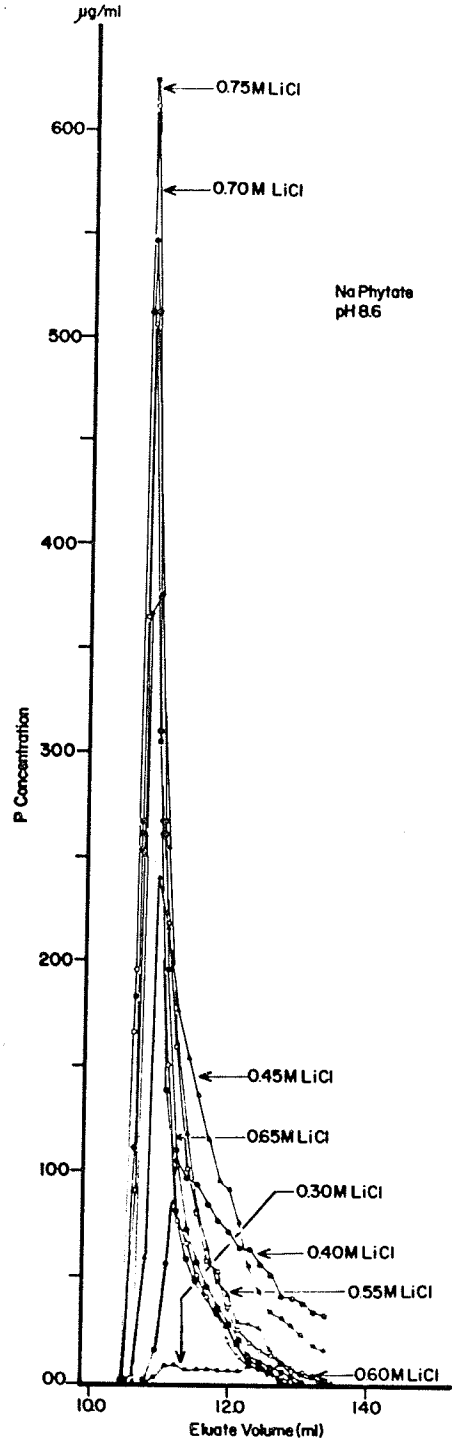


FIGURE III·7

The elution pattern obtained when Na Phytate, pH 8.6, was eluted from a Dowex-1-Cl anion exchange column using either 0.30, 0.40, 0.45, 0.55, 0.60, 0.65, 0.70, or 0.75 M LiCl as eluent. Fractions eluted were monitored for organic P.

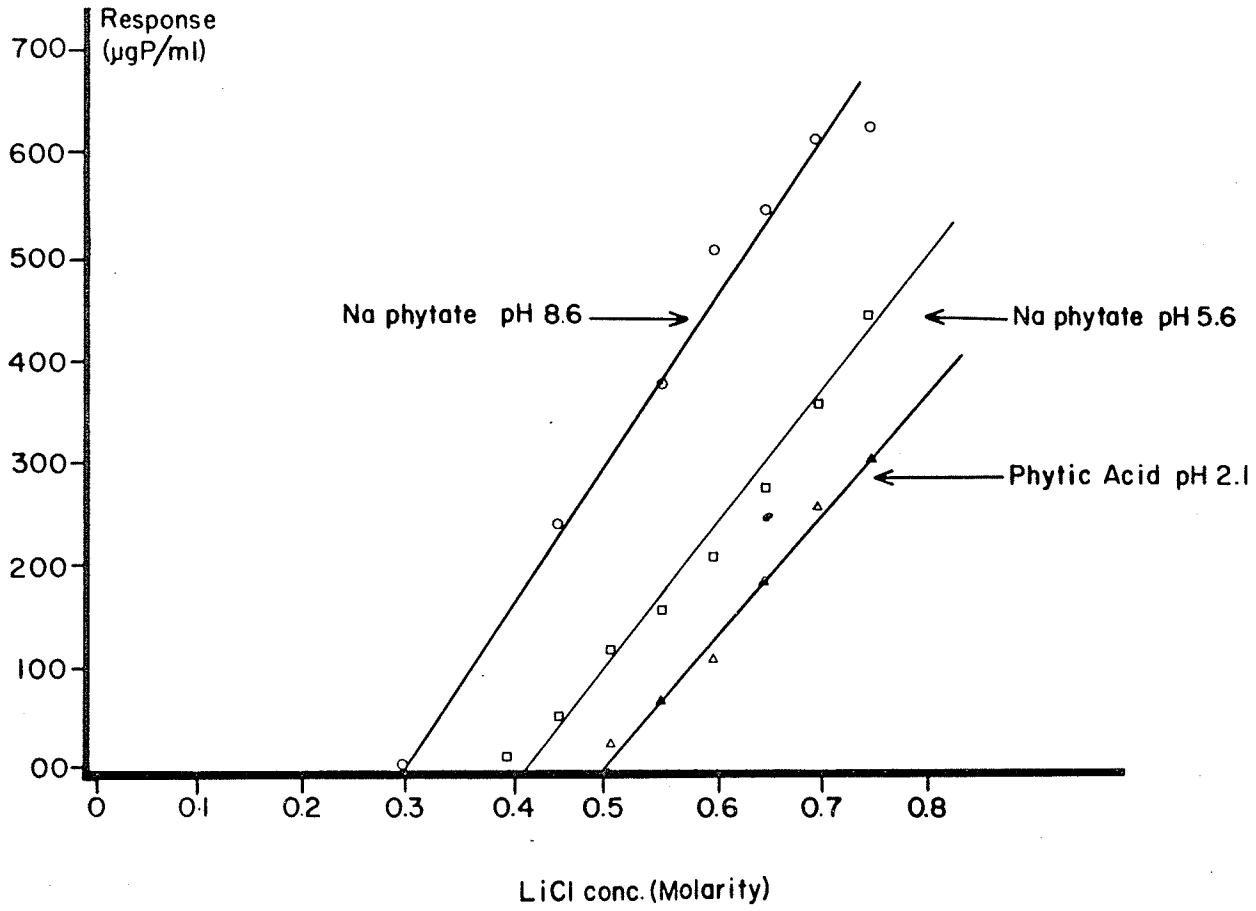


FIGURE III·8

Maximum phytate concentration reached in the eluate with each LiCl concentration as a function of the LiCl concentration for phytate, pH 2.1, 5.6 and 8.6.

line relationship is observed with each pH level. The effect of pH is evident from this diagram.

3) Changes in pH, Na^+ and Cl^- Associated with Putting Phytate and IMP onto the Dowex-1-Cl Column

Changes in pH and in Na^+ and Cl^- concentrations in the eluate associated with the putting of phytate and IMP onto the column are shown in Figs. III·9 and III·10 respectively.

With phytate at pH 5.6 and 8.6 and with IMP at pH 8.6, the elution peaks for Na and Cl were similar (when comparing any one pH level) and occurred with small elution volumes (Fig. III·9 a and b; Fig. III·10 a and b).

With phytate at pH 5.6 and 8.6, the pH change from the base-line level was similar, occurring in a larger volume of eluent than that in which the corresponding Na and Cl ions were eluted (Fig. III·9 c). With IMP, pH 8.6, the displacement of the pH above the base-line occurred over a similar volume to that in which the corresponding Na and Cl ions were eluted (Fig. III·10 c).

With phytate at pH 2.1 and IMP at pH 2.4, elution of Cl was irregular and required a larger volume of eluent (Fig. III·9 a and III·10 a respectively) and the corresponding displacement in the pH was opposite in direction and was spread over a much larger volume than for Na phytate or Na IMP (Figs.

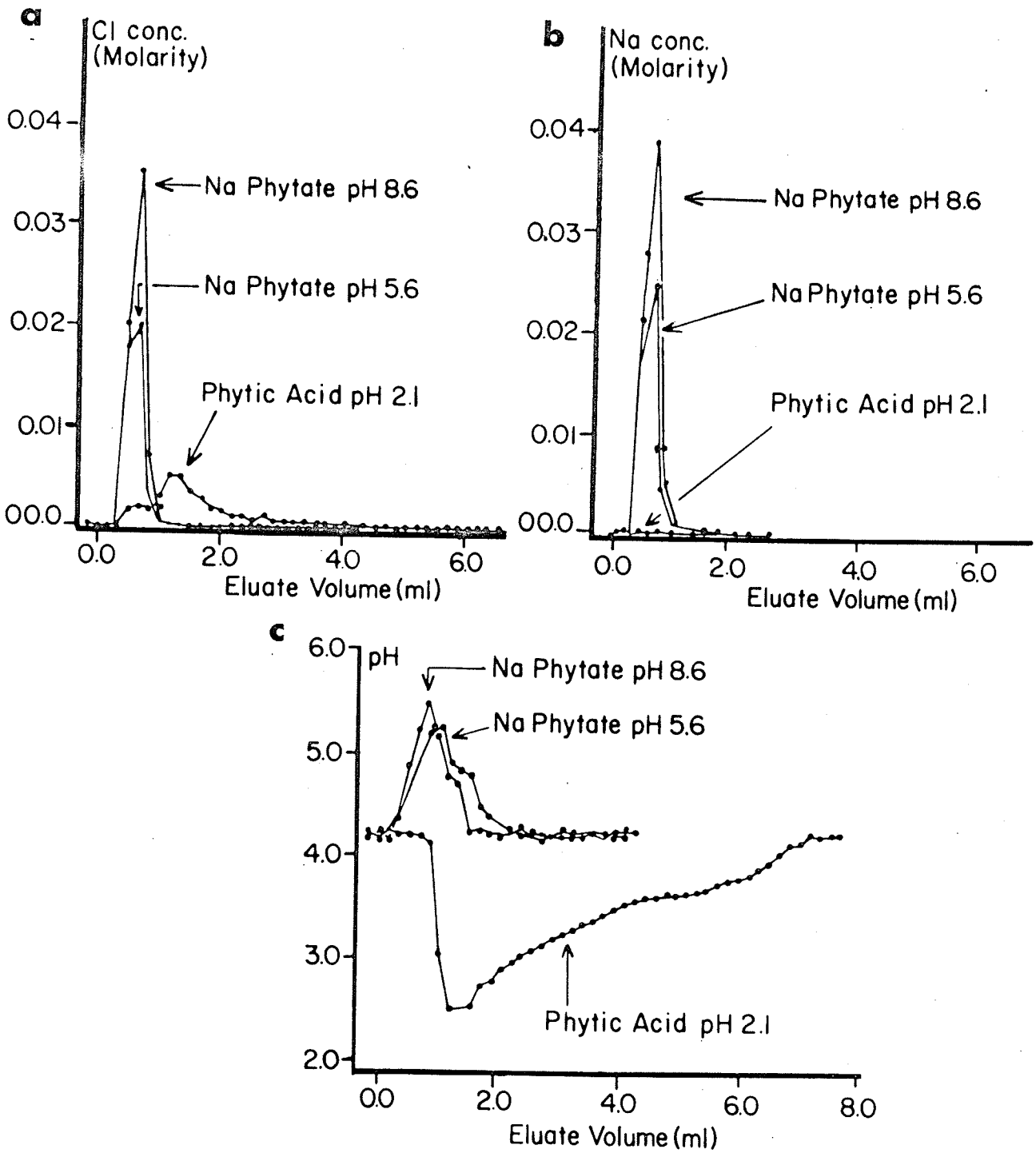


FIGURE III·9

Changes in pH, Na and Cl concentrations associated with putting phytate at either pH 2.1, 5.6, or 8.6 onto a Dowex-1-Cl anion exchange resin prior to LiCl elution.

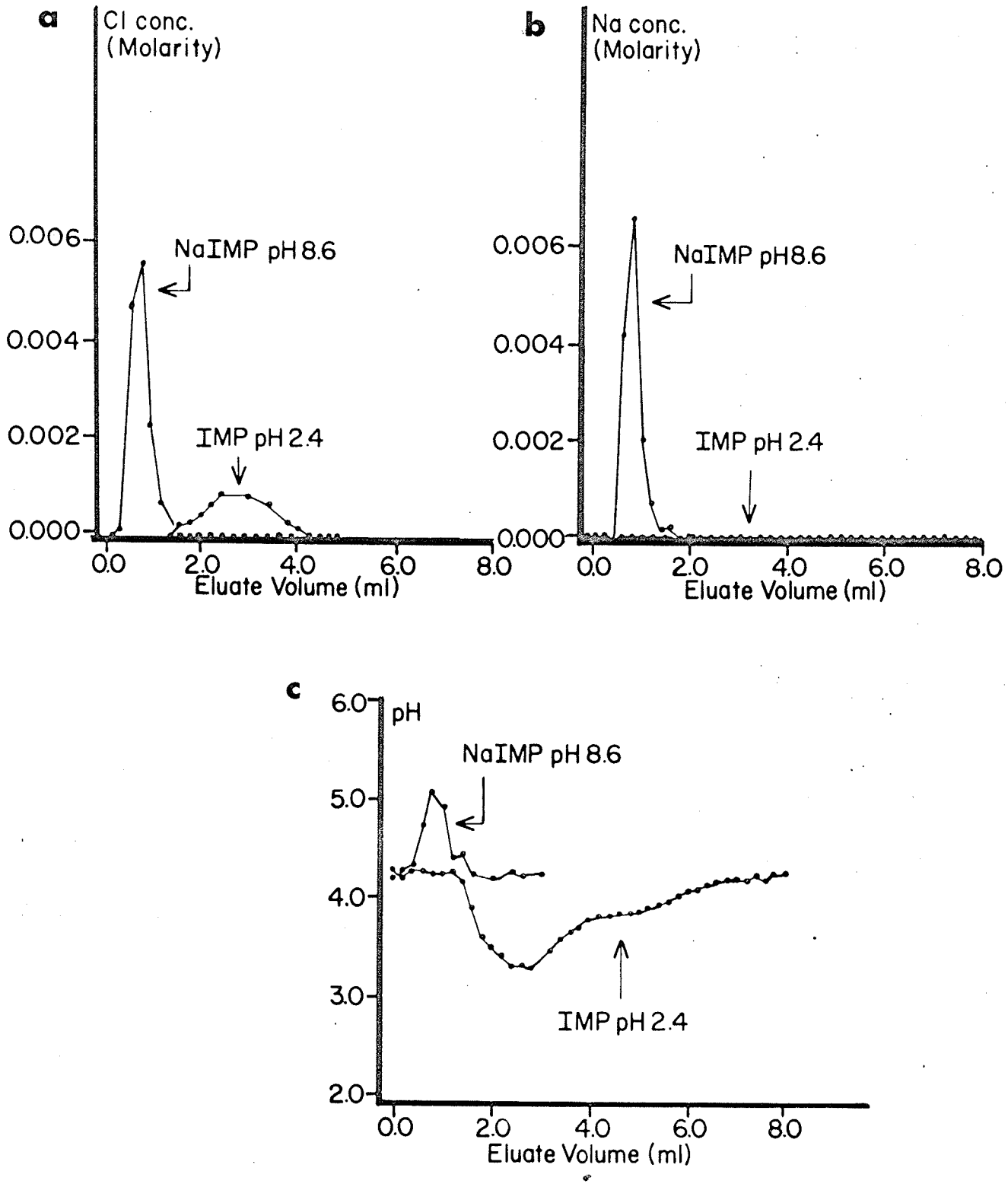


FIGURE III·10

Changes in pH, Na and Cl concentrations associated with putting IMP at either pH 2.4 or 8.6 onto a Dowex-1-Cl anion exchange resin prior to LiCl elution.

III·9 c and III·10 c respectively), indicating that the behaviour of the acidic forms of phytate and IMP must be different than that of the Na salts of these compounds.

The number of microequivalents of acid or base, Na^+ and Cl^- in the eluate which were released when phytate and IMP were adsorbed to the resin are shown in Tables III·1 and III·2 respectively.

The increase in the Cl^- concentration in the eluate with the more ionized forms of phytate and IMP (i.e. samples at higher pH), is indicative of the exchange that has gone on between the phosphate groups of the phytate or IMP and the chloride of the resin; more exchange occurring at higher pH. This is supported by the fact that the concentration of titratable hydrogen released into the eluate, when phytate at pH 2.1 or IMP at pH 2.4 was adsorbed to the resin, was the same as the concentration of Cl^- displaced from the resin. Also the concentration of sodium eluted, when Na phytate (pH 5.6 or 8.6) or NaIMP (pH 8.6) was put onto the column, was about the same as the concentration of chloride displaced (i.e. there is an approximate balance of charges in all cases).

The displacement in the pH from the base-line level, when phytate pH 8.6 is adsorbed to the resin, is approximately the same as that for phytate pH 5.6 (see Fig. III·9 c and Table

TABLE III·1

Microequivalents of Acid or Base, Na^+ and Cl^- Released into the Eluate when Phytate is Adsorbed to Dowex-1-Cl

	μ Equivalents Eluted		
	pH 2.1	pH 5.6	pH 8.6
Acid	5.6	0.0	0.0
Base	0.0	1.5	1.7
Na^+	0.0	8.9	12.6
Cl^-	6.0	7.1	10.7

TABLE III·2

Microequivalents of Acid or Base, Na^+ and Cl^- Released into the Eluate when IMP is Adsorbed to Dowex-1-Cl

	μ Equivalents Eluted	
	pH 2.1	pH 8.6
Acid	1.4	0.0
Base	0.0	0.3
Na^+	0.0	2.8
Cl^-	1.3	2.7

III·1). When IMP, pH 8.6 is adsorbed, a smaller pH displacement occurs than with the Na phytate salts, as can be seen when Tables III.1 and III·2 are compared.

With each of the sodium salts, the amount of sodium eluted was the same as the sodium content of the sample.

Of interest is the fact that approximately four hydrogen ions out of a possible twelve are released per molecule of phytate (pH 2.1) adsorbed to the resin, whereas, one hydrogen ion out of a possible two is released when IMP (pH 2.4) is adsorbed. These values were obtained by comparing the number of microequivalents of titratable acid released upon adsorption of phytate or IMP onto the resin (Tables III·1 and III·2 respectively) to the total number of microequivalents of phytate (pH 2.1) or IMP (pH 2.4) put onto the column.

Discussion

When anions are adsorbed to Dowex-1-Cl, chloride ions are displaced, the amount displaced reflecting the amount of exchange that has taken place. In the present study, with increase in pH, both for phytate and for IMP, more chloride was displaced indicating as one might have expected that more exchange occurred with the more ionized forms of either molecule.

With the salt forms of phytate and IMP, the Cl^- displaced

was accompanied by Na^+, whereas with their acidic forms, the Cl^- displaced was accompanied by H^+ (see Table III·1 and III·2).

Since ion exchange is a chemical reaction, adsorption of phytate and IMP resulting from such a reaction is by chemisorption (Glasstone, 1947).

If chemisorption were the only type of adsorptive force holding the phytate or IMP molecules to the resin, then at high pH a higher LiCl concentration would be required for their displacement from the resin than that required at low pH. In the present study, this was true for IMP but not for phytate. The phytate showed a reverse relationship in that it was more strongly adsorbed to the resin in its acidic than in its more basic forms.

Since there are only two types of adsorption, that involving physical forces and that involving chemical forces, in addition to chemisorption substantial physical adsorption of phytate to the Dowex resin must be involved (Glasstone, 1947). The large number of polar hydroxyl groups in the phytate molecule, should they be in their unionized forms, would favour such adsorption (La Mer and Smellie, 1956a, b; La Mer and Healy, 1963).

Since phytate, at pH 2.1, loses 4 hydrogen ions upon its

adsorption to the resin (see results), theoretically it still has 8 unionized OH groups which can partake in physical adsorption. Phytate at pH 5.6 and 8.6, on the other hand, only has 6 and 4 unionized OH groups respectively, which do not appear to take part in the exchange process that occurs when phytate is adsorbed to the resin. Consequently physical adsorption of phytate at these higher pH levels would be less than for phytate at pH 2.1. In other words, at high pH, adsorption of phytate to the resin by chemisorption would be favoured, whereas physical adsorption would be favoured at low pH.

The number of hydroxyl groups that may be available for physical adsorption at all three pH levels studied may actually be less than the theoretical number discussed above because some of the hydrogen atoms of these groups appear to be bound between adjacent phosphates. Since this would probably be the same number at all three pH levels, the above argument is not affected (see Chapter IV).

Since phytate is adsorbed more strongly at low than at high pH (Fig. III·8) the sum of the chemical and physical adsorptive forces must be correspondingly greater at low than at high pH. The reverse appears to be true for IMP, since this substance is adsorbed more strongly at high than at low pH

(Fig. III·3).

These findings together with similar findings on condensed phosphates (Beukenkamp et al, 1954) suggest that the number of phosphates in the molecule may determine whether the sum of the physical and chemical forces is greater at high or at low pH.

The observation that both the acidic and salt forms of IMP were eluted with lower LiCl concentrations than the acidic and salt forms of phytate (cf. Fig. III·2 to Fig. III·1) indicates that phytate adsorbs more strongly to the Dowex resin than IMP and can be attributed to the greater number of charged groups on the phytate molecule. The finding that when phytate and IMP are eluted stepwise phytate shows an irregular elution pattern whereas IMP does not, suggests different degrees of binding of the phytate but not of the IMP molecules. This difference may result from variation in the way in which the phytate molecules are adsorbed to sites on the resin because of spatial considerations not present with the much simpler IMP molecule.

The observations by Goring and Bartholomew (1950) and by Anderson and Arlidge (1962) that the adsorption of phytate to clay minerals increased with decrease in pH, can now be accounted for from the findings in this chapter.

As pointed out in the introduction, phytate, in acetic acid buffer, has been shown by Jenkins et al (1959) to protect the teeth against decalcification. The fact that this protective effect is retained following the washing of the teeth with water suggested that phytate was adsorbed to the enamel surface. The results in this chapter indicate that this adsorption would be favoured by the low pH of the system.

CHAPTER IV

A COMPARATIVE STUDY ON SODIUM AND CALCIUM BINDING BY PHYTIC ACID AND ITS INOSITOL PHOSPHATE DERIVATIVES AND THE SOLUBILITY OF THEIR CALCIUM SALTS

Introduction

As pointed out in Chapter I, reduction of calcium absorption in he intestine when phytate was present in the diet, was attributed by McCance and Widdowson (1942a,b) to the formation of calcium phytate, a salt which they found was less soluble than calcium phosphate under conditions likely to prevail in the small intestine.

Their data, however, indicated that phytic acid can be hydrolyzed in the intestine, a phenomenon which would be hard to explain if phytate was present as insoluble calcium phytate.

It was therefore considered necessary to re-examine this point by determining the effect of pH on the solubility of calcium phytate. Also, because conditions in the intestine were such that phytate underwent hydrolysis and formed inositol phosphate intermediates (in the experiments of McCance and Widdowson), the effect of pH on the solubility of the calcium salts of these intermediates was determined in this thesis.

The binding and solubility characteristics of phytate and its inositol phosphate derivatives were determined by titrating

these compounds with NaOH and $Ca(OH)_2$, differences in the titration curves reflecting differences in binding (Schwarzenbach, Biedermann and Bangerter, 1946; Schwarzenbach and Biedermann, 1948; Schwarzenbach, 1952), and differences in the pH at which precipitation occurred, reflecting differences in solubility.

Methods

100 µl aliquots of an 0.02 N solution of the acid form of each of IMP, IDP, ITriP, ITetraP, IP'P, and phytic acid were titrated between approximately pH 2.0 and 11.5 with both NaOH and $Ca(OH)_2$. The $Ca(OH)_2$ was a saturated solution prepared by adding CaO in excess to distilled water and allowing the mixture to stand at room temperature for several days. An aliquot was removed and filtered and aspirated into an Agla micro-burette (Burroughs Wellcome and Co., London) which was sufficiently air-tight to prevent contamination by carbon dioxide in the atmosphere.

The NaOH and $Ca(OH)_2$ were added as 1 to 5 µl aliquots, with the total volume of base added during a titration not exceeding 66 µl. Aliquots of the NaOH and $Ca(OH)_2$ were standardized against oxalic acid.

Water was also titrated with NaOH and $Ca(OH)_2$ so that the titration curves obtained for the inositol phosphates could be corrected for water.

The pH during the titrations was measured as described in Chapter II, with the electrode assembly, consisting of a glass electrode and KCl salt bridge, continually held in each solution.

To ensure rapid and complete mixing of the tube contents, nitrogen gas was constantly bubbled through the solution by means of a fine polyethylene tube leading from a nitrogen tank to the solution.

Because the pK_1 value obtained for each of the inositol phosphates in these titration experiments was higher than that observed earlier by Barré, Courtois and Wormser (1954) for phytic acid, experiments were carried out to determine whether their most acidic forms were behaving more like strong acids than weak ones. This was tested by determining whether the pK_1 values for IMP, ITetraP, and phytic acid changed with concentration. Because we were interested in whether the pK_2 values were also concentration dependent, $Ca(OH)_2$ rather than NaOH was used to titrate 100 µl samples of 0.02 and 0.21 N IMP, 0.01 and 0.02 N ITetraP, and 0.02 and 0.07 N phytic acid. The titration curves obtained from these experiments were also corrected for water.

Results

The pH titration curves for each of the six inositol

phosphates with NaOH and $Ca(OH)_2$ are shown in Figs. IV.1 and IV.2. To enable comparison between the curves for the different inositol phosphates, the titration curve of IMP with NaOH is reproduced in each of the figures in Fig. IV.1 and IV.2.

The titration curves for IMP with NaOH and $Ca(OH)_2$ were identical (Fig. IV.1a). IDP, ITriP, ITetraP, IP'P, and phytic acid, when titrated with $Ca(OH)_2$, all showed curves similar to that for IMP. However the titration curves for the same phosphates with NaOH deviated from their corresponding $Ca(OH)_2$ curves above a pH between approximately 3.0 and 5.0. This deviation became progressively more evident with increase in the phosphorus/inositol ratio of the inositol phosphate, the greatest deviation being observed with IP'P and phytic acid.

To enable more quantitative comparison of this deviation, the pH for each inositol phosphate was plotted when it was 25% and 75% neutralized with NaOH and with $Ca(OH)_2$ (Fig. IV.3). Whereas little or no difference was observed at 25% neutralization, at 75% neutralization, IHP and IP'P showed a very marked deviation, ITetraP and ITriP showed a less marked deviation but were similar to each other, IDP showed an even less marked deviation, and IMP showed no deviation.

With all of the inositol phosphates except IMP, a fine

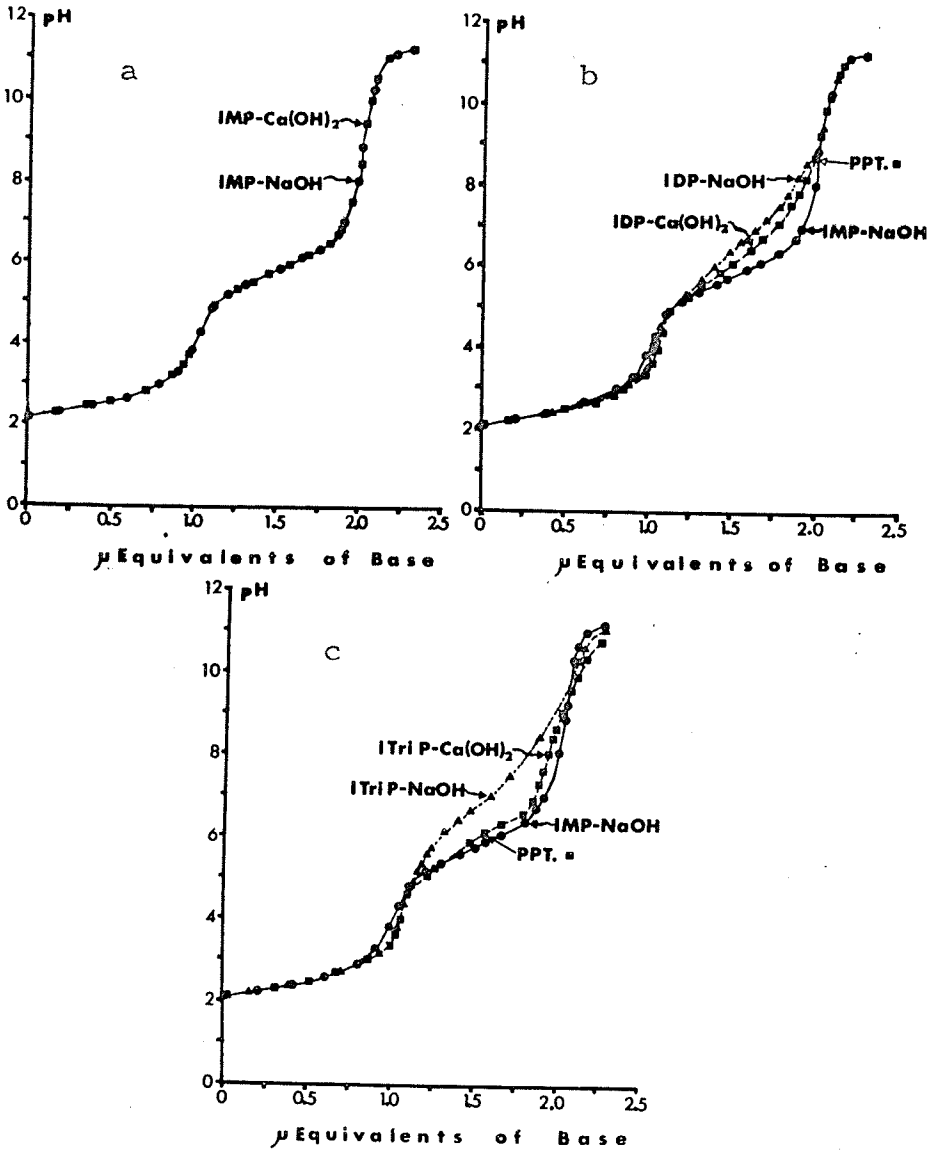


FIGURE IV·1

Titration curves for each of IMP (a), IDP (b), and ITriP (c) with NaOH and $Ca(OH)_2$. The titration curve for IMP with NaOH is reproduced in each figure for comparison purposes.

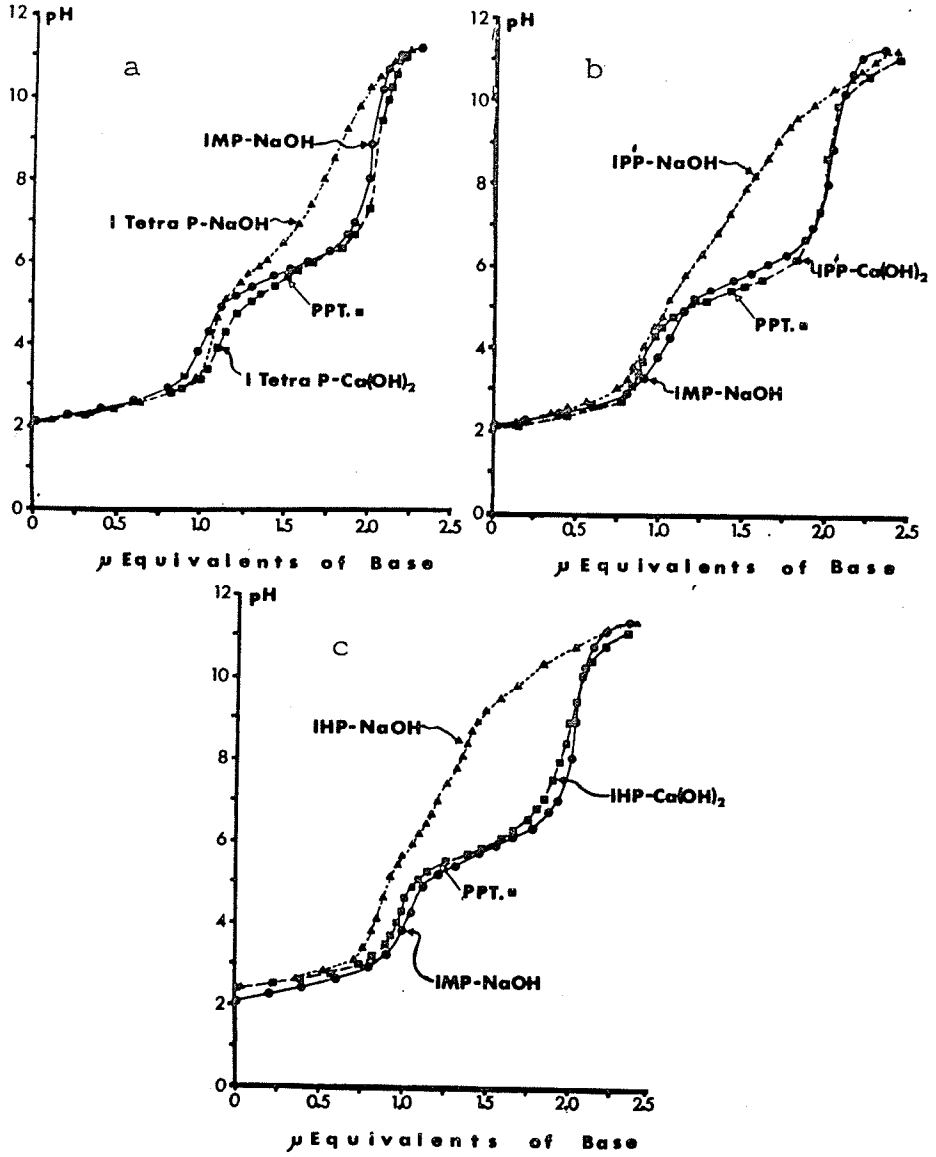


FIGURE IV·2

Titration curves for each of ITetraP (a), IP'P (b), and IHP (c) with NaOH and $Ca(OH)_2$. The titration curve for IMP with NaOH is reproduced in each figure for comparison purposes.

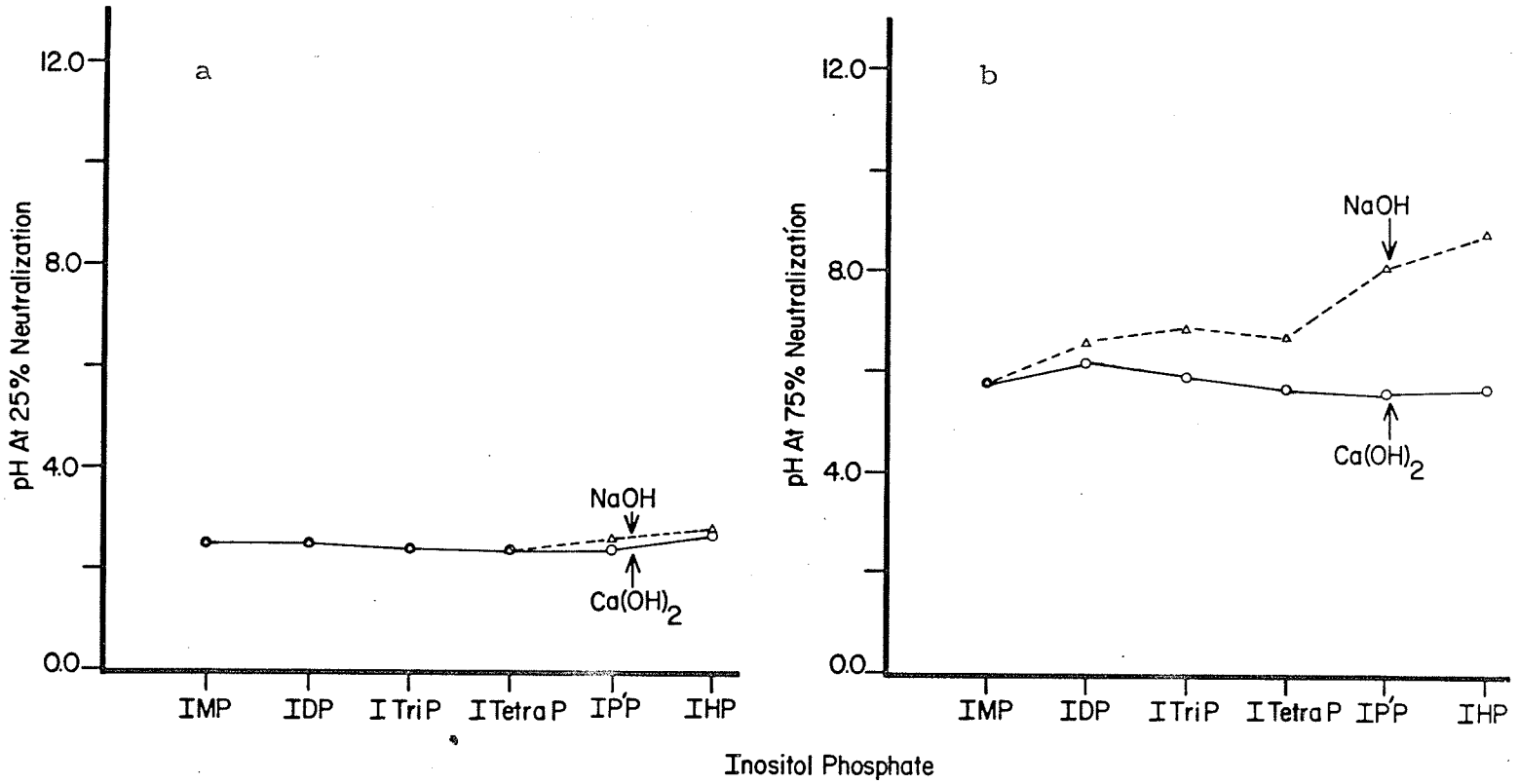


FIGURE IV·3

The pH of each inositol phosphate when it is 25% (a) and 75% (b) neutralized with NaOH and with $Ca(OH)_2$.

suspension was formed which remained throughout the $Ca(OH)_2$ titration. Centrifugation at 600 x g was the gravitational force necessary to sediment the suspended material. As expected, no suspension occurred with any of the inositol phosphates with NaOH as titrant. The pH in the titration at which each suspension was first visible is shown in Fig. IV.4. This pH varied inversely as the P/I ratio of the inositol phosphate.

In the experiments to determine the effect of concentration on the titration curves in the region of the first pK (Fig. IV.5), the curves shifted to a lower pH with increase in concentration.

The portions of each titration curve in the neutral region of the pH scale did not change with alteration in concentration however.

The differences in the pH curves in the high pH range can be attributed to differences in the total volume of titrant and acid being titrated and therefore the $Ca(OH)_2$ concentration at the end of the titrations. This is not easily avoided because the limited solubility of $Ca(OH)_2$ does not permit concentrations above approximately 0.04N to be used. The difference in this volume is no problem in the acid region because there is no excess $Ca(OH)_2$ to affect the pH.

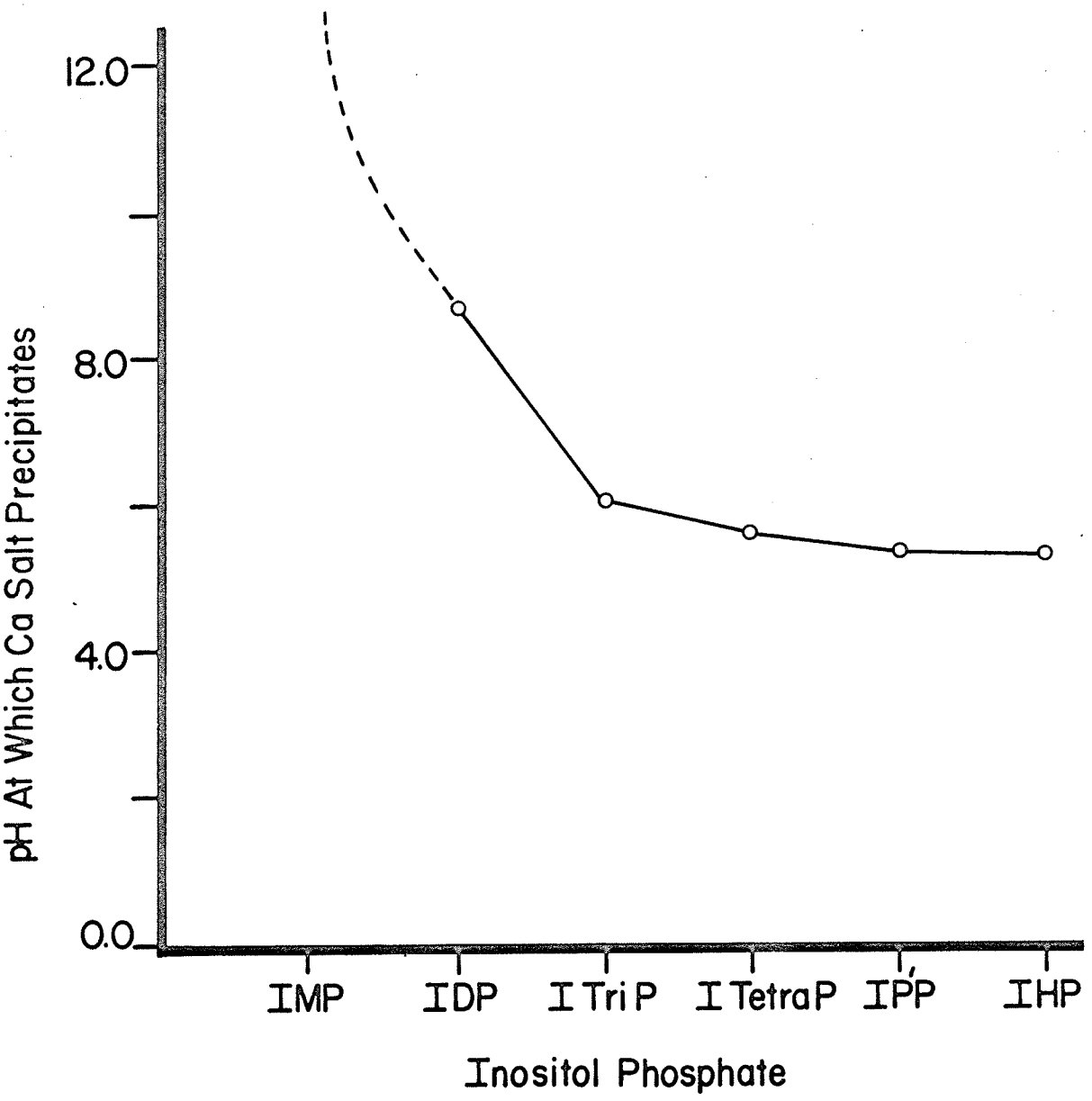


FIGURE IV·4

The pH at which a suspension is first visible with each of IMP, IDP, ITriP, ITetraP, IP'P, and IHP when titrated with $Ca(OH)_2$.

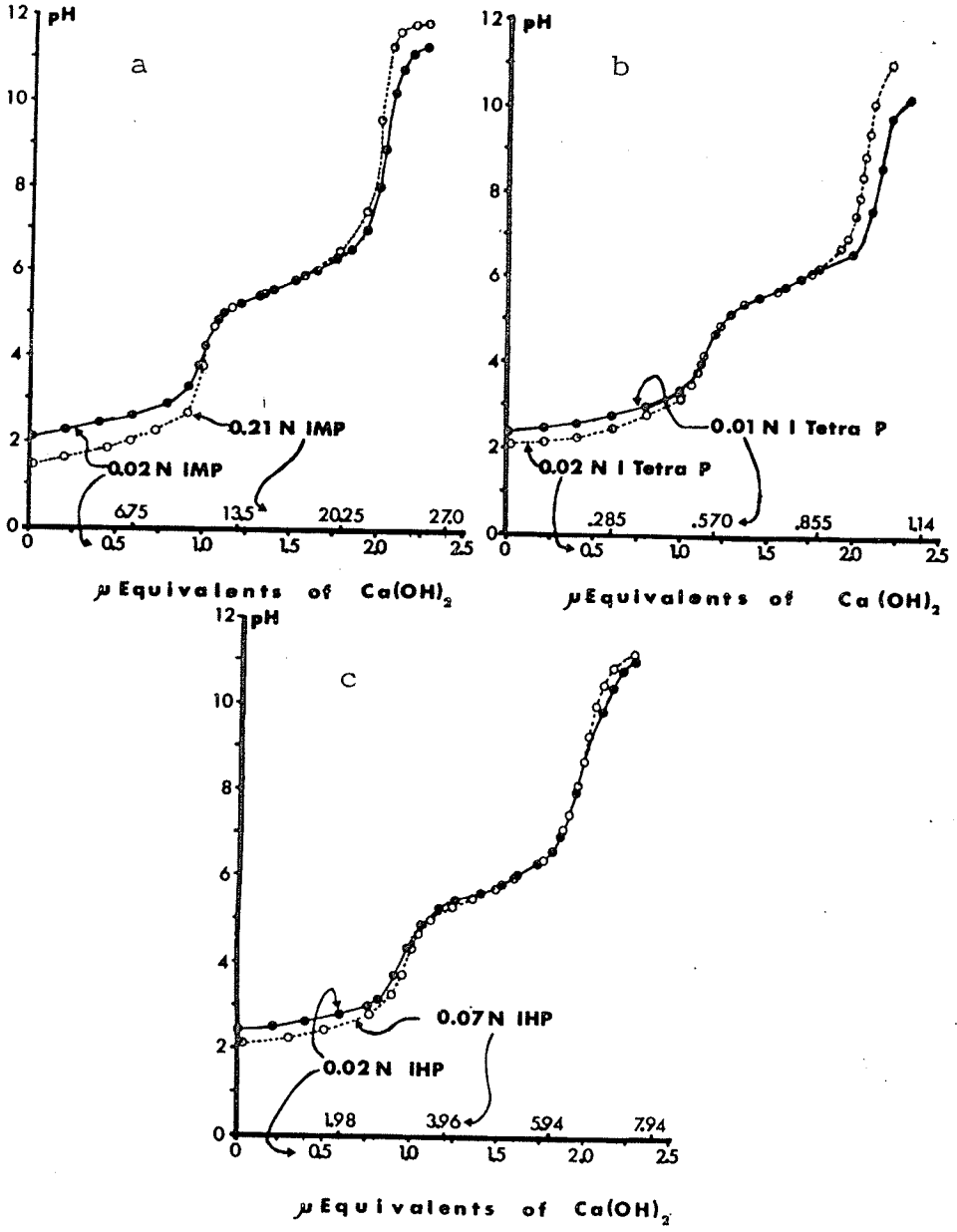


FIGURE IV·5

Titration curves for two different concentrations of each of IMP (a), ITetraP (b), and IHP (c) with $Ca(OH)_2$.

Discussion

When an acid, such as any one of the various inositol phosphoric acids in the present chapter, is titrated with either NaOH or $Ca(OH)_2$, hydroxyl ions from the added base are neutralized by hydrogen ions from the acid to form molecules of water, thus preventing a rise in pH. When a rise in pH occurs during such titration, insufficient hydrogen ions are available to neutralize the hydroxyl ions from the added base. Consequently for the same amount of added base, any deviation between the titration curves of an acid with NaOH and $Ca(OH)_2$ would be a reflection of the ease with which Na^+ or Ca^{++} facilitates the ionization of hydrogen ions from the acid (Martell, 1957).

Such deviation was observed in the present study for all of the inositol phosphoric acids except IMP in the region of 50 to 100% neutralization (Figs. IV·1, IV·2 and IV·3). Little or no deviation was observed in the region of 0 to 50% neutralization.

In the region of 0 to 50% neutralization, the region in which one of the two hydrogens on each phosphate group ionizes, Na^+ can replace hydrogen ion as readily as Ca^{++}. However, in the region of 50 to 100% neutralization, where the second hydrogen on each phosphate group ionizes, Ca^{++} replaces hydrogen

ion with equal facility in all of the inositol phosphates, whereas with Na^+, hydrogen ion is replaced with progressively greater difficulty as the P/I ratio of the inositol phosphate increases. Only in IMP, can Ca^{++} and Na^+ displace hydrogen ion equally.

The greater affinity for calcium than for sodium of each inositol phosphate with a P/I ratio greater than that for IMP, strongly indicates that the hydrogen ions that were not displaced by sodium are each held between two phosphate groups, most probably by hydrogen bonding (cf. Barré et al, 1954).

Since inositol phosphates with higher P/I ratios would have more phosphates, more hydrogens can be bound in this way, thus explaining the larger difference between their NaOH and $Ca(OH)_2$ titration curves than those for inositol phosphates having lower P/I ratios.

The fact that the titration curves for IMP with $Ca(OH)_2$ and NaOH were identical is consistent with this conclusion, since IMP does not possess the minimum of two phosphate groups needed for hydrogen bonding to occur.

Because IDP does show a difference between the titration curves with sodium and calcium hydroxide, as do the higher inositol phosphates, phosphates linked by bound hydrogens must still be present, indicating that such phosphates resisted

acid hydrolysis, the process used to prepare the various inositol phosphates from phytic acid.

This is in agreement with the results of Tomlinson and Ballou (1962) who showed that during the sequential hydrolysis of phytic acid by wheat bran phytase, (pH optimum 5.2) such phosphate groups are preserved (Fig. IV·6). This suggests that the number of isomers that can occur during an acidic or enzymic hydrolysis is restricted.

pK Values for the Inositol Phosphates

In the pH range corresponding to 50 to 100% neutralization of each inositol phosphate molecule, pK values with $Ca(OH)_2$ were different from those with NaOH. With $Ca(OH)_2$, the pK_2 values for the phosphate groups in the different inositol phosphates (corresponding to 75% neutralization of each molecule) were approximately the same - 5.6 to 6.2 (Fig. IV·3) and are lower than those generally observed for inorganic phosphate compounds without ring structure such as glycerophosphate (West et al, 1966b).

Because of the closeness of these pK_2 values for the various inositol phosphates with $Ca(OH)_2$ and because of the similarity of their titration curves with this base, the ionization of hydrogens from the various phosphate groups must be very similar. In other words, the pK_2 values with $Ca(OH)_2$

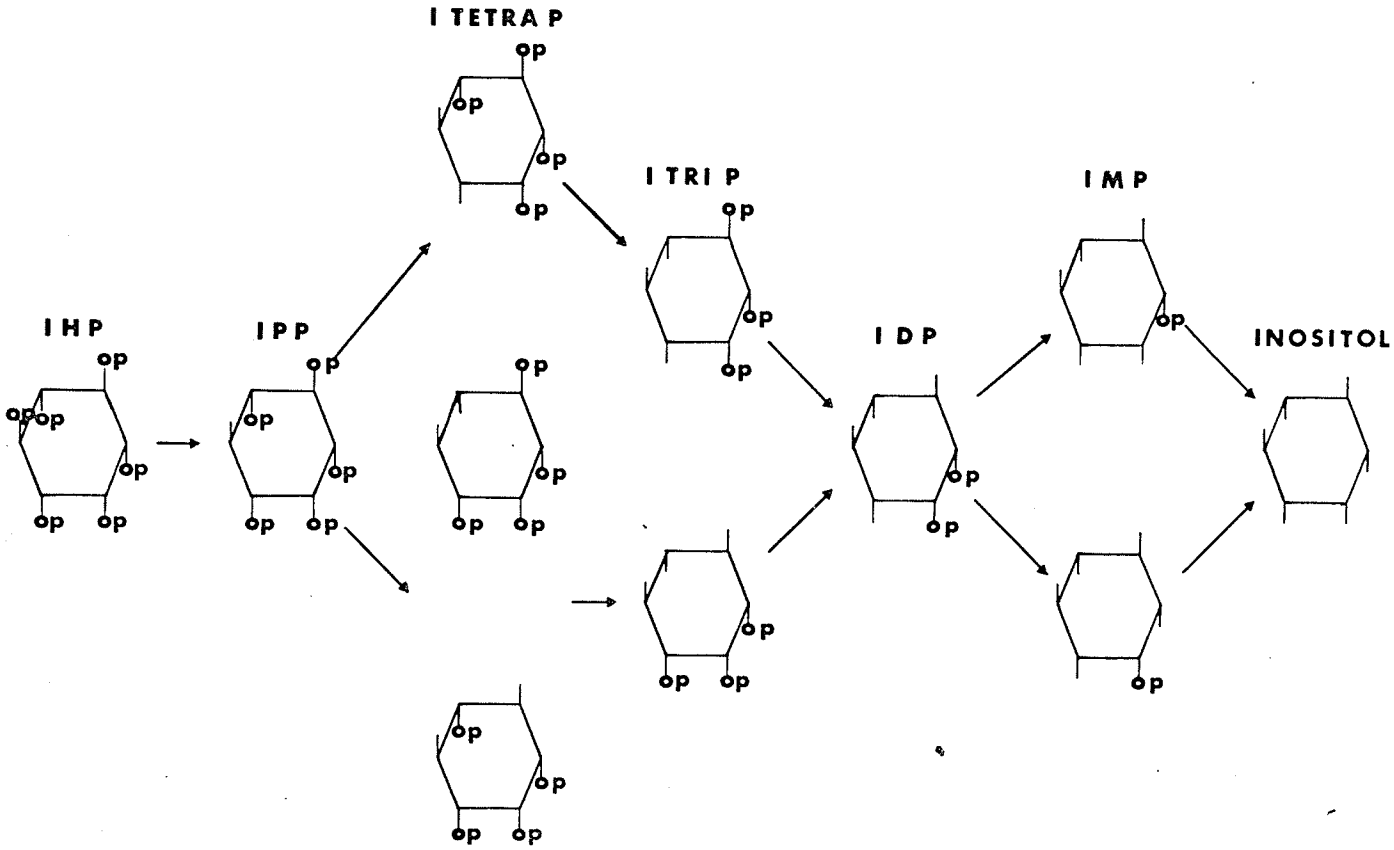


FIGURE IV·6

Scheme proposed by Tomlinson and Ballou (1962) for the sequential hydrolysis of phytic acid (IHP) with phytase.

are mean values, each representing an average of the pK values for the second ionizable hydrogens on each phosphate group in an inositol phosphate.

With NaOH, because of the binding of hydrogens between phosphates, pK_2 values determined from their titration curves would reflect the second ionizable hydrogens of those phosphate groups not involved in such binding. The pK_2 values vary with the type of inositol phosphate and are higher than those observed with $Ca(OH)_2$, with the difference increasing with the P/I ratio of the inositol phosphate. With phytate and IP'P, these pK_2 values do not correspond with the 75% neutralization point.

The pH at which the inositol phosphates are 25% neutralized should be the pH that corresponds to the first pK of each of their phosphate groups. Since this value was shown to be dependent upon the concentration of the inositol phosphate being titrated (Fig. IV·5), these compounds must be behaving more like strong acids than weak ones in the region of the first pK. Consequently, the pK_1 values that have been reported (Barré et al, 1954; Andrews and Herrarte, 1951) are meaningless.

Effect of pH on the Solubilities of the Calcium Salts of the Various Inositol Phosphates

Inositol phosphates with higher P/I ratios formed insoluble calcium salts at lower pH levels than did those with lower P/I ratios (Fig. IV·4).

The value obtained for the initial formation of a precipitate of calcium phytate was 5.4, which is higher than the value of 3.0 reported by McCance and Widdowson (1942a) and of approximately 4.0 reported by Barré et al (1954). It agrees more closely however with the value of 5.0 obtained by Hoff-Jorgensen (1944). The reasons for these differences are not readily apparent; the main difference between the experiments in the present study and the experiments of these other workers is that chloride was not present in the former.

However, because calcium phytate forms a colloidal rather than a flocculent precipitate above pH 5.4, it may remain suspended, and if this occurred in such areas as the gastrointestinal tract it might, in spite of its insolubility, still be available for hydrolysis by phytases.

CHAPTER V

HYDROLYSIS OF PHYTIC ACID AND ITS INOSITOL PHOSPHATE DERIVATIVES BY ACID AND ALKALINE PHOSPHATASES

Introduction

Because phytate is hydrolyzed sequentially by enzymes called phytases, and little is known about the factors that may be involved during such hydrolysis, in this chapter, the effect of pH, enzyme, substrate, and P_i concentrations on the enzymic hydrolysis of phytic acid and its various inositol phosphate intermediates by acid and alkaline phosphatases has been examined. In the introduction (Chapter I) and in the methods chapter (Chapter II, sections (1)-iv and (1)-v), the reasons for selecting these enzymes has been given.

Initially, pH activity curves with both enzymes were determined for each of the inositol phosphates including phytic acid. The pH optima from these curves were used to select one pH with acid phosphatase and one with alkaline phosphatase at which to determine the Km values for each inositol phosphate-enzyme combination. Comparing the Km values permitted the relative rates of hydrolysis of phytic acid and its intermediates to be evaluated.

pH activity and Km values for glycerophosphate were also

determined to ensure that characteristics of the enzyme preparations that were used were comparable to the characteristics of those reported by other workers.

Because the activity with either enzyme might be related to the net charge, the isoelectric point of each enzyme was determined; this was done spectrophotometrically.

Because inorganic phosphate is a product of the sequential hydrolysis of phytic acid, and because it is a known inhibitor of the hydrolysis of organic phosphates by acid and alkaline phosphatase (Burstone, 1962; Gezelius and Wright, 1965; Lazdunski and Oullet, 1962; Garen and Leventhal, 1960; Shaw, 1966; Scott, 1966), a series of experiments was carried out to permit the effect of inorganic phosphate on the relative rates of hydrolysis of phytic acid and its intermediates to be compared.

Methods

The acid and alkaline phosphatases and the phytic acid intermediates (other than IMP) used in the experiments in the present chapter were prepared by the procedures described in Chapter II. IMP was obtained from Calbiochem, Los Angeles, U.S.A. The glycerophosphate used in the experiments to determine the pH activity curves and Km values for the various substrate-enzyme combinations was obtained from Eastman Organic

Chemicals.

1) Determination of the pH Optima of the Various Enzyme-Substrate Combinations

The pH activity curves for acid and alkaline phosphatase with each of IMP, IDP, ITriP, ITetraP, IP'P, phytic acid and β-glycerophosphate were determined between pH 1.5 and 11.0. Each of the pH curves was determined in a separate experiment. In each experiment, a final concentration of 0.003 N substrate was used. This concentration provided equimolar levels of phosphate for all substrates studied.

In each experiment, three enzyme-substrate mixtures at each of 24 different pH levels between 1.5 and 10.5, were prepared in 10 x 75 mm pyrex test tubes as follows. 50 µl of substrate, 25 µl of buffer (dimethylglutaric acid-NaOH between pH 1.5 and 7.0, and glycine-NaOH between pH 7.0 and 11.0), and 25 µl of enzyme (0.02 mg acid phosphatase or 0.006 mg alkaline phosphatase per ml of reaction mixture) were introduced into each tube.

Enzyme and substrate controls were prepared by replacing either enzyme or substrate respectively with distilled water. This was done at one pH since earlier experiments in this laboratory showed that the pH had little or no effect in the controls.

The enzyme-substrate mixtures were incubated at 37°C for two hours, and one mixture from each group of three was used to determine the pH at which the incubation took place. In the other two mixtures, the inorganic phosphate hydrolyzed during the incubation was measured. Preliminary experiments established that the pH hardly changed during the incubation period.

The reaction in the mixtures used for measurement of the inorganic phosphate released by enzymic hydrolysis was stopped by adding 25 µl of 25% (W/V) trichloracetic acid (TCA). The tubes were then centrifuged in a Clinical Centrifuge (International Equipment Co., Boston) at 1100 x g for 15 minutes to remove any enzyme that was precipitated by the addition of TCA. Then, 50 µl aliquots were removed from the supernatants for analysis for inorganic phosphate.

Due to the reported effect of substrate concentration on the pH optimum for alkaline phosphatase activity (Folley and Kay, 1935; Ross, Ely and Archer, 1951; Morton, 1957; Motzok and Branion, 1961; Motzok, 1963; Dabich and Neuhaus, 1966), pH activity curves were determined for several concentrations of each of phytic acid and its inositol phosphate intermediates.

2) Determination of the Km Values for the Various Enzyme-Substrate Combinations

Km values for each of IMP, IDP, ITriP, ITetraP, IP'P, and phytic acid with acid and alkaline phosphatases were determined from a Lineweaver-Burk plot (Lineweaver and Burk, 1934) of rates of hydrolysis as a function of substrate concentration. The rates were determined from time curves obtained with each of several substrate concentrations (same reference). The pH at which Km values were determined with acid phosphatase was 5.0 since at this pH, this enzyme showed optimal activity with each of the inositol phosphates.

However, with alkaline phosphatase, the pH optima were different (see Table V·1). Consequently, the pH selected for the determination of the corresponding Km values with alkaline phosphatase was 9.0, which was the average of the optimal pH values obtained from the pH activity curves for the various inositol phosphate substrates.

Subsequent experiments showed that the differences in pH optima were the result of differences in the molar substrate concentrations selected for determination of their pH activity curves.

The Km for β-glycerophosphate with acid phosphatase was determined at pH 5.5 rather than 5.0, since this was the pH of optimal activity for this enzyme-substrate combination. With alkaline phosphatase the Km was determined at pH 9.0.

TABLE V·1

pH Optima Values for the Inositol Phosphates and Glycerophosphate with Acid and Alkaline Phosphatases

SUBSTRATE (0.003N)	pH OPTIMA	
	ACID PHOSPHATASE	ALKALINE PHOSPHATASE
GLYCEROPHOSPHATE	5.5	9.0
INOSITOL MONOPHOSPHATE	5.0	9.1
INOSITOL DIPHOSPHATE	5.0	9.2
INOSITOL TRIPHOSPHATE	5.0	9.1
INOSITOL TETRAPHOSPHATE	5.0	9.0
INOSITOL PENTAPHOSPHATE'	5.0	8.8
INOSITOL HEXAPHOSPHATE	5.0	8.8

The final concentrations of DMG-NaOH buffer used with acid phosphatase and the glycine-NaOH buffer used with alkaline phosphatase were 3.75×10^{-2} and 5.0×10^{-2} M respectively. These were necessary to ensure that, with the wide variation in substrate concentration, the respective pH levels were constant. Earlier experiments in this laboratory showed that DMG does not interfere with acid phosphatase activity. Glycine does not interfere with alkaline phosphatase activity unless the concentration is greater than 0.05 M (Babson et al, 1966).

In each experiment, 6 enzyme-substrate mixtures at each of five different substrate concentrations (1.2×10^{-4} M to 1.3×10^{-3} M) were prepared in 10 x 75 mm pyrex tubes, such that each of the 30 mixtures contained 50 µl of substrate, 25 µl of buffer, and 25 µl of enzyme (see Table V·2 for enzyme concentration used). Two mixtures from each group of six served as zero time controls while the four remaining tubes in each group were incubated in a water bath at 37°C. The length of time during which reaction velocities were measured varied anywhere from 0 to 20 minutes depending upon the rate of release of P_i from the substrate under study (Table V·2). The total time period chosen for each enzyme-substrate combination was that most suitable for measurement of initial

TABLE V·2

Enzyme Concentrations and Times Used for Km Determinations

SUBSTRATE	ACID PHOSPHATASE		ALKALINE PHOSPHATASE	
	TIME (Min.)	CONCENTRATION (mg/ml reaction mixture)	TIME (Min.)	CONCENTRATION (mg/ml reaction mixture)
IMP	0 20 40	0.08	0 5 10	0.04
IDP	0 5 10	0.10	0 5 10	0.01
ITriP	0 5 10	0.10	0 5 10	0.02
ITetraP	0 10 20	0.09	0 5 10	0.01
IP'P	0 10 20	0.05	0 5 10	0.04
IHP	0 20 40	0.03	0 5 10	0.02
GLYCERO-PHOSPHATE	0 5 10	0.08	0 5 10	0.01

velocities (Lineweaver and Burk, 1934).

At the appropriate times, the reaction in each mixture was stopped by the addition of 25 µl of a 25% solution of TCA. As previously, each mixture was centrifuged at 1100 x g for 15 minutes, and duplicate 50 µl samples were removed from the supernatants for inorganic phosphate analyses.

Km values were then determined for each enzyme-substrate combination from a Lineweaver-Burk plot of rates of hydrolysis as a function of substrate concentration.

3) Isoelectric Point Determinations for Acid and Alkaline Phosphatases

In the present study, the isoelectric points of acid and alkaline phosphatase were determined by measuring the pH at which each enzyme is least soluble (really most turbid) in an aqueous solution (White, Handler, and Smith, 1964). Since, with increased turbidity a solution absorbs more visible light, changes in optical density of the solution reflect changes in solubility (Nichols & Kindt, 1950). Consequently for the determination of the isoelectric points of acid and alkaline phosphatase, the pH of 15 to 17, 200 µl aliquots of each enzyme was adjusted with NaOH or HCl between approximately 2.0 and 11.0 and the optical densities measured in a Beckman DU Spectrophotometer at 700 mµ. The pH at which maximum absorbance

occurred was taken as the isoelectric point.

4) The Effect of Inorganic Phosphate on the Activity of Acid and Alkaline Phosphatases

IMP is the only one of the different inositol phosphates studied which upon hydrolysis does not result in the formation of another inositol phosphate which in turn can be further hydrolyzed. The inhibitory effect of P_i on the hydrolysis of IMP would therefore be expected to be less complex than that for the hydrolysis of the higher inositol phosphate derivatives. IMP was therefore considered separately from the other inositol phosphates in this series of experiments.

The concentrations of DMG-NaOH and glycine-NaOH buffers were the same for these experiments as in those in section (2) in this chapter.

i) IMP

The inhibitory effect of P_i at each of two different concentrations (see below) on the hydrolysis of IMP by acid and alkaline phosphatase was examined as follows. The experiments were carried out in the same way as those in section (2) except that instead of one group of 30 mixtures, three groups of 30 mixtures were prepared. The contents of the mixtures were exactly the same as in section (2) except that to one of the groups, the control group, 5 µl of distilled water was

added while to each of the other two groups 5 µl of P_i was added to give a final P_i concentration of 0.84×10^{-4} M and 1.7×10^{-4} M respectively. The duration of the experiments and the enzyme concentrations used are shown in Table V·3.

ii) IDP, ITriP, ITetraP, IP'P, and IHP

The inhibitory effect of P_i at each of four different concentrations on the hydrolysis of each of IDP, ITriP, ITetraP, IP'P, and IHP by acid and alkaline phosphatase was examined in separate experiments, one enzyme-substrate combination per experiment.

In each experiment thirty mixtures, each containing enzyme, substrate, buffer and either distilled water or inorganic phosphate, were prepared in 10 x 75 mm pyrex test tubes. All contained 50 µl of one of the substrates (0.003 N final concentration), 25 µl of either DMG-NaOH or glycine-NaOH buffer, and 25 µl of enzyme preparation (see Table V·3 for enzyme concentrations used). Each group of six mixtures contained 5 µl of either distilled water or one of four solutions of inorganic phosphate to give final concentrations of P_i between 0.47×10^{-2} M and 7.5×10^{-2} M.

From each group of six mixtures, two mixtures served as zero time controls, while the four remaining tubes in each group were incubated in a water bath at 37°C for time periods

TABLE V·3

Enzyme Concentrations and Times Used for P_i Inhibition Experiments

SUBSTRATE	ACID PHOSPHATASE		ALKALINE PHOSPHATASE	
	TIME (Min.)	CONCENTRATION (mg/ml reaction mixture)	TIME (Min.)	CONCENTRATION (mg/ml reaction mixture)
IMP	0 20 40	0.08	0 5 10	0.04
IDP	0 5 10	0.11	0 5 10	0.01
ITriP	0 5 10	0.04	0 5 10	0.02
ITetraP	0 10 20	0.11	0 5 10	0.01
IP'P	0 5 10	0.09	0 5 10	0.04
IHP	0 10 20	0.04	0 5 10	0.02

found suitable for initial velocity determinations (Table V·3). The subsequent handling of the mixtures was as in section (2).

Results

1) Determination of the pH Optima for the Various Enzyme-Substrate Combinations

The pH optima determined from pH activity curves (Figs. V·1 and V·2) for acid and alkaline phosphatase with IMP, IDP, ITriP, ITetraP, IP'P, phytic acid and β-glycerophosphate are shown in Table V·1. The pH optima with acid phosphatase were the same whereas the pH optima with alkaline phosphatase were not.

In the experiments in which the effect of substrate concentration on the optimal pH for alkaline phosphatase activity was examined, the pH optimum decreased linearly as the log of the substrate concentration decreased (Fig. V·3).

The absence of reports in the literature on the effect of substrate concentration on the pH optimum for acid phosphatase, coupled with our findings of a constant pH optimum for several different molar concentrations of inositol phosphate substrates (Table V·1) suggested that the pH optimum for acid phosphatase is unaffected by substrate concentration. However, the effect of substrate concentration was checked with IMP in an experiment identical to those in which the effect

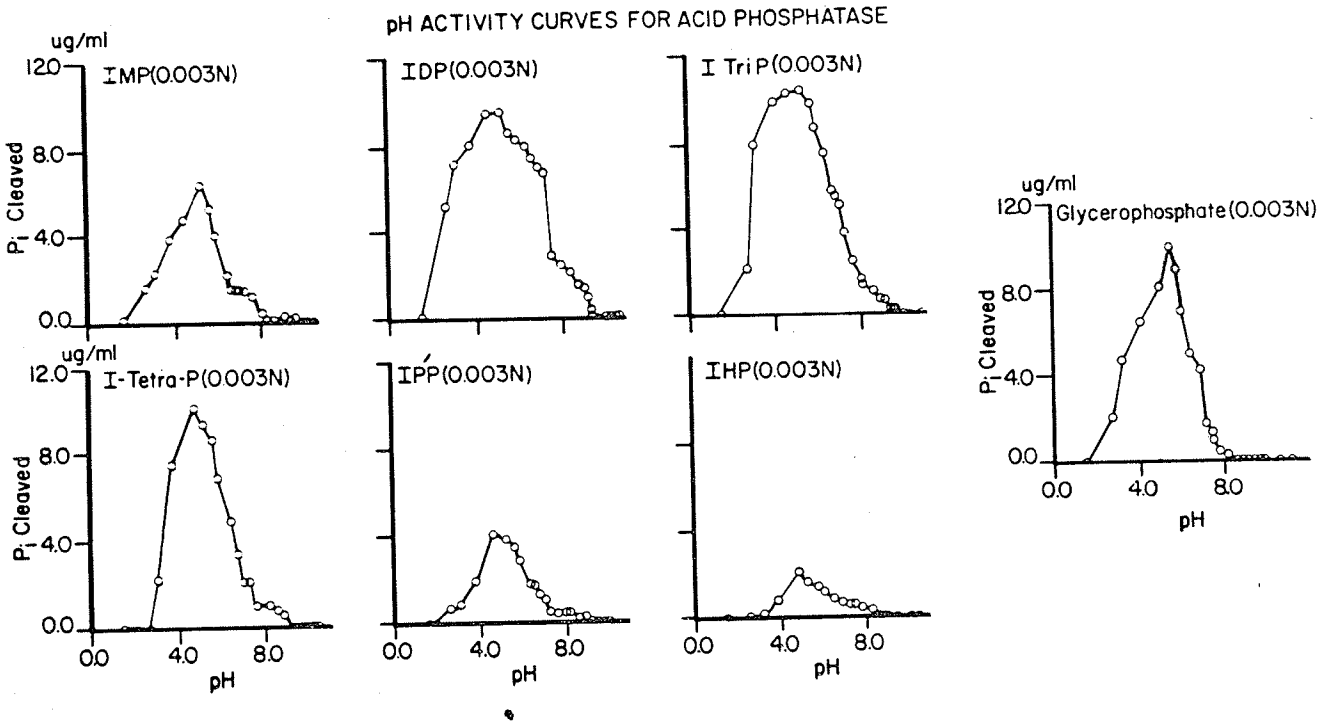


FIGURE V·1

Acid phosphatase activity as a function of pH with each of 0.003N IMP, IDP, ITriP, ITetraP, IP'P, IHP, and glycerophosphate as substrate. Enzyme activity: µg P_i liberated/ml of reaction mixture in a 2 hour period.

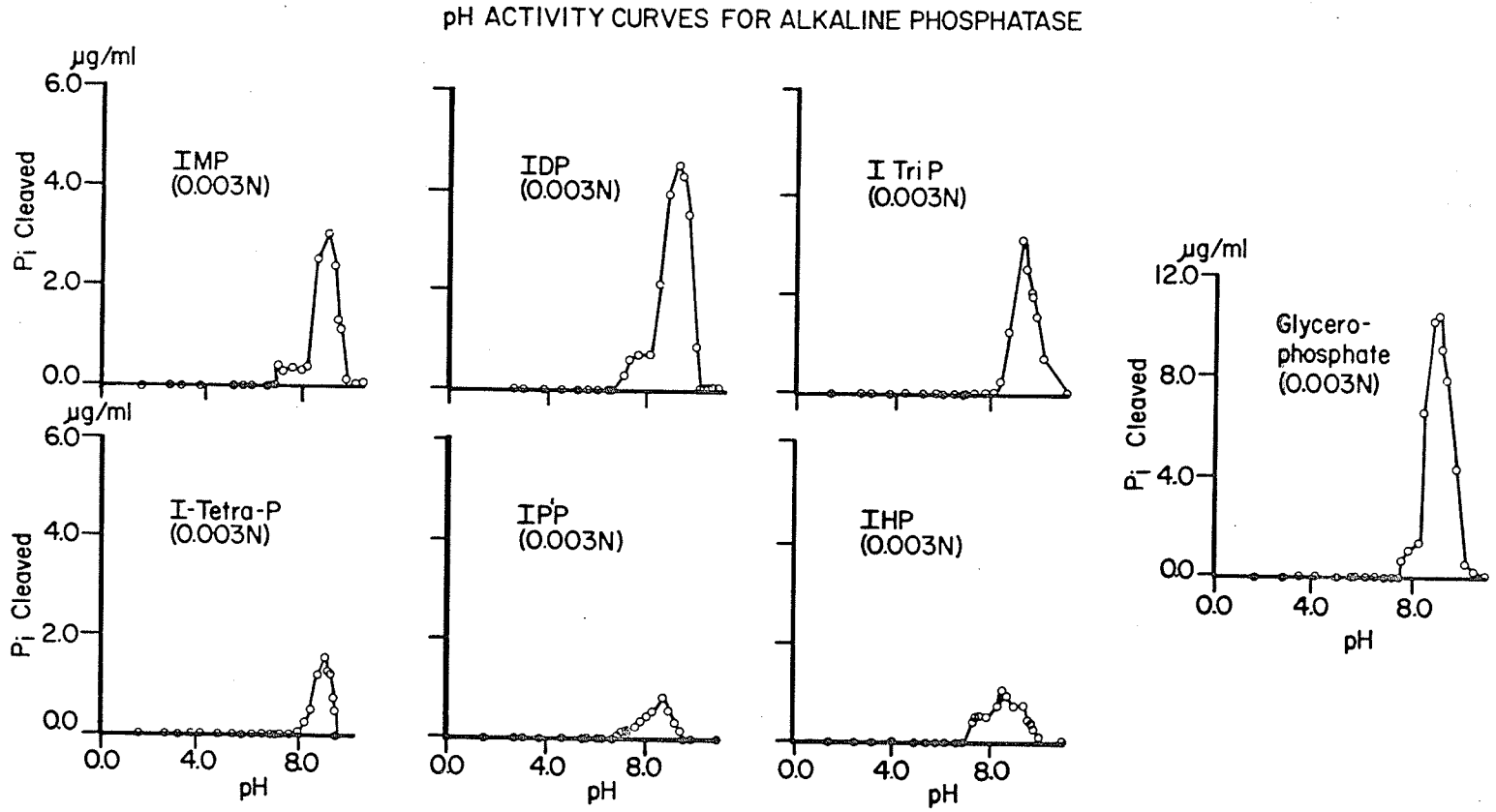


FIGURE V·2

Alkaline phosphatase activity as a function of pH with each of 0.003N IMP, IDP, ITriP, ITetraP, IP'P, IHP, and glycerophosphate as substrate. Enzyme activity: µg P_i liberated/ml of reaction mixture in a 2 hour period.

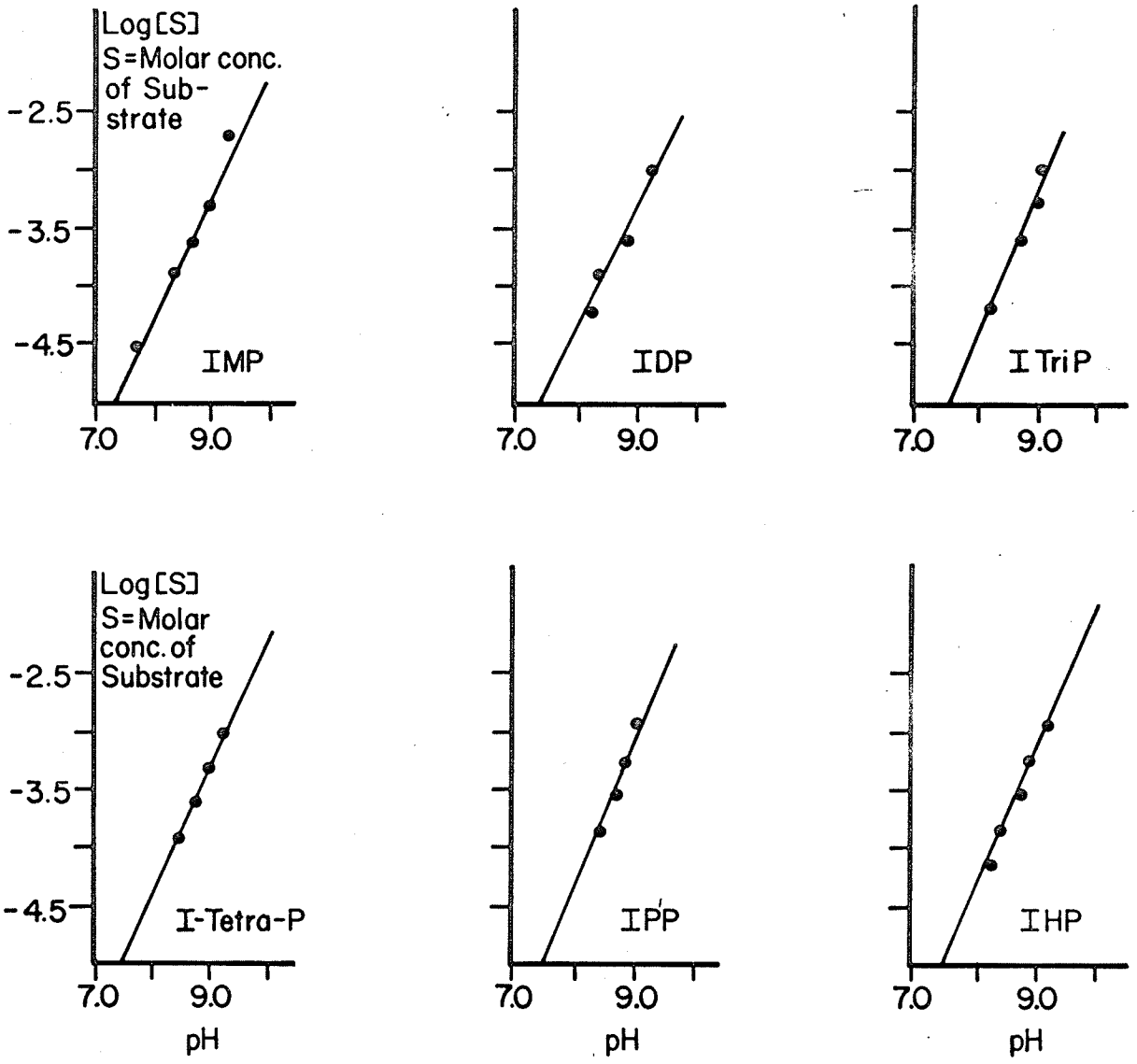


FIGURE V·3

Relationship between pH for optimum alkaline phosphatase activity and the log of the concentration of each of IMP, IDP, ITriP, ITetraP, IP'P, and IHP.

of inositol phosphate concentration on the pH optimum for alkaline phosphatase was determined, and the pH optimum was shown not to vary with substrate concentration.

2) Determination of the Km Values for the Various Enzyme-Substrate Combinations

The Km values for each of IMP, IDP, ITriP, ITetraP, IP'P, phytic acid and β-glycerophosphate with acid and alkaline phosphatases are shown in Fig. V·6 and were obtained from the Lineweaver-Burk plots shown in Figs. V·4a, V·4b, and V·5.

The data in Fig. V·6 show that the Km values for the inositol phosphates with acid phosphatase decrease asymptotically as the number of phosphate groups per molecule increase. The Km values also decreased with alkaline phosphatase with increase in the P/I ratio of the inositol phosphate, but this decrease was only slight.

When Km values were calculated on a molar basis with respect to phosphate, with acid phosphatase these values decreased as the number of phosphate groups per inositol phosphate molecule increased; but with alkaline phosphatase this relationship was reversed (Fig. V·7).

When the Km with acid phosphatase for each inositol phosphate is divided by the corresponding Km with alkaline phosphatase, the Km ratios thus obtained (Fig. V·8) showed different

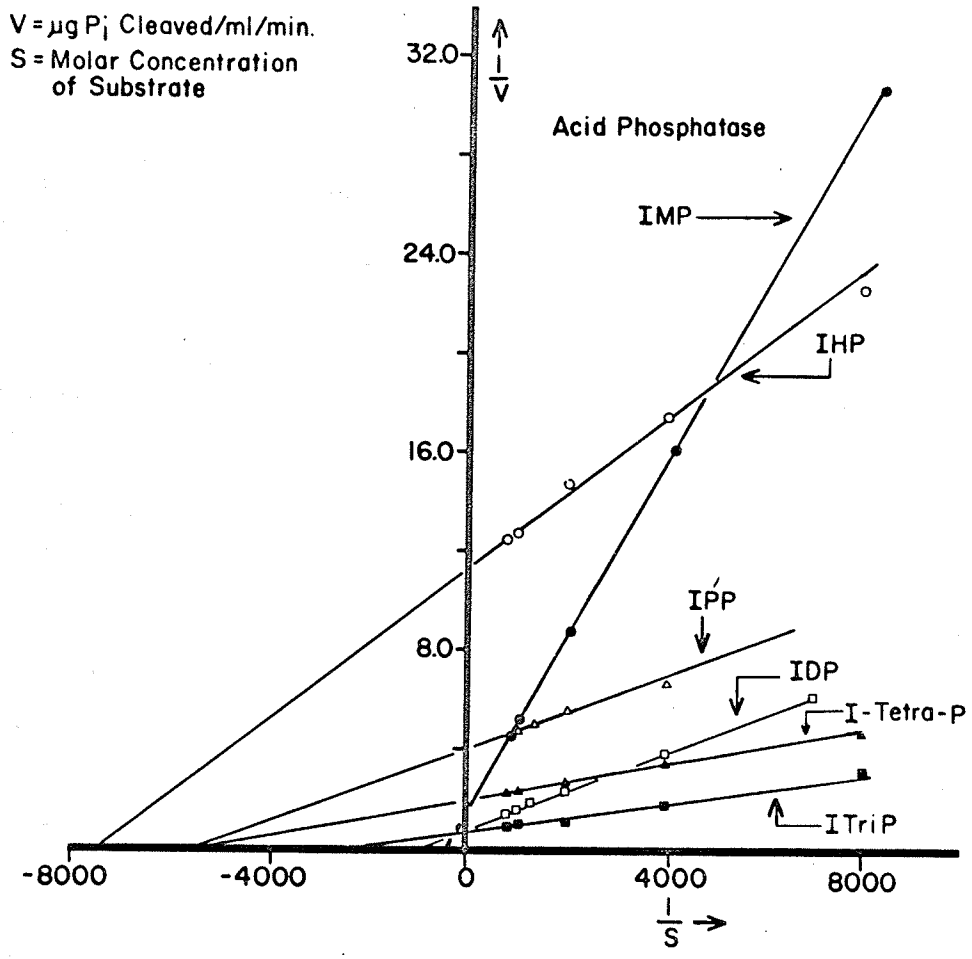


FIGURE V·4a

Lineweaver-Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of the substrate concentration) for acid phosphatase with IMP, IDP, ITriP, ITetraP, IP'P, and IHP as substrates at pH 5.0.

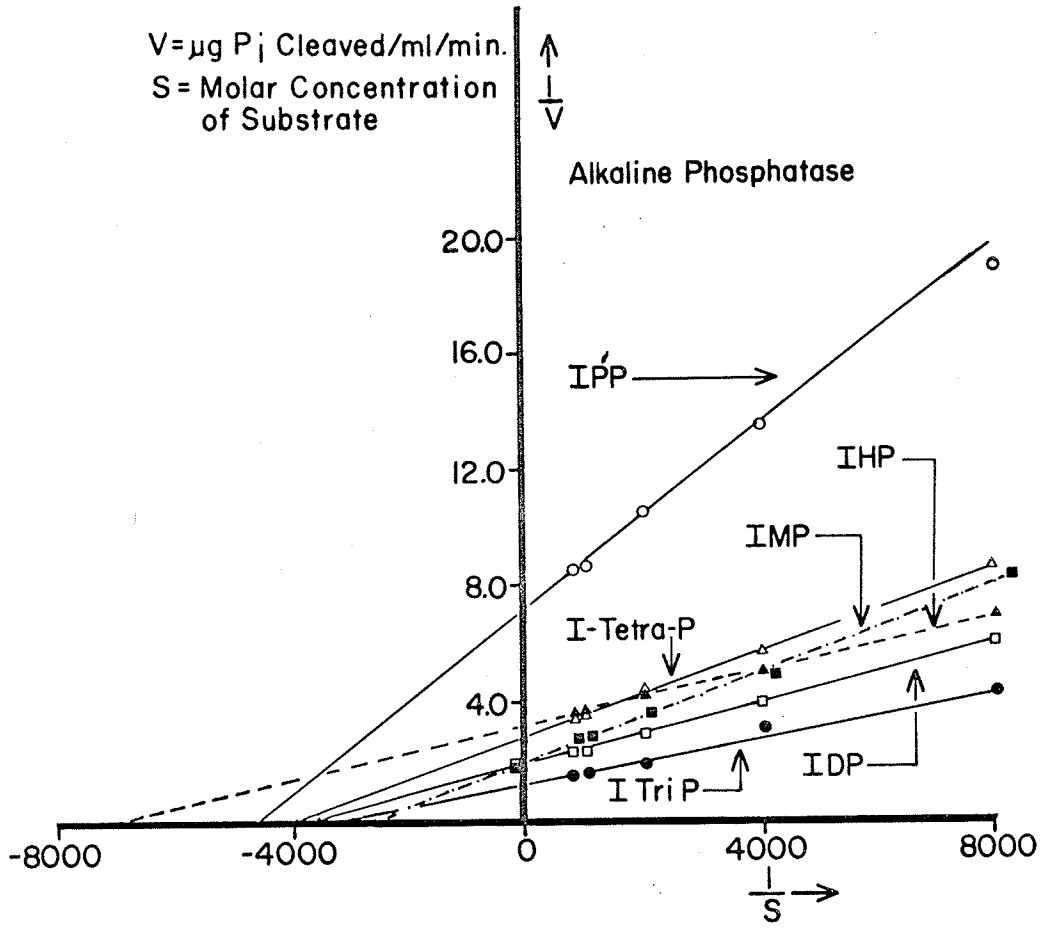


FIGURE V·4b

Lineweaver-Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of the substrate concentration) for alkaline phosphatase with IMP, IDP, ITriP, ITetraP, IP'P, and IHP as substrates at pH 9.0.

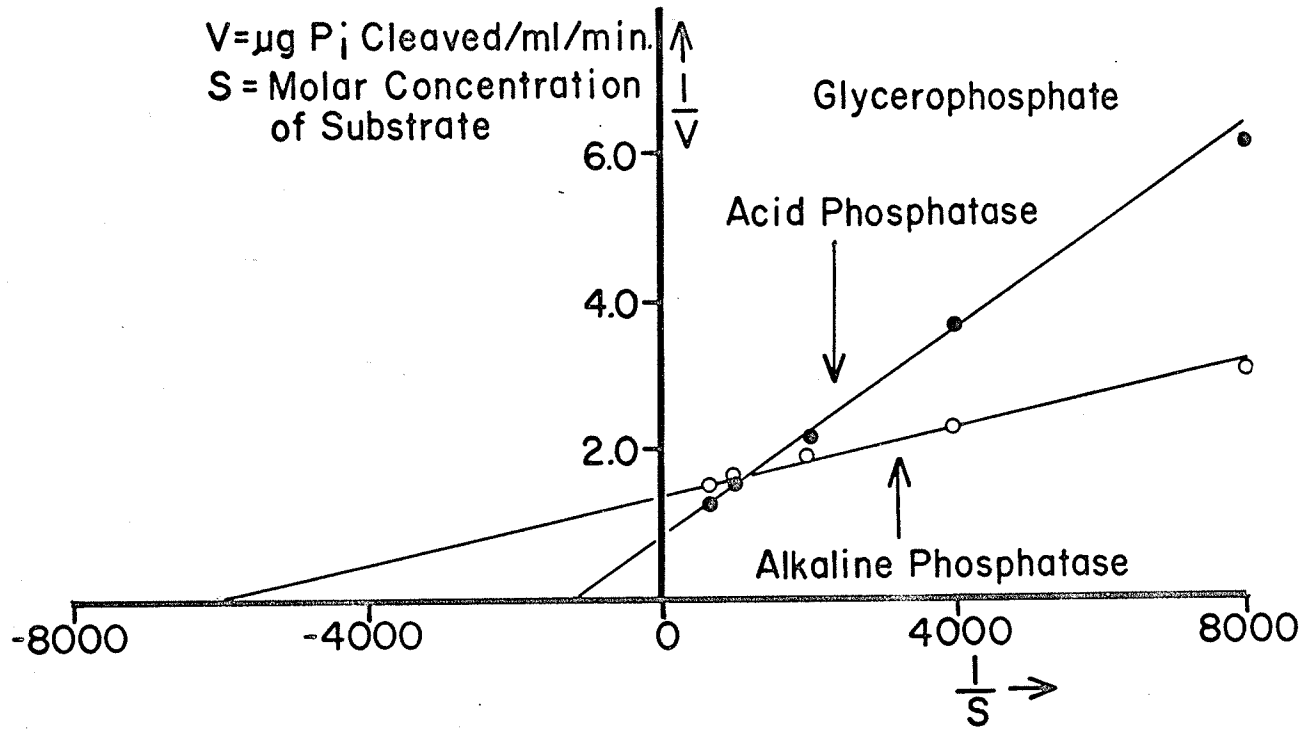


FIGURE V·5

Lineweaver-Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of substrate concentration) for acid phosphatase at pH 5.5 and alkaline phosphatase at pH 9.0 with glycerophosphate as substrate.

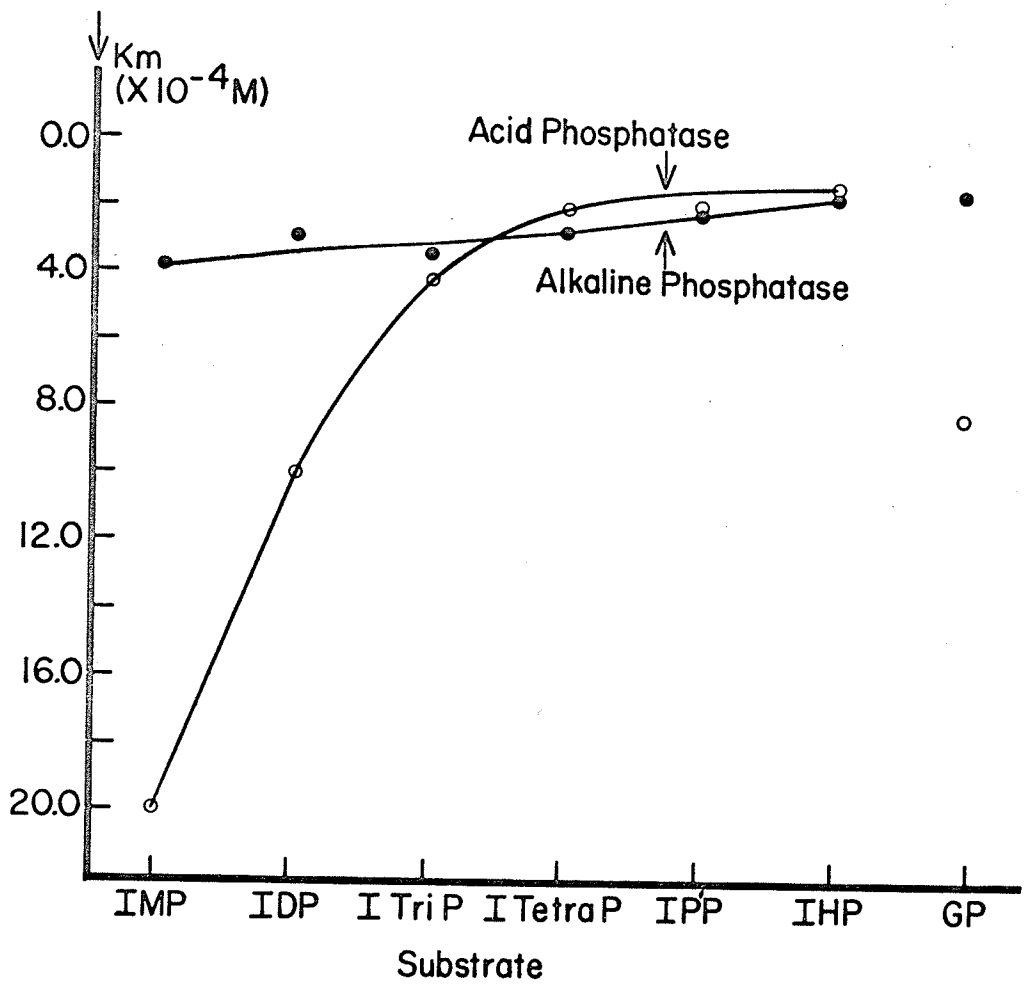


FIGURE V·6

Km values for acid and alkaline phosphatase with each of IMP, IDP, ITriP, ITetraP, IP'P, IHP, and glycerophosphate (GP).

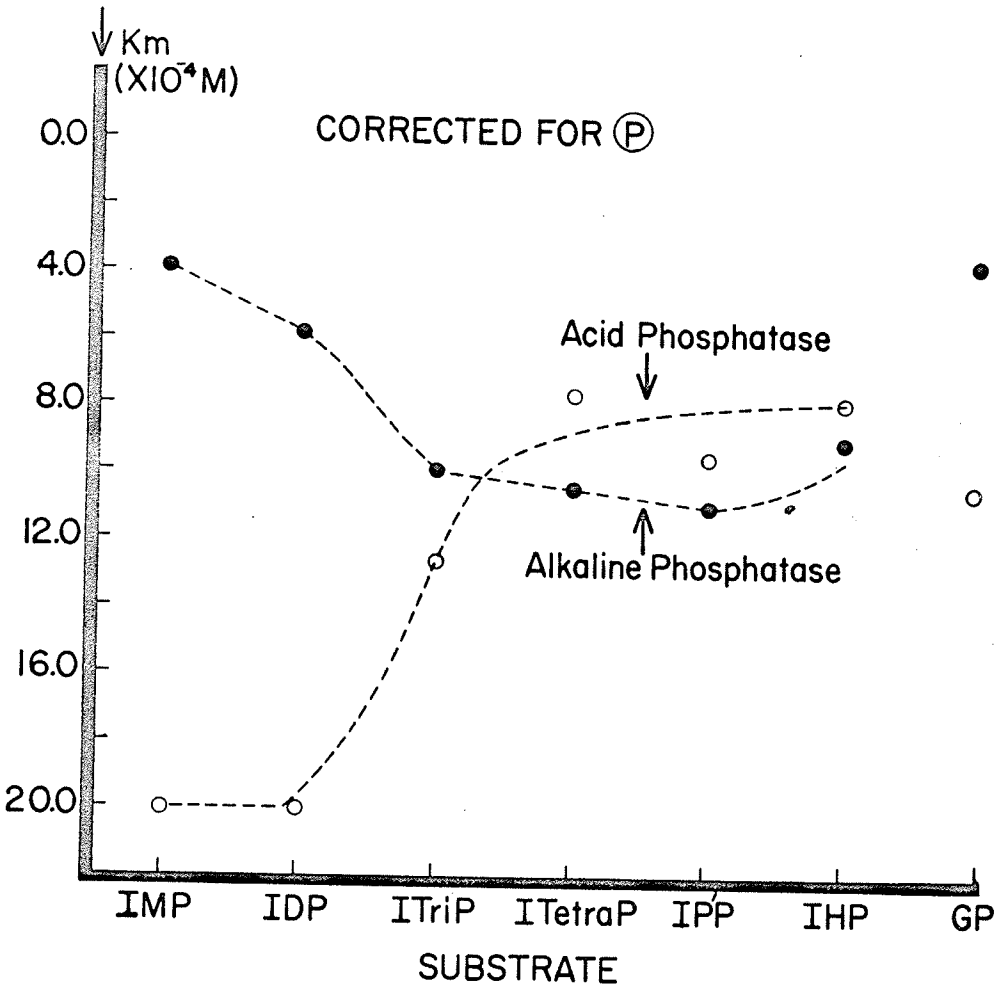


FIGURE V·7

Km values for acid and alkaline phosphatase calculated on a molar basis with respect to phosphate with each of IMP, IDP, ITriP, ITetraP, IP'P, IHP, and GP.

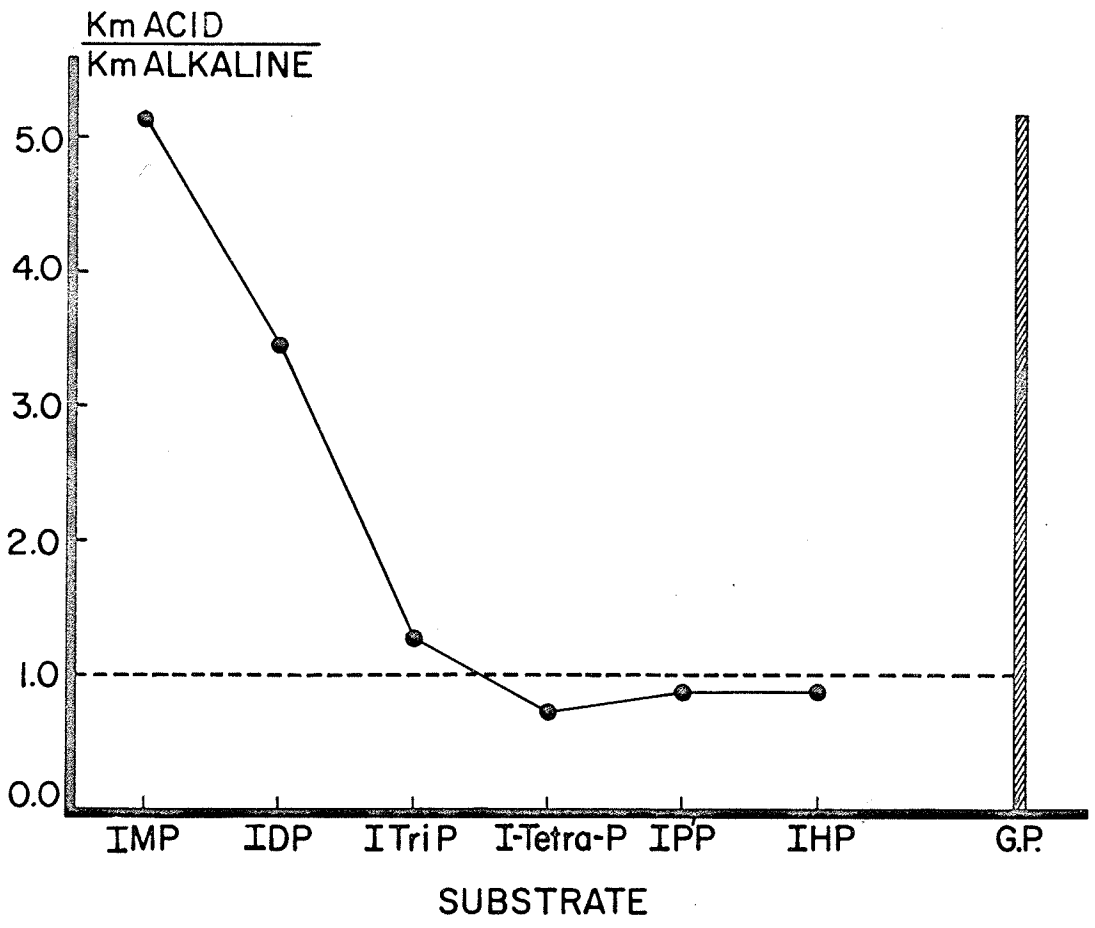


FIGURE V·8

Km ratios (Km with acid phosphatase divided by the corresponding Km with alkaline phosphatase) for each enzyme-substrate combination.

values for IMP, IDP, and ITriP which were each greater than one, and similar values for ITetraP, IP'P, and IHP which were slightly less than one.

Glycerophosphate (a monophosphate) showed a Km ratio similar to that for IMP (a monophosphate).

From the plots in Figs. V·4a and V·4b, Vmax values for each of the twelve enzyme-substrate combinations (and glycerophosphate) were determined for a constant enzyme concentration (0.05 mg/ml) and these are shown in Fig. V·9. With both enzymes, highest Vmax values were observed with IDP and ITriP with both acid and alkaline phosphatases.

3) Isoelectric Point Determinations for Acid and Alkaline Phosphatases

The experiments in which optical determination of the isoelectric points of acid and alkaline phosphatase was made, showed that the isoelectric points of acid and alkaline phosphatase were approximately 5.8 and 3.5 respectively (Figs. V·10 and V·11).

4) The Effect of Inorganic Phosphate on the Activity of Acid and Alkaline Phosphatases

Inorganic phosphate inhibited the hydrolysis of IMP by both acid and alkaline phosphatase and this inhibitory effect is shown by means of Lineweaver-Burk plots in Figs. V·12 and V·13. Since the presence of P_i did not affect the Vmax

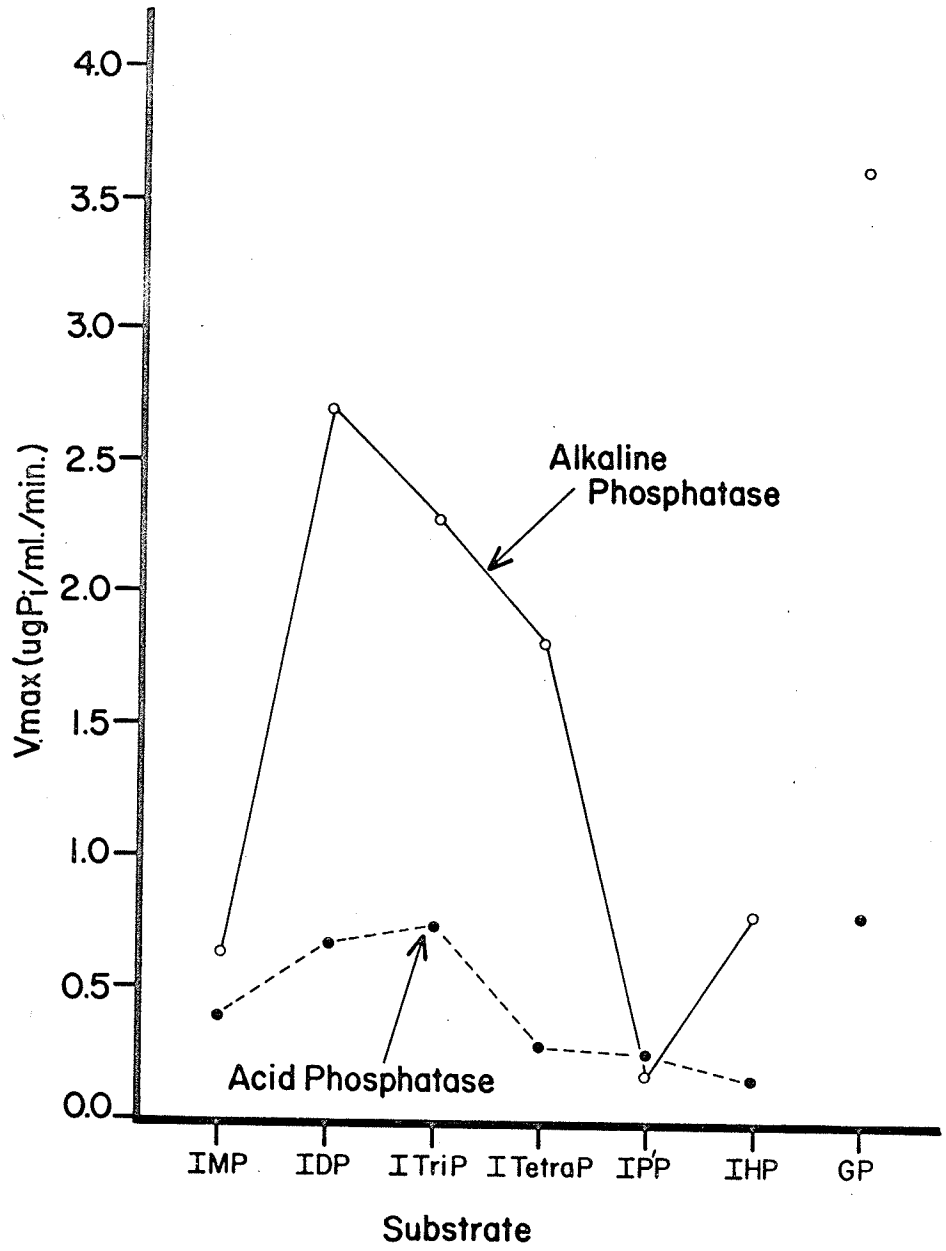


FIGURE V·9

Vmax values for each enzyme-substrate combination.

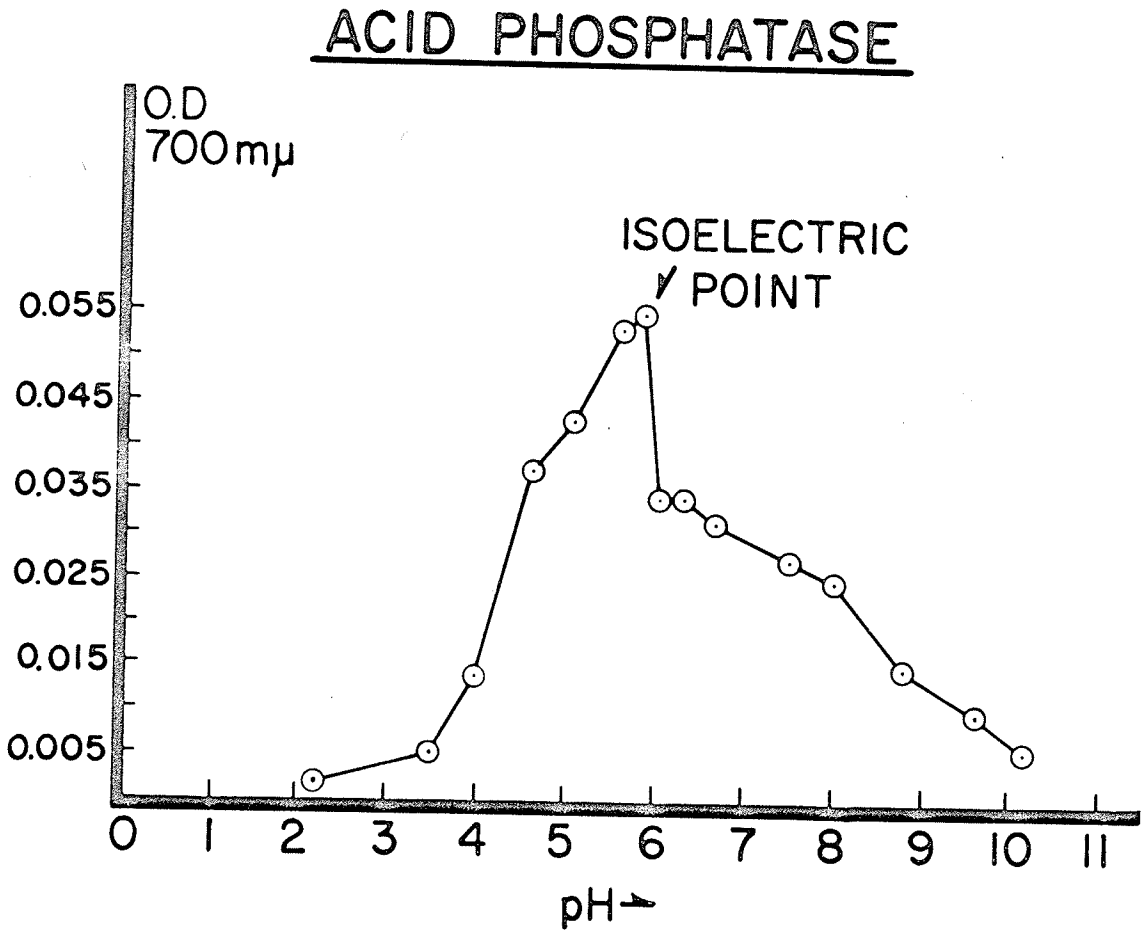


FIGURE V·10

Isoelectric point of acid phosphatase: Changes in optical density of acid phosphatase at 700 mu are plotted as a function of pH.

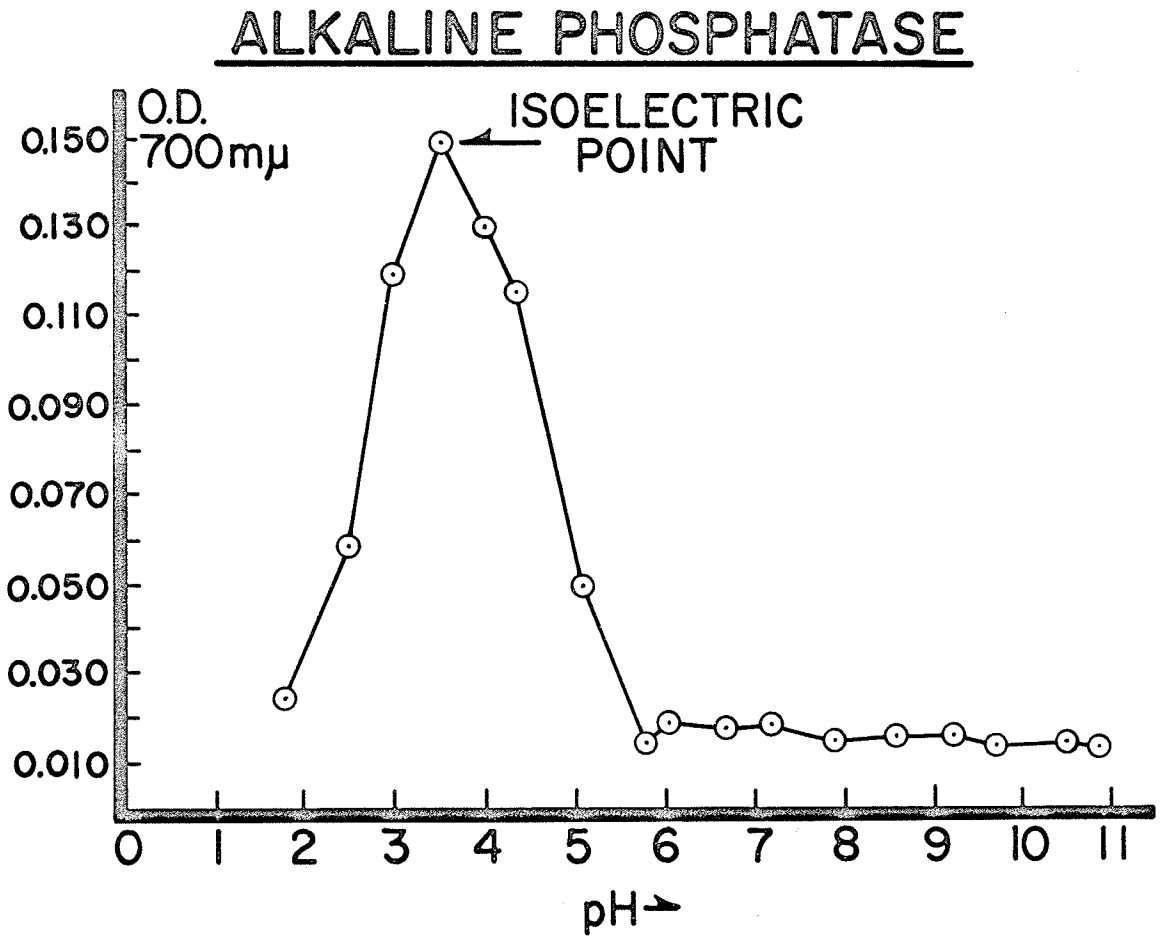


FIGURE V·11

Isoelectric point of alkaline phosphatase: Changes in optical density of alkaline phosphatase at 700 mu are plotted as a function of pH.

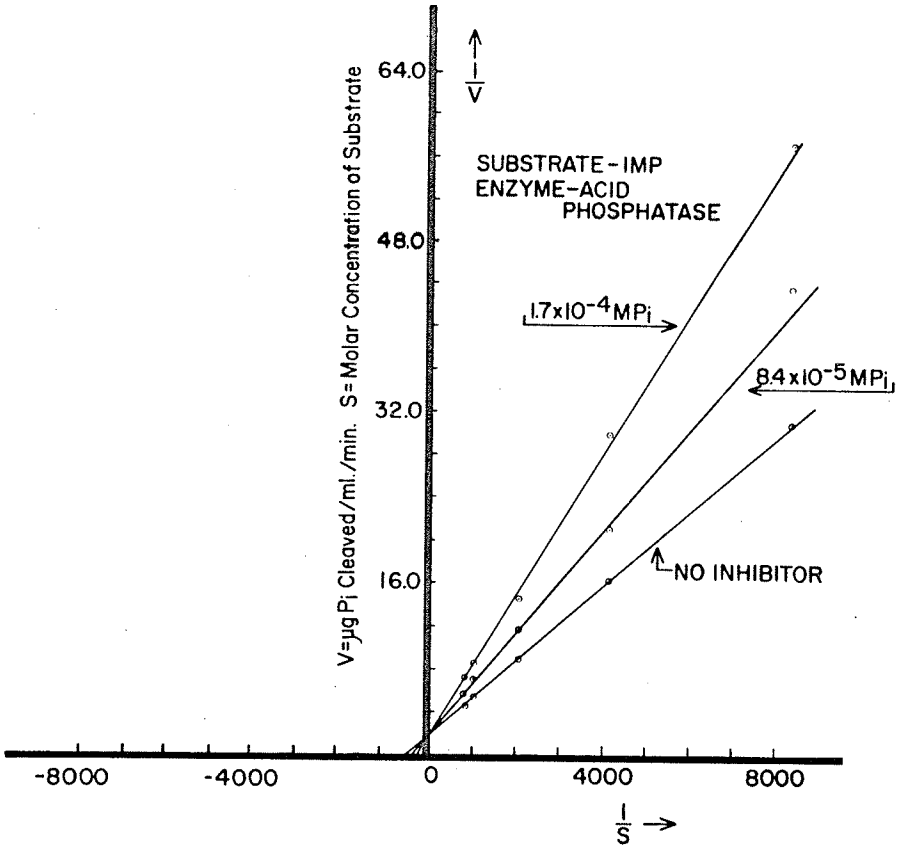


FIGURE V·12

Lineweaver-Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of the substrate concentration) for acid phosphatase at pH 5.0 with IMP both in the absence of P_i and in the presence of either 0.84×10^{-4} M P_i or 1.7×10^{-4} M P_i.

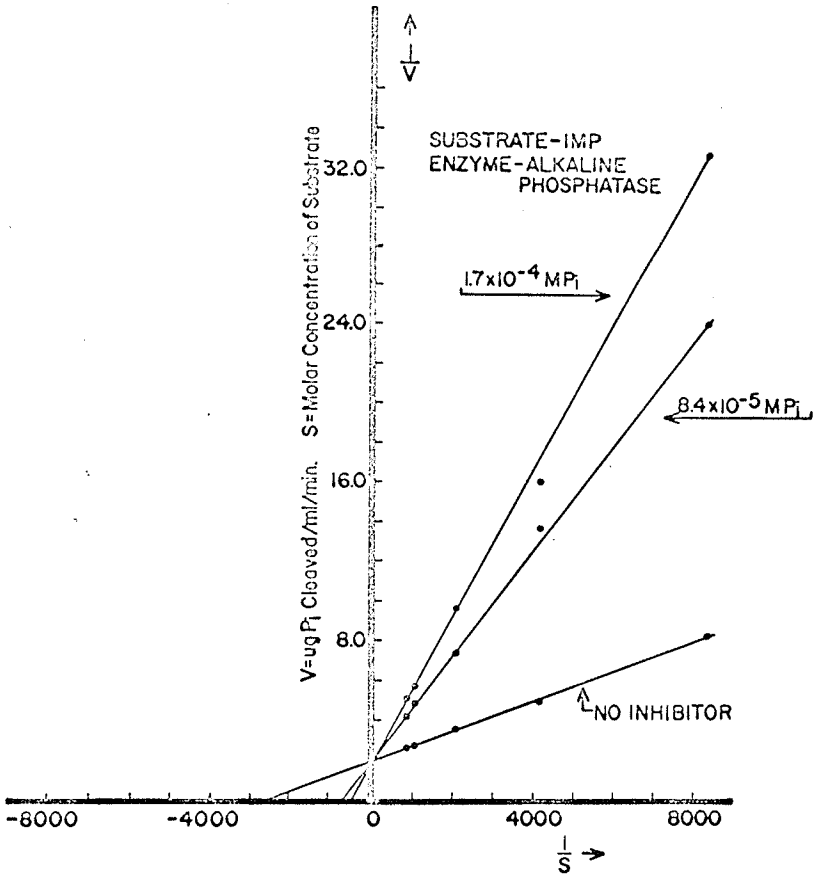


FIGURE V·13

Lineweaver-Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of the substrate concentration) for alkaline phosphatase with IMP at pH 9.0 both in the absence of P_i and in the presence of either 0.84×10^{-4} M P_i or 1.7×10^{-4} M P_i.

values for the two enzyme-substrate combinations, but did alter their Km values, competitive inhibition of the hydrolysis of IMP is indicated (Lineweaver and Burk, 1934).

Inorganic phosphate inhibited hydrolysis of all the other inositol phosphates and this is shown for phytic acid with different substrate concentrations in Figs. V·14 and V·15, and for a single substrate concentration and different P_i levels with IDP, ITriP, ITetraP, IP'P, and IHP in Figs. V·16 and V·17.

From Figs. V·14 and V·15, it is obvious that phytate does not show the same classical competitive inhibition with P_i that was shown with IMP. This does not necessarily mean that the inhibition with P_i was not the same as for IMP since with inositol phosphates having more phosphate groups per molecule than IMP, P_i inhibition involves more than one substrate. A second inositol phosphate, the hydrolysis of which can also be inhibited by P_i, forms as soon as hydrolysis of the initial inositol phosphate begins.

To permit comparison of the degree to which each inositol phosphate enzyme combination was inhibited by P_i, the slope of each of the lines in Figs. V·16 and V·17 ($\Delta\frac{1}{V}/\Delta I$) was determined and presented in Fig. V·18. The values for $\Delta\frac{1}{V}/\Delta I$ for IMP shown in Fig. V·18 were calculated from the data in Figs. V·12

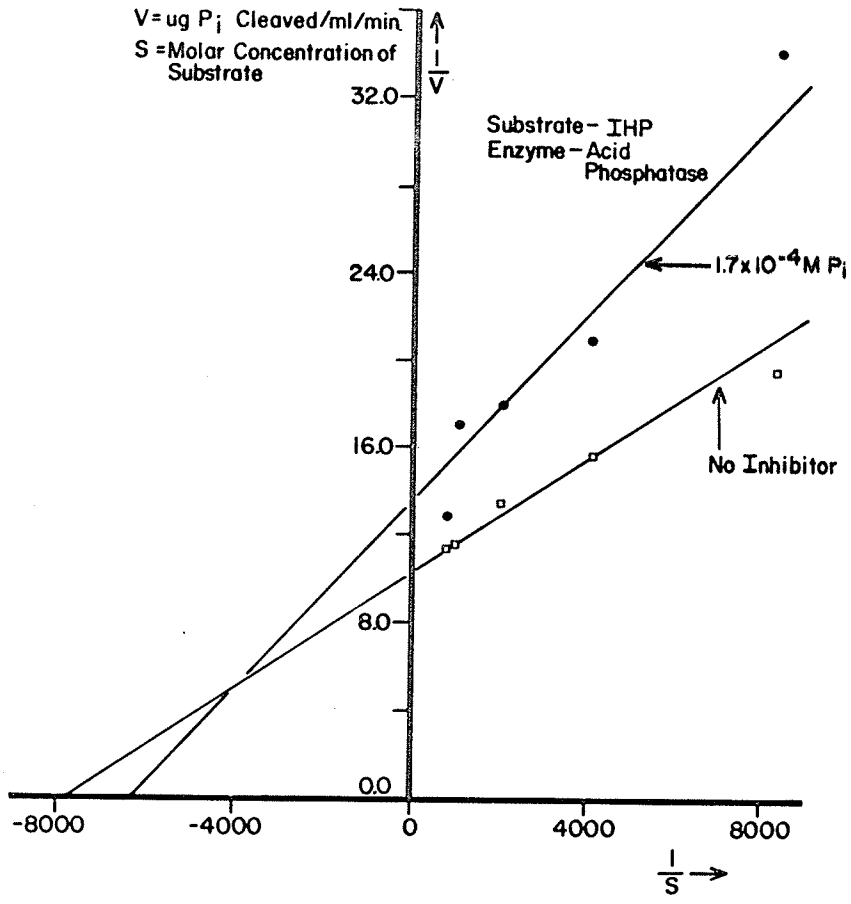


FIGURE V·14

Lineweaver-Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of the substrate concentration) for acid phosphatase at pH 5.0 with phytate both in the absence of P_i and in the presence of 1.7×10^{-4} M P_i.

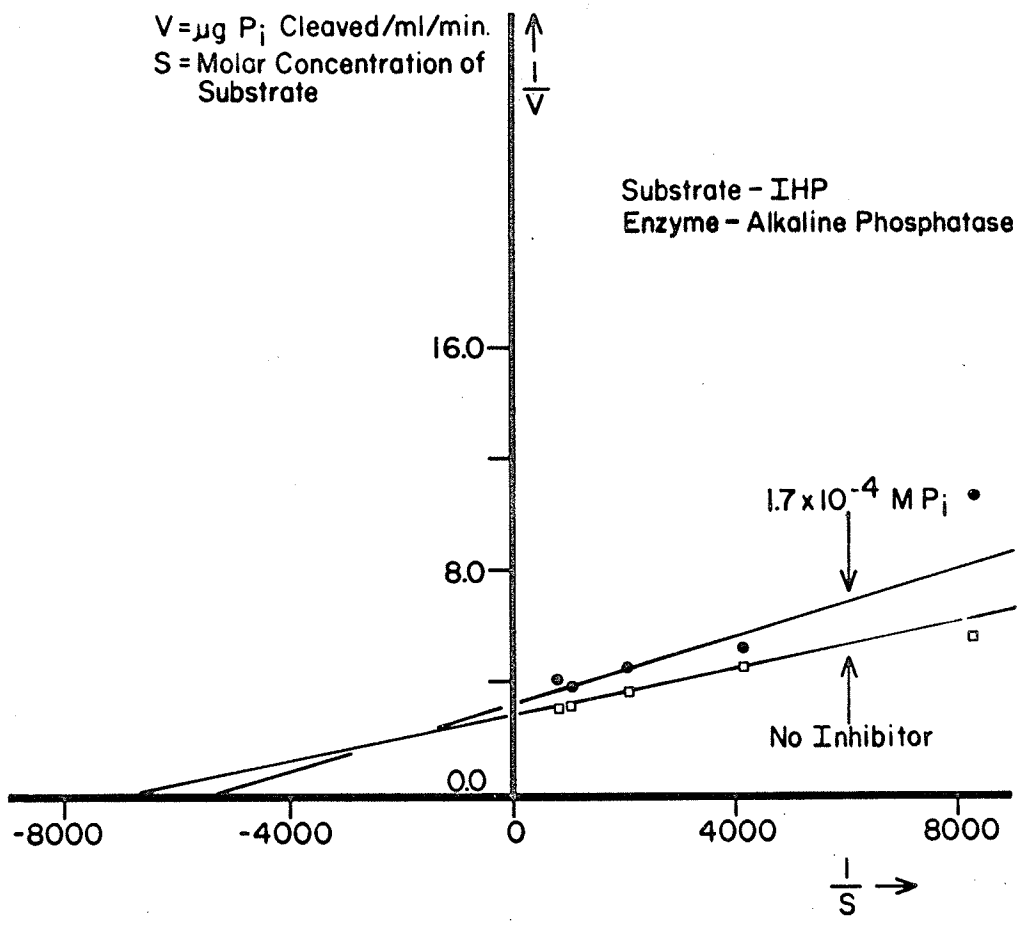


FIGURE V·15

Lineweaver-Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of the substrate concentration) for alkaline phosphatase at pH 9.0 with phytate both in the absence of P_i and in the presence of 1.7×10^{-4} M P_i.

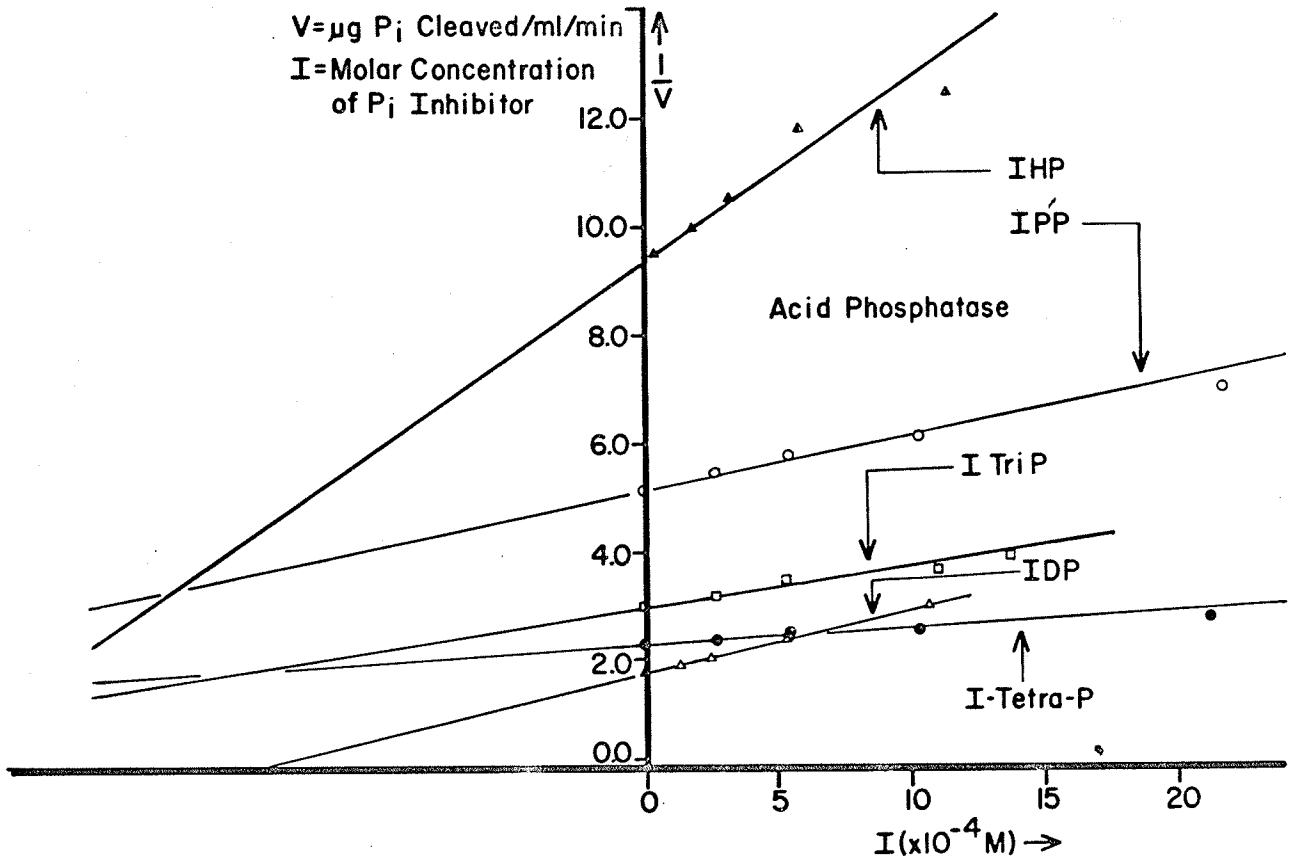


FIGURE V·16

Inhibition by orthophosphate of acid phosphatase activity with each of IDP, ITriP, ITetraP, IP'P, and IHP as substrate.

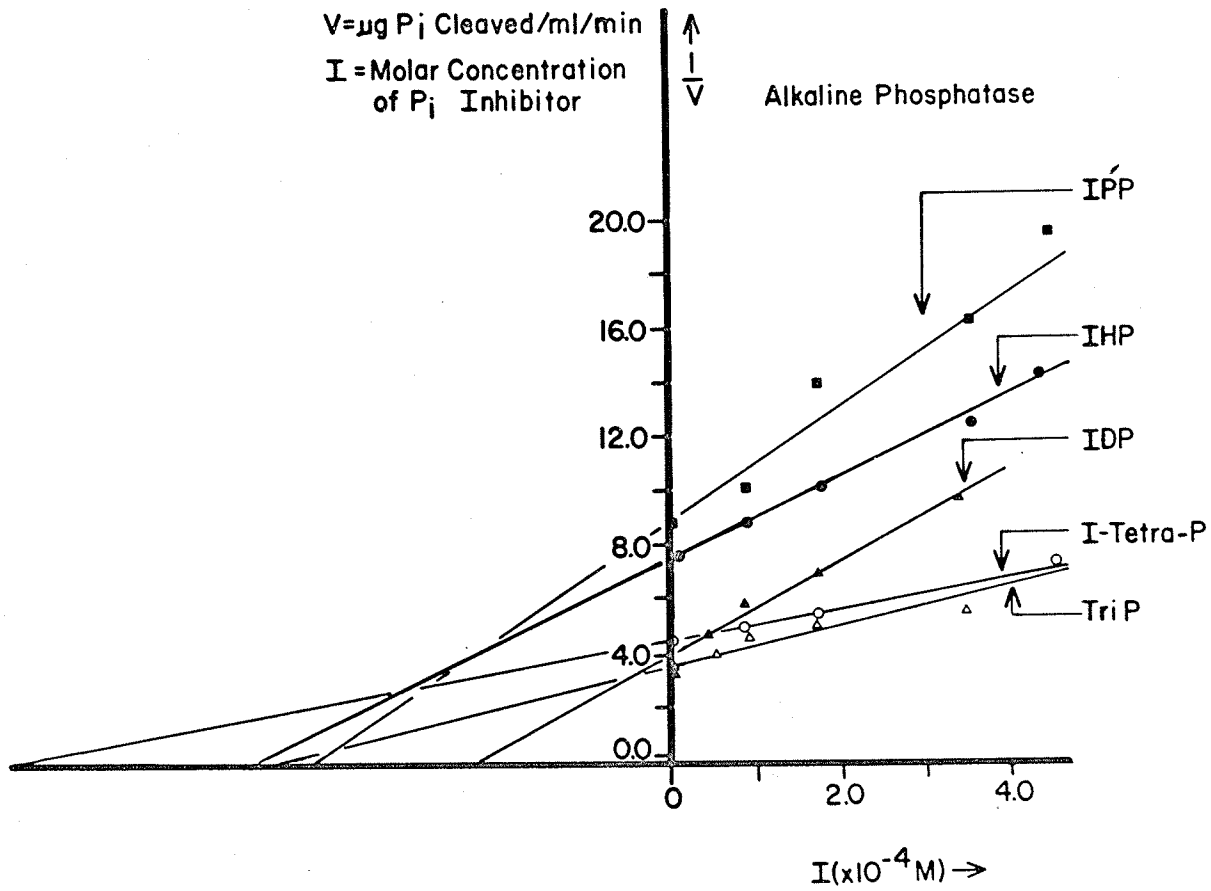


FIGURE V·17

Inhibition by orthophosphate of alkaline phosphatase activity with each of IDP, ITriP, ITetraP, IP'P, and IHP as substrate.

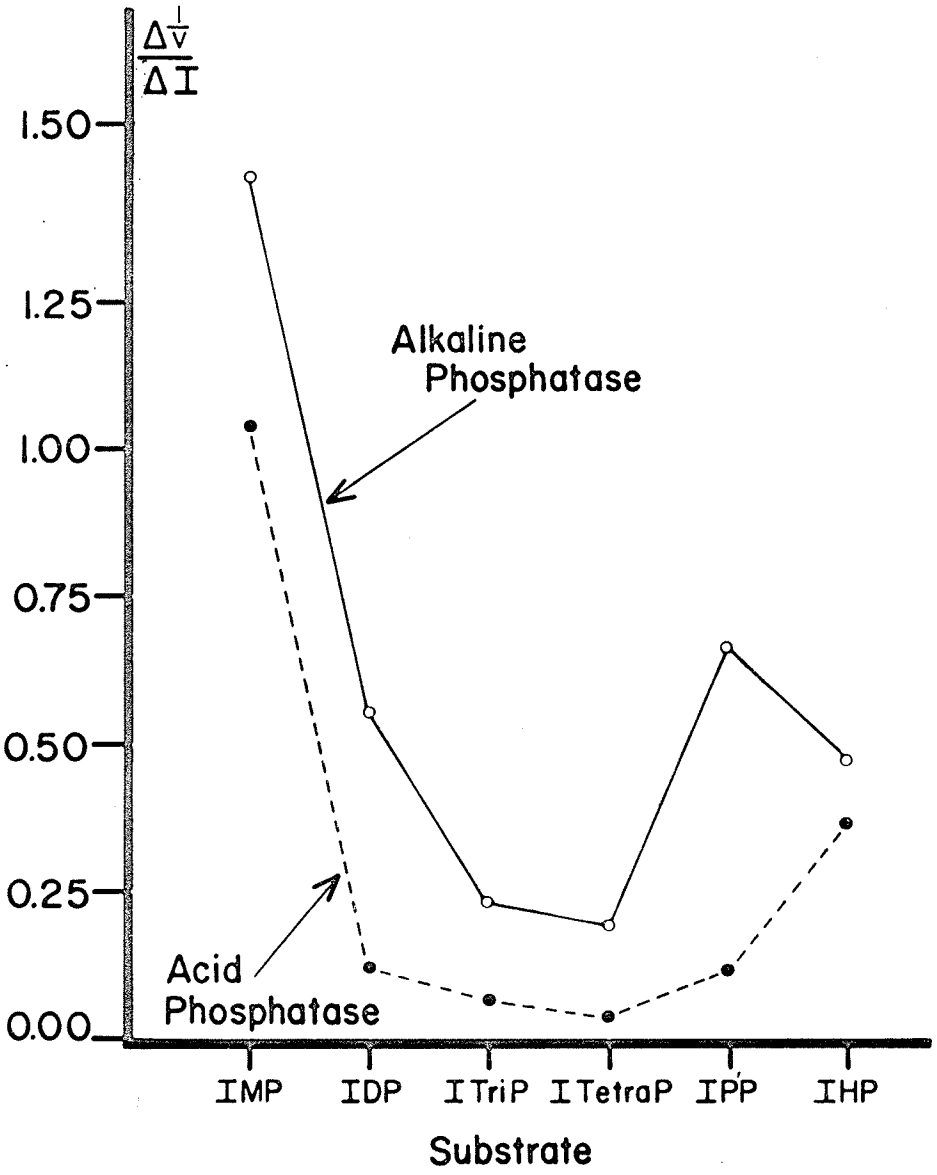


FIGURE V·18

Change in the reciprocal of the reaction velocity per unit change in inhibitor concentration ($\Delta\frac{1}{v}/\Delta I$) for each enzyme-substrate combination.

and V·13.

With each substrate, alkaline phosphatase was more sensitive to P_i inhibition than acid phosphatase. With both enzymes, the ITriP and ITetraP-enzyme combinations were least sensitive to P_i inhibition.

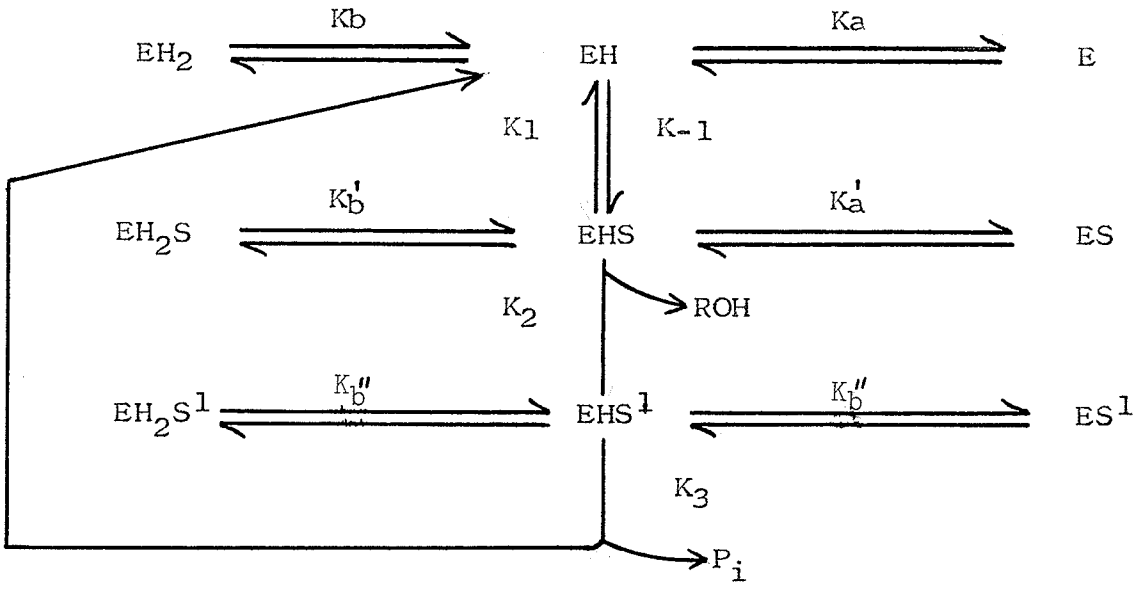
Discussion

Experiments in this chapter showed, as was shown earlier with other organic phosphate substrates (Folley and Kay, 1935; Ross et al, 1951; Morton, 1957; Motzok and Branian, 1961; Motzok, 1963; Dabich and Neuhaus, 1966), that the pH optimum for each of the various inositol phosphates with alkaline phosphatase increases with increasing substrate concentration. Shift in the pH optimum of an enzyme with change in the substrate concentration has been attributed by Krupka and Laidler (1960) and Laidler (1963) to alteration in the ionization of the charged groups at the active site of the enzyme in response to change in pH. According to Laidler (1963), the active centre of the enzyme contains two such groups, one more acidic than the other (i.e. different pK's) so that three states of ionization can exist upon change in pH. These states can be represented as EH_2, EH, and E, where EH_2 is the most acidic and E the most basic form of the active centre of the enzyme.

When a substrate is hydrolyzed, the active centre is in

the EH form and a simultaneous nucleophilic attack by the basic group (i.e. the one deficient in hydrogen) upon and a proton transfer from the acidic group to the substrate occurs. This dual change between enzyme and substrate is referred to by Laidler as the "push-pull" mechanism for enzyme catalysis.

According to a general theory proposed by Krupka and Laidler (1960) to account for the influence of pH on enzyme reaction rates, the effect of pH on the hydrolysis of an organic phosphate by alkaline phosphatase can be explained as follows;



where EH_2, EH, and E represent free enzyme with the active centre in each of its three ionized forms, EH_2S, EHS, and ES the corresponding enzyme-substrate intermediates, and EH_2S^1,

EHS^1, and ES^1 the corresponding enzyme-P_i intermediates; ROH and P_i are the products of the hydrolysis. The K values are the rate constants for the various reactions.

With increase in pH, ionization and therefore the forms to the right of the scheme would be favoured. With increase in substrate concentration, the formation of EHS would be favoured. Formation of EH_2S and ES can be neglected since, as pointed out above, only enzyme in the EH form is capable of catalyzing the hydrolysis of the substrate.

The rate of formation of the products ROH and P_i will depend upon the level of EHS which is dependent upon both pH and substrate concentration. Consequently, the pH resulting in the maximal level of EHS will be the pH optimum.

EHS, in addition to forming products, can, depending upon the rate constants involved, (i) release hydrogen ion to produce ES or (ii) accept a proton to form EH_2S. With increase in substrate concentration, not only would more products be formed but also more ES and/or EH_2S.

If the formation of ES over EH_2S is favoured hydrogen ions will be released from the active centre, so that to return to the previous optimal state of the active centre, the pH must be raised. Shift in the pH optimum to higher levels in response to increase in substrate concentration was observed

for alkaline phosphatase in the present and in earlier studies and can be explained in this way. If the fomation of EH_2S over ES is favoured the reverse occurs and shift in the pH optimum with increase in substrate concentration would be to lower pH levels. If there is no formation of ES and EH_2S or their formation is equal, no shift in the pH optimum would occur with alteration in substrate concentration as was observed with acid phosphatase in the present study.

With alkaline phosphatase the pH at which the enzyme is active are above the isoelectric point which is 3.5, and therefore enzyme and substrate will each be negatively charged. Charge repulsion between the whole enzyme molecule and substrate would make it difficult for the substrate to approach the active centre of the enzyme, which would become more difficult with increase in pH, since enzyme and substrate would both become more negatively charged. On the other hand, with acid phosphatase the isoelectric point was 5.8, which is above the pH levels at which this enzyme is active so that attraction rather than repulsion between this enzyme and its substrates would occur.

Repulsion between the whole alkaline phosphatase molecule and P_i has been demonstrated in the experiments in Chapter VI, although near the active centre, the presence of positively

charged groups such as zinc may facilitate attraction between the enzyme molecule and its substrates in this region (Schwartz, 1963).

Charge repulsion between enzyme and substrate might be less with increase in substrate concentration since substrate molecules would be present as larger aggregates (Knapp, 1922). Such aggregation might reduce the facility with which the attachment of individual substrate molecules to the active site can take place, but by increasing the pH and thus increasing the ionization of the substrate molecules, such aggregation could be offset.

The Km, which is a combined rate constant reflecting the rate at which a molecule of substrate is broken down by an enzyme, although higher in value for inositol phosphates with smaller P/I ratios, was similar for each of ITriP, ITetraP, IP'P and IHP with both acid and alkaline phosphatases. However, for IMP and IDP, Km values were considerably higher with the acid than with the alkaline phosphatase enzyme; with the alkaline enzyme, Km values were slightly higher than for the inositol phosphates with greater P/I ratios (Fig. V·6 and V·8).

When Km values were calculated on a molar basis with respect to phosphate, the Km values for ITriP, ITetraP, IP'P,

and IHP were still quite close, and those for IMP and IDP were still very different. Km values for acid phosphatase still decreased as the P/I ratios increased, whereas with alkaline phosphatase the order was reversed (Fig. V·7).

These findings indicate that inositol phosphate molecules, in which the P/I ratios are between 3 and 6, will all be hydrolyzed at approximately the same rate at the active sites on both acid and alkaline phosphatases. IMP and IDP will be hydrolyzed at different rates reflecting differences in characteristics between the two enzymes since glycerophosphate shows the same relationship. Failure to show this difference in activity between the two enzymes with the higher inositol phosphate derivatives may be the result of resistance to hydrolysis with alkaline phosphatase of phosphate groups linked by hydrogen bonds (see Chapter IV).

The maximum rate of an enzyme-catalyzed reaction (Vmax) is a reflection of the maximum association between the substrate and the active site of an enzyme. The number of sites and, as a result, Vmax can be increased by increasing the concentration of enzyme.

The results in the present study suggest that at constant enzyme concentration, the number of active sites available to the different inositol phosphates are different, since the

Vmax values of these inositol phosphates at constant enzyme concentration were different (Fig. V·9). IDP and ITriP showed the largest Vmax values with both acid and alkaline phosphatases indicating that the active sites are more accessible to these compounds than to the other inositol phosphates.

Since the rate of release of product in an enzyme-catalyzed reaction is dependent upon both the rate of turnover of a molecule at an enzyme site (reflected by the Km) and the number of sites available for that molecule (reflected by the Vmax), of the various inositol phosphates examined in the present study, IDP and IMP would be hydrolyzed at the most rapid rate.

P_i competitively inhibits the hydrolysis of many organic phosphates by acid and alkaline phosphatases (Gezeluis and Wright, 1965; Lazdunski and Ouellet, 1962; Garen and Levinthal, 1960; Shaw, 1966; Scott, 1966; Burstone, 1962). In the present study, IMP hydrolysis was competitively inhibited by P_i when either acid or alkaline phosphatase was used, but with the other inositol phosphates this type of inhibition could not be demonstrated since one is dealing with substrates that upon hydrolysis form another inositol phosphate which in turn can be hydrolyzed. However, since P_i inhibition is inhibition of

the enzyme, it is quite probable that this type of inhibition also occurs with these substrates.

With each inositol phosphate, alkaline phosphatase was more sensitive to P_i inhibition than acid phosphatase and with both enzymes, ITriP and ITetraP hydrolysis was least affected by P_i.

The Km, Vmax, and P_i inhibition data taken together suggest that IDP, ITriP, and ITetraP of the various inositol phosphates would on balance yield the most phosphate upon hydrolysis by acid and alkaline phosphatases.

In a sequential hydrolysis of phytate by these enzymes, complete depletion of P_i would be resisted, since IMP, the terminal inositol phosphate in such a hydrolysis, is quite sensitive to P_i inhibition (Figs. V·12 and V·13), and is comparatively slowly hydrolyzed because of its low Vmax and high Km values (Figs. V·6 and V·9).

CHAPTER VI

THE USE OF A GEL FILTRATION PROCEDURE TO STUDY THE INTERACTION BETWEEN INORGANIC PHOSPHATE AND ALKALINE PHOSPHATASE

Introduction

In Chapter V it was observed that inorganic phosphate inhibited the hydrolysis of inositol phosphates by acid and alkaline phosphatases and that this inhibition varied with the type of inositol phosphate used. Since P_i inhibits such hydrolysis by interacting with these enzymes to inhibit their activity, in the present chapter experiments were carried out to examine some of the variables that might be involved in the interaction between P_i and alkaline phosphatase.

The variables examined were (i) pH, (ii) ionic strength, (iii) enzyme concentration, and (iv) P_i concentration.

The interaction was examined by a similar gel filtration technique to that used by Hummel and Dryer (1962) to determine the association between the substrate, cytidylic acid, and the enzyme, ribonuclease. In this technique, a chromatographic column containing sephadex (G-25) or a similar resin, is initially equilibrated with a solution containing a substrate. An aliquot of this solution, containing a given amount of enzyme, is then added to the column and eluted with the substrate

solution. The eluate is monitored for both enzyme and substrate and if the concentration of substrate in the eluate does not change then no interaction between enzyme and substrate has occurred. If it has changed, then interaction has taken place and may, as shown by Hummel and Dryer in their experiment with cytidylic acid and ribonuclease, be used to determine the number of moles of substrate bound per mole of enzyme. In the present study, P_i replaced the substrate.

An attempt was also made to examine by this gel filtration technique the interaction between phytate and alkaline phosphatase, but this failed because even at temperatures close to 0^oC this substrate is hydrolyzed by alkaline phosphatase.

However, in a series of experiments carried out to examine the behaviour of P_i alone and in the presence of enzyme on columns not previously equilibrated with solutions of P_i, the behaviour of phytate alone was also examined.

Experiments were also carried out to determine whether the small amount of P_i, which could not be removed during the purification of alkaline phosphatase, was involved in the interaction between Pi and alkaline phosphatase.

Methods

The gel filtration experiments carried out in the present study were carried out at 4^oC using glass columns containing

Bio-Gel P-4 (50-150 mesh) and using either distilled water or aqueous solution of NaCl, P_i, or both as eluents. Fractions from these columns were collected in 10 x 75 mm pyrex test tubes and monitored where applicable (see individual sections) for alkaline phosphatase (by O.D. measurements at 280 mμ), P_i, phytate-P and ^{32}P.

1) Experiments to Determine the Elution Characteristics of Inorganic Phosphate and Phytate on Bio-Gel P-4 with (i) Distilled Water and (ii) 0.1 M NaCl as Eluent

To study the elution characteristics of different amounts of P_i on Bio-Gel P-4, not equilibrated with P_i solution, 3 ml of either a 1.4, 5.2, 10.0 or 54.0 μg/ml sodium phosphate solution (pH 7.0) was put onto a column of Bio-Gel P-4 (0.8 x 60 cm), previously equilibrated with distilled water, and the P_i eluted with the distilled water. Fractions were collected and analyzed for inorganic phosphate.

A similar series of experiments was carried out in which the P_i samples were made up in a solution of 0.1 M NaCl which also served as eluent.

To compare the elution characteristics of phytate to that of P_i, similar experiments were carried out in which Na phytate, pH 7.0 replaced the Na phosphate, pH 7.0. The only difference was that the Na phytate concentrations used were either 10, 25

or 100 μgP/ml, which on a molar basis are within the range studied with sodium phosphate.

2) Experiments to Determine the Elution Characteristics on Bio-Gel P-4 of P_i and Alkaline Phosphatase when (i) Distilled Water and (ii) 0.1 M NaCl was Used as Eluent

Alkaline phosphatase solutions containing the same P_i concentrations as in section (i) were prepared, and 3 ml aliquots of each solution, were, in separate experiments, put on columns containing Bio-Gel and eluted with either water or NaCl as before. Eluate fractions were monitored for the enzyme at 280 mμ and also analyzed for P_i.

The protein concentrations used in these experiments were 0.67 mg/ml when water was used as eluent and 0.54 mg/ml when NaCl was used as eluent.

3) Experiments to Determine the Factors Affecting the Elution Characteristics of P_i and Alkaline Phosphatase on Bio-Gel P-4 Equilibrated with P_i Solutions of Different Concentrations which Also Served as Eluents

The effect of pH in the presence and absence of sodium chloride, P_i concentration, and concentration of alkaline phosphatase on the elution of P_i and alkaline phosphatase from Bio-Gel P-4, equilibrated with solutions containing P_i, were examined as follows.

In each experiment, a column (0.8 x 35 cm) containing Bio-Gel P-4 was equilibrated with one level of P_i. One ml of enzyme solution containing the same concentration of P_i as that with which the column was equilibrated, was put onto the column and eluted also with the same solution of P_i that was used to equilibrate the column. The eluate was monitored for P_i and for enzyme as in earlier experiments.

i) Effect of pH in the Presence and Absence of NaCl

To determine the effect of pH in the presence and absence of NaCl, the column was equilibrated with a 3 µg/ml solution of P_i at either pH 2.0, 5.0, 7.0, or 10.0 and the same P_i solution was used as eluent. The enzyme concentration was constant (0.12 mg/ml) and the samples were at the same pH and contained P_i at the same concentration as in the eluent. To determine the effect of NaCl, a parallel series of experiments was carried out in which the eluents and samples were prepared in 0.1 M NaCl.

ii) Effect of Enzyme Concentration

In these experiments, the pH of the samples and eluent was kept constant at 7.0, the P_i concentration was again 3 µg/ml, and the enzyme concentration in the samples was either 0.07, 0.13, 0.19, 0.26, or 0.65 mg/ml.

iii) Effect of P_i Concentration

In these experiments, the pH of the samples and eluent was again 7.0, the enzyme concentration was 0.13 mg/ml, and the P_i concentration in the eluent and in the samples was varied between 0.8 and 10.4 μg/ml.

iv) Use of ^{32}P to Determine where Phosphate is Eluted Relative to Alkaline Phosphatase in Equilibration Type Experiments

To determine whether phosphate is eluted in these equilibration type of experiments in the same position relative to the enzyme as in the experiments in section (2) of this chapter, the following experiments were carried out.

A Bio-Gel P-4 column was equilibrated with P_i at a concentration of 3 μg/ml and one ml of enzyme, equilibrated with the same P_i solution (pH 7.0), added as before.

0.4 μC of ^{32}P was added either to the one ml of eluent (i) following the enzyme (ii) containing the enzyme, (iii) immediately preceding the enzyme, and (iv) 2 ml ahead of the enzyme. The enzyme concentration in all experiments was 0.14 mg/ml. The eluent was monitored for enzyme, P_i and ^{32}P.

4) Studies Concerned with the Residual P_i in the "Purified" Alkaline Phosphatase Preparation Used in the Experiments in this Thesis

3 ml of the enzyme (1.18 mg/ml) was equilibrated with 0.4 μC ^{32}P and passed through a 0.8 x 60 cm Bio-Gel P-4 column to determine whether ^{32}P could exchange with the small amount of P_i bound to alkaline phosphatase, and eluate fractions monitored for enzyme, P_i, and ^{32}P. Presence of ^{32}P in the P_i associated with the enzyme would indicate that exchange had taken place.

Because low levels of P_i are eluted with a Ve/Vo ratio slightly greater than the enzyme, which is eluted in the void volume, the above experiment was repeated using alkaline phosphatase containing 25 μg P_i/ml as carrier. This higher level results in a greater separation of P_i and enzyme.

This binding of P_i by enzyme was studied in another type of experiment in which 0.4 μC of ^{32}P was incubated at 4°C with 500 μl of alkaline phosphatase for 15 minutes (cf. Schwartz and Lipmann, 1961), the mixture dialyzed against distilled water and the dialyzing solution monitored for ^{32}P. When the distilled water was free of radioactivity, an aliquot of the contents of the dialysis tubing (Visking, $\frac{1}{4}$ inch) was transferred to an aluminum planchet and counted for ^{32}P activity.

Results

(1) Elution Characteristics of Inorganic Phosphate and Phytate on Bio-Gel P-4 with (i) Distilled Water and (ii) 0.1

M NaCl as Eluent

The results of the elution of P_i and Na phytate from Bio-Gel P-4 using distilled water and NaCl as eluents are shown in Figs. VI·1 and VI·2. Alkaline phosphatase is shown on these graphs to mark the void volume. This was determined separately. At low P_i concentration and in the absence of NaCl, P_i was eluted as a symmetrical peak and with a Ve/Vo ratio slightly greater than 1. With increasing P_i concentration the curves became progressively more asymmetrical. Increased skewing to the left and decreased skewing to the right was observed. Also, with increasing P_i concentration, elution of P_i occurred at higher Ve/Vo ratios.

In the presence of NaCl, all elution curves were approximately symmetrical. The P_i, regardless of concentration, was eluted in the same position and at a higher Ve/Vo ratio than in the absence of NaCl.

Na phytate, at low and high concentration, and in the presence and in the absence of NaCl, was eluted essentially as a symmetrical peak (Fig. VI·2). As with P_i in the presence of NaCl, elution of Na phytate occurred at higher Ve/Vo ratios but was not as high as for P_i.

(2) Elution Characteristics on Bio-Gel P-4 of P_i and Alkaline Phosphatase when (i) Distilled Water and (ii) 0.1 M

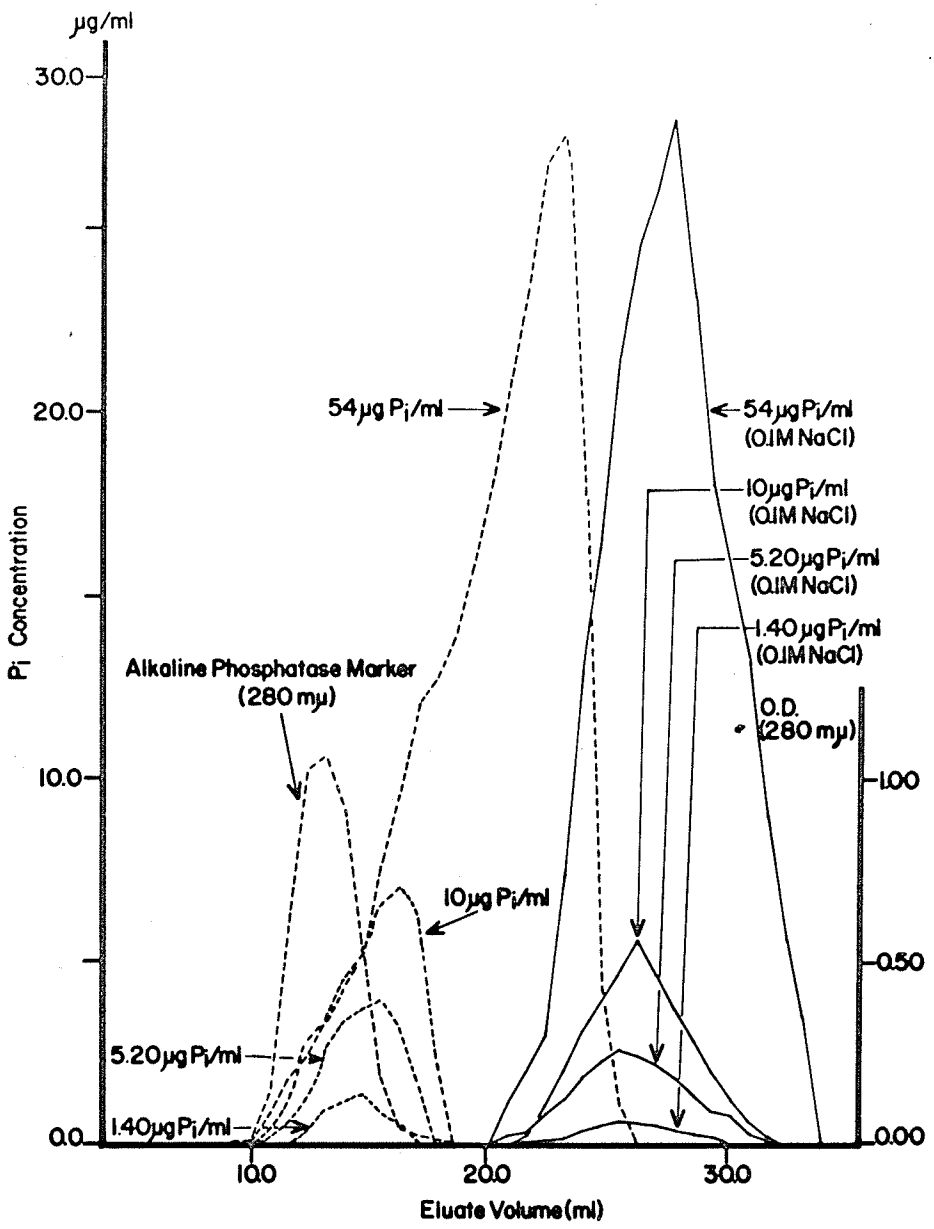


FIGURE VI·1

The elution pattern obtained on gel filtration on a Bio-Gel P-4 column (equilibrated with distilled water or 0.1 M NaCl) of different concentrations of P_i using distilled water or 0.1 M NaCl respectively as eluent. Eluted fractions were analyzed for P_i. The position of elution of alkaline phosphatase is also shown to mark the void volume.

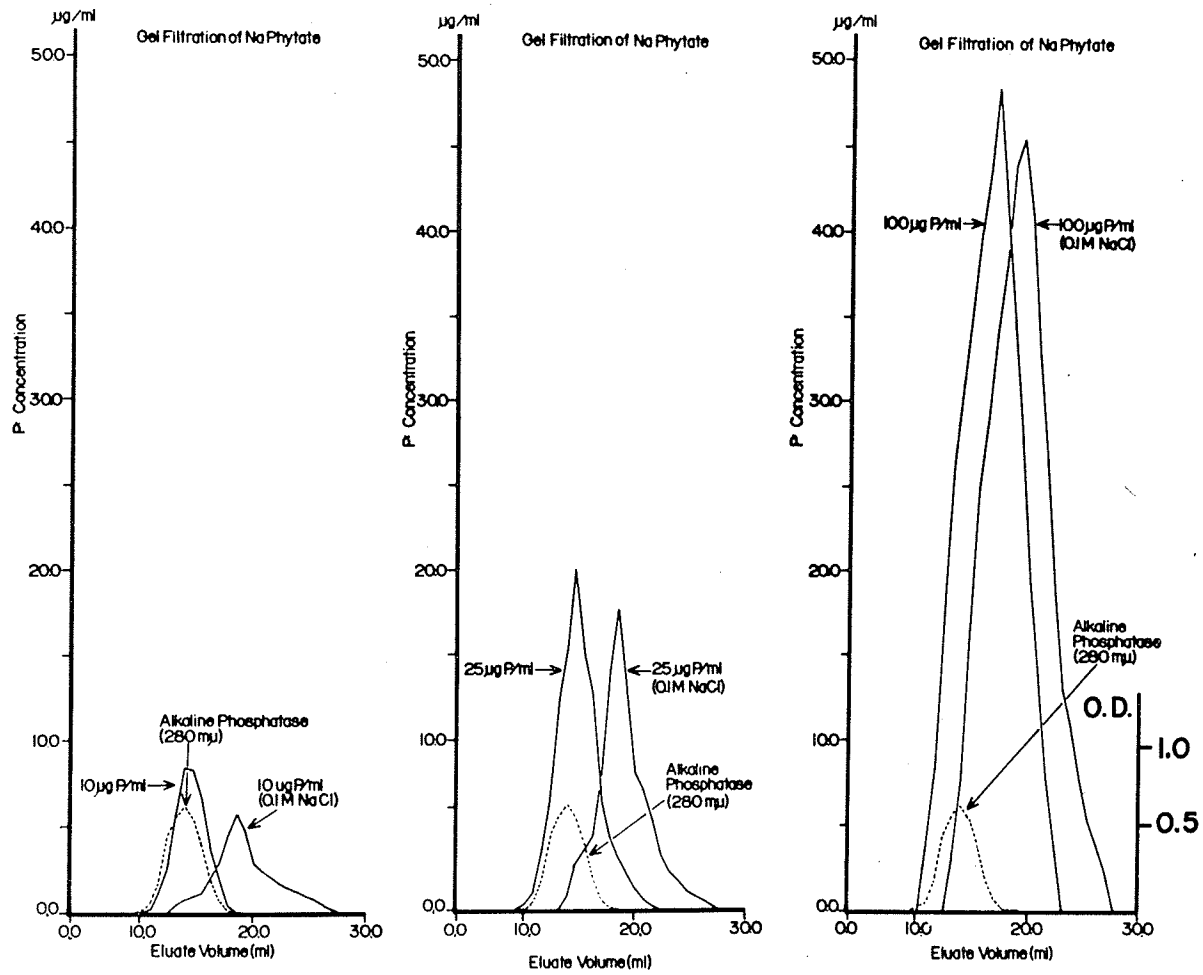


FIGURE VI·2

The elution pattern obtained on gel filtration on a Bio-Gel P-4 column (equilibrated with distilled water or 0.1 M NaCl) of different concentrations of Na phytate using either distilled water or 0.1 M NaCl respectively as eluent. Eluted fractions were analyzed for organic P. The position of elution of alkaline phosphatase is also shown to mark the void volume.

NaCl was Used as Eluent

When P_i was eluted together with alkaline phosphatase from Bio-Gel P-4 in the absence of NaCl (Fig. VI·3), the shapes of the P_i elution curves were similar to those for P_i alone except that they were displaced to the right.

When NaCl was used as eluent (Fig. VI·4) the curves were similar in shape and location to those curves obtained in the absence of the enzyme (cf. Fig. VI·4 to Fig. VI·1).

(3) Factors Affecting the Elution Characteristics of P_i and Alkaline Phosphatase on Bio-Gel P-4 Equilibrated with P_i Solution of Different Concentrations which Also Served as Eluents

i) Effect of pH in the Presence and Absence of NaCl

The effect of pH in the presence and absence of NaCl on Bio-Gel P-4 equilibrated with a constant level of P_i is shown in Figs. V·5 and V·6.

At pH-2.0, the level of P_i in the eluate did not deviate from 3.0 µg/ml both in the presence and absence of NaCl.

At pH-5.0, there was a decrease in P_i concentration corresponding to the location of the enzyme. This decrease was followed by a subsequent increase in P_i concentration. This effect was opposite to that shown by Hummel and Dryer (1962) and was indicative of repulsion rather than binding (see dis-

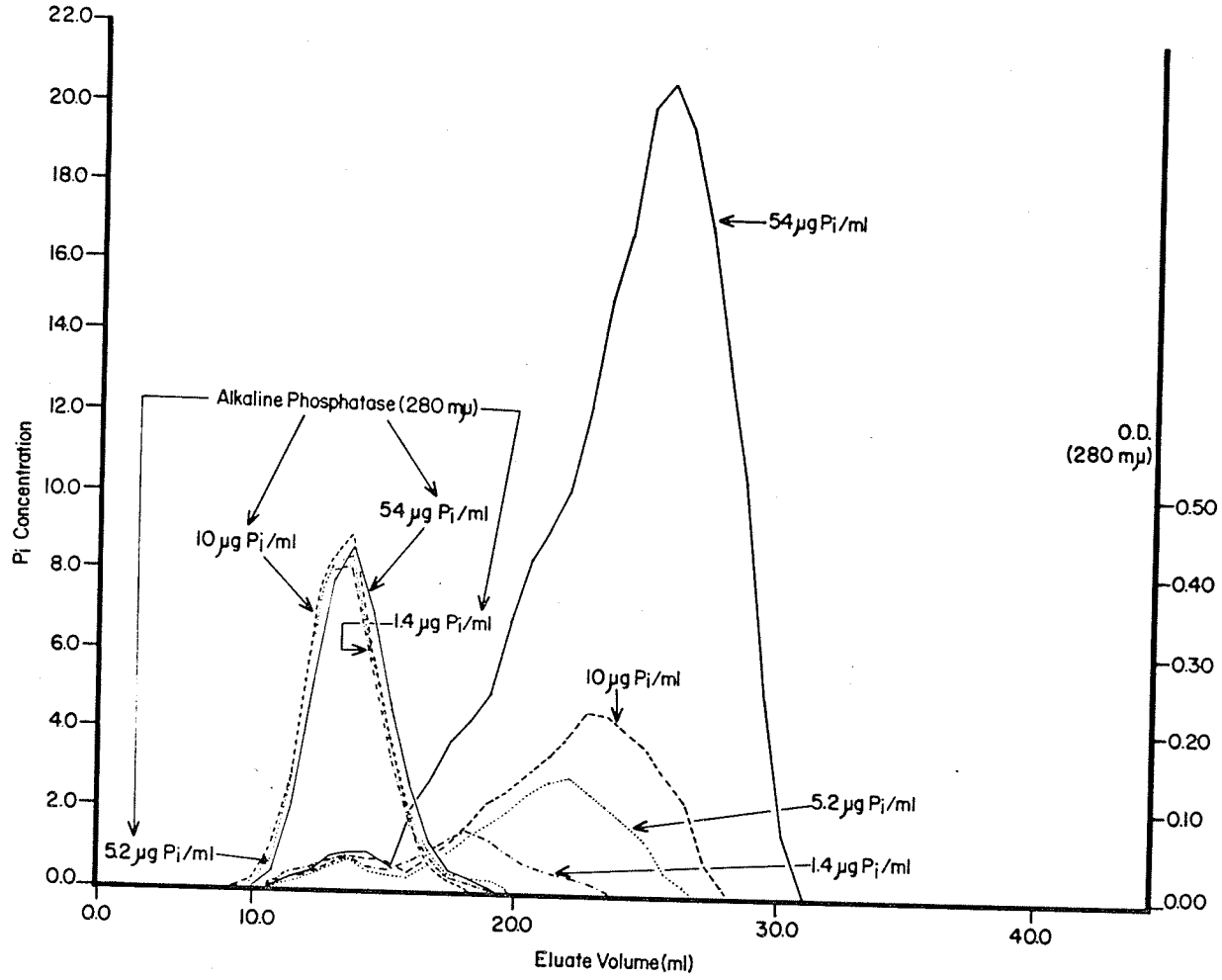


FIGURE VI·3

The elution pattern obtained on gel filtration on a Bio-Gel P-4 column (equilibrated with distilled water) of different concentrations of P_i mixed with a fixed concentration of alkaline phosphatase using distilled water as eluent. Fractions eluted were assayed for P_i and monitored at 280 mμ for enzyme.

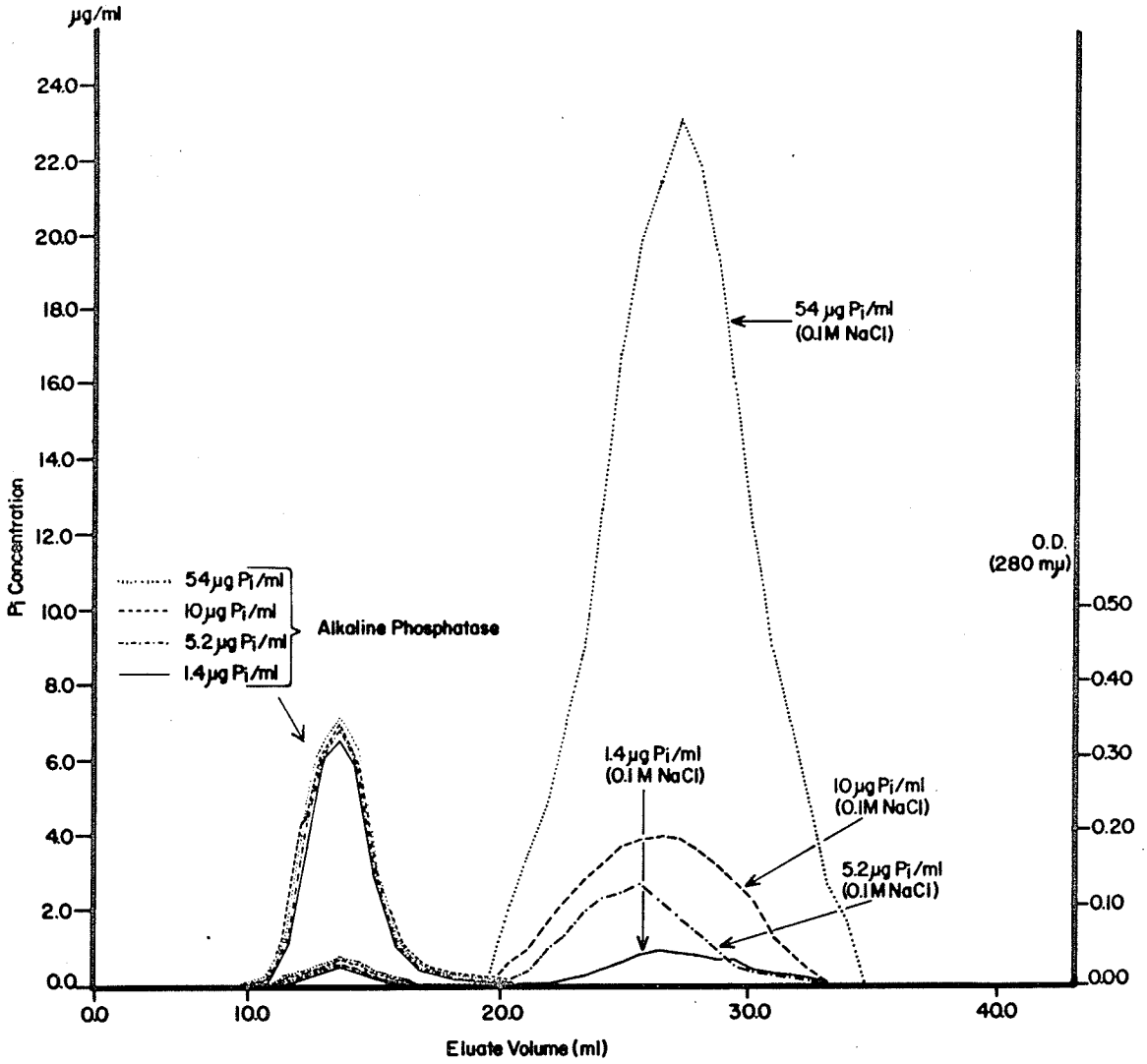


FIGURE VI·4

The elution pattern obtained on gel filtration on a Bio-Gel P-4 column (equilibrated with 0.1 M NaCl) of different concentrations of P_i mixed with a fixed concentration of alkaline phosphatase using 0.1 M NaCl as eluent. Fractions eluted were assayed for P_i and monitored at 280 mµ for enzyme.

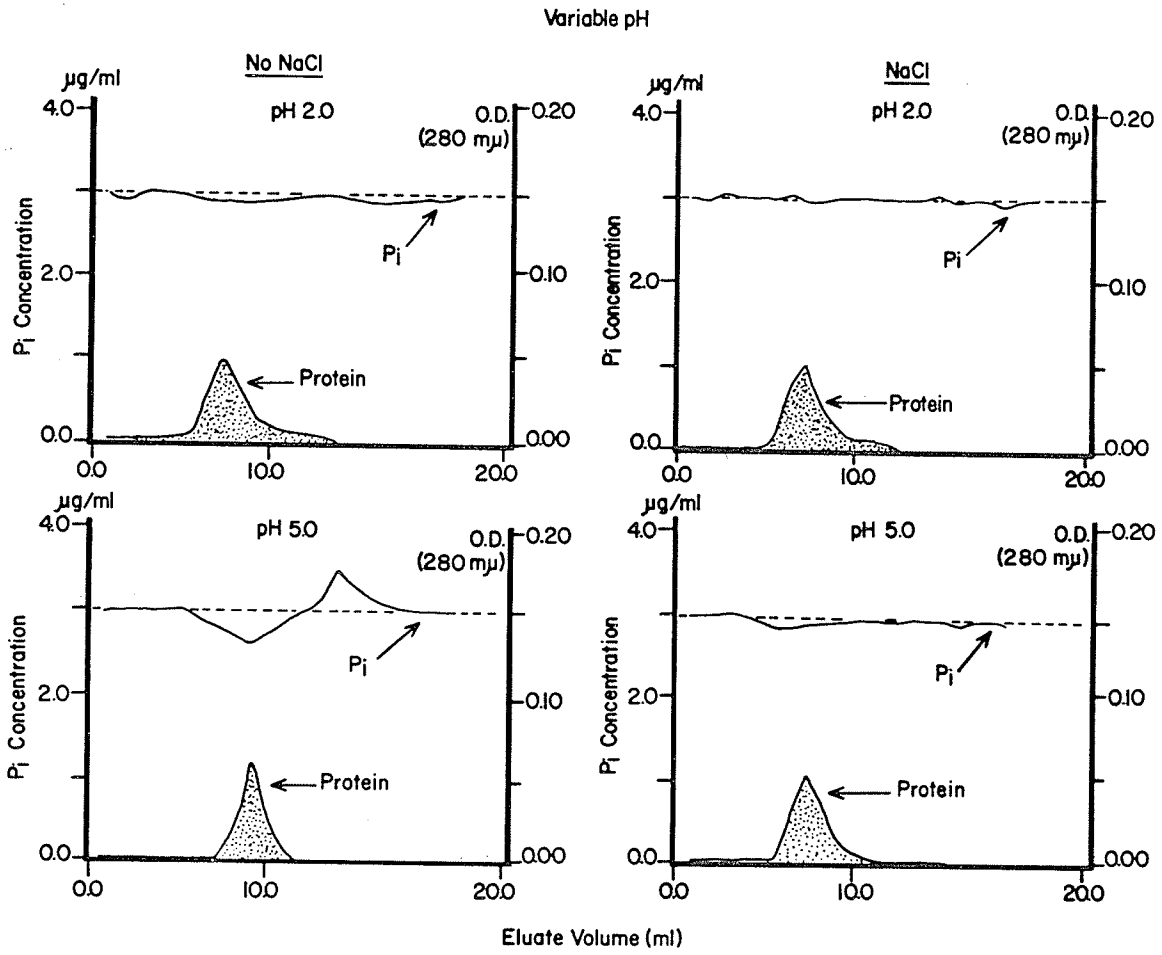


FIGURE VI·5

Effect of pH on the elution characteristics of P_i and alkaline phosphatase on Bio-Gel P-4 equilibrated with 3 μg P_i/ml or 3 μg P_i/ml in 0.1 M NaCl using either 3 μg P_i/ml or 3 μg P_i/ml in 0.1 M NaCl as eluent. Fractions eluted were monitored for both enzyme and P_i.

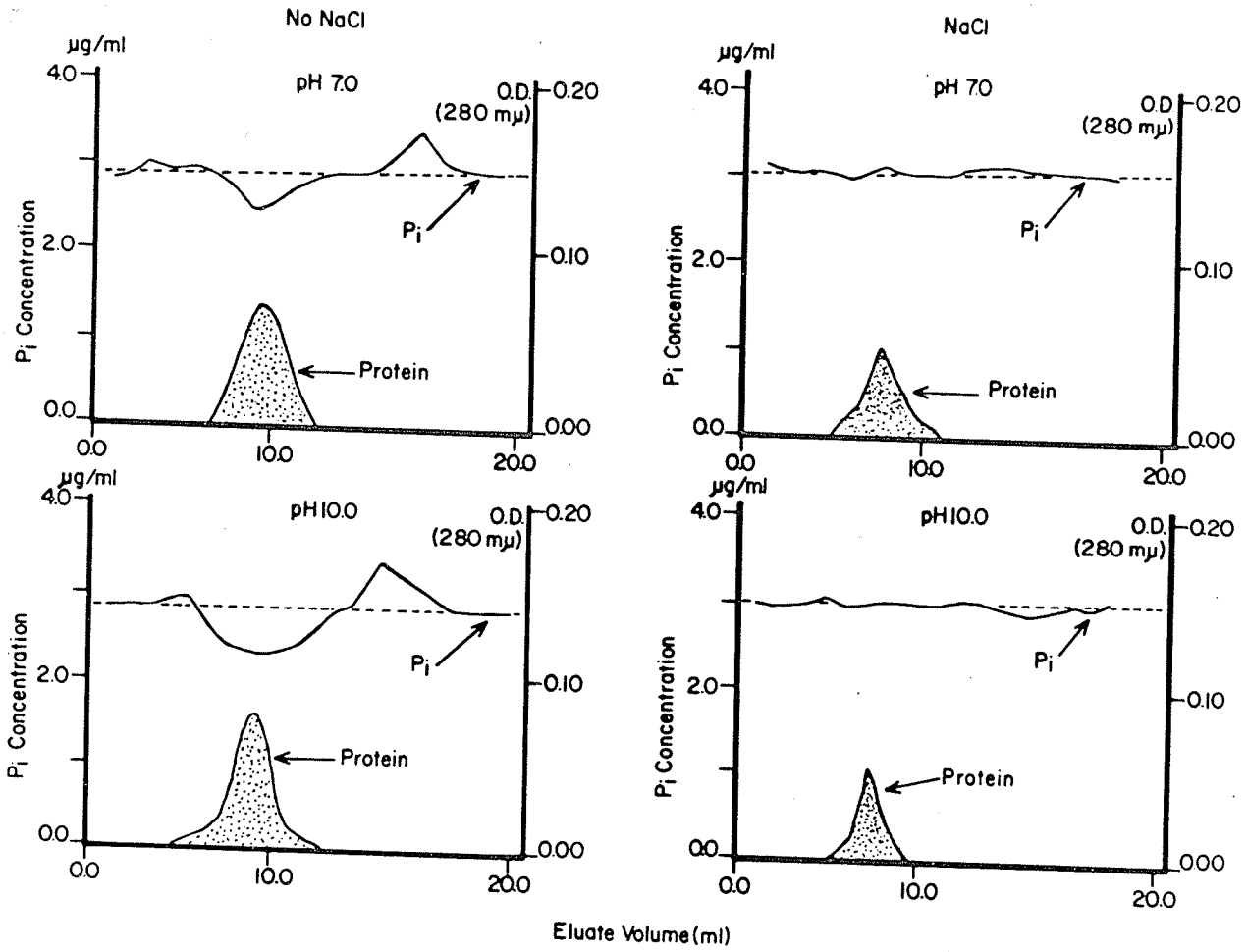


FIGURE VI·6

Effect of pH on the elution characteristics of P_i and alkaline phosphatase on Bio-Gel P-4 equilibrated with 3 µg P_i/ml or 3 µg P_i/ml in 0.1 M NaCl using either 3 µg Pi/ml or 3 µg Pi/ml in 0.1 M NaCl as eluent. Fractions eluted were monitored for both enzyme and P_i.

cussion below). The effect disappeared when NaCl was present.

When the pH was increased to 7.0 or to 10.0, the effect was more marked (i.e. a more pronounced dip and rise in P_i occurred). These effects once more disappeared in the presence of NaCl.

ii) Effect of Enzyme Concentration

As the enzyme concentration was increased, the dip and rise became more pronounced (Fig. VI·7).

iii) Effect of P_i Concentration

With higher P_i concentrations the P_i elution curves showed larger dips but the subsequent rising portions were not as pronounced relative to the dips (Fig. VI·8).

iv) Use of ^{32}P to Determine where Phosphate is Eluted Relative to Alkaline Phosphatase in Equilibration Type Experiments

The results of these experiments are shown in Fig. VI·9 and VI·10.

Whether ^{32}P was added to the column before, at the same time as, or after the enzyme, its elution volume was approximately the same, indicating that it was not affected by the enzyme.

(4) Studies Concerned with the Residual P_i in the "Purified"

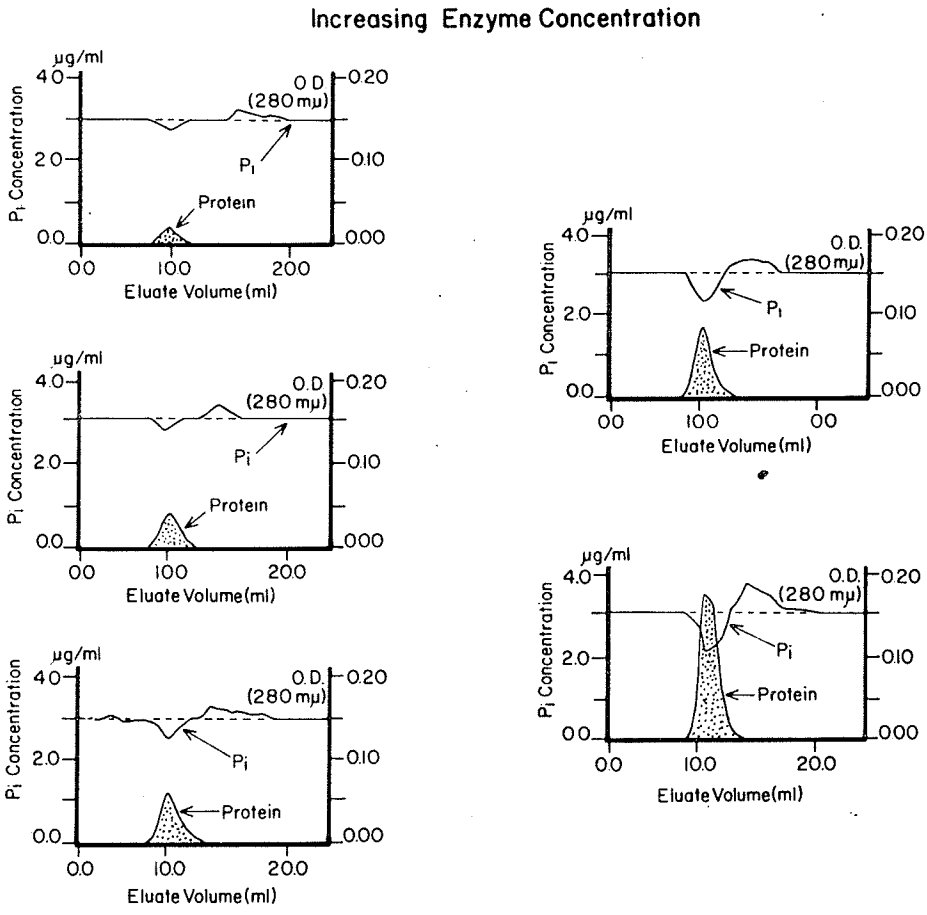


FIGURE VI·7

Effect of different enzyme concentrations on the elution characteristics of P_i and alkaline phosphatase on Bio-Gel P-4 equilibrated with 3 µg P_i/ml at pH 7.0. Samples were eluted with 3 µg P_i/ml, pH 7.0 and fractions monitored for enzyme and P_i.

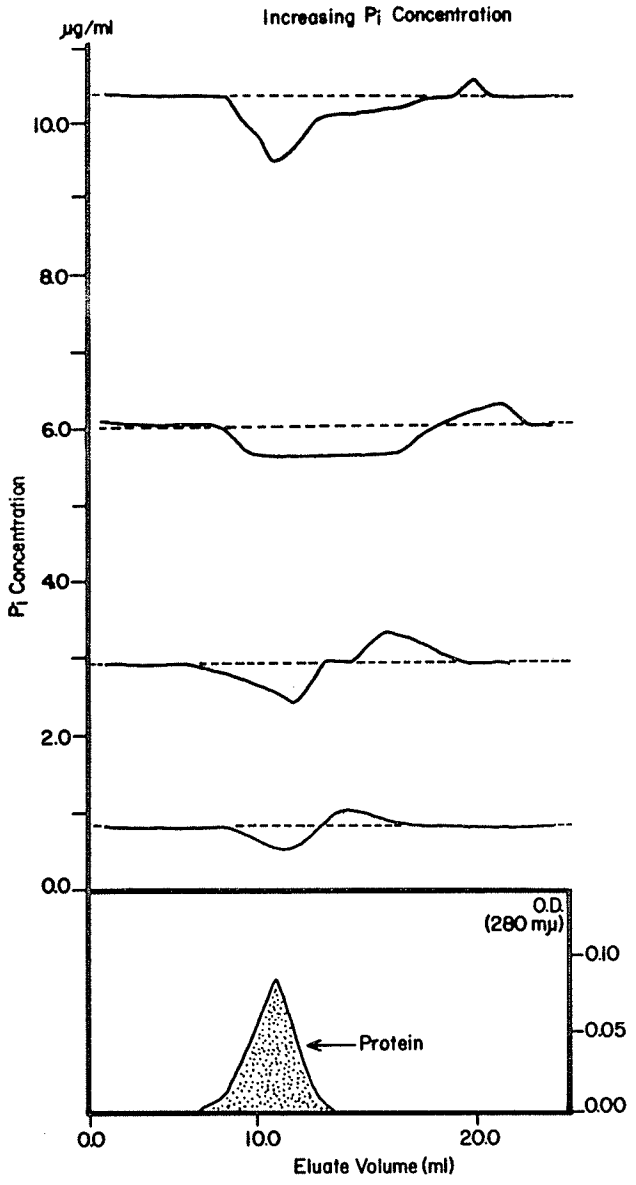


FIGURE VI·8

Effect of increasing P_i concentration on the elution characteristics of P_i and alkaline phosphatase on Bio-Gel P-4 equilibrated with a solution containing P_i in the same concentration as the sample, at pH 7.0. A constant enzyme concentration was used, and samples, eluted with the equilibrating P_i solution, were monitored for P_i and enzyme.

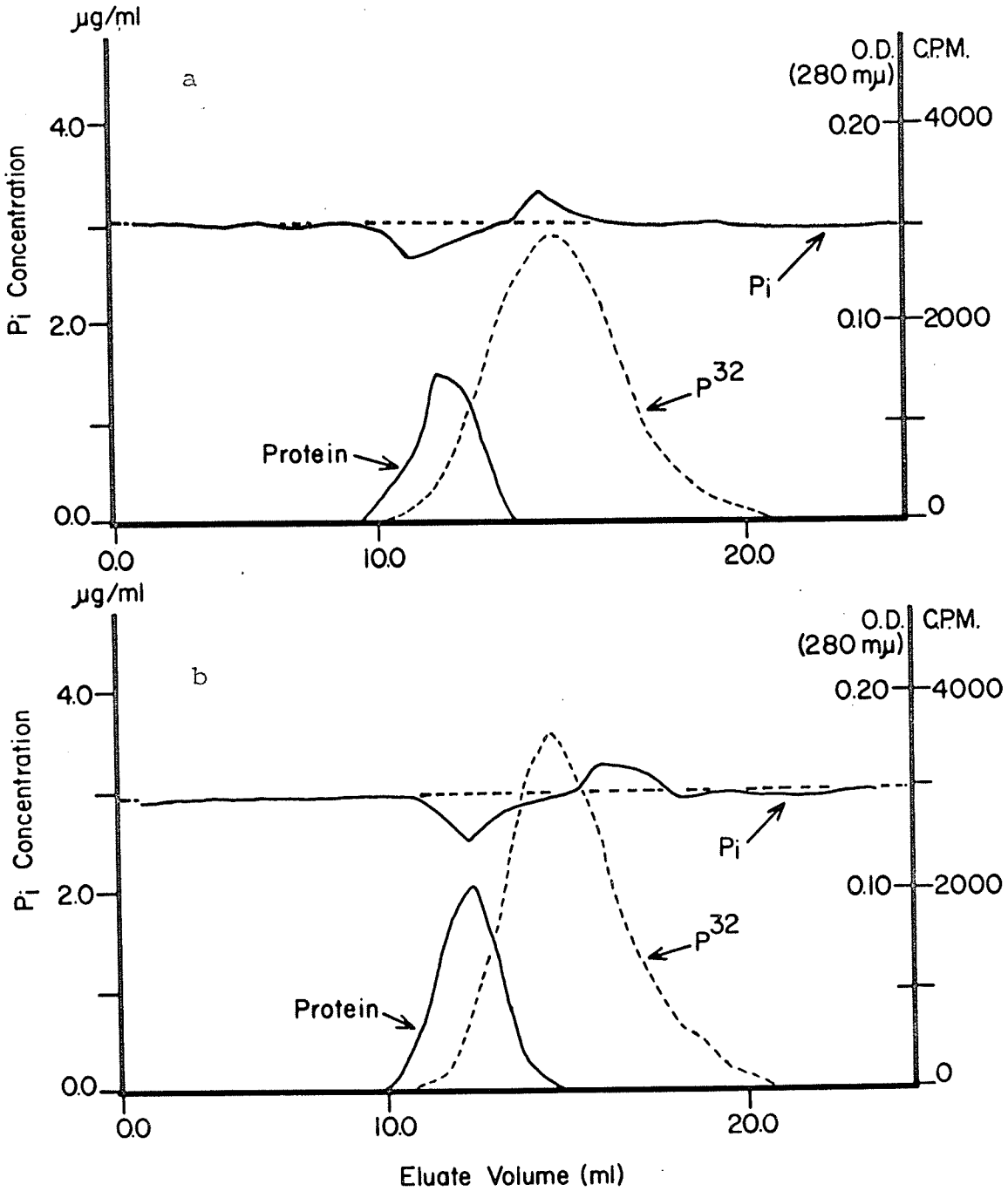


FIGURE VI·9

Elution of ^{32}P and alkaline phosphatase from a Bio-Gel P-4 column equilibrated with 3 µg P_i/ml, pH 7.0 when the ^{32}P was added to the 1 ml of eluent (a) following the enzyme and (b) containing the enzyme, using the equilibrating solution as eluent. Fractions eluted were monitored for enzyme, P_i, and ^{32}P.

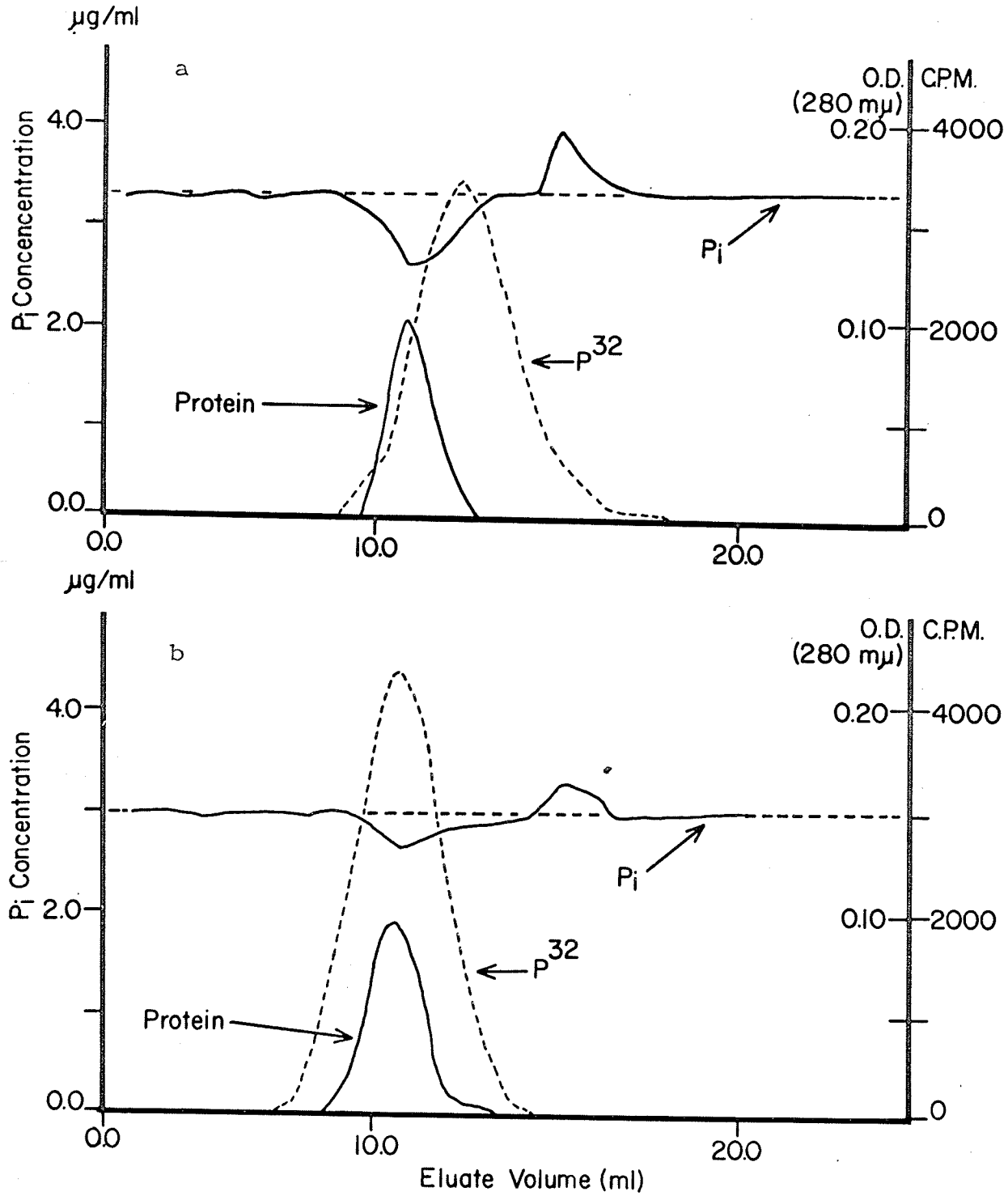


FIGURE VI·10

Elution of ^{32}P from a Bio-Gel P-4 column equilibrated with 3 µg P_i/ml, pH 7.0 when the ^{32}P was added to the 1 ml of eluent a) immediately preceeding the enzyme and (b) 2 ml ahead of the enzyme, using the equilibrating solution as eluent. Fractions eluted were monitored for enzyme, P_i, and ^{32}P.

Alkaline Phosphatase Preparation

When ^{32}P was added to the enzyme and eluted from a P-4 Bio-Gel column with water, ^{32}P was eluted later than P_i, the elution of which occurred at the same Ve/Vo ratio as the enzyme (Fig. VI·11).

When the same experiment was repeated with P_i added, elution of ^{32}P occurred with the added P_i, indicating, as in the previous type of experiment, that ^{32}P had not associated with the enzyme (Fig. VI·12). However this was not conclusive since the elution curves overlapped that of the P_i associated with the enzyme.

Addition of ^{32}P to the enzyme and subsequent dialysis did not remove all of the ^{32}P suggesting that a small amount was still attached.

Discussion

Molecules having molecular weights greater than approximately 3000 cannot penetrate the pores of the Bio-Gel P-4 resin used in the present study so that they pass through the column in the liquid phase around and between the resin particles. As a result, these molecules are eluted from the column first and their elution volume is referred to as the void volume (Flodin, 1962).

Since inorganic phosphate has a molecular weight much

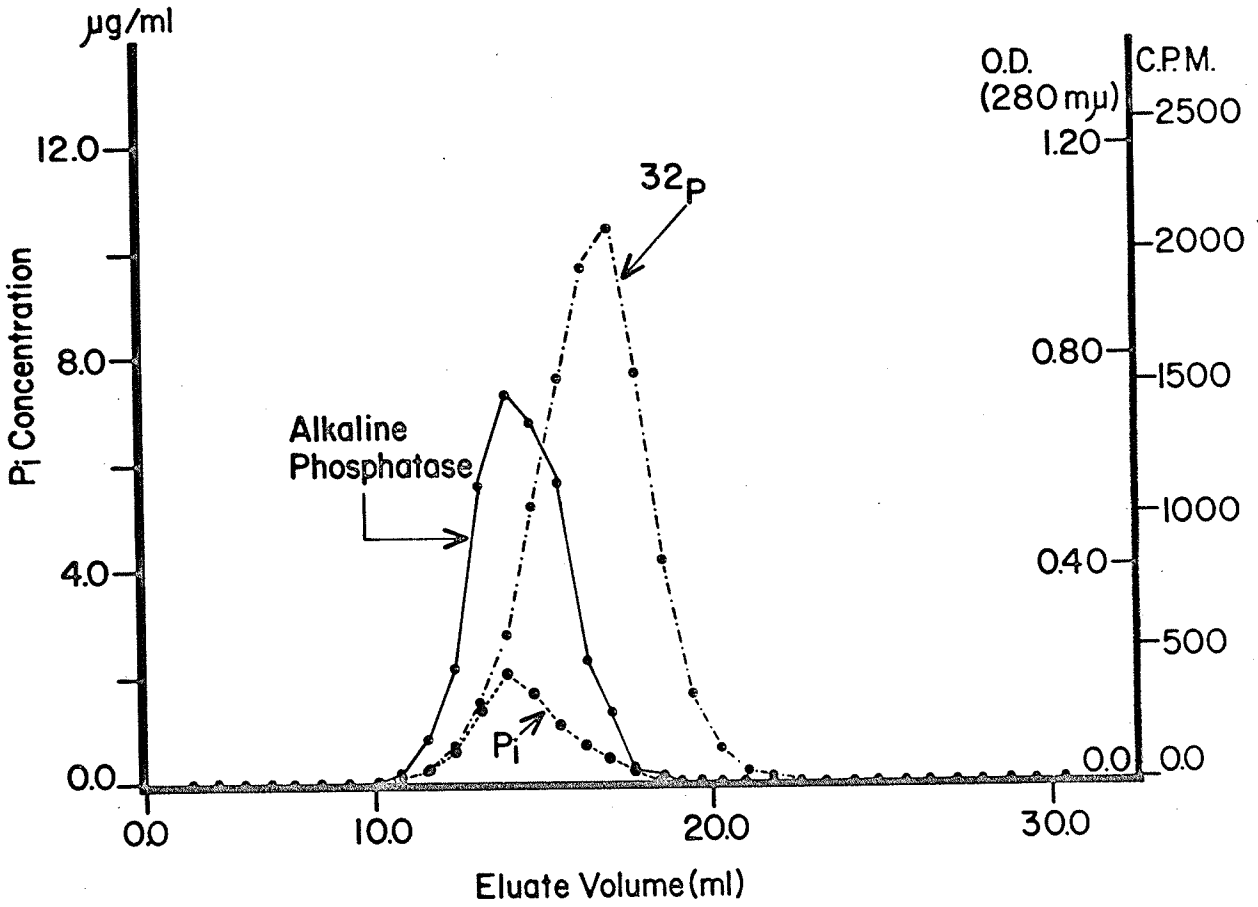


FIGURE VI·11

Elution of ^{32}P and alkaline phosphatase from a Bio-Gel P-4 column equilibrated with distilled water. ^{32}P was added to alkaline phosphatase which contained a small amount of bound P_i and this sample was eluted with distilled water. Eluate fractions were monitored for enzyme, P_i, and ^{32}P.

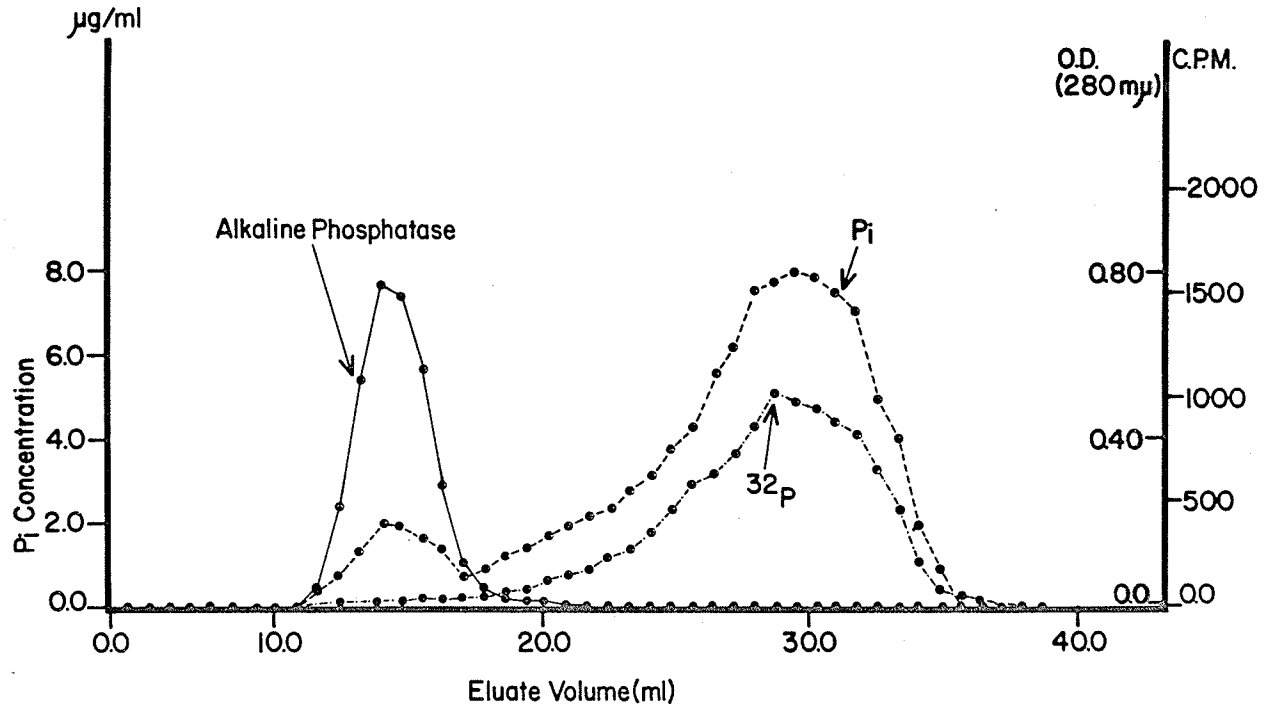


FIGURE VI·12

Elution of ^{32}P and alkaline phosphatase from a Bio-Gel P-4 column equilibrated with distilled water. ^{32}P was added to alkaline phosphatase containing, along with a small amount of bound P_i, 25 µg P_i/ml, and this sample was eluted with distilled water. Eluate fractions were monitored for enzyme, P_i, and ^{32}P.

less than 3000, it is sufficiently small to penetrate the pores of the gel, and based upon its size should be eluted from the column with an elution volume much larger than that of the void volume (i.e. Ve/Vo ratio greater than 1). This was not observed, however, in the present study; the elution volume for P_i was only slightly greater than the void volume and the Ve/Vo ratio increased with increase in P_i concentration.

The elution of P_i much sooner than one might have expected because of its small size, suggests that some negative charge is present within the pores, preventing by charge repulsion the entry of the negatively charged P_i molecule.

The increase in elution volume with increase in P_i concentration suggests that the P_i has penetrated the pores to a greater extent than at low concentration as would occur if the charge on each P_i molecule were reduced. With increase in concentration, such is the case with any electrolyte (Glasstone, 1947a), since with increase in concentration, the ionic strength is increased.

The increased skewing with increased P_i concentration can be attributed to the P_i molecules in the leading and trailing edges diffusing more rapidly than those in the bulk portion of the P_i peak, those at the leading edge pulling ahead of, while those at the trailing edge "banging-up" against the bulk

molecules. This is commonly seen in sedimentation studies on the ultracentrifuge with molecules that do not sediment independently of one another (Williams, 1963). The bulk molecules would be at a higher concentration than those at the edges and therefore subject to a higher ionic strength. With increase in P_i concentration these effects would be larger.

With NaCl, elution of P_i occurs later and the skewing disappears; both effects can be attributed to the reduction in charge on each P_i molecule (enabling increased penetration) which is sufficient to minimize differences in ionic strength between those molecules at the edges and those in the bulk portion of a P_i peak.

With sodium phytate the effects were similar to P_i except that with increase in phytate concentration the increase in elution volume was less. This may have been because of phytate being a more highly charged molecule than P_i.

In the experiments with P_i and alkaline phosphatase in which the column was not equilibrated with P_i, elution of P_i occurred later than corresponding P_i levels in the absence of enzyme. Since these experiments were carried out at pH7, a pH at which the enzyme is negatively charged (Fig. V·11), this retardation of P_i can be attributed to charge repulsion. This conclusion is supported by the observation that, in the

presence of NaCl which would reduce the effective charge on both P_i and enzyme molecules, this effect was eliminated (Fig. VI·4).

In the experiments with P_i, and alkaline phosphatase, in which the columns were equilibrated with P_i, the P_i concentration in the portion of the eluate in which the enzyme was eluted, fell below the base line level (referred to in this chapter as the P_i dip), once more suggesting that repulsion of P_i by the enzyme had occurred. This conclusion is supported by the many experiments in which the effect of pH, enzyme concentration, P_i concentration and ionic strength, on this phenomenon was examined.

Those conditions which favoured an increase in negative charge on either or both enzyme and P_i molecules and therefore their repulsion, resulted in a more marked fall in the P_i concentration of the eluate associated with the enzyme.

For example, when the pH was above that of the isoelectric point of the enzyme (pH 3.5), the P_i dip was evident and more so with increase in the pH. When the P_i and enzyme concentrations were increased, the P_i dip also increased.

On the other hand, when the pH was 2.0 (i.e. a pH at which the enzyme would be positively charged), no dip was observed. The P_i dip was also eliminated when NaCl, which would reduce

the effective charge on both enzyme and P_i, was present.

The P_i rise, that was associated with and occurred later than the P_i dip, was also observed when the dip was present, but a large dip did not necessarily correspond with a large rise. This suggests that the P_i that was displaced may have been partly displaced into the pores of the gel.

Experiments with ^{32}P indicate that the P_i that is missing in the dip is not the P_i that is present in the rise.

The small amount of P_i, that could not be separated from the enzyme, does not appear to be exchangeable from the experiments in which ^{32}P was added to the enzyme and partial separation on the Bio-Gel, P-4 carried out. Yet when the ^{32}P was added to the enzyme and dialyzed, ^{32}P remained associated with the enzyme, suggesting that binding had taken place.

In the first of these two types of experiments, if the bound phosphate were covalently linked to serine at the active centre of the enzyme, as in the scheme proposed by Schwartz (1963) for the phosphorylation of this enzyme with P_i, then exchange would not take place unless the pH were raised and subsequently lowered.

In the equilibrium dialysis experiment the binding of ^{32}P may have taken place at a site other than serine, which in the phosphorylation scheme of Schwartz (1963) is probably Zn^{++}

and is a step which preceeds the binding of phosphate to the serine site. The binding shown in the equilibrium dialysis experiments has also been demonstrated by other workers (Schwartz, 1963).

In addition to providing information on the relationship between P_i and alkaline phosphatase, this study also demonstrated that the procedure of Hummel and Dryer can be used for studying repulsion between enzyme and product as well as attraction between enzyme and substrate.

CHAPTER VII

RELATION OF FINDINGS IN THIS THESIS TO DENTAL CARIES, THE INTESTINAL ABSORPTION OF CALCIUM AND OTHER BIOLOGICAL PHENOMENA

The experiments in this thesis have enabled some of the results of investigators concerned with the role of phytate in dental caries, the intestinal adsorption of calcium, and other biological phenomena to be related.

Dental Caries

Jenkins et al (1959) suggested that phytate can adsorb to the surface of enamel to form a protective coat and thus reduce its solubility. The results in Chapter III indicate that should such adsorption to enamel occur, this would be favoured by an acid pH. Since, according to the acid decalcification theory for dental caries, acid formed by the bacterial breakdown of carbohydrate is responsible for the initiation of the caries process, the presence of phytate in the carbohydrate (if it is unrefined) would provide phytate at the right time and pH to most effectively prevent such dissolution of the enamel.

However, the experiments in Chapter III were carried out using the anion resin Dowex-1-Cl, and, as pointed out in the

introduction to Chapter III, the testing of whether this pH effect occurs with tooth structure awaits investigation. This is of course likely to be much more complex, since with changes in pH, calcium phosphate changes in form (Gray, Frances and Griebstein, 1962).

Because phytate and its inositol phosphate intermediates can be hydrolyzed with both acid and alkaline phosphatases, these phosphate compounds would, should these enzymes be present, be susceptible to hydrolysis over the wide pH range found in the dental plaque (Kleinberg and Jenkins, 1964). During the bacterial breakdown of carbohydrate by dental plaque, the pH falls from a neutral or slightly basic pH to one between approximately 5.0 to 6.0. Since the pH optimum with acid phosphatase was 5.0 with both phytate and its inositol phosphate intermediates (Table V·1), the rate of release of phosphate would progressively increase during such a plaque pH fall. This would provide, at the right time, P_i, which may lower the critical pH in the plaque sufficiently to protect the tooth from dissolution by the acids formed during the bacterial breakdown of the carbohydrate. This of course assumes the presence of an acid phosphatase in plaque, (originating either from the plaque bacteria or saliva, Afonsky, 1961), since phytase in the foodstuff would likely

be inactive because of prior cooking (Jenkins et al, 1959).

In summary, the experiments in this thesis provide evidence for the possibility that phytate may protect the dental hard tissues from acid formed in the plaque (i) by phytate adsorbing to the tooth surface forming a protective layer suggested by Jenkins et al (1959) and (ii) by providing P_i to lower the critical pH.

The Intestinal Absorption of Calcium

The results in the present study suggest that under some conditions, hydrolysis of phytate will occur more readily in the proximal than in the distal portion of the small intestine while under other conditions the location of optimal hydrolysis will be reversed.

In the small intestine the pH is lower (Cantarow and Schepartz, 1958), and the calcium and phosphate in solution (Cantarow and Trumper, 1956) and the levels of alkaline phosphatase (Triantaphyllopoulos and Tuba, 1959) are higher in the proximal than in the distal regions, the pH progressively rising and the Ca, P_i, and alkaline phosphatase levels falling as the distal portions are approached. The higher calcium and phosphate levels in the proximal portion of the small intestine have been attributed to the lower pH in this region, since at low pH, calcium phosphate is more soluble than at

high pH (Cantarow and Trumper, 1956).

Considering only the alkaline phosphatase levels, one would expect more hydrolysis of organic phosphates such as phytate to occur at the proximal than at the distal end of the small intestine. However, since P_i levels are higher in the proximal region, more inhibition of alkaline phosphatase activity would be expected to occur. This inhibition would be enhanced in the proximal region because repulsion between P_i and enzyme is less (see Chapter VI) and better phosphate fixation can take place at the lower pH (Schwartz, 1963). Conversely, in the distal region of the small intestine where the pH is higher, even though the level of alkaline phospha--tase is lower, less inhibition of its activity by P_i would be expected to occur. Therefore, when one considers the data available on pH, P_i, and alkaline phosphatase levels in the small intestine, the rate of hydrolysis of organic phosphate would probably be similar in all regions of the small intest-ine.

This does not however take into account the effect of variation in the concentration of phytate.

If phytate were present in low concentration, because the optimal pH for its hydrolysis with alkaline phosphatase would be lower than if it were present in high concentration,

hydrolysis would be favoured in the proximal rather than the distal portion of the small intestine. The reason for this is that in the proximal region, the pH may be sufficiently low and the presence of competing cations such as Na^{+}, K^{+}, and Mg^{++} sufficiently high, that precipitation of calcium phytate may not occur. Should this be so, then hydrolysis of the phytate molecule and the formation of its various inositol phosphate derivatives would be favoured. Since the calcium salts of these products are more soluble than calcium phytate, absorption of calcium, even at the higher pH levels in the more distal regions of the small intestine, would presumably be enhanced. On the other hand, if phytate were present in high concentration, then the pH optimum with alkaline phosphatase would be shifted to higher pH levels and its hydrolysis in the proximal portion of the small intestine would be hindered, whereas in the more alkaline distal portions, it would be favoured.

If calcium phytate should form, as for example might occur with high calcium levels in the diet, reduction in the availability of calcium would probably result, but not necessarily phosphate availability from phytate (McCance and Widdowson, 1942a). Precipitation of calcium phytate in the intestine would lower the phytate concentration in solution, and, as

argued above, more effective hydrolysis of the phytate in solution might occur due to reduction in the pH optimum for alkaline phosphatase to a level closer to that found in most of the small intestine.

It would appear therefore that one combination of factors will favour phytate hydrolysis while another combination will favour the reverse. Which conditions apply, would presumably have a major influence on whether the absorption of calcium remains the same as, or is altered from, some base-line condition.

Other Biological Phenomena

The effect of phytate upon the absorption of heavy metals (such as Zn^{++} and Fe^{++}) is probably very similar to that of calcium, although the binding of these metals by phytate and the solubilities of the salts so formed occur at different pH levels (Vohra, Gray and Kratzer, 1965).

The same factors that determine the availability of cations for intestinal absorption would also apply to the availability for plant nutrition of these cations from soils. Hydrolysis of phytate by soil bacteria or plant phytases in the soil would as in the small intestine result in the formation of inositol phosphate intermediates which would probably produce more soluble salts with these various metals,

increasing their availability.

However, in soil an additional factor would apply, since at low pH, although phytate is very soluble, phytate availability is poor (Goring and Bartholomew, 1950; Anderson and Arlidge, 1962). In the present study, adsorption of phytate to Dowex-1-Cl increased with decrease in pH, which is probably analogous and would account for this observation with soils since soils contain inorganic materials to which phytate can adsorb.

With respect to the inositides, the inositol phosphate containing compounds found in nerve tissue and in the membranes of cells, tetraphosphoinositides (i.e. phosphatidylinositoltriphosphate) have never been isolated, and there is uncertainty as to whether or not they exist (Santiago-Calvo, Mulé and Hokin, 1963; Santiago-Calvo et al, 1964). This may be because this compound is too easily hydrolyzed to exist or if present, to exist in more than in small quantity. The studies on the hydrolysis of the various inositol phosphates with acid and alkaline phosphatases suggest this possibility, since ITriP is one of the most easily hydrolyzed of the inositol phosphates and one of those most insensitive to P_i inhibition (see Chapter V).

CHAPTER VIII

SUMMARY

The factors involved in the hydrolysis of phytate and its inositol phosphate derivatives and the properties of these compounds particularly as they may relate to dental caries and nutrition were examined.

The inositol phosphates studied were phytic acid, inositol monophosphate, inositol diphosphate, inositol triphosphate, inositol tetraphosphate, and inositol pentaphosphate. IDP, ITriP, ITetraP, and IP'P were prepared from an acid hydrolysis of phytic acid and were identified from their P/I ratios. IMP was purchased.

Experiments were carried out to determine the effect of pH on the adsorption and elution of phytate from a column containing Dowex-1-Cl anion exchange resin and by analogy to teeth and soils. Similar experiments were also carried out using IMP for comparison.

Phytate at each of three pH levels (viz. 2.1, 5.6, and 8.6) and IMP at pH 2.4 and 8.6 were put onto the column and eluted with LiCl under a variety of conditions. In some experiments, following application of the sample, the eluate, containing material eluted with distilled water, was collected and monitored for pH, Na^+ and Cl^- to determine the changes

which occur when phytate and IMP are adsorbed to the resin.

An analysis of the data obtained when pH, Na^+ and Cl^- were monitored showed that both phytate and IMP were, as expected, more negatively charged at high than at low pH, indicating that chemisorption would be greater at higher pH levels. Therefore, one would have expected phytate and IMP to be adsorbed more strongly at high than at low pH. Such was the case with IMP, but with phytate, pH had a reverse effect. This effect with phytate was attributed to the large number of polar OH groups on the phytate molecule which would favour physical adsorption to the resin. Since chemisorption also occurs, the sum of the physical and chemical adsorptive forces must be greater at low than at high pH. Because IMP does not contain a large number of polar OH groups, physical adsorptive forces would be weak, and the sum of the physical and chemical adsorptive forces would be greater at high than at low pH.

These results may explain why, with decrease in pH, the adsorption of phytate to soils is increased (Goring and Bartholomew, 1950; Anderson and Arlidge, 1962) and might also explain why phytate may have adsorbed to enamel at the low pH levels used in the experiments of Jenkins et al (1959).

Experiments were carried out to compare sodium and calc-

ium binding by phytic acid and its inositol phosphate derivatives by comparing differences between their titration curves when each compound was titrated with NaOH and with $Ca(OH)_2$. At the same time, the effect of pH on the solubility of their calcium salts was determined by noting during the titration with $Ca(OH)_2$, the pH at which a precipitate first formed.

A diphasic curve, typical of that obtained when a diprotic acid is titrated with base, was obtained when IMP was titrated with NaOH, and when IMP and the other inositol phosphates were titrated with $Ca(OH)_2$. The IMP-NaOH curve and all of the $Ca(OH)_2$ curves were similar.

Between 50-100% neutralization of each inositol phosphate, the NaOH curves showed progressively larger deviations from the $Ca(OH)_2$ curves as the P/I ratio increased from 2 to 6. This difference was attributed to the possibility that some of the hydrogens on these molecules (other than IMP) are bound between the oxygen atoms on adjacent phosphate groups and that, whereas Ca^{++} can displace these hydrogens easily, Na^{+} cannot.

pK_2 values determined from the mid-point of the second portion of the diphasic curves with $Ca(OH)_2$ were between approximately 5.6 and 6.2, values lower than those for organic

phosphates without a ring structure such as glycerophosphate. Those values determined with NaOH were higher than those observed with $Ca(OH)_2$ with the difference increasing with increase in P/I ratio of the inositol phosphate. Because Na^+ does not appear able to displace all of the hydrogens on the inositol phosphates other than IMP, the pK_2 values determined from NaOH titration curves probably only reflect the second ionizable hydrogens of those phosphate groups not involved in the hydrogen bonding discussed above.

pK_1 values determined by other workers for the inositol phosphates have been shown to be meaningless since when two concentrations of each of IMP, ITetraP, and IHP were titrated, the pH curve in the 0 to 50% neutralization region was lower for the higher concentration of inositol phosphate. These experiments indicated that these compounds behave more like strong acids than weak ones in the 0 to 50% neutralization region.

The pH at which a precipitate formed when these compounds were titrated with $Ca(OH)_2$ decreased as the P/I ratio increased, indicating that calcium inositol phosphate salts with higher P/I ratios are less soluble than those with lower ratios.

Because phytate can be hydrolyzed sequentially by enzymes

called phytases, and little is known about the factors that may be involved during such hydrolysis, the effect of pH, enzyme, substrate, and P_i concentrations on the enzymic hydrolysis of phytic acid and its various inositol phosphate derivatives by acid and alkaline phosphatases was examined.

The pH optima for alkaline phosphatase were shown to decrease with decreasing substrate concentration for all of the inositol phosphates examined whereas those for acid phosphatase did not. This was explained in terms of a scheme proposed by Krupka and Laidler (1960) and also in terms of the relative charges on the enzymes and substrates involved.

The Km values for each inositol phosphate-enzyme combination (and for glycerophosphate included for comparative purposes) were determined from Lineweaver-Burk plots of the reciprocal of enzyme reaction velocity as a function of the reciprocal of the substrate concentration. From the same data, Vmax values were also calculated at constant enzyme concentration (0.05 mg/ml).

Km values were similar with both enzymes for the inositol phosphates with P/I ratios between 3 and 6, whereas with IMP, IDP, and glycerophosphate, Km's were higher with acid phosphatase than with alkaline phosphatase.

Vmax values were higher with alkaline phosphatase than

with acid phosphatase (except for IP'P) and with both enzymes, were highest with IDP and ITriP.

Km data indicated that whereas molecules with P/I ratios between 3 and 6 would all be hydrolyzed at approximately the same rate at the active sites on both acid and alkaline phosphatases, IMP and IDP would be hydrolyzed at slower rates with acid phosphatase. The same relationship occurs with glycerophosphate. Failure to show this difference in activity between the two enzymes with the higher inositol phosphates may be the result of resistance to hydrolysis with alkaline phosphatase of phosphate groups linked by hydrogen bonds.

The Vmax data suggested that the number of active sites available on the acid and alkaline phosphatase molecules to the different inositol phosphates are different, the sites being more accessible to IDP and ITriP than to the other inositol phosphates.

P_i was shown to inhibit to varying degrees the hydrolysis by acid and alkaline phosphatase of all six inositol phosphates studied. Competitive inhibition of IMP was demonstrated but with the other intermediates such competitive inhibition could not be demonstrated. This was attributed to the fact that with inositol phosphates with P/I ratios greater than that for IMP another inositol phosphate forms upon

hydrolysis which can in turn be hydrolyzed.

With each inositol phosphate, alkaline phosphatase was more susceptible to P_i inhibition than acid phosphatase and with both enzymes, hydrolysis of ITriP and ITetraP was least affected by P_i.

The Km, Vmax, and P_i inhibition data taken together suggest that IDP, ITriP, and ITetraP would yield, on balance, more phosphate upon enzymic hydrolysis than the other inositol phosphates. In a sequential hydrolysis of phytate by these enzymes, complete depletion of organic P would be resisted since IMP, the terminal substrate in such a hydrolysis, is quite sensitive to P_i inhibition and is comparatively slowly hydrolyzed as is indicated by its low Vmax and high Km values.

Experiments were carried out, using a gel filtration procedure, to study the variables that might be involved in the interaction between P_i and alkaline phosphatase.

Different amounts of P_i at pH 7.0 were put onto a Bio-Gel P-4 column equilibrated with either distilled water or 0.1 M NaCl and eluted with distilled water or 0.1 M NaCl respectively. Low levels of P_i (in the absence of NaCl) were eluted with a Ve/Vo ratio only slightly greater than one and as the P_i concentration was increased, the elution

curves became progressively skewed to the left and P_i was eluted with larger Ve/Vo ratios. With NaCl, P_i was eluted with a larger elution volume and the peaks were symmetrical. These experiments indicated that, since P_i was eluted much sooner than one might have expected because of its small size, the negative P_i molecules cannot enter the pores of the gel (which probably also have negative charges) unless this negative charge is reduced, either by increasing the concentration of P_i or using NaCl, both of which would reduce the activities of the charges on the P_i.

A similar series of experiments using Na phytate showed similar effects to P_i except that with increase in phytate concentration the increase in elution volume was less probably because phytate is a more highly charged molecule than P_i.

In experiments in which different concentrations of P_i (pH-7.0) were put onto Bio-Gel columns equilibrated with either distilled water or 0.1 M NaCl along with alkaline phosphatase and eluted with distilled water or NaCl respectively, elution of P_i occurred later with distilled water as eluent than corresponding P_i levels in the absence of enzyme, indicating that, since both enzyme (the isoelectric point of which was determined spectrophotometrically to be 3.5) and

P_i would be negatively charged at the pH of the experiment, this effect was due to charge repulsion. That a charge effect is involved was supported by the finding that these effects were eliminated with NaCl, P_i being eluted in a similar location to P_i in the absence of enzyme.

A series of experiments was carried out in which Bio-Gel P-4 columns were equilibrated with P_i at a fixed pH. A sample containing alkaline phosphatase and P_i in the same concentration and at the same pH as the equilibrating solution was applied to the column and eluted with the P_i solution used to equilibrate the column. The eluate was monitored for P_i and protein. When the pH was above the isoelectric point of alkaline phosphatase the P_i concentration in the portion of the eluate in which the enzyme was eluted fell below the base-line level (referred to as the P_i dip) suggesting that repulsion between P_i and enzyme had occurred. Similar experiments in which either the pH level, the enzyme concentration, or the P_i concentration were increased, conditions which would favour an increase in the negative charge of either or both enzyme and P_i, showed a more marked P_i dip, indicating increased repulsion. This repulsion effect was eliminated at a pH below the isoelectric point of alkaline phosphatase (where the enzyme would be positively charged) and also with

0.1M NaCl present in the eluent. The NaCl would reduce repulsion by reducing the activities of the charges on the enzyme and P_i.

The P_i rise that was associated with and occurred later than the P_i dip did not necessarily correspond in size to the dip, suggesting that the P_i displaced may have been partly displaced into the pores of the gel.

Similar experiments to the above in which ^{32}P was included in the sample indicated that the P_i that was missing in the dip was not the P_i present in the rise.

To determine whether the residual P_i in the alkaline phosphatase preparation used in this thesis was exchangeable, ^{32}P was added to the enzyme and this sample was put onto a Bio-Gel P-4 column and eluted with distilled water. Monitoring for enzyme, P_i, and ^{32}P showed that ^{32}P had separated from the P_i bound to the enzyme, indicating that exchange had not occurred.

In another type of experiment, when ^{32}P was added to the enzyme and dialyzed against distilled water, binding of ^{32}P was shown to take place.

These experiments suggested that the P_i associated with the enzyme was probably covalently linked (possibly to serine), therefore not being exchangeable; whereas, the ^{32}P bound to

the enzyme in the equilibrium dialysis experiment probably was bound to a different site (e.g. Zn^{++}). These findings are consistent with the hypothesis of Schwartz (1963) that two sites exist on the alkaline phosphatase molecule for P_i binding, one involving electrostatic forces (probably with Zn^{++}) and the other involving covalent bonding (probably to serine).

In Chapter VII, the relation of the findings in this thesis to dental caries, the intestinal absorption of calcium and other biological phenomena has been discussed.

BIBLIOGRAPHY

BIBLIOGRAPHY

Afonsky, D. 1961. Phosphatase, p.175-182. In D. Afonsky, Saliva and its Relation to Oral Health. University of Alabama Press, Alabama.

Anderson, G. and Arlidge, E. Z. 1962. The adsorption of inositol phosphates and glycerophosphate by soil clays, clay minerals, and hydrated sesquioxides in acid media. J. Soil Sci. 13, 216-224.

Anderson, R. J. 1915. Concerning the organic phosphoric acid compound of wheat bran. IV. The occurrence of inosite triphosphate in wheat bran. J. biol. Chem. 20, 463-473.

Andlaw, R. J: 1960a. Acid production and enamel decalcification by fermenting foodstuffs. J. dent. Res. 39, 705-706.

Andlaw, R. J. 1960b. The relationship between acid production and enamel decalcification in salivary fermentations of carbohydrate foodstuffs. J. dent. Res. 39, 1200-1209.

Andrews, J. C. and Herrarte, E. 1951. Studies on phytin. J. Elisha Mitchell Sci. Soc. 67, 45-53.

Arnold, P. W. 1956. Paper ionophoresis of inositol phosphates, with a note on the acid hydrolysates of phytic acid. Biochim. biophys. Acta 19, 552-554.

Asada, K. and Kasai, Z. 1959. Formation of phytin and its role in the ripening process of rice plant. Mem. Res. Inst. Food Sci., Kyoto Univ. 18, 32-40.

Azoulay, E. and Couchoud-Beaumont, P. 1965. Étude de la cytochrome-oxidase de Pseudomonas aeruginosa. Biochim. biophys. Acta 110, 301-311.

Babson, A. L., Greeley, S. J., Coleman, C. M. and Phillips, G. E. 1966. Phenolphthalein monophosphate as a substrate for serum alkaline phosphatase. Clin. Chem. 12, 482-490.

Bandurski, R. S. and Axelrod, B. 1951. The chromatographic identification of same biologically important phosphate

esters. J. biol. Chem. 193, 405-410.

Barré, R., Courtois, J. E. and Wormser, G. 1954. Étude de l'action de quelques cations divalents sur les combinaisons acide phytique-protéine. Ann. Acad. Scient. Fennicae. Ser. A, II, No. 60, 104-133.

Bather, R., Sebastian, I. and Webb, S. J. 1964. Inositol and the protection of cells. II. The release of nucleic acids from normal and malignant cells. Can. J. Biochem. 42, 167-177.

Beadle, G. W. 1944. An inositolless mutant strain of Neurosporra and its use in bioassays. J. biol. Chem. 156, 683-689.

Behal, F. J. and Center, M. 1965. Heterogeneity of calf intestinal alkaline phosphatase. Archs Biochem. Biophys. 110, 500-505.

Beukenkamp, J., Rieman 111, W. and Lindenbaum, S. 1954. Behaviour of condensed phosphates in anion-exchange chromatography. Analyt. Chem. 26, 505-512.

Bibby, B. G. and Andlaw, R. J. 1961. Effects of some food factors on enamel solubility. J. dent. Res. 40, 729-730.

Bonham Carter, R. E., Dent, C. E., Fowler, D. I. and Harper, C. M. 1955. Calcium metabolism in idiopathic hypercalcaemia of infancy with failure to thrive. Archs Dis. Childh. 30, 399-404.

Bower, C. A. 1945. Separation and identification of phytin and its derivatives form soils. Soil Sci. 59, 277-285.

Burstone, M. S. 1962. Enzyme Histochemistry and its Application in the Study of Neoplasms (Academic Press, New York and London). Ch. 5, 160-292.

Byrd, C. A. and Matrone, G. 1965. The chemical basis of Zn-Ca-phytate interaction in biological systems. Proc. Soc. exp. Biol. Med. 119, 347-349.

Cantarow, A. and Schepartz, B. 1958. Intestinal juice (succus entericus), P.262-263. In A. Cantarow and B. Schepartz, Biochemistry, 2nd ed. W. B. Saunders Co., Philadelphia.

Cantarow, A. and Trumper, M. 1956. Calcium and inorganic phosphate metabolism, p.224-247. In A. Cantarow and M. Trumper,

Clinical biochemistry, 5th ed., W. B. Saunders Co., Philadelph.

Charalampous, F. C. 1959. Biochemical studies on inositol. V. Purification and properties of the enzyme that cleaves inositol to D-glucuranic acid. J. biol. Chem. 234, 220-227.

Conrad, J. P. 1939. Retention of some phosphorus compounds by soils as shown by subsequent plant growth. J. agric. Res. 59, 507-518.

Courtois, J. E. and Manet, L. 1952. Recherches sur la phytase. XVII. Les phytases du colibacille. Bull. Soc. Chim. biol. 34, 265-278.

Cowan, J. W., Esfahani, M., Salji, J. P. and Azzam, S. A. 1966. Effect of phytate on iron absorption in the rat. J. Nutr. 90, 423-427.

Dabich, D. and Neuhaus, O. W. 1966. Purification and properties of boivine synovial fluid alkaline phosphatase. J. biol. Chem. 241, 415-420.

Dawes, C. and Shaw, J. H. 1965. Dietary phosphate supplementation and its effects on dental caries and salivary and serum concentrations of calcium and inorganic phosphate in the rat. Archs oral Biol. 10, 567-577.

Eagle, H., Oyama, V. I., Levy, M. and Freeman, A. E., 1957. Myo-inositol as an essential growth factor for normal and malignant human cells in tissue culture. J. biol. Chem. 226, 191-205.

Eisenberg, F., Jr. and Bolden, A. H. 1965. D-myo-inositol-1-phosphate, an intermediate in the biosynthesis of inositol in the mammal. Biochem. biophys. Res. Commun. 21, 100-105.

Flodin, P. 1962. Theory of gel filtration. p.35-41. In P. Flodin, Dextran gels and their applications in gel filtration, Pharmacia Uppsala, Sweden; Halmstad 1962. Meijels Bokindustri.

Folley, S. J. and Kay, H. D. 1935. CCXV. The alkaline phosphomonoesterase of the mammary gland. Biochem. J. 29, 1837-1850.

Forfar, J. O., Balf, C. L., Maxwell, G. M. and Tompsett, S.L. 1956. Idiopathic hypercalcaemia of infancy. Clinical

and metabolic studies with special reference to the aetiological role of vitamin D. Lancet I, 981-988.

Fosdick, L. S. and Starke, A. C. 1939. Solubility of tooth enamel in saliva at various pH levels. J. dent. Res. 18, 417-430.

Friedli, H. 1924. Absorption des rayons ultraviolets par les dérivés de l'émoglobine. Contribution à l'étude de la constitution chimique des matières colorantes du sang. Bull. Soc. Chim. biol. 6, 908-934.

Garcia-Buñuel, L. and Garcia-Buñuel, V. M. 1964. Enzymic determination of free myoinositol in human cerebrospinal fluid and plasma. J. Lab. clin. Med. 64, 461-468.

Garen, A. and Levinthal, C. 1960. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of E. coli. 1. Purification and characterization of alkaline phosphatase. Biochim. biophys. Acta 38, 470-483.

Gavin, G. and McHenry, E. W. 1941. The effects of biotin upon fat synthesis and metabolism. J. biol. Chem. 141, 619-625.

Gezelius, K. and Wright, B. E., 1965. Alkaline phosphatase in Dictyostelium discoideum. J. gen. Microbiol. 38, 309-327.

Glasstone, S. 1947. Surface phenomena. Adsorption, p.1194-1210. In S. Glasstone, Textbook of physical chemistry, 2nd ed. D. Van Nostrand and Co., Inc., New York. MacMillan and Co., Ltd., London.

Glasstone, S. 1947a. Activity coefficients, p.954-974. In S. Glasstone, Textbook of physical chemistry, 2nd ed. D. Van Nostrand and Co. Inc., New York. MacMillan and Co., Ltd., London.

Goring, C. A. I. and Bartholomew, W. V. 1950. Microbial products and soil organic matter. III. Adsorption of carbohydrate phosphates by clays. Soil Science Soc. Am. Proc. 15, 189-194.

Gray, J. A., Francis, M. D. and Griebstein, W. J. 1962.

Chemistry of enamel dissolution, p.164-179. In R. F. Sognnaes, Chemistry and prevention of dental caries, Charles C. Thomas. Publisher. Springfield, Illinois.

Greaves, M. P., Anderson, G. and Webley, D. M. 1967. The hydrolysis of inositol phosphates by Aerobacter aerogenes. Biochim. biophys. Acta 132, 412-418.

Grenby, T. H. 1967a. Flour, bread and wheat grain fractions in decalcification tests. Archs oral Biol. 12, 513-521.

Grenby, T. H. 1967b. Wheat bran factors in decalcification tests. Archs oral Biol. 12, 523-529.

Grenby, T. H. 1967c. Phytates in decalcification tests in vitro. Archs oral Biol. 12, 531-537.

Harrison, D. C. and Mellanby, E. 1939. CCVIII. Phytic acid and the rickets-producing action of cereals. Biochem. J. 33, 1660-1680.

Hawthorne, J. N. (1960). The inositol phospholipids. J. Lipid Res. I, 255-280.

Hawthorne, J. N. 1964. The biochemistry of the inositol lipids. Vitams Horm. 22, 57-79.

Henneman, P. H., Carroll, E. L. and Albright, F. 1956. The suppression of urinary calcium and magnesium by oral sodium phytate: a preliminary report. Ann. N.Y. Acad. Sci. 64, 343-350.

Hoff, J. E. 1963. An effect of the sample pH on the separation of phosphates by ion-exchange chromatography. J. Chromatog. 12, 544-546.

Hoff-Jorgensen, E. 1944. Investigations on the solubility of calcium phytate. Det. Kgl. Danske Videnskabernes Selskab, Mat.-fys. Meddelelser 21, No. 7, 27pp.

Hokin, M. R. and Hokin, L. E. 1961. The role of phosphatidic acid in transmembrane transport. Lab. Invest. 10, 1151-1161.

Horie, S. and Morrison, M. 1963. Cytochrome c oxidase com-

ponents. III. Spectral properties of cytochromes a and a_3. J. biol. Chem. 238, 2859-2865.

Hummel, J. P. and Dreyer, W. J. 1962. Measurement of protein-binding phenomena by gel filtration. Biochim. biophys. Acta 63, 530-532.

Jenkins, G. N. and Smales, F. C. 1966. The potential importance in caries prevention of solubility reducing and antibacterial factors in unrefined plant products. Archs oral Biol. II, 599-608.

Jenkins, G. N., Forster, M. G., Speirs, R. L. and Kleinberg, I. - 1959. The influence of the refinement of carbohydrates on their cariogenicity. I. In vitro experiments on white and brown flours. Brit. dent. J. 106, 195-208.

Johnson, M. J. 1949. A rapid micromethod for estimation of non-volatile organic matter. J. biol. Chem. 181, 707-711.

Keilin, J. 1960. Nature of the Haem-binding groups in native and denatured haemoglobin and myoglobin. Nature 187, 365-371.

Kerr, S. E. and Kfoury, G. A. 1962. Chromatographic separation and identification of myo-inositol phosphates. Archs. Biochem. Biophys. 96, 347-353.

Kleinberg, I. and Jenkins, G. N. 1964. The pH of dental plaques in the different areas of the mouth before and after meals and their relationship to the pH and rate of flow of resting saliva. Archs oral Biol. 9, 493-516.

Knapp, L. F. 1922. The solubility of small particles and the stability of colloids. Trans. Faraday Soc. 17, 457-465.

Krupka, R. M. and Laidler, K. J. 1960. The influence of pH on the rates of enzyme reactions. Part 5. The case of several enzyme-substrate intermediates. Trans. Faraday Soc. 56, 1467-1476.

Kuttner, T. and Cohen, H. R. 1927. Micro colorimetric studies. 1. A molybdic acid, stannous chloride reagent. The micro-estimation of phosphate and calcium in pus, plasma, and spinal fluid. J. biol. Chem. 75, 517-531.

Laidler, K. J. 1963. Catalysis by enzymes, p.71-81. Enzyme catalysis, p.108-111. In K. J. Laidler, Reaction kinetics. Volume two. Reactions in solution, Pergamon

Press Ltd., Oxford.

LaMer, V. K. and Healy, T. W. 1963. Adsorption-flocculation reactions of macromolecules at the solid-liquid interface. Revs. Pure and Appl. Chem. 13, 112-133.

LaMer, V. K. and Smellie, R. H., Jr. 1956a. Flocculation, subsidence, and filtration of phosphate slimes. I. General. J. Colloid Sci. 11, 704-709.

LaMer, V. K. and Smellie, R. H., Jr. 1956b. Flocculation, subsidence, and filtration of phosphate slimes. II. Starches as agents for improving flocculation, subsidence, and filtration of phosphate slimes. J. Colloid Sci. 11, 710-719.

Lazdunski, M. and Ouellet, L. 1962. Inhibition of intestinal alkaline phosphatase. Can. J. Biochem. 40, 1619-1639.

Likuski, H. J. A. and Forbes, R. M. 1965. Mineral utilization in the rat. IV. Effects of calcium and phytic acid on the utilization of dietary zinc. J. Nutr. 85, 230-234.

Lineweaver, H. and Burk, D. 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56, 658-666.

MacDonald, W. B. and Stapleton, T. 1955. Ideopathic hypercalcaemia of infancy. Studies on the mineral balance. Acta paediat., Stockh. 44, 559-576.

Maddaiah, V. T., Kurnick, A. A. and Reid, B. L. 1964. Phytic acid studies. Proc. Soc. exp. Biol. Med. 115, 391-393.

Martell, A. E. 1957. The chemistry of metal chelates in plant nutrition. Soil Sci. 84, 13-26.

Matile, P. 1966. Inositol deficiency resulting in death, an explanation of its occurrence in Neurosporra crassa. Science, N. Y. 151, 86-88.

McCance, R. A. and Widdowson, E. M. 1942a. Mineral metabolism of healthy adults on white and brown bread dietaries. J. Physiol. 101, 44-85.

McCance, R. A. and Widdowson, E. M. 1942b. Mineral metabolism of dephytinized bread. J. Physiol. 101, 304-313.

McCance, R. A., Edgecombe, C. N. and Widdowson, E. M. 1943. Phytic acid and iron absorption. Lancet 2, 126-128.

McClure, F. J. 1960. The cariostatic effect in white rats of phosphorous and calcium supplements added to the flour of bread formulas and bread diets. J. Nutr. 72, 131-136.

McClure, F. J. 1963. Further studies on the cariostatic effect of organic and inorganic phosphates. J. dent. Res. 42, 693-699.

McClure, F. J. 1965. Cariostatic effect of dietary vs. intubated phosphate. Archs oral Biol. 10, 1011-1013.

McClure, F. J. and Muller Jr., A. 1959. Further observations on the cariostatic effect of phosphates. J. dent. Res. 38, 776-781.

Mellanby, E. 1921. Experimental rickets. Spec. Rep. Ser. Med. Res. Counc., No. 61, 78pp.

Mellanby, E. 1925. Experimental rickets. Effect of cereals and their interaction with other factors of diet and environment in producing rickets. Spec. Rep. Ser. med. Res. Counc., No. 93, 66pp.

Mirsky, A. E. and Anson, M. L. 1936. Sulfhydryl and disulfide groups of proteins. III. Sulfhydryl groups of native proteins-hemoglobin and the proteins of the crystalline lens. J. gen. Physiol. 19, 439-450.

Morgan, H. G., Mitchell, R. G., Stowers, J. M. and Thomson, J. 1956. Metabolic studies on two infants with idiopathic hypercalcaemia. Lancet 1, 925-931.

Morton, R. K. 1957. The kinetics of hydrolysis of phenyl phosphate by alkaline phosphatase. Biochem. J. 65, 674-682.

Motzok, I. 1963. Studies on alkaline phosphatases. 4. Influence of dietary minerals on pH optima and reaction velocities of phosphatases of fowl. Biochem. J. 87, 172-180.

Motzok, I. and Branion, H. D. 1961. Studies on alkaline phosphatases. 3. Influence of age of fowl and mammals on pH optima. Biochem. J. 80, 5-9.

Neifakh, S. A. and Kazakova, T. B. 1963. Actomyosin-like protein in mitochondria of mouse liver. Nature 197, 1106.

Neilands, J. B. 1958. Biological Oxidations. Ann. Rev. Biochem. 27, 455-488.

Nichols, M. L. and Kindt, B. H. 1950. Titrations with a photoelectric titrimeter. Anal. Chem. 22, 785-790.

Nielson, F. H., Sunde, M. L. and Hoekstra, W. G. 1966. Effect of dietary amino acid source on the zinc-deficiency syndrome in the chick. J. Nutr. 89, 24-34.

Nikaido, H. 1962. Phospholipid as a possible component of carrier system in β-galactoside permease of Escherichia coli. Biochem. biophys. Res. Commun. 9, 486-492.

Oberleas, D., Muhrer, M. E. and O'Dell, B. L. 1966. Dietary metal-complexing agents and zinc availability in the rat. J. Nutr. 90, 56-62.

Ohnishi, T. and Ohnishi, T. 1962a. Extraction of contractile protein from liver mitochondria. J. Biochem., Tokyo 51, 380-381.

Ohnishi, T. and Ohnishi, T. 1962b. Extraction of actin- and myosin-like proteins from liver mitochondria. J. Biochem, Tokyo 52, 230-231.

Osborn, T. W. B. and Noriskin, J. N. 1937. The relation between diet and caries in the South African Bantu. J. dent. Res. 16, 431-441.

Osborn, T. W. B., Noriskin, J. N. and Staz, J. 1937. A comparison of crude and refined sugar and cereals in their ability to reproduce in vitro decalcification of teeth. J. dent. Res.16, 165-171.

Peers. F. G. 1953. The phytase of wheat. Biochem. J. 53, 102-110.

Pileggi, V. J. 1959. Distribution of phytase in the rat. Archs Biochem. Biophys. 80, 1-8.

Pileggi, V. J., De Luca, H. F. and Steenbock, H. 1955. The role of vitamin D and intestinal phytase in the prevention of rickets in rats on cereal diets. Archs Biochem. Biophys. 58, 194-204.

Platt, B. S. and Glock, G. E. 1943. The estimation of inositol in animal tissues. Biochem. J. 37, 709-712.

Posternak, T. 1965a. Fungi, p.292-296. In T. Posternak, The cyclitols. Holden-Day, Inc., San Francisco, California, U.S.A.

Posternak, T. 1965b. Bacteria. p.292. In T. Posternak, The cyclitols. Holden-Day Inc., San Francisco, California, U.S.A.

Posternak, T. 1965c. Biosynthesis. p.363-364. In T. Posternak, The cyclitols. Holden-Day Inc., San Francisco, California, U.S.A.

Posternak, T. 1965d. General properties of the inositol-phosphoric acids. p.223. In T. Posternak, The cyclitols. Holden-Day Inc., San Francisco, California, U.S.A.

Posternak, T. 1965e. Phytases and the hydrolyses they cause. p.365-367. In T. Posternak, The cyclitols. Holden-Day Inc., San Francisco, California, U.S.A.

Rapoport, S., Leva, E. and Guest, G. M. 1941. Phytase in plasma and erythrocytes of various species of vertebrates. J. biol. Chem. 139, 621-632.

Rhaney, K. and Mitchell, R. G. 1956. Idiopathic hypercalcaemia of infants. Lancet 1, 1028-1033.

Roberts, R. N., Johnston, J. A. and Fuhr, B. W. 1965. A method for the quantitative estimation of myoinositol by gas-liquid chromatography. Analyt. Biochem. 10, 282-289.

Rogers, H. T., Pearson, R. W., and Pierre, W. H. 1940. Absorption of organic phosphorus by corn and tomato plants and the mineralizing action of exo-enzyme systems of growing

roots. Soil Sci. Soc. Amer. Proc. 5, 285-291.

Ross, M. H., Ely, J. O. and Archer, J. G. 1951. Alkaline phosphatase activity and pH optima. J. biol. Chem. 192, 561-568.

Saio, K. 1964. Change in inositol phosphates during the ripening of rice grains. Plant Cell Physiol. (Tokyo) 5, 393-400.

Santiago-Calvo, E., Mulé, S. J. and Hokin, L. E. 1963. A new phosphoinositide containing four phosphates per inositol. Biochim. biophys. Acta 70, 91-93.

Santiago-Calvo, E., Mulé, S. J., Redman, C. M., Hokin, L. E. and Hokin, M. R. 1964. The chromatographic separation of phosphoinositides and studies on their turnover in various tissues. Biochim. biophys. Acta 84, 550-562.

Schwartz, J. H. 1963. The phosphorylation of alkaline phosphatase. Proc. natn. Acad. Sci. U.S.A. 49, 871-878.

Schwartz, J. H. and Lipmann, F. 1961. Phosphate incorporation in alkaline phosphatase of E. coli. Proc. natn. Acad. Sci. U.S.A. 47, 1996-2005.

Schwarzenbach, G. 1952. Chelate complex formation as a basis for titration processes. Anal. Chim. Acta 7, 141-155.

Schwarzenbach, G. and Biedermann, W. 1948. 53. Komplexone VII. Titration von metallen mit nitrilotriessigsäure H_3X. Endpunktsindikation durch P_H-Effekte. Helv. chim. Acta 31, 331-340.

Schwarzenbach, G, Biedermann, W. and Bangerter, F. 1946. 101. Komplexone VI. Neue einfache titriermethoden zur bestimmung der wasserhärte. Helv. chim. Acta 29, 811-818.

Scott, E. M. 1966. Kinetic comparison of genetically different acid phosphatases of human erythrocytes. J. biol. Chem. 241, 3049-3052.

Shaw, J. G. 1966. Acid phosphatase from tobacco leaves. Archs Biochem. Biophys. 117, 1-9.

Sidwell, A. E., Jr., Munch, R. H., Barron, E. S. G. and Hogness, T. R. 1938. The salt effect on the hemoglobin-oxygen equilibrium. J. biol. Chem. 123, 335-350.

Slater, E. C. 1961. The cytochrome system. p.622-636. In C. Tong, E. J. King and W. M. Sperry, Biochemists' Handbook, E. and F. N. Spon Ltd., London.

Soret, J. L. 1878. Recherches sur l'absorption des rayons ultra-violets par diverses substances. Arch. Sci. phys. nat. 61, 322-359.

Stapleton, T., MacDonald, W. B. and Lightwood, R. 1956. Management of "idiopathic" hypercalcaemia in infancy. Lancet 1, 932-934.

Staz, J. 1938. Dental caries in South Africa. S. Afr. J. med. Sci. (supp.) 3, 1-63.

Strittmatter, C. F. and Ball, E. G. 1954. The intracellular distribution of cytochrome components and of oxidative enzyme activity in rat liver. J. cell. comp. physiol. 43, 57-78.

Tomlinson, R. V. and Ballou, C. E. 1962. Myoinositol polyphosphate intermediates in the dephosphorylation of phytic acid by phytase. Biochemistry, N.Y. 1, 166-171.

Tria, E. and Barnabei, O. 1963. Presence of a phosphatidopeptide fraction in liver cell membrane and its possible role in the active transport of amino acids. Nature 197, 598-599.

Triantaphyllopoulos, E. and Tuba, J. 1959. Studies on the distribution and kinetics of the alkaline phosphatase of rat small intestine. Can. J. Biochem. Physiol. 37, 699-708.

Tsukagoshi, S., Lembach, K. and Charalampous, F. C. 1966. Metabolic functions of myo-inositol. III. Utilization of purine bases and nucleosides for nucleic acid biosynthesis in inositol-deficient KB cells. J. biol. Chem. 241, 388-399.

Vagelos, R. and Henneman, P. H. 1957. Effect of sodium phytate on the hypercalciuria of acute quadriplegia due to poliomyelitis. Preliminary report. New Engl. J. Med. 256, 773-776.

Vignais, P. M., Vignais, P. V. and Lehninger, A. L. 1963.

Restoration of ATP-induced contraction of "aged" mitochondria by phosphatidyl inositol. Biochem. biophys. Res. Commun. 11, 313-318.

Vohra, P. and Kratzer, F. H. 1966. Influence of various phosphates and other complexing agents on the availability of zinc for turkey pouls. J. Nutr. 89, 106-112.

Vohra, P., Gray, G. A. and Kratzer, F. H. 1965. Phytic acid-metal complexes. Proc. Soc. exp. Biol. Med. 120, 447-449.

Walker, A. R. P., Fox, F. W. and Irving, J. T. 1948. Studies in human mineral metabolism. 1. The effect of bread rich in phytate phosphorus on the metabolism of certain mineral salts with special reference to calcium. Biochem. J. 42, 452-462.

Webb, S. J. 1960a. Factors affecting the viability of air-borne bacteria. II. The effect of chemical additives on the behaviour of air-borne cells. Can. J. Microbiol. 6, 71-87.

Webb, S. J. 1960b. Factors affecting the viability of air-borne bacteria. III. The role of bonded water and protein structure in the death of air-borne cells. Can. J. Microbiol. 6, 89-105.

Webb, S. J. 1961. Factors affecting the viability of air-borne bacteria. V. The effect of dessication on some metabolic systems of Escherichia coli. Can. J. Microbiol. 7, 621-632.

Webb, S. J. 1963. Factors affecting the viability of air-borne bacteria. VI. The action of inositol on lactose oxidation by dessicated Escherichia coli. Can. J. Bio-chem. Physiol. 41, 455-460.

Webb, S. J. and Dumasia, M. D. 1964. Bound water, inositol, and the effect of X-rays on Escherichia coli. Can. J. Microbiol. 10, 877-885.

Webb, S. J., Bather, R. and Hodges, R. W. 1963. The effect of relative humidity and inositol on air-borne viruses. Can. J. Microbiol, 9, 87-92.

Webb, S. J., Cook, C. A. M. and Bather, R. 1964. Inositol and the protection of cells. I. The release of nucleic acids and viruses from bacterial and mammalian cells.

Can. J. Biochem. 42, 157-166.

Weissbach, A. 1958. The enzymic determination of myoinositol. Biochim. biophys. Acta 27, 608-611.

West, E. S., Todd, W. R., Mason, H. S. and Van Bruggen, J. T. 1966b. Dissociation constants of phosphate esters. Table 9-6. p366. In E. S. West, W. R. Todd, H. S. Mason and J. T. Van Bruggen, Textbook of biochemistry, 4th ed. The MacMillan Co., New York, Collier-MacMillan Co., Toronto, Ontario.

White, A., Handler, P. and Smith, E. L. 1964. Solubility. p.128-131. In A. White, P. Handler and E. L. Smith, Principles of biochemistry, 3rd ed. McGraw-Hill, Inc., New York.

Whiting, A. L. and Heck, A. F. 1926. The assimilation of phosphorus from phytin by oats. Soil Sci. 22, 477-493.

Williams, J. W. 1963. Ultracentrifugal analysis. Academic Press, New York, London. 282p.

Wiss, O. 1950. Microbiological determinations of vitamins and amino acids. Mitt. Lebensm. Hyg. 41, 225-258.

Woolley, D. W. 1941. Identification of the mouse antialopecia factor. J. biol. Chem. 139, 29-34.

Woolley, D. W. 1942. Synthesis of inositol in mice. J. exp. Med. 75, 277-284.