INHIBITION STUDIES ON THE INTESTINAL ALKALINE PHOSPHATASE

by

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INTRODUCTION

The modern period of phosphate biochemistry began with the work of Büchner and of Harden and Young, who laid the foundations of present knowledge of phosphorus metabolism. Both Büchner, and Harden and Young observed the disappearance of inorganic phosphate which accompanies fermentation of sugar by cell-free yeast preparations (10). Those findings stimulated extensive investigation of phosphate esters in living things; the intimate relationship between phosphate and carbohydrate metabolism has subsequently been unfolding.

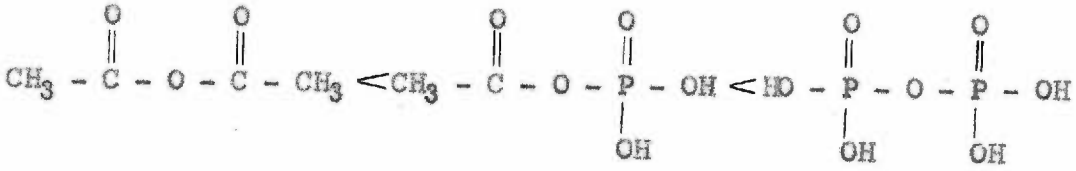
Phosphorylated compounds are widely associated with the energy utilization fundamental to life. The sources of energy for living organisms are extremely diverse; in all cases, even among autotrophs, the energy is obtained by oxidation of some primary compound.

Exergonic (energy-producing) oxidative reactions are coupled with endergonic syntheses of organic phosphates from inorganic phosphate. Subsequent syntheses of new cell material are coupled with utilization of these organic phosphates. Not only does the phosphate modification provide the unique chemical form required for the metabolic alterations, but the chemical energies arising from the metabolic reactions can be converted into a common currency of potential chemical energy, the 'high energy' phosphate derivatives. These derivatives become the driving substances for the numerous energy-requiring reaction of syntheses, growth, active absorption, sensory stimulation and responses, etc. We are largely indebted to Lipmann for the development of this concept (50).

It is not obvious why phosphate esters rather than esters of

other inorganic acids predominate in biological systems. Neither sulphates nor arsenates have comparable importance; the latter compounds are usually toxic to living cells. It is probably significant that phosphate anhydrides combine high activation energies of non-enzymic hydrolysis with large negative free energies of hydrolysis. This permits controlled enzymic cleavage of the anhydride rather than spontaneous hydrolysis.

This stabilizing effect of phosphate is well illustrated by the increasing stability of hydrolysis in the following series of compounds;

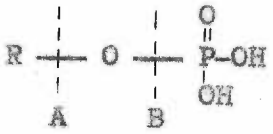


Acetic anhydride is hydrolyzed rapidly in neutral conditions, acetyl phosphate is slightly more stable and pyrophosphate is quite resistant to hydrolysis at pH 7. Phosphates are probably protected from hydroxyl attack by their negative charge (10).

Dissociation constants of phosphates are such that a change of pH within the physiological range alters their charge, and thus modifies their interaction with enzymes. This provides a control mechanism by which cells may adjust reaction rates in response to changes in environment. Almost all reactions in the living cell take place at useful rates because they are catalyzed by enzymes.

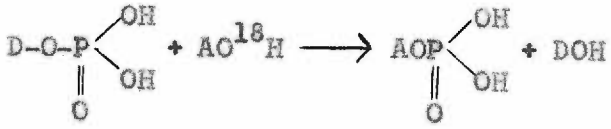
Phosphoryl and Phosphate Transfer Reactions

Phosphates, with the general formula shown,



may be cleaved at A or B. Cleavage at A results in transfer of the phosphate group (-O-P(=O)(OH)-OH) in which oxygen and phosphorus atoms are transferred together. When the break occurs at B, the oxygen atom is not transferred with the phosphorus; this is called phosphoryl (-P(=O)(OH)-OH) transfer. The site of cleavage can be most readily recognized by the use of compounds containing O^{18} and P^{32} (13).

Considering the following reaction;

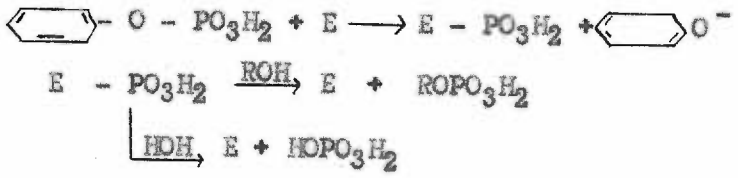


DOH will be unlabeled if cleavage occurs at O-P, but it will be labeled if cleavage is at D-O. In the first case a phosphoryl transfer has ensued, while in the second case a phosphate transfer has occurred. Both reactions would involve different mechanisms, although the end products would be chemically indistinguishable. This has been experimentally illustrated by the pioneering work of Cohn, who elucidated the mechanisms of several reactions involving the cleavage of glucose-1-phosphate using O^{18} (17). She found that the acid-catalyzed hydrolysis proceeds through a C $\frac{|}{|}$ O-P cleavage, while in the enzymatic hydrolysis catalyzed by alkaline phosphatase as well as by acid phosphatase, the cleavage occurs between the oxygen and phosphorus (C-O $\frac{|}{|}$ P). Such a mechanism was also demonstrated by

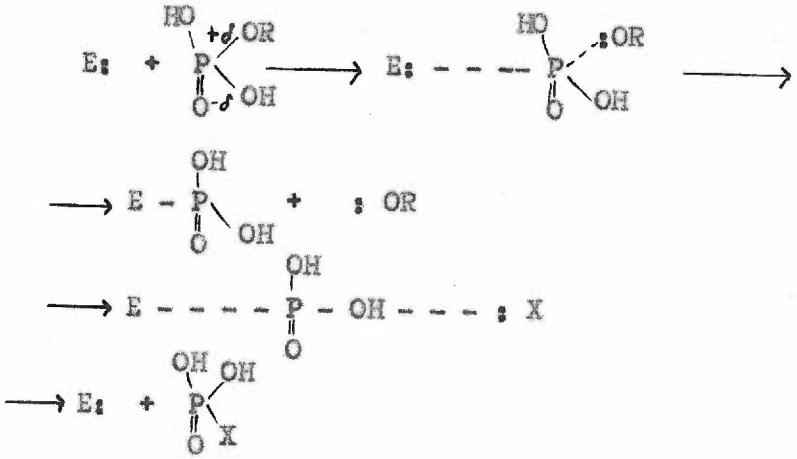
Stein and Koshland, using alkaline phosphatase and a variety of substrates with H_2O^{18} (81).

In connection with the phosphoryl bond-breaking enzymes it seems reasonable to suppose that a transition stage exists in which this group can be held intact between the time it is on the acceptor and the time it is placed on the donor. The suggestion of this type of behavior comes from the transfer reaction noted with phosphatase (13,64,67).

The reaction is most suitably explained on the following basis:



and in terms of the usual mechanistic reactions the enzyme may be regarded as an attacking nucleophilic reagent, as follows:



The nature of X is determined by the specificity of the enzyme; it may be water or any of a number of hydroxyl compounds. The enzyme may function by increasing the positive charge on the phosphorus atom and thus favoring such cleavage, or that the point of attachment of

the substrate on the enzyme is a nucleophilic region. The phosphorus atom is partially polarized in a positive manner owing to the disproportionate sharing of its electrons with the oxygen atoms (13).

The Phosphotransferase Action of "Non-specific" Phosphatases

Although there are kinases which catalyze the formation of almost all known phosphorylated metabolites the existence of other phosphoryl transfer reactions should not be overlooked.

The "non-specific" phosphatases (acid and alkaline phosphatases of different tissue origin), unlike the specific phosphatases such as glucose-6-phosphatase which are more selective in their substrates, act upon a wide variety of aromatic and aliphatic alcohols (30). In their action, these non-specific phosphatases were considered to set free equivalent amounts of orthophosphate and hydroxy compound. It was found, however, that when some phosphatase preparations of widely differing origins act on monophosphate esters in the presence of certain alcohols, the expected equivalence is not observed, and the orthophosphate was in deficit (9,11). This resulted from the phosphorylation of the added alcohol concurrent with the breakdown of the monophosphate ester. The two types of reactions catalyzed by the phosphatases are as follows:

Reaction 1 $RO\text{-}PO_3H_2 + HOH \longrightarrow ROH + H_3PO_4$

Reaction 2 $RO\text{-}PO_3H_2 + R'OH \longrightarrow ROH + R'OPO_3H_2$

In reaction 1, the enzyme acts as hydrolase with the cleavage of the P-O bond and removal of the phosphoryl group to inorganic phosphate. Reaction 2, describes the phosphotransferase activity (transfer of

phosphoryl group to the acceptor ROH) and does not involve the intermediate formation of inorganic phosphate (12).

It has been generally assumed that reation 1, characterizes the action of alkaline phosphatase in its natural environment and all methods of estimating the activity of the enzyme are based on its hydrolytic property.

Whether the enzyme preferentially catalyzes reaction 1, or 2, seems to depend upon competition between water and other hydroxyl-containing compounds for sites at the surface of the enzyme donor complex and upon pH of the environment, relative concentrations of the participating compounds, etc. (68). These factors are of importance in considering the obscure role of alkaline phosphatase in nature. Since, ester formation depends on the relative concentration of the organic acceptor and water, hydrolysis may be predominant under most physiological conditions.

Hoffmann-Ostenhof (35) in commenting on Morton's report (61) pointed out that the transferring activity of some hydrolases is probably not only an incidental quality of these catalysts, but it may also have some biological implications. It may be assumed that some hydrolases act as transferring enzymes in vivo, this action being favored by the relationship of their respective acceptor substrates within the cell. By bringing the enzyme into solution the close association of enzyme, donor substrate, and acceptor is broken down and, in vitro the enzyme action usually observed is hydrolytic.

Yagi and Okuda, reporting on the in vitro synthesis of flavin mononucleotide from riboflavin by dog intestinal alkaline

phosphatase, suggested that the phosphorylation of riboflavin in the small intestine may be due to the transferase action of alkaline phosphatase; this implies a biological function of phosphatase in coenzyme synthesis (87).

The synthesis of organic phosphates by enzymic transfer of the phosphoryl group from organic "donor" compounds to a suitable alcohol was first demonstrated by Axelrod (11) and by Appleyard (9). Axelrod established that the synthesis of the new phosphate ester by the citrus fruit acid phosphatase preparation did not involve the intermediary formation of inorganic phosphate (12).

Synthesis of organic phosphates by reversal of hydrolysis catalyzed by the phosphomonoesterases had been demonstrated in vitro. Kay using phosphatases derived from duodenal or kidney extracts found that these tissue extracts were capable of esterifying phosphoric acid in the presence of high concentrations of methyl or ethyl alcohol, ethylene glycol or glycerol (39). A table compiled by Atkinson and Morton shows the great variety of acceptors and donors that have been investigated with respect to the phosphotransferase action of phosphatases on inorganic phosphate and organic phosphate esters (10).

Meyerhof and Green subsequently showed that partially purified alkaline phosphatase from calf intestinal mucosa catalyzed a phosphoryl transfer between a number of donors (phosphocreatine, acetyl phosphate, p-nitrophenyl phosphate etc.) to acceptors such as glucose, glycerol, fructose, etc. These workers also showed that the speed of enzymatic synthesis of glycerophosphate was increased 2 to 4-fold in

the presence of phosphate compounds of higher energy content such as, phosphocreatine, and acetyl phosphate (33,54,55). Meyerhof and Green, by the use of radioactive phosphorus, also demonstrated that the transferase reaction of alkaline phosphatase involves direct transfer of the phosphoryl group from the donor without the intermediary formation of inorganic phosphate (55).

At that time, however, it was not clear whether the same enzyme catalyzed both reactions that is, phosphoryl transfer to water (hydrolysis) and to organic acceptor (transferase activity). Appleyard had proposed that a "transphosphorylase", distinct from the phosphatase, was present in the extract of prostate gland that he used to demonstrate phosphotransferase activity of phosphatase (9).

Morton has contributed a great part of today's knowledge of the kinetic behavior of alkaline and acid phosphatases with respect to hydrolytic and transferase activities (59,64,65,66,67,68). The conclusions drawn by Morton's studies on purified intestinal alkaline phosphatase are as follows:

a) The phosphotransferase activity is a property of mucosal phosphatase itself. The failure of other phosphatases, such as, hexose diphosphatase, 5'-nucleotidase and myosin-adenosine triphosphatase etc., to catalyze phosphoryl transfer, were grouped by Morton into specific hydrolases in that water is the only phosphate acceptor, as compared to those which are transferases as well as hydrolases (alkaline and acid phosphatases).

b) The rates of synthesis of glucose and glycerol phosphates

by intestinal alkaline phosphatase are not related to the free energy of hydrolysis of the donor, but rather are determined primarily by the affinity of the enzyme for the particular donor used.

c) Various acceptors, including water are adsorbed at specific sites of the enzyme surface.

A mechanism for the transferase action of intestinal alkaline phosphatase as well as acid phosphatase, has been proposed by Morton in an attempt to better understand the in vitro behavior of the enzyme (59,64). The proposed reactions are as follows:

1. $E + RO.P \rightleftharpoons RO.P.E \rightleftharpoons E.P + RO.H$

2. $E.P + HOH \rightleftharpoons E.P.W \longrightarrow E + P.OH$

3. $E.P + H.OR' \rightleftharpoons E.P.A \longrightarrow E + P.OR'$

E represents the phosphatase.

P the appropriately-ionized group of the phosphate ester.

RO.P.E the phosphatase-substrate (donor) complex.

E.P the "phosphorylated enzyme" intermediate.

E.P.W. and E.P.A. the complexes of the "phosphorylated enzyme" with water (E.P.W.) or other acceptor (E.P.A.).

Adsorption of the donor compound occurs at the enzyme surface, which gives rise to an intermediate "phosphorylated enzyme". The complex E.P may represent the "active enzyme" which contains a reactive phosphate group (possibly formed by phosphorylation of the phenolic hydroxyl of a tyrosine residue). When acceptors such as water or glucose, are activated at the enzyme surface by forming the complexes shown in equation 2 and 3, the reactive phosphate group

passes to the acceptors thus, forming either inorganic phosphate (P.OH equation 2) or a new ester (P.OR' equation 3).

Stein and Koshland, who studied the exchange reaction of alkaline phosphatase using H_2O^{18}, concluded that the oxygen-phosphorus bond of the ester is broken simultaneously with the formation of a new bond to the phosphorus atom (either a P-OH or P-enzyme bond), (81). The hypothesis of formation of a covalent bond as proposed by Stein and Koshland, has recently been strengthened by the reports of Engström, and Ågren (2,26,27). These workers incubated purified intestinal alkaline and bone phosphatase with inorganic phosphate, P^{32}-labeled, and following hydrolysis of the enzyme, they were able to isolate P^{32}-labeled phosphoryl serine. The results indicated that inorganic phosphate is incorporated into the phosphatase molecule by a covalent bond. This might also suggest the formation of a "phosphorylated enzyme" intermediate. The possibility that the incorporation of phosphate into the molecule of phosphatase is nonspecific and independent of the enzyme function has not been ruled out (27).

In addition to the significance of phosphoryl serine isolation, Ågren et al., observed a rapid incorporation and high affinity of phosphate to the bone phosphatase, which may imply a possible role of this enzyme as a transphosphorylating agent in bone tissue (2).

Purification and Properties of Intestinal Alkaline Phosphatase

Many of the intracellular enzymes are closely associated with well-defined cell components (76). In order to obtain these enzymes in true solution to permit purification, various forms of proteolysis,

such as digestion with trypsin (75) or treatment with butanol (57,60) have been employed. Morton purified the intestinal alkaline phosphatase, by butanol treatment of washed microsomes, followed by acetone precipitation, heating and charcoal adsorption (60). This enzyme is associated with microsomal particles of the intestinal mucosa and with the butanol treatment it is released quantitatively into true solution (60,83). Homogeneous preparations of the intestinal alkaline phosphatase have also been reported by other investigators (72,77).

The enzyme, as reported by Schramm and Ambruster, was found to behave as a single component both on electrophoresis and on sedimentation, and has a molecular weight of 60,000 (77). Mathies and Goodman, working with relatively impure enzyme preparations, reported molecular weights of 500,000 to 1 million with the best estimation being 800,000 for kidney and intestinal phosphatases (52).

The tyrosine and tryptophan content of intestinal alkaline phosphatase was reported by Morton to be 26 and 11 moles per 10^5 grams respectively (62).

The reported values for the carbohydrate content of this enzyme are at variance. Schmidt and Thannhauser (75) found that their partially purified preparation of the intestinal enzyme contained about 20% of polysaccharide (reported as glucose), and Portmann (72) reported that his highly purified enzyme contained sugar and hexosamine about 54 and 33 mgs. per 100 mg. N enzyme, respectively. Portmann observed that an increase of the hexosamine content paralleled the increase in the specific activity in all stages of

purification of the intestinal enzyme; he could not decide if hexosamine is a structural unit of the phosphatase molecule (72). Morton (62), and Schramm and Armbruster (77), however, found approximately 2% and less than 5% carbohydrate respectively, in their highly active preparations.

Morton calculated the turnover number of intestinal alkaline phosphatase to be about 236,000 moles of phenyl phosphate hydrolyzed per minute per mole of enzyme at 38°C., assuming a molecular weight of 60,000 (66).

Activators, Coenzymes, and Inhibitors of Alkaline Phosphatase

The influence of bivalent metals and of amino acids on the activity of alkaline phosphatase has been the subject of numerous investigations; only few of these studies will be mentioned here. It has been shown that the activity of many alkaline phosphatases, even when partially purified, is influenced by a number of metal cations (8,14,36,62,83). Magnesium and to a lesser extent manganese increased the activity of intestinal alkaline phosphatase and maximum activity was observed with a concentration of 10^{-2} and 10^{-5} M metal respectively (62).

The results suggested, as in the case of pyrophosphatases and peptidases, that, metal is necessary for the formation of active phosphatase substrate complexes (62), and that it is a metalloprotein (73). Zinc and beryllium, at concentrations greater than 10^{-6} M, and calcium at concentrations greater than 10^{-4} M, inhibited the intestinal enzyme (62).

Conflicting reports, concerning the effect of amino acids on the

activity of intestinal phosphatase, have appeared in the literature (3,5,14,62). These differences of amino acid activation may be due to removal of metal inhibitors from the enzyme (62).

The presence of a prosthetic group or a coenzyme in the alkaline phosphatase has been claimed by many investigators. Akamatsu and Aso, stated that the apo-enzyme combines reversibly with one atom of magnesium and three molecules of histidine or two molecules of other amino acids or histamine to become active (4,5).

Alvarez and Lora-Tamayo, had suggested that a nucleotide derived from uridylic acid, was a component of alkaline phosphatase (7,51). In a recent report by Ahmed et al., a dialyzable coenzyme was present in kidney alkaline phosphatase prepared by autolysis (3).

The variety of different compounds that have been claimed or suggested as possible "coenzymes" of alkaline phosphatase may be due to the method of purification of these enzymes and actually no true coenzyme has as yet been shown to be associated with intestinal alkaline phosphatase (62).

The known relatively unspecific inhibitors of alkaline phosphatase, have been classified by Morton (65) as follows:

a) Metal-binding agents such as $\alpha\alpha'$- dipyridyl and ethylenediamine-tetracetic acid.

b) Inhibitory metal salts such as zinc and beryllium chlorides.

c) Amino-binding agents such as keten, phenyl isocyanate, nitrous acid and formaldehyde, and

d) competitive inhibitors of hydrolysis such as inorganic phosphate.

The effect of oxidants and reductants on phosphatase was investigated by Sizer (80). He showed that, oxidizing agents, such as potassium permanganate and iodine, and reducing agents, such as cysteine and related thiol compounds, inhibit the activity of alkaline phosphatase. The inhibition by iodine and potassium permanganate was partly reversible with sodium sulfide. Sizer suggested that the inactivation of phosphatase by strong oxidants might be due to the reaction of these compounds with substituent amino acids in the enzyme molecule rather than to oxidation of -SH groups or protein denaturation.

Beryllium poisoning has been a subject of considerable importance as a result of the frequent use of beryllium compounds in industrial processes. This element has been reported as a potent inhibitor of alkaline phosphatases by many investigators (25,34,41, 78,84). Zinc salts, which are relatively nontoxic when applied externally, strongly inhibit intestinal alkaline phosphatase (62).

Polymeric phosphates of phloretin and phloroglucinol have been described as potent inhibitors of alkaline phosphatase and other enzymes as well (20,28). The inhibition of alkaline phosphatase by these compounds could be reversed by basic proteins (protamine, methylated gelatin), indicating the possibility of an electrostatic interaction (20). Polymeric phosphates of synthetic estrogens have also been reported as potent inhibitors of alkaline phosphatases (6,21,29). The type of inhibition produced by such compounds is

competitive (21).

In a recent report by Walters, a pigment with a molecular weight of 2500 was isolated from normal human male urines, and it was described as a truly competitive inhibitor of purified ox kidney alkaline phosphatase. Walters suggested that this pigment inhibits the enzyme through an interaction of acidic groups in the pigment with the primary amino groups of alkaline phosphatase (86).

Substrate Specificity and General Kinetic Aspects of Intestinal Alkaline Phosphatase

In 1943, Schmidt and Thannhauser showed that their purified enzyme preparation from calf intestinal mucosa hydrolyzed, in addition to monophosphate esters, pyrophosphates (pyrophosphoric and adenyl-pyrophosphoric acids) and phosphate diesters (75). Morton, on the other hand, using intestinal phosphatase of higher purity and a variety of different phosphate compounds, was able to clarify the substrate spectrum for this enzyme. Morton found that all true orthophosphate monoesters were hydrolyzed by intestinal alkaline phosphatase as well as the orthophosphoamide, phosphocreatine, and phosphoenol-pyruvate. He also pointed out that the thiophosphates may be hydrolyzed by this enzyme, since the (-S-P) bond is more analogous to the (-O-P) bond in orthophosphates than is the (-N-P) bond in phosphoamides (65). The orthophosphate diesters (diphenyl phosphate), triesters (trimethyl and triphenyl phosphate), polyphosphates (sodium pyrophosphate, diphenyl pyrophosphate, ATP, ADP, DPN, etc.), were not hydrolyzed by the enzyme (65). This implies absence of pyrophosphatase diesterase activity from Morton's enzyme

preparation. TPN, having the third phosphate group esterified to ribose was hydrolyzed by the intestinal alkaline phosphatase and quantitatively converted into DPN (63).

Melander et al., using a commercial sample of purified intestinal alkaline phosphatase, demonstrated that synthetic o-phosphorylated serine peptides (monoesters) were readily hydrolyzed by the enzyme; diesters and triesters were resistant to this enzyme. Such, or similar phosphorylated peptides believed to be constituent of tissue proteins, are valuable model compounds for the study of the biological function of phosphoproteins (53).

Delory and King examined a series of phosphate esters (substituted aromatic and aliphatic esters) with respect to the rate of hydrolysis by intestinal alkaline phosphatase and concluded that with decreasing pK of the second acid group of the phosphoric acid, the rate of hydrolysis increases progressively and the enzyme is optimally active at a more alkaline pH (19). The Michaelis constant (Km) decreased with decreasing pK of the substrate. Morton observed that there is a variation of rate of hydrolysis with different substrates (65). The Km values for adenosine 3'- and 5'- phosphates, 2.0×10^{-4} and 1.1×10^{-2}M respectively, differ considerably, although these two compounds are closely related. Indeed, the pK values (19) of phenyl phosphate and β-glycerolphosphate (5.73 and 6.34 respectively) and that of phosphocreatine (4.6), (16), do not show any direct relationships with the reported Km values (65) for these substrates (8.6×10^{-4}M, for phenyl phosphate; 1.7×10^{-2}M for β-glycerolphosphate and 3.5×10^{-2}M for phosphocreatine). Differences in

experimental conditions (pH and purity of enzyme) used by these workers might account for the above observations.

The pH optimum for alkaline phosphatases from various tissues has been shown by many investigators to vary with initial substrate concentrations, type of substrate and buffer used (8,31,66,69,70,74, 90). Ross et al., using rat intestinal phosphatase and a variety of phosphorylated metabolites, demonstrated that at the optimum pH the rate of hydrolysis was found to be lower when the concentration of substrate was lower, but the small amount of phosphate released represented a greater proportion of available phosphate (74).

The optimum pH values obtained in vitro with β-glycerol-phosphate and some naturally occuring organic phosphates are considerably lower than the values obtained with phenyl phosphate. These observations suggested that alkaline phosphatases may be quite active at physiological pH values, which are near neutrality (19,65, 74).

Intracellular alterations of pH combined with activators and inhibitors present in the cells would offer an effective means of controlling phosphatase activity and therefore intracellular concentrations of phosphorylated metabolites (66,74). They observed, for example, that at a concentration of 2.7×10^{-6} M ribonucleic acid as a substrate the pH optimum for the enzyme was 7.6.

The purpose of this investigation was to obtain information concerning the hydrolytic and transferase site(s) of the intestinal alkaline phosphatase, through the use of inhibition kinetics. As a prerequisite to this, the purification of the enzyme, its kinetic

behavior towards pH and substrate, and the mechanism of inhibition by cysteine and iodine with respect to hydrolytic activity, were studied.

MATERIAL AND METHODS

General. The experimental conditions for the enzyme reaction vessels are given in detail under each Figure and Table. Throughout these experiments, the enzyme reaction vessels were set up in duplicate..

Control vessels were incorporated in every experiment. These control vessels contained the same components as the assay reaction vessel, except that the enzyme activity was arrested before the addition of substrate or the particular substance by which the reaction was commenced.

The enzyme reaction was stopped either by; (a) the addition of Folin and Ciocalteu's phenol reagent, (b) by perchloric acid, or (c) by heating the reaction vessel in a boiling water bath for 30 seconds.

(a) Following the indicated reaction time under each experiment, one ml. of diluted Folin and Ciocalteu's phenol reagent was added to the reaction tube, and the contents of the tube were analyzed for phenol.

(b) In some experiments, 0.1 ml. of 50% (v/v) perchloric acid was used to stop the reaction. Aliquots then, were taken from the reaction vessel for phenol and phosphorus analyses. This reagent had no interfering effect on phenol and phosphorus determinations.

(c) In all experiments dealing with the hydrolytic and phosphotransferase activities of intestinal alkaline phosphatase the reaction was stopped by heating the tube in a boiling water bath for 30 seconds. This method of stopping the enzymic reaction was selected because of the interfering

effect of perchloric acid and trichloracetic acid on the enzymic assay of glucose-6-phosphate.

The control tubes incorporated in the experiments in which glucose-6-phosphate, inorganic phosphate and phenol were determined, contained all the components of the reaction except the substrate, which was added just prior to heating the control tube in a boiling water bath for 30 seconds. After stopping the enzyme reaction, both the experimental and control reaction tubes were placed in an ice-water bath until aliquots were taken for the various analyses.

The low concentration of inhibitors, used in these studies, iodine and cysteine, had no interfering effect on phenol, inorganic phosphate and glucose-6-phosphate determinations.

All the concentrations of reactants including the inhibitors, were expressed as molar concentrations based on the total volume of the reaction mixture.

Purification of the Intestinal Alkaline Phosphatase. The commercially available alkaline phosphatase "purified" from calf intestinal mucosa, was used as the starting material for the following two techniques of purification:

1. Purification by continuous flow electrophoresis (Spinco Model C P). Two grams of intestinal alkaline phosphatase (batch No. P29-78) were dissolved in 50 ml. demineralized water and dialyzed against water for 15 hours at 4°C and then lyophilized. One gram of the lyophilized material was dissolved in 30 ml. barbital buffer pH 8.6 (0.736 gram of diethyl barbituric acid and 4.107 gram of sodium diethyl

barbiturate per liter of water), ionic strength (μ) of .02 and placed in the left hand feed tab of the curtain in the refrigerated (4°C) flow electrophoresis, using barbital pH 8.6 (μ=.02) as the developing buffer. Other conditions of the electrophoresis were: Overflow and wick feed 6.9. Feed rate 2.5. 50 milliamperes and 740 volts.

The fractions collected were screened qualitatively for the presence of the enzyme by adding 0.1 ml. solution from each fraction to 2 ml. of a solution containing 0.075 M ethanolamine pH 10 and 1×10^{-2} M p-nitrophenyl phosphate. An intense yellow color, due to p-nitrophenol liberated by the action of alkaline phosphatase on the substrate, appeared within one minute. The fractions containing most of the enzyme activity were in tubes 15 to 22. Following dialysis and lyophilization of each fraction the specific activity (μmoles phenol per mg. enzyme protein per 5 minutes) was determined.

2. Purification with acetone fractional precipitation. Two grams of intestinal alkaline phosphatase were dissolved in 100 ml. of water and pH adjusted to 6.4 with .05 M acetic acid. Thereafter, the enzyme containing solution was subjected to acetone fractionation using stages 4, 5 and 6 of Morton's technique (60). Following stage 6 the enzyme solution was lyophilized and stored in an evacuated desiccator at refrigerator temperature (approximately 5°C).

All enzyme solutions were made with demineralized water (pH 6.5) and stored in the refrigerator (5°C). No loss of

enzymic activity occurred for a period of 10 days when enzyme solution was kept at refrigerator temperature.

This enzyme preparation was employed in all experiments described in this thesis, unless otherwise indicated.

Protein Determination. The protein content for all the enzyme solutions employed, unless otherwise indicated, was determined spectrophotometrically using Kalckar's equation (48).

Protein concentration (mg. per ml.) = $1.45\ D_{280} - 0.74\ D_{260}$ where D_{280} and D_{260} are optical densities at 280 and 260 mμ respectively.

Phenol. This was estimated using the Folin and Ciocalteu's phenol reagent. The reagent was made according to King (40) and can also be purchased from Fisher Scientific Co.; one volume of reagent was diluted with two volumes of water, before use. The determination of phenol was as follows:

An aliquot of the enzymic reaction mixture or standard phenol solution was added to a test tube containing sufficient water to make a total volume of 3.0 ml. To this solution, 1.0 ml. of diluted Folin and Ciocalteu's phenol reagent was added and mixed well. Following this 1.0 ml. of 2 M Na_2CO_3 was added, mixed and the color development took place in a water bath at 38°C for 20 minutes (65).

The optical density was measured in a Beckman DU Spectrophotometer at 760 mμ using a 1 cm (light path) cuvette. The reagent blank contained 3 ml. of water in a place of phenol containing solution 1.0 ml. of phenol reagent and 1.0 ml. of Na_2CO_3. The

760 mμ wavelength was found to be optimum, giving maximum sensitivity and good reproducibility. A calibration curve was constructed by using varying quantities of phenol standard solution. The optical density for one μmole phenol in a total volume of 5 ml., at a wavelength of 760 mμ, was 2.25 units.

It was found, however, that in the presence of glucose the phenol color was enhanced by a factor related to the quantity of glucose present. The optical density readings obtained with control tubes, containing glucose (varying quantities), phenol reagent, and Na_2CO_3 but no standard phenol solution, although proportional to glucose concentration in the vessel, could not be used to correct the higher optical density readings obtained when standard phenol solution was present. The estimation of phenol, therefore, when glucose was present in the enzyme reaction vessel was compared with standard phenol solution run under the same conditions as the unknown.

Inorganic Phosphate. This was determined according to Taussky and Shorr (82).

To a suitable aliquot, sample or standard phosphate solution (KH_2PO_4), enough 0.7 N H_2SO_4 was added to make a total volume of 1.5 ml. To this, 1.5 ml. of ferrous sulfate-ammonium molybdate reagent (made up freshly before use) was added, mixed well, and the optical density reading was obtained at 720 mμ using a Beckman DU Spectrophotometer.

Glucose-6-Phosphate. Glucose-6-Phosphate was determined enzymatically using glucose-6-phosphate dehydrogenase and TPN according to

Horecker and Wood (37). The reaction can be described as follows:

$$\text{G-6-P} + \text{TPN} \xrightarrow{\text{G-6-P dehydrogenase}} \text{6-phosphogluconic acid} + \text{TPNH}$$

In the experiments where glucose-6-phosphate was formed by intestinal alkaline phosphatase 1.0 ml. aliquot was taken from the enzymic reaction vessel and placed in a test tube containing 1.5 ml. of .25 M glycylglycine buffer pH 7.6. To this, 0.10 ml. of 0.1 M $MgCl_2$, 0.60 µmole TPN and 0.2 Kornberg unit (43) were added and made to a final volume of 3.0 ml. with water. The solution in the tube was mixed, covered with parafilm and left at room temperature (about 21°C) for approximately 40 minutes until the reaction was completed. The optical density of the solution was obtained at 340 mµ in a 1 cm (optical path) cuvette using a Beckman DK-1 Spectrophotometer. The reference cell contained water in place of the aliquot. It was found that the reaction was complete after 40 minutes standing at room temperature.

The amount of glucose-6-phosphate present in the aliquot taken was calculated as follows:

$$\text{G-6-P (µmoles/aliquot used)} = \frac{\text{O.D. sample} - \text{O.D. control}}{\text{2.07 density units/µmole of TPNH}}$$

The molar absorbancy index (a_m) for reduced TPN is $6.22 \times 10^6 \text{cm}^2 \times \text{mole}^{-1}$ at 340 mµ (45). By using known quantities of glucose-6-phosphate (disodium salt) an optical density of 2.08 to 2.2 per µmole of glucose-6-phosphate was obtained. These correspond to a molar absorbancy index of 6.24 to $6.6 \times 10^6 \text{cm}^2 \times \text{mole}^{-1}$ respectively. Kornberg pointed out that higher values of molar absorbancy index were

observed with glucose-6-phosphate dehydrogenase preparations contaminated with 6-phosphogluconic acid dehydrogenase activity. By aging the glucose-6-phosphate dehydrogenase solution 24 hours at 3°C this interference was removed (44) and a molar absorbancy index of $6.24 \times 10^6 cm^2 mole^{-1}$ was obtained using different amounts of glucose-6-phosphate.

Buffers. The pH measurements for the ethanolamine-HCl, $NaHCO_3$-Na_2CO_3 and glycylglycine buffers employed were made at room temperature (approximately 21°C) using a Beckman G-pH meter.

The pH of the buffered-acceptor solution, used for the studies on the transferase activities, was adjusted to pH 10 (at 21°C) with NaOH and this solution contained .06 M $NaHCO_3$-Na_2CO_3 and 1.6 M glucose, unless otherwise indicated.

Substrate. Phenyl phosphate disodium salt was used throughout these experiments, unless otherwise indicated. No free phenol was detected in this reagent when 1.0 ml. of 0.1 M phenyl phosphate was tested for phenol. All solutions of the substrate were made with demineralized water and stored in the refrigerator.

Inhibitors. The preparation of a stock solution containing iodine .05 M and .15 M KI was made according to Pierce and Haenisch (71), and stored in a low actinic flask. Dilutions of the stock solution were made with demineralized water and appropriate aliquots were taken for the inhibition studies to be reported.

A stock solution of 1.6 mM of DL-CySH.HCl (neutralized with weak NaOH solution) was made weekly and kept in the refrigerator. Proper dilutions of the stock solution were made with demineralized water.

Biochemical Reagents

Source: Sigma Chemical Company, 3500 DeKalb Street, St. Louis 18, Missouri.	Source: Mann Research Laboratories, Inc., 136 Liberty Street, New York 6, New York.
Phosphatase, Alkaline "Purified" Calf	Triphosphopyridine Nucleotide Sodium Salt
Sigma 104 Phosphatase Substrate (p-nitrophenyl phosphate disodium)	Glycylglycine, C.P.
Glucose-6-Phosphate Disodium Salt	DL-Cysteine Hydrochloride (P)
Glucose-6-Phosphate Dehydrogenase, Type II	Monophenol Phosphate Disodium
	D (+) Dextrose Anhydrous

RESULTS

Purification of Intestinal Alkaline Phosphatase. Before attempting to study the inhibition of intestinal alkaline phosphatase by cysteine and iodine, it was found necessary to further purify the commercially available phosphatase preparation. This enzyme had been purified by Sigma Chemical Company (personal communication) according to the method of Schmidt and Thannhauser (75).

In the present study a further purification of the purchased enzyme preparation was attained by continuous flow electrophoresis and fractional precipitation with acetone. The specific activities and the degree of purification of different fractions obtained through the above mentioned procedures are presented in Table I.

The fractions collected in tubes 18, 19 and 20, (Table I-A), demonstrated higher specific activities and the degree of purification was 3.6 and 3.4 respectively. The combined fractions 18, 19, and 20, showed a typical protein ultraviolet absorption spectrum with a maximum at 278-280 mμ. The absorbancy at 280 mμ of a water solution containing .956 mg. per ml. was 1.05 units. No further attempt was made to purify the flow electrophoresis preparation.

The acetone fractional precipitation technique utilizing stages 4, 5 and 6 of Morton's method (60) produced a 9.1 fold purification over the starting phosphatase solution (Table I-B). The 32 to 40% (v/v) acetone fraction was more than twice the purity obtained from the electrophoretic preparation. Ultraviolet absorption spectra of the acetone preparation and the commercial preparation are shown in Figure 1-a, and 1-b, respectively. Both preparations showed a

TABLE I

Purification of Intestinal Alkaline Phosphatase

A. Continuous Flow Electrophoresis

The reaction mixture (3.0 ml) contained ethanolamine-HCL buffer .05 M at pH 10.2, phenyl di-sodium phosphate 2.5×10^{-2}M and .01 M magnesium acetate. The reaction was commenced by adding a suitable aliquot of each enzyme solution to the reaction mixture.

Reaction time 5 minutes at 38°C. Reaction stopped by addition of 1.0 ml of phenol reagent. Control tube contained all components, and under the same conditions, except that the reaction was stopped with 1.0 ml of phenol reagent before addition of enzyme.

The protein content of the enzyme solution was determined by the biuret method. *

B. Fractional Precipitation with Acetone

The reaction volume (2.5 ml) contained ethanolamine-HCL .06 M buffer pH 10.0, magnesium acetate 1.2×10^{-2}M, enzyme, and p-nitrophenyl phosphate 1.22×10^{-2}M. The reaction was commenced by addition of substrate following temperature equilibration of the reaction vessel at 38°C for 15 minutes. The reaction time was 5 minutes at the same temperature and the reaction was stopped by adding 1.0 ml of 0.5 N NaOH. The p-nitrophenol liberated was determined spectrophotometrically at 410 mμ.

* The assistance of Miss Ann Pearly on the protein analyses of the different electrophoretic fractions is appreciated.

TABLE I

Purification of Intestinal Alkaline Phosphatase

A. Continuous Flow Electrophoresis

Fraction corresponding to tube No.:	µmoles phenol per mg enzyme protein per 5 minutes	Purification
15	7.33	<1
16	22.9	<1
17	70.2	1.9
18	133	3.6
19 & 20	125	3.4
21 & 22	25.9	<1
Starting enzyme solution	36.7	1

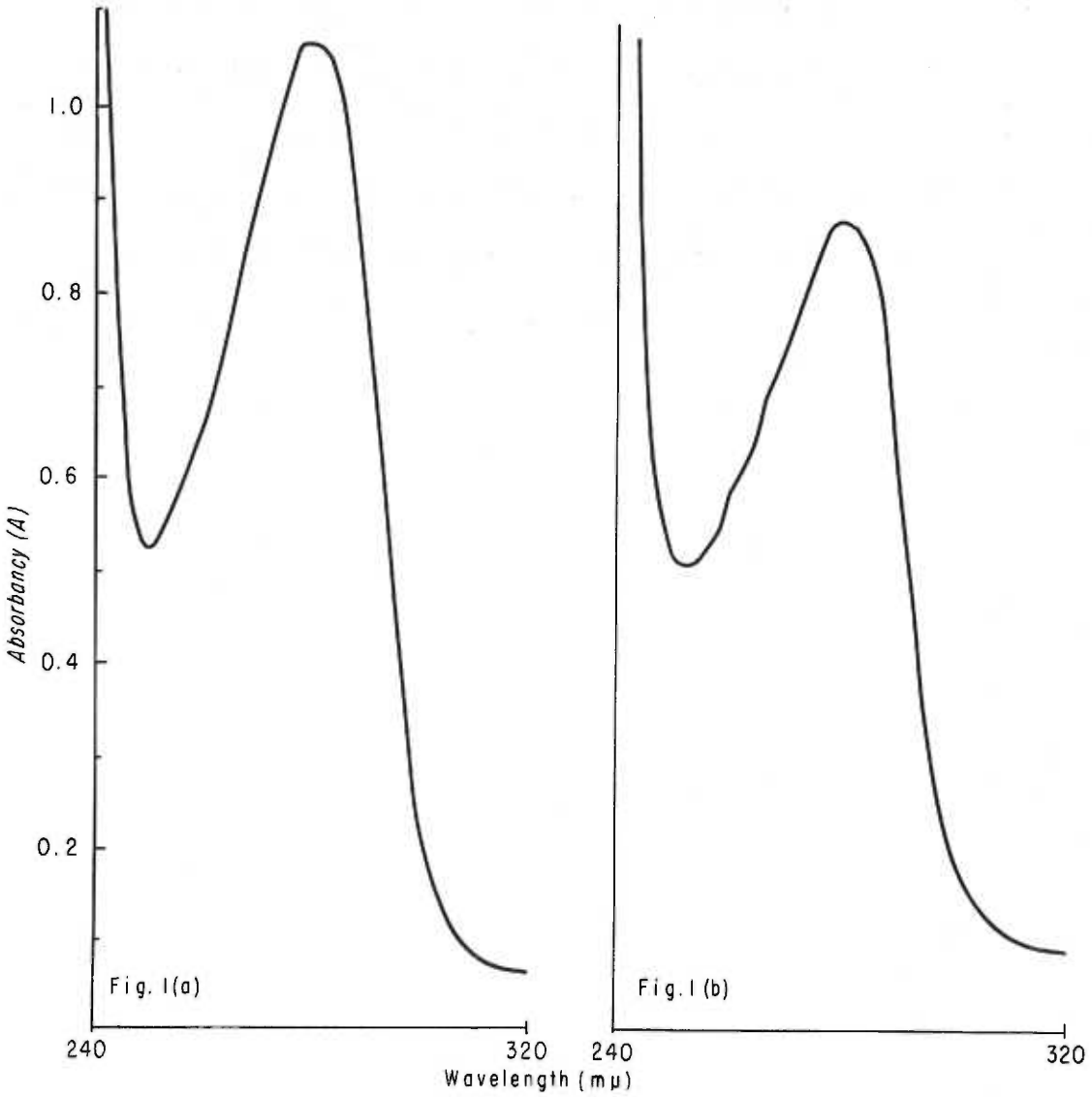
B. Fractional Precipitation with Acetone

Step	Acetone fraction % (V/V)	µmoles p-nitro phenol per mg enzyme protein per 5 minutes	Purification
1.	60	12.3	1.1
2.	35-48	27.5	2.4
3. After heating to 48° for 2 min.			
a.	32-40	106	9.1
b.	40-50	20.1	1.7
Starting phosphatase solution		11.6	1.0
Electrophoretic preparation		45.8	3.9

Figures 1-a and 1-b. Ultraviolet-absorption spectra of phosphatase preparations in demineralized water (pH 6.5).

Figure 1-a. Phosphatase preparation obtained by fractional precipitation with acetone. Solution contained 1.147 mg of the material per ml.

Figure 1-b. Commercial phosphatase preparation following drying over P_2O_5 under vacuum for 24 hours at 21°C. Solution contained 1.026 mg of the material per ml.


1.0
0.8
0.6
0.4
0.2
Absorbancy (A)
Fig. 1(a)
240
320
Fig. 1 (b)
240
320
Wavelength (mμ)

maximum absorption at 278-280 mμ. The phosphatase preparation corresponding to acetone fraction 32 to 40% (v/v) gave an absorbancy, Figure 1-a, at 280 mμ of .94 per mg. of material per ml. of water solution. The commercial phosphatase preparation, Figure 1-b, on the other hand gave an absorbancy at 280 mμ of .83 per mg. of material per ml. Using Kalckar's formula for estimating the enzyme protein content of a solution, (see experimental, under protein determination), it was found that the acetone preparation contained 93% protein, while the commercial preparation contained only 82% protein.

Paper electrophoresis of the different preparations, Figures 2-a, 2-b, and 2-c, revealed that, the commercial preparation consisted of more than three components, Figure 2-a, the electrophoresis preparation contained only one component, Figure 2-b, and the acetone fraction (32 to 40%) contained two components. The fraction carrying the phosphatase activity (shaded area), in all preparations, was found to be in the same region. Ahmed et al., found that the electrophoretic mobility of dog intestinal alkaline phosphatase, prepared by autolysis and also by butanol extraction, corresponded to that of α_2-globulin (3). In the present study however, when the electropherogram was compared to a normal serum protein pattern, the active region corresponded to β-globulin. This was only comparative and no attempt was made to verify the position or calculate the mobility of the active component. The phosphatase preparation obtained by acetone fractionation was employed in all the experiments to be described in this thesis; the reason being that it had the highest specific activity (μmoles p-nitrophenol per mg. enzyme

Figures 2-a, 2-b and 2-c. Paper electrophoresis* of intestinal alkaline phosphatase preparations. Shaded area shows the fraction where phosphatase activity was detected.**

Figure 2-a. Commercial Phosphatase preparation (.04 ml/strip of a 5.7 percent (w/v) water solution).

Figure 2-b. Phosphatase purified by continuous flow electrophoresis (.04 ml/strip of a 4.2 percent (w/v) water solution).

Figure 2-c. Phosphatase preparation obtained by fractional precipitation with acetone. (.04 ml/strip of a 4.6 percent (w/v) water solution)

* The paper electrophoretograms were developed (veronal buffer pH 8.6, μ=.075; 16 hours at room temperature) and stained (bromphenol blue in methanol) by Miss Glaydis Basinger according to Spinco Model R paper electrophoresis system - Instruction Manual (RIM-5). Her contribution is acknowledged.

** Phosphatase activity was demonstrated on a duplicate strip (not stained) with p-nitrophenyl phosphate in glycine buffer pH 10.

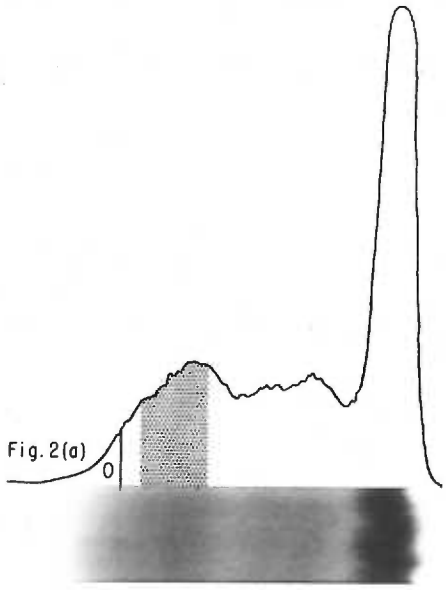


Fig. 2(a)

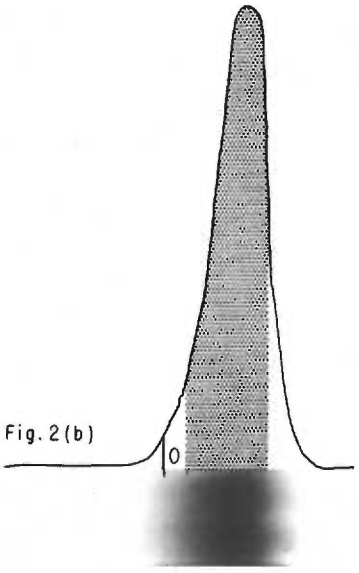


Fig. 2(b)

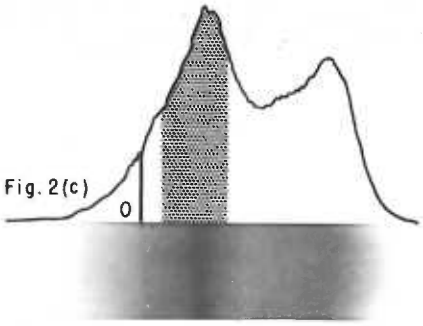


Fig. 2(c)

TABLE II

Influence of Added $MgCl_2$ on the Hydrolytic Activity of Phosphatase

Experiment 1. Reaction commenced by addition of substrate.

Experiment 2. Reaction commenced by addition of enzyme.

The reaction mixture (3.0 ml) consisted of .05 M buffer ($NaHCO_3$-Na_2CO_3) pH 9.6, 2.5×10^{-2} M phenyl phosphate, varying $MgCl_2$ concentrations, 2.01 µg enzyme protein. Following a 15 minute preincubation of the reaction vessel at 38°C, the reaction was commenced by adding either substrate or enzyme as indicated above. Reaction period 10 minutes at 38°C; the reaction was stopped with 1.0 ml of phenol reagent.

TABLE II

Influence of Added $MgCl_2$ on the Hydrolytic Activity of Phosphatase

$MgCl_2$ concentration (M)	µmole phenol/tube/10 min.	
	Experiment 1.	Experiment 2.
---	.442	.450
$1x10^{-2}$	.526	.539
$5x10^{-3}$	.548	.558
$1x10^{-3}$	.562	.549
$1x10^{-4}$	.548	.487
$5x10^{-5}$	.531	.456
$1x10^{-5}$	.526	.456
$1x10^{-6}$	.473	.451
$5x10^{-7}$	.473	---

proteins per 5 minutes), although paper electrophoresis revealed two components present in this preparation.

Before embarking into the kinetic behavior of phosphatase towards phenyl phosphate, the influence of Mg^{++} on the hydrolytic activity of this enzyme preparation was investigated. The results of such an experiment are presented in Table II. An increase in the hydrolytic activity of phosphatase was produced in the presence of $1x10^{-3}$M $MgCl_2$. This increase was about 25% higher over the activity of the reaction vessel to which $MgCl_2$ was not added. In experiment 1, the enzyme was preincubated with $MgCl_2$ at pH 9.6 and the reaction was commenced by adding the substrate. In experiment 2, the reaction was commenced by adding the enzyme without preincubation with $MgCl_2$. The hydrolytic activity (µmole phenol per 10 minutes) in both experiments was not significantly different within the range of $MgCl_2$ concentration of $1x10^{-3}$ to $1x10^{-2}$M. A slight decrease in activity was noted in experiment 2, as compared to experiment 1, when $1.0x10^{-5}$ to $1x10^{-4}$M $MgCl_2$ was present. No significant change in activity was observed between experiment 1 and experiment 2 when $MgCl_2$ was not added.

From the results presented in Table II, the $MgCl_2$ concentration employed in subsequent experiments ranged from $1.0x10^{-3}$ to $1.5x10^{-3}$M.

The Kinetic Behavior of Alkaline Phosphatase towards Phenyl Phosphate. The relationship of initial velocity to substrate concentration at different pH values was investigated and the data are presented in Table III. The buffer employed for all the pH values was $NaHCO_3$-Na_2CO_3. At pH 8.3 the buffer capacity of this system $NaHCO_3$-Na_2CO_3 is very low, since the pK_2 of H_2CO_3 at 25°C is 10.36. In order to keep the same buffer throughout the pH spectrum, $NaHCO_3$ adjusted to pH 8.3 with Na_2CO_3 was employed in the reaction mixture.

The results in Table III were expressed as µmole phenol liberated per 5 minutes and were calculated based on initial velocities. It was found that the initial velocity of the hydrolysis of phenyl phosphate (1.0×10^{-3} to 2.5×10^{-2}M) by phosphatase was linear up to 12% hydrolysis of the substrate. With low substrate concentrations, Figure 3, the initial rate of hydrolysis was about 14% for 7.5×10^{-4}M, 18% and 15% for 5.0×10^{-4}M and 1×10^{-4}M substrate, respectively. The reaction time, where low substrate concentrations were employed, was such that the hydrolysis of phenyl phosphate by the enzyme never exceeded 12%.

Representative plots of the initial velocity against pH at various substrate concentrations, Figure 4-a, demonstrated the marked dependence of pH optimum on substrate concentration. Such a change of pH-optimum with substrate concentration has been reported by other workers (66,69,74). With low substrate concentration, the pH optimum (D and E plot of Figure 4-a and 7.5×10^{-5}M substrate of Table III) was not well defined.

In Figure 4-b, the reciprocal of initial velocity was plotted

TABLE III

Relationship of Initial Velocity to Substrate Concentration and pH.

The reaction vessels (total volume of 3.0 ml) contained .05 M buffer ($NaHCO_3$-Na_2CO_3) at the indicated pH, 1.0×10^{-3} M $MgCl_2$, and 7.83 µg of enzyme protein. The reaction was commenced by the addition of substrate (phenyl phosphate), following a preincubation period of 15 minutes at 38°C. The enzymic reaction having proceeded for an appropriate time interval within the limits of initial rate, was stopped with Folin and Ciocalteu's phenol reagent and thereafter analyzed for phenol.

TABLE III

Relationship of Initial Velocity to Substrate Concentration and pH.

Substrate concentration (M)	pH								
	8.30	8.90	9.25	9.40	9.60	9.82	10.1	10.2	10.4
	μmole phenol per 5 minutes								
7.5×10^{-2}	.267	.390	.523	.588	.715	.907	1.17	1.17	1.31
2.5×10^{-2}	.304	.513	.718	.787	.902	1.06	1.17	1.14	1.04
7.5×10^{-3}	.362	.613	.771	.848	.937	.991	.915	.782	.554
5.0×10^{-3}	.402	.658	.768	.876	.912	.928	.778	.646	.444
2.5×10^{-3}	.485	.656	.736	.877	.855	.701	.553	.398	.254
1.0×10^{-3}	.473	.588	.627	.622	.582	.388	.285	.182	.102
7.5×10^{-4}	.476	.564	.573	.560	.472	.342	.221	.148	.0840
5.0×10^{-4}	.450	.507	.502	.450	.378	.250	.158	.112	.0612
3.33×10^{-4}	.397	.410	.363	.317	.250	.167	.100	.0653	.0357
2.0×10^{-4}	.342	.316	.268	.225	.170	.107	.0680	.0393	.0268
1.0×10^{-4}	.223	.194	.143	.107	.0895	.0535	.0268	.0143	—
7.5×10^{-5}	.210	.180	.130	—	.0650	.0320	—	—	—
PKm*	3.78	3.66	3.47	3.29	3.12	2.80	2.53	2.32	—
pKm**	3.71	3.60	3.30	3.20	3.00	—	—	—	—

* pKm was calculated as the substrate concentration for one-half the apparent maximum velocity at each pH.

** pKm calculated from Lineweaver-Burk plots ($\frac{1}{v}$ against $\frac{1}{s}$).

against the reciprocal of substrate concentration according to Lineweaver and Burk (49) for various pH values of the enzyme reaction. The equation developed by Lineweaver and Burk for the determination of enzyme dissociation constants, describes a linear relationship when $\frac{1}{v}$ is plotted against $\frac{1}{S}$.

$$\frac{1}{v} = \frac{Ks}{Vmax\ (S)} + \frac{1}{Vmax}$$

v = initial velocity.

(S) = substrate concentration.

Vmax = maximum velocity obtained when the enzyme is saturated with substrate.

Ks = equilibrium constant of the dissociation of ES into E and S.

Morton, using a wider range of substrate concentration, showed that the Lineweaver and Burk plots at several pH values were non-linear (66). In the present study, the non-linearity of such plots is shown in Figure 4-b, for pH 9.82 and 10.09. The plots ($\frac{1}{v}$ vs $\frac{1}{S}$) for pH 8.3 and 8.9, not shown in Figure 4-b, were linear. The pKm (-log Km) values therefore were calculated from such plots and presented in Table III. Those (pKm values) corresponding to pH 9.4 and 9.6 were calculated from the best fit line drawn between points covering the substrate concentration of 2.5×10^{-3} to $1. \times 10^{-4}$M.

From the well-known Michaelis equation $v = \frac{Vmax}{1 + \frac{Km}{S}}$ the following equation was derived (24):

$$p\ (S) = pKm + \log \frac{Vmax - v}{v}$$

When v is equal to $\frac{Vmax}{2}$ then p (S) = pKm. Plots of the initial velocity of hydrolysis against the negative logarithm of substrate

Figure 3. Reaction-time curves for phenol liberation by intestinal alkaline phosphatase at low substrate concentrations.

Conditions of experiment were the same as in Table III.

Curve A. Reaction at pH 9.25 and 7.5×10^{-4}M substrate

Curve B. Reaction at pH 8.90 and 5.0×10^{-4}M substrate

Curve C. Reaction at pH 8.90 and 1×10^{-4}M substrate

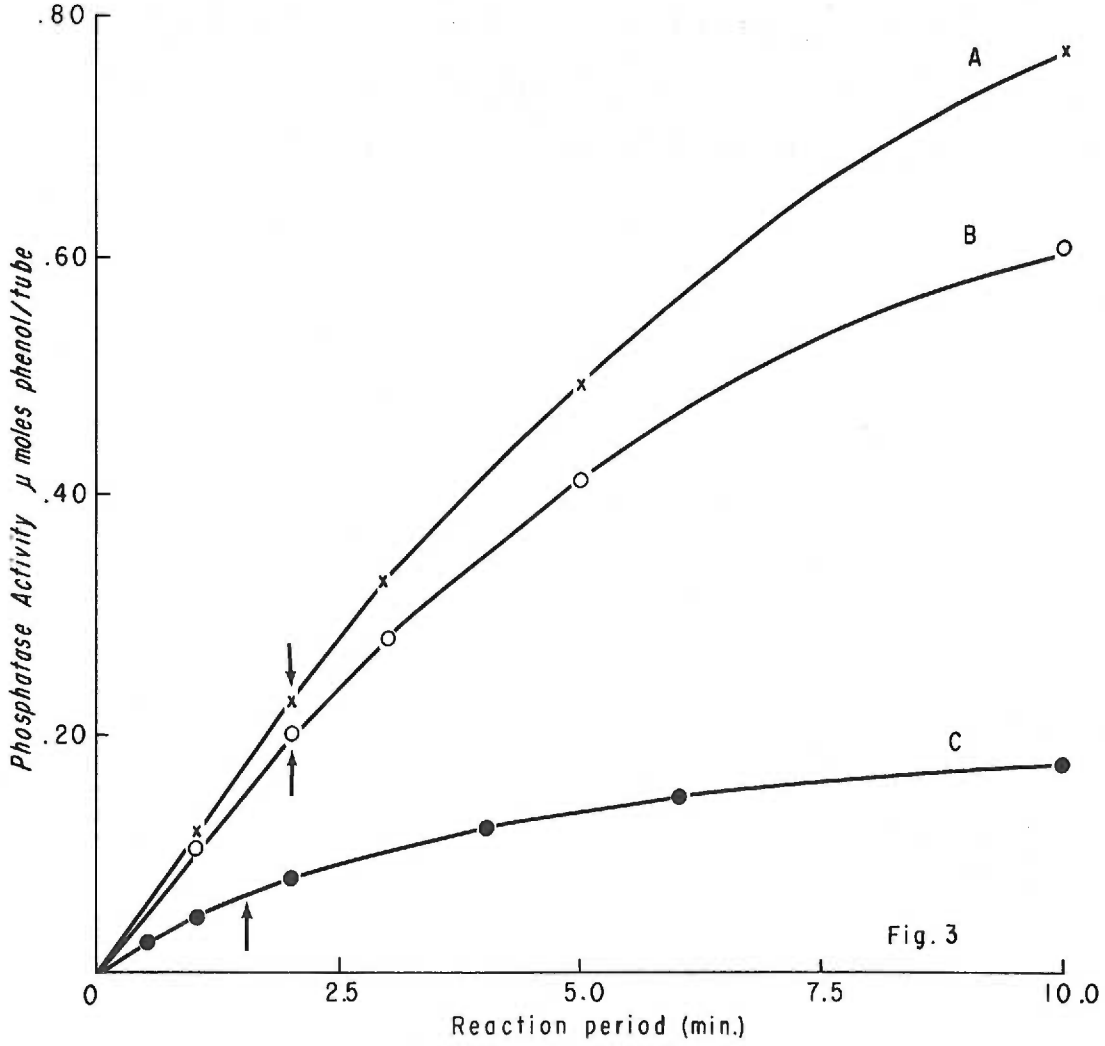

.80
.60
.40
.20
0
2.5
5.0
7.5
10.0
Phosphatase Activity μ moles phenol/tube
Reaction period (min.)
A
B
C

Fig. 3

Figure 4-a. Plots of initial rates of hydrolysis of phenyl phosphate at different concentrations, against pH.

Curve A. Substrate concentration 2.5×10^{-2}M

Curve B. Substrate concentration 5.0×10^{-3}M

Curve C. Substrate concentration 1.0×10^{-3}M

Curve D. Substrate concentration 5.0×10^{-4}M

Curve E. Substrate concentration 1.0×10^{-4}M

Figure 4-b. Reciprocal plots of the initial rate of hydrolysis ($\frac{1}{v}$), against the reciprocal of substrate concentration ($\frac{1}{s}$) at various pH values of the enzyme reaction.

Figures 4-a and 4-b, were derived from the data presented in Table III.

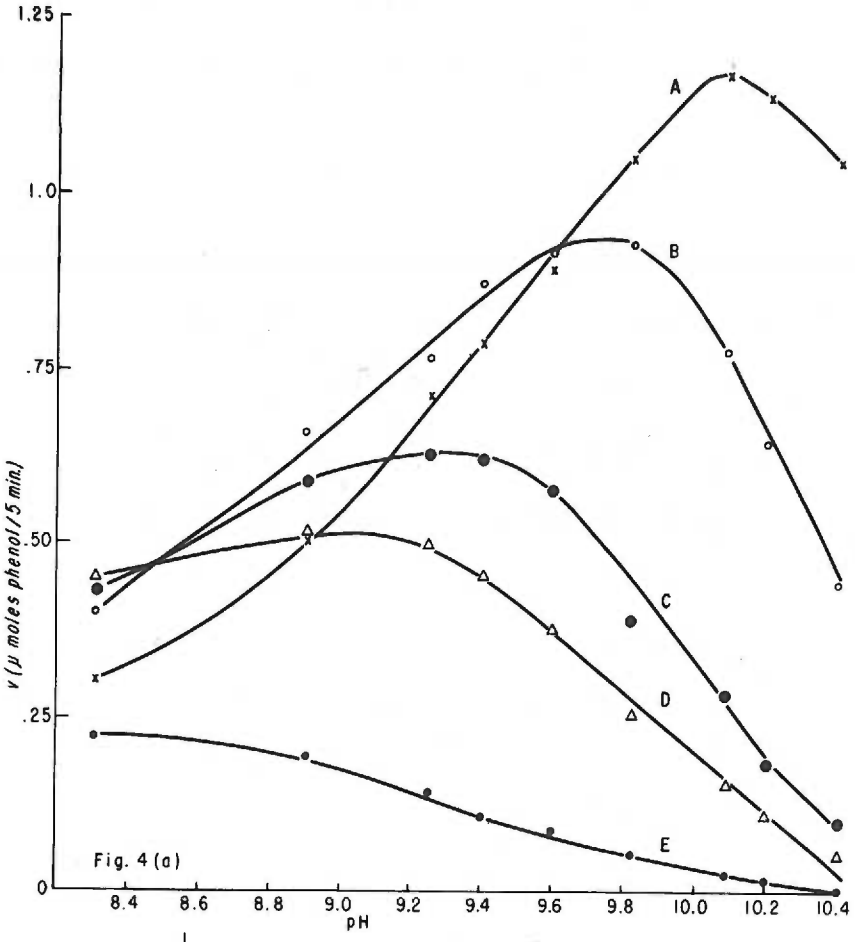

1.25
1.0
.75
.50
.25
0
v (μ moles phenol/5 min.)
A
B
C
D
E
8.4
8.6
8.8
9.0
9.2
9.4
9.6
9.8
10.0
10.2
10.4
pH

Fig. 4 (a)

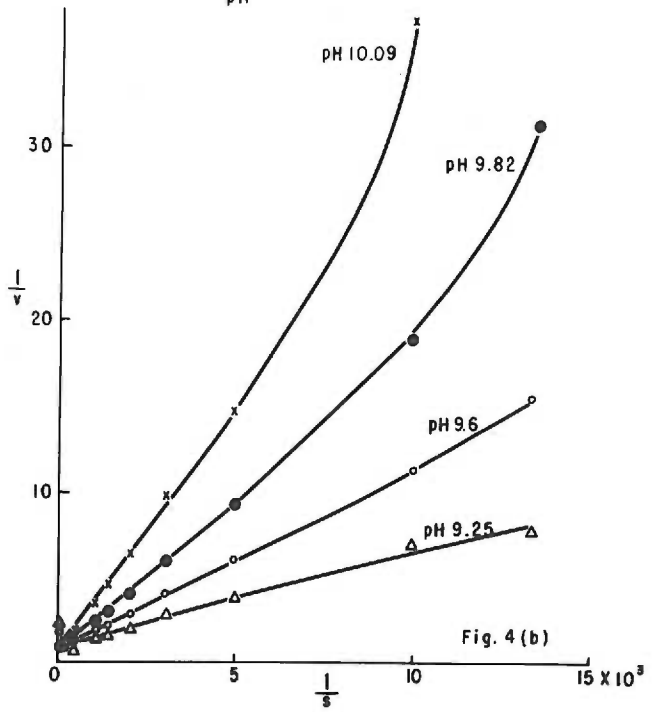

pH 10.09
pH 9.82
pH 9.6
pH 9.25
30
20
10
0
5
10
15 X 10³
1/v
1/s

Fig. 4 (b)

Figure 5-a. Variation of initial velocity (v) with p(s) at various pH values. $p(s) = -\log (s)$

Figure 5-b. Dixon plot (pKm against pH) for the hydrolysis of phenyl phosphate by intestinal alkaline phosphatase. $pKm = -\log Km$

Figure 5-c. Linear relationship of $\frac{(S)}{Umax}$ against (S). In this case Umax is the apparent maximum rate of hydrolysis of phenol phosphate observed at each substrate concentration (Table III, figure 4-a).

Figures 5-a, 5-b and 5-c, were derived from the data presented in Table III.

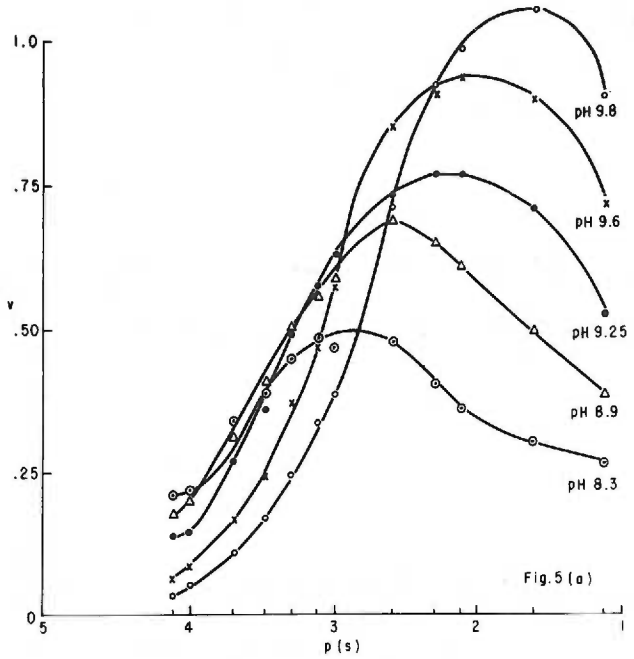


Fig. 5 (a)

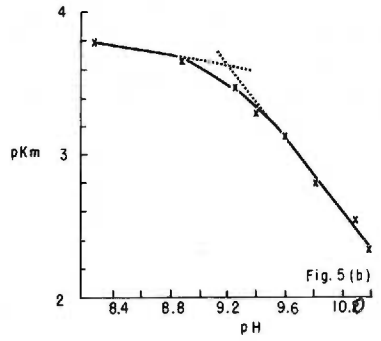


Fig. 5 (b)

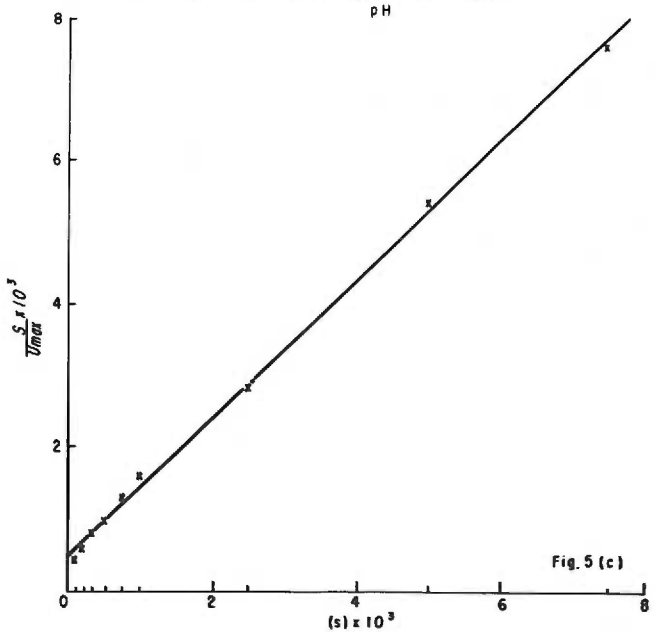


Fig. 5 (c)

concentration, p (S), for different pH values are shown in Figure 5-a. Such plots were used to calculate the pKm values for the range of pH 8.3 to 10.2 (66). The pKm for each pH plot is equal to p (S) at the point where the initial velocity (v) is one-half the apparent maximum velocity. These plots, Figure 5-a, showed that the intestinal alkaline phosphatase had a narrow range of substrate concentration over which optimal activity was obtained. Considerable inhibition of the rate of hydrolysis was also noted with higher concentrations of phenyl phosphate.

The relation of pKm, calculated from Figure 5-a, with pH is shown in Figure 5-b. This was plotted according to Dixon (23). He suggested that pKm - pH plots might yield information concerning: (a) the nature of the enzyme-substrate link, deduced mainly from the slope of the curve; (b) information about the nature of the substrate-binding groups of the enzyme, deduced from their pK values as determined from the position of the discontinuities of the curve; (c) information about the nature of the activation process, deduced from the ionizations of the enzyme-substrate complex. In the present plot, Figure 5-b, the pKm did not change considerably over the pH range 8.3 to 9.0 and the straight line drawn through those points had a slope of about -0.16. The pKm at pH values greater than 9.2 changed considerably and the line had a slope of -1.2. A discontinuity was observed at about pH 9.2. When the pkm values (calculated from Lineweaver and Burk plots) of Table III were plotted against the corresponding pH a similar change in slope (from 0 to -1 unit) was observed, and a discontinuity occurred at pH 9.1.

Other investigators have demonstrated such a variation of pKm with pH for intestinal and milk alkaline phosphatase (66,90) and for placental phosphatase (8).

Figure 5-c was plotted according to the equation $\frac{S}{v} = \frac{S}{Vmax} + \frac{Km}{Vmax}$ where Vmax was substituted with Umax which is the apparent maximum observed velocity for each substrate concentration tested at different pH values (Figure 4-a and Table III). From the slope of the line which is equal to $\frac{1}{Umax}$ and the value of $\frac{Km}{Umax}$ (when S approaches 0) the Km was found to be 4.8×10^{-4}M.

Inhibition of the Hydrolytic Activity of Phosphatase by Cysteine and Iodine. Figure 6 demonstrates the course of the hydrolytic activity in the presence of cysteine (curve C), and iodine (curve B and D), and in the absence of inhibitor (curve A), with time (in minutes). With curve A the hydrolytic activity was linear, as expected. In curves B and D the inhibition of hydrolysis by 1.25×10^{-5}M iodine is apparent, and to a greater degree in curve D. The only difference between these two curves was that in the experiment shown by curve D, the iodine was added to the reaction vessel which contained the enzyme, the buffer pH 9.8 and $MgCl_2$, while in the experiment (shown by curve B), the iodine was already present in the reaction vessel (containing the buffer and $MgCl_2$), when the enzyme was added.

For curve D, the hydrolysis of phenyl phosphate is linear up to 15 minutes reaction time and thereafter the slope of the line changes with time, while for curve B, the hydrolytic activity is linear with reaction time. The decrease of hydrolysis of phenyl phosphate after the 15 minutes reaction period might be due to a further interaction of the iodine with the enzyme.

Curve C of Figure 6, represents the experiment in which 8.0×10^{-6}M cysteine was present in the reaction mixture. The hydrolytic activity in this case was linear with reaction time up to 20 minutes and thereafter a slight increase in activity occurred. This might be due to oxidation of cysteine by air. There was no difference in activity when cysteine was added to the reaction mixture already containing the enzyme, or when the enzyme was added to cysteine.

In all subsequent experiments, unless otherwise indicated, the

Figure 6. Progress curves for the hydrolysis of phenyl phosphate by intestinal alkaline phosphatase in presence and absence of inhibitors.

The reaction mixture (2.0 ml) contained .056 M $NaHCO_3$-Na_2CO_3 buffer pH 9.8, 1.5×10^{-3}M $MgCl_2$, 45.6 µg enzyme, the inhibitor as indicated below, and phenyl phosphate 5×10^{-2}M. The reaction was preincubated at 38°C for 20 minutes and was commenced by adding the substrate. At the indicated time interval, the reaction was stopped with perchloric acid and analyzed for phenol.

Curve A. No inhibitor added.

Curve B. In presence of 1.25×10^{-5}M iodine in KI solution (in this experiment the iodine was added to the reaction vessel before the enzyme addition).

Curve C. In presence of 8.0×10^{-6}M cysteine (cysteine added to the reaction vessel following the enzyme addition).

Curve D. In presence of 1.25×10^{-5}M iodine in KI solution (in this experiment the iodine was added to the enzyme, buffer and $MgCl_2$ mixture).

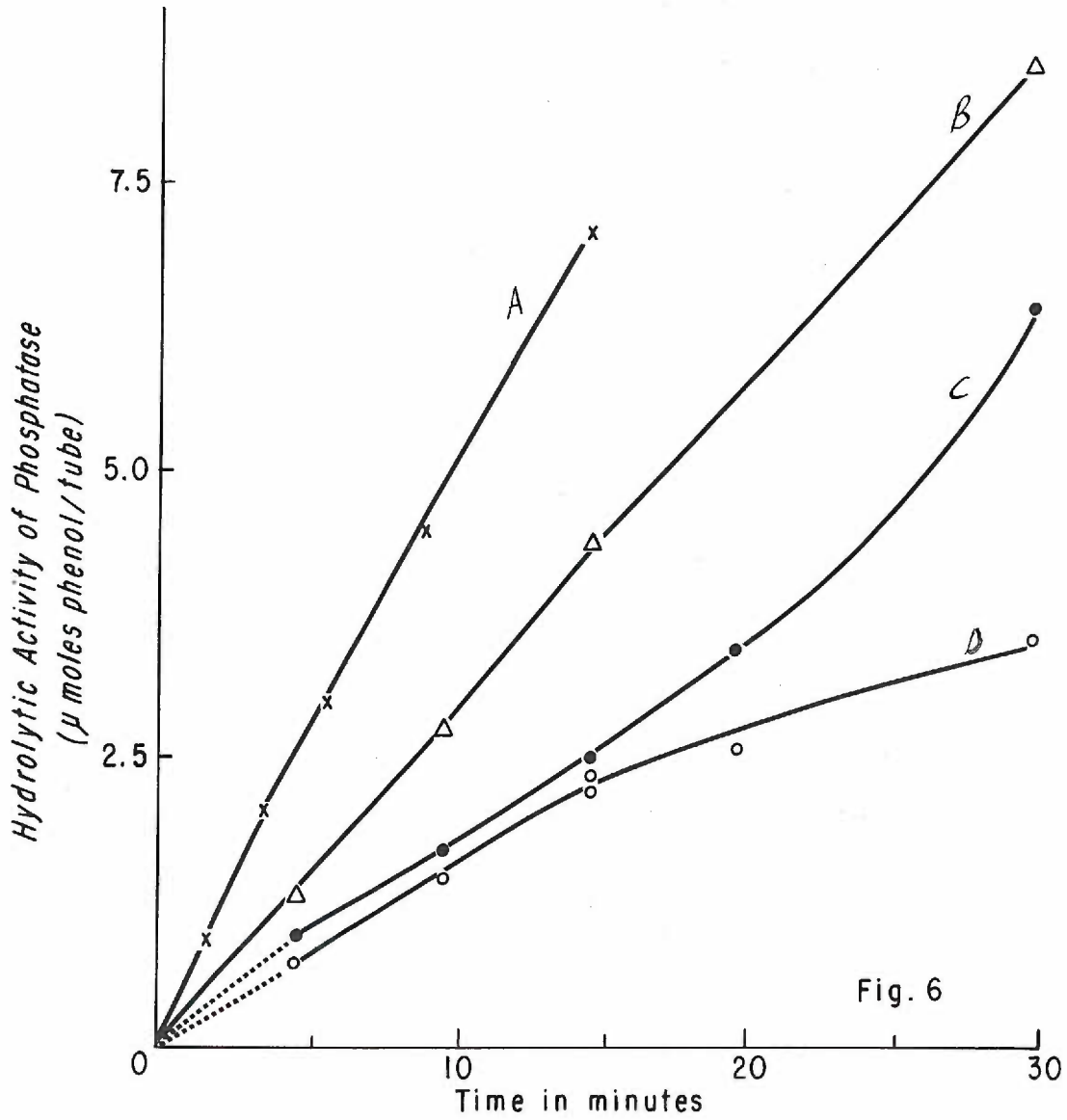

Hydrolytic Activity of Phosphatase
(μ moles phenol / tube)
7.5
5.0
2.5
0
10
20
30
Time in minutes
A
B
C
D

Fig. 6

inhibitor was always added to the reaction mixture containing the enzyme and the reaction period never exceeded 5 minutes.

The inhibition (reversible, irreversible) produced by iodine and cysteine was investigated according to the method of Ackerman and Potter (1). These investigators showed that for an irreversible inhibitor (the enzyme is converted to a form which cannot be converted back into active enzyme), a plot of the velocity of reaction against different concentrations of the enzyme intercepted the enzyme axis when extrapolated to zero velocity, and the amount of inhibition varied with enzyme concentration. The K_i, which is the dissociation constant of the enzyme-inhibitor complex or the reciprocal of the affinity of the enzyme for the inhibitor, will not be constant and it would depend on the enzyme concentration used in the experiment. For a reversible inhibitor (the enzyme recovers its activity on removal of inhibitor) the plot of rate against enzyme concentration is a straight line passing through the origin. Plots of enzyme activity against enzyme concentrations are shown in Figure 7-a and 7-b.

These plots revealed that the inhibition by iodine, Figure 7-a, is irreversible, since the slope of the lines (□ - □, ■ - ■) are the same as that of the control (x - x), and the enzyme axis is cut at a point which is proportional to the amount of inhibitor (1). With cysteine, Figure 7-b, the inhibition seemed to be reversible, since the slope of the line (△ - △) is less than in the case of the control (x - x) and the line is passing through the origin. In the presence of 7.7×10^{-6} M cysteine (▲ - ▲) the curve was linear, and

Figure 7-a. The hydrolysis rate of phenyl phosphate against enzyme concentration, in presence and absence of iodine.

(x - x) µmoles phenol and P_i (● - ●) in the absence of iodine.

(□ - □) in the presence of 6.25×10^{-6}M iodine.

(■ - ■) in the presence of 1.25×10^{-5}M iodine.

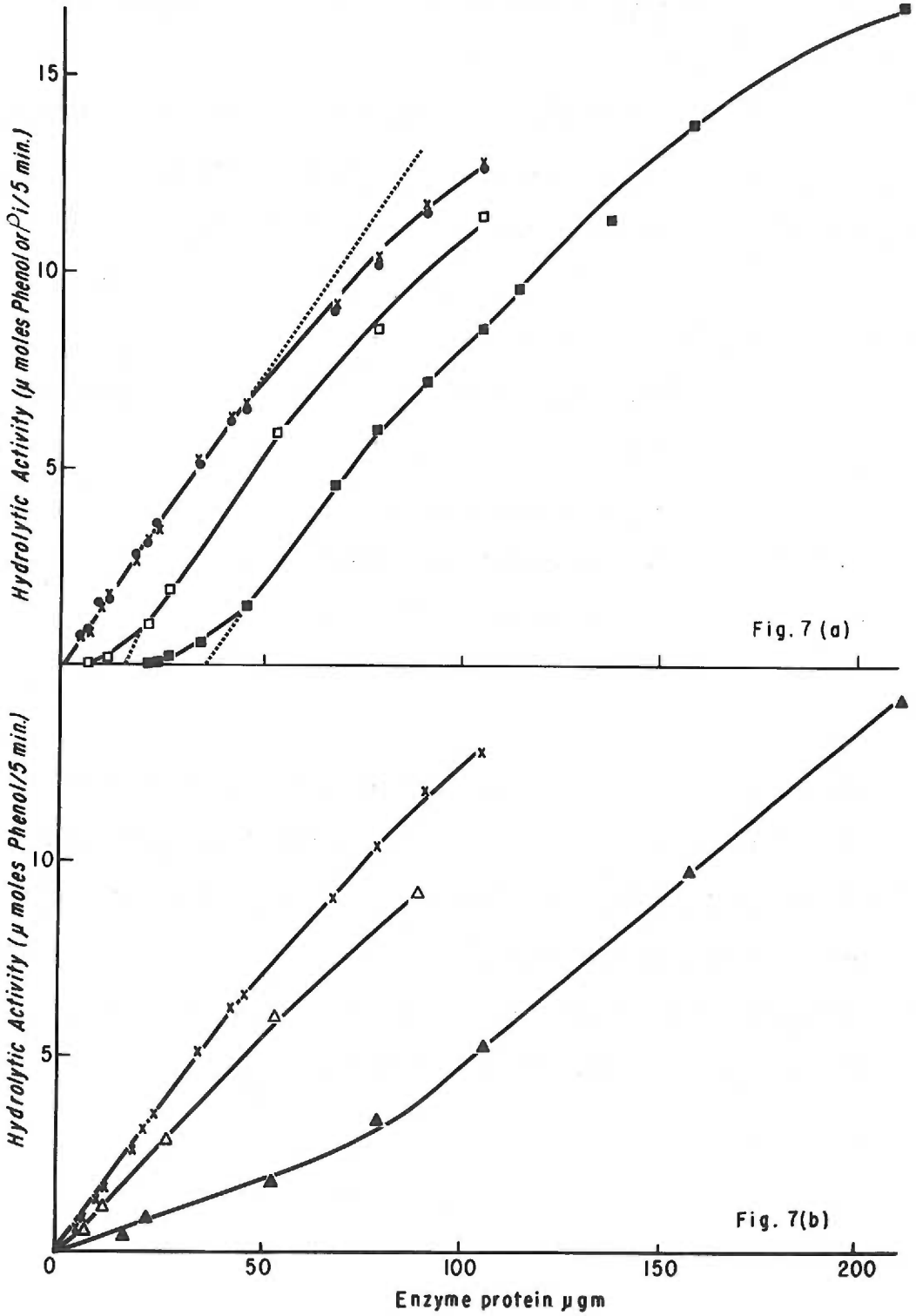
Figure 7-b. The hydrolysis rate of phenyl phosphate against enzyme concentration, in presence and absence of cysteine.

(x - x) no cysteine present.

(Δ - Δ) in the presence of 3.8×10^{-6}M cysteine.

(▲ - ▲) in the presence of 7.7×10^{-6}M cysteine.

Figures 7-a and 7-b. The reaction mixture (2.0 ml) contained .056 M buffer ($NaHCO_3$-Na_2CO_3) pH 9.8, 1.5×10^{-3}M $MgCl_2$, enzyme (indicated in the abscissa), inhibitor, and substrate 2.5×10^{-2}M. The reaction vessel was preincubated at 38°C for 20 minutes, and the reaction was commenced by adding the substrate. Reaction time 5 minutes at 38°C. The reaction was stopped with perchloric acid.


Hydrolytic Activity (μ moles Phenol or Pi/5 min.)
15
10
5
Fig. 7 (a)
Hydrolytic Activity (μ moles Phenol/5 min.)
10
5
0
50
100
150
200
Enzyme protein μgm
Fig. 7(b)

passing through the origin, up to 70 µg enzyme protein and with higher enzyme concentrations there was an increase in slope. Such a change in slope of the line with the higher enzyme concentration, might be attributed to a reversal of inhibition (oxidation of cysteine to cystine) by a contaminant present in the enzyme solution.

Shuster investigated the effect of different concentrations of cysteine on the hydrolysis of 3'-adenylic acid by 3'-nucleotidase and suggested that the inhibition produced by cysteine was non-competitive (79).

In the present study, plots of the reciprocal initial velocity ($\frac{1}{v}$) against various inhibitor concentrations (22) and also against the reciprocal of substrate concentration, were made in an attempt to elucidate the type of inhibition produced by these inhibitors. These plots are shown in Figure 8-a, 8-b, and 8-c.

The plot of Figure 8-a, for cysteine and that of Figure 8-b, for iodine did not show any linear relationship between the reciprocal of initial velocity ($\frac{1}{v}$) against inhibitor concentration. Therefore, these inhibitors could not be classified as non-competitive or competitive, according to Dixon's equations which describe either case (22). Instead, a mixed type of inhibition was obtained, Figure 8-c, when $\frac{1}{v}$ was plotted against $\frac{1}{S}$ in the presence of cysteine ($\triangle$-$\triangle$) and iodine (● - ●), and in the absence of inhibitor (O - O). These inhibitors may act on both Vmax and Km thus giving a mixture of competitive and non-competitive effects (24).

The data of Table IV describe the effect of cysteine and iodine

Figure 8-a. Reciprocal plot of the initial rate of hydrolysis of phenyl phosphate by intestinal alkaline phosphatase at pH 10.2 at various substrate concentrations against concentrations of cysteine.

(x - x) substrate concentration 2.5×10^{-2} M

(O - O) substrate concentration 1.0×10^{-2} M

(Δ - Δ) substrate concentration 5×10^{-3} M

Figure 8-b. Reciprocal plot of the initial rate of hydrolysis of phenyl phosphate by intestinal alkaline phosphatase at pH 10.2 at various substrate concentrations against concentrations of iodine.

(O - O) substrate concentration 2.5×10^{-2} M

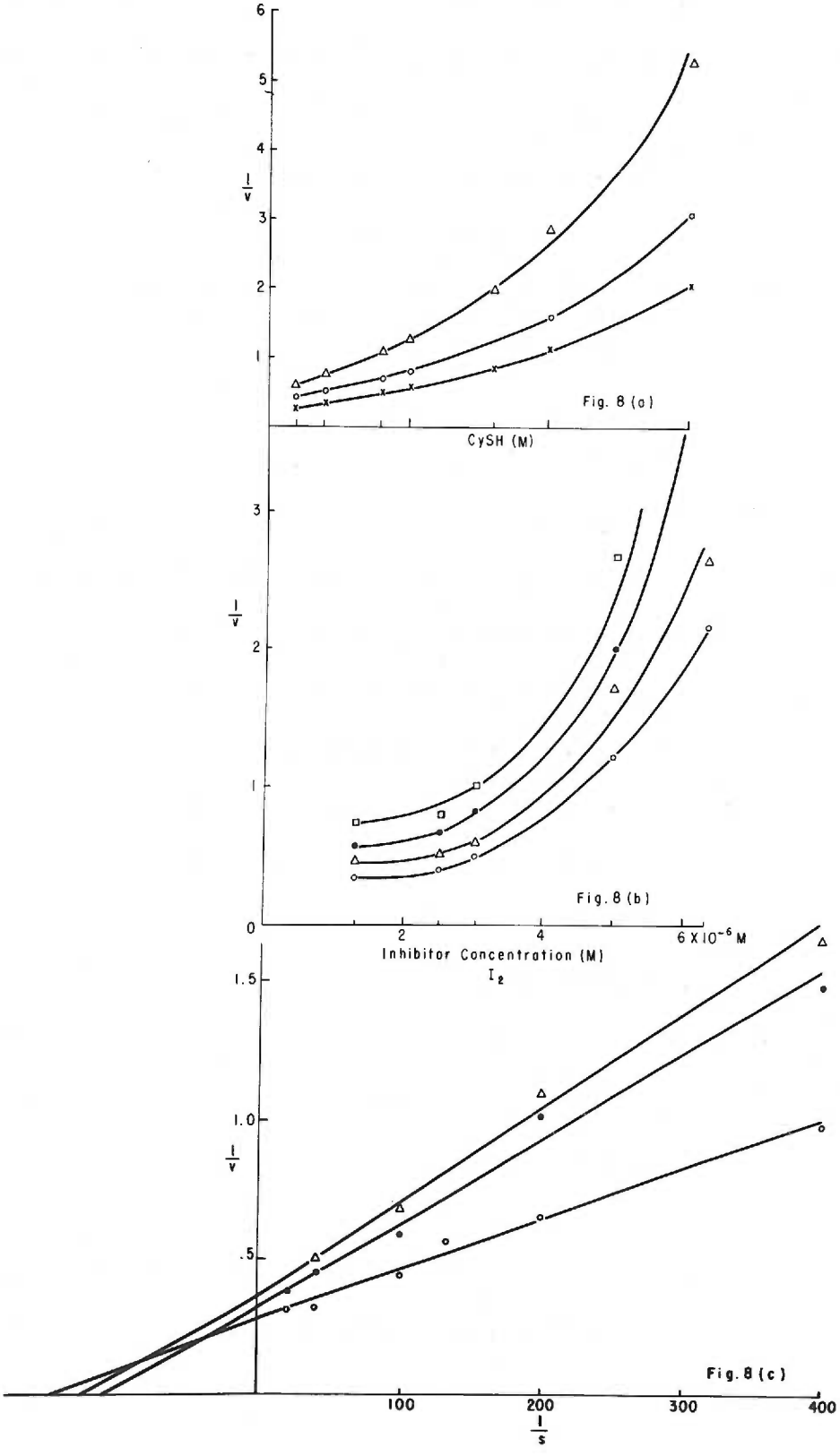
(Δ - Δ) substrate concentration 1.0×10^{-2} M

(● - ●) substrate concentration 7.5×10^{-3} M

(□ - □) substrate concentration 5.0×10^{-3} M

Figure 8-c. Lineweaver-Burk plots ($\frac{1}{v}$ vs $\frac{1}{s}$) at pH 10.2 in the absence of inhibitor (O - O), in the presence of 3.12×10^{-6} M iodine (● - ●), and in the presence of 1.6×10^{-6} M cysteine (Δ - Δ).

The experimental conditions and concentrations of reactants (except for changes in substrate concentrations) were the same as in Table IV.


6
5
4
3
2
1
1/v
Fig. 8 (a)
CySH (M)
3
2
1
0
1/v
Fig. 8 (b)
2
4
6 X10⁻⁶ M
Inhibitor Concentration (M)
I₂
1.5
1.0
.5
1/v
Fig. 8 (c)
100
200
300
400
1/s

on the enzyme when both were added to the reaction mixture, iodine being added before cysteine. The percent inhibition was compared to that calculated from the percent inhibition produced by each inhibitor. The percent inhibition produced by 2.5×10^{-3}mM iodine and 1.6×10^{-3}mM cysteine, both added to the same reaction vessel, was 52.9. When iodine 2.5×10^{-3}mM was added alone, the remaining enzyme activity in the reaction vessel was 2.401 µmoles phenol. Now, when cysteine 1.6×10^{-3}mM added to the remaining enzyme activity (2.401 µmoles phenol) one would expect this inhibitor to produce a 35.8% inhibition (obtained from the experiment when cysteine was added alone to the enzyme); therefore, 2.401x0.358 = .86 µmole phenol will be inhibited by the cysteine alone.

The calculated percent inhibition should be equal to $\frac{(0.86 + .717) \times 100}{3.118} = 50.6$. The percent inhibition, obtained when both inhibitors were present in the reaction vessel, was almost identical to that calculated from individual runs.

The effect of pH on the relative activity of the phosphatase in the presence of cysteine and iodine is shown in Table V. The increased inhibition by cysteine (7.7×10^{-6}M) with increasing pH (8.9 to 10.4) is apparent. At pH 7.5 the inhibitory effect of cysteine (8.0×10^{-6}M) is almost abolished, and at pH 6.1 there is a reversal of the effect of cysteine from inhibition to activation. The increase in pH therefore seems to produce the same result as that obtained by increasing the cysteine concentration at a particular pH. These results corroborate with those reported by Shuster, who suggested that the substance responsible for inhibition may be an

TABLE IV

Additive Effect of Inhibitors on the Hydrolytic Activity of Alkaline Phosphatase

The reaction vessel (total volume 2.0 ml), contained .056 M buffer ($NaHCO_3$-Na_2CO_3) pH 10.2, 1.5×10^{-3} M $MgCl_2$, 18.1 µg enzyme protein, the indicated concentration of inhibitor, and 2.5×10^{-2} M substrate.

The inhibitor was added following the addition of the enzyme. Prior to commencing the reaction with the substrate, the reaction vessel was preincubated at 38°C for 20 minutes. Reaction time 5 minutes at 38°C; the reaction was stopped with perchloric acid.

In the case where both inhibitors were added, the iodine was added to the reaction mixture containing the buffer pH 10.2, $MgCl_2$, and the enzyme, and the reaction vessel was left at room temperature for 10 minutes. To this, cysteine was added and the reaction vessel was preincubated at 38°C for 20 minutes. The reaction was started by adding the substrate.

TABLE IV

Additive Effect of Inhibitors on the Hydrolytic Activity of Alkaline Phosphatase

Cysteine ($mMx10^3$)	Iodine ($mMx10^3$)	µmoles phenol/tube	% Inhibition	Calculated % inhibition
-	-	3.12	-	-
0.8	-	2.82	9.43	-
1.6	-	2.00	35.8	-
2.0	-	1.64	47.3	--
3.2	-	1.11	64.4	-
4.0	-	0.892	71.4	-
-	2.5	2.40	23.0	-
-	3.1	2.06	33.8	-
-	5.0	0.817	73.8	-
-	6.2	0.412	86.8	-
0.8	2.5	2.04	34.4	30.2
1.6	2.5	1.47	52.9	50.6
2.0	3.1	1.04	66.6	65.1
3.2	5.0	0.254	91.9	90.7
4.0	6.2	0.107	96.6	96.2
1.6	5.0	0.441	85.9	83.2
3.2	2.5	0.842	73.0	72.6
4.0	2.5	0.638	79.5	77.6

TABLE V

Inhibition of Alkaline Phosphatase by

Cysteine and Iodine in Relation to pH

Reaction mixture (2.0 ml) contained .056 M ($NaHCO_3$-Na_2CO_3) buffer for pH 8.9 to 10.4, or .056 M $NaHCO_3$ buffer (pH range 6.1 to 7.5) adjusted to the indicated pH with dilute HCL, 1.5×10^{-3}M $MgCl_2$, 21.1 and 22.6 µg enzyme protein for pH 8.9 to 10.4 and pH 6.1 to 7.5 respectively.

Following the enzyme addition the inhibitor was added and preincubated at 38°C for 20 minutes. Reaction was commenced by adding substrate (final concentration 2.5×10^{-2}M phenyl phosphate) and stopped 5 minutes later at same temperature with perchloric acid.

TABLE V

Inhibition of Alkaline Phosphatase by Cysteine and Iodine in Relation to pH

pH	Control* (no inhibitor)	In presence of cysteine (M)	activity*	In presence of 6.25×10^{-6}M iodine*	Relative Activity (%) Cysteine	Iodine
10.4	3.140	7.7×10^{-6}	.340	.924	10.8	29.4
10.2	2.961	"	.246	.718	8.31	24.2
10.	3.423	"	.892	.756	26.1	22.1
9.8	3.255	"	1.180	1.188	36.2	36.5
9.6	2.845	"	1.302	.726	45.8	25.5
9.5	2.373	"	1.596	1.142	67.3	48.1
9.3	2.026	"	1.613	.901	79.6	44.5
9.1	1.659	"	1.428	.863	86.1	52.0
8.9	1.396	"	1.260	.638	90.3	45.7
7.5	.970	8.0×10^{-6}	.902	.192	93.0	19.8
-	-	3.2×10^{-5}	.485	-	50.0	-
7.3	.855	4.0×10^{-5}	.714	-	83.5	-
6.9	.687	4.0×10^{-5}	.634	-	92.3	-
6.5	.550	4.0×10^{-5}	.657	-	119	-
6.1	.323	8.0×10^{-6}	.492	.128	152	39.6
6.1	-	4.0×10^{-5}	.519	-	161	-
6.1	-	8.0×10^{-5}	.491	-	152	-
6.1	-	6.4×10^{-4}	.207	-	64.1	-
6.1	.317	2.92×10^{-3}	.151	-	47.6	-
6.1	.573**	9.75×10^{-4}	.433**	-	75.6	---
6.1	-	1.95×10^{-3}	.346**	-	60.4	-

* µmoles phenol per 5 minutes

** Reaction time 9 minutes

TABLE VI

Inhibition of Phosphatase by Mercaptoethanol and Thioglycolic Acid

Reaction mixture (2.0 ml) contained .056 M buffer ($NaHCO_3$-Na_2CO_3) pH 10.2, 1.5×10^{-3}M $MgCl_2$, 18.1 µg enzyme protein, inhibitor at the indicated concentration, and phenyl phosphate 2.5×10^{-2}M. Prior to the addition of substrate to commence the reaction, the mixture was pre-incubated at 38°C for 20 minutes.

The reaction time was 6 minutes and it was stopped with perchloric acid.

TABLE VI

Inhibition of Phosphatase by Mercaptoethanol and Thioglycolic Acid

Inhibitor concentration (M)	Hydrolytic Activity µmoles phenol/tube	Relative activity %
None	3.53	100
Mercaptoethanol		
2.96×10^{-5}	3.24	91.7
1.48×10^{-4}	2.95	83.6
2.96×10^{-4}	2.49	70.4
5.93×10^{-4}	1.78	50.4
Thioglycolic Acid		
1.32×10^{-5}	2.72	77.0
2.65×10^{-5}	2.49	70.4
1.32×10^{-4}	0.233	6.60

ion whose relative concentration increases with increasing pH (79).

The inhibition of phosphatase by iodine did not show such dependence of inhibition on pH. An increase in relative activity however, was observed when the pH of the reaction mixture was less than 9.6.

Inhibition of phosphatase by other mercapto-compounds is shown in Table VI. Mercaptoethanol and thioglycolic acid were also tested and found to be potent inhibitors of the enzyme.

Thioglycolic acid (1.32×10^{-4}M) seemed to be a more potent inhibitor (relative activity = 6.6%) when compared to mercaptoethanol (1.48×10^{-4}M, relative activity = 84%) but less effective when compared to cysteine (Table V, pH 10.2). Such differences in the potency of these inhibitors might be due to the relative occurrence of RS^- species which result from differences in the pK (-SH) values of these compounds.

Phosphotransferase Activity of Intestinal Alkaline Phosphatase

a) General Properties. The properties of the transferase reaction of phosphatase have been thoroughly investigated by Morton, using phosphocreatine as the donor and a variety of acceptors (67).

In the present study, some of the important properties of the transferase reaction were reinvestigated using phenyl phosphate as the donor and glucose as the acceptor.

Figure 9, shows the rate of formation of phenol (curve A), inorganic phosphate (curve B), and of glucose-6-phosphate (curve C), in the presence of 1.5 M glucose. Curve D of Figure 9, represents the percent phosphate transfer from phenyl phosphate to glucose $\left(\frac{\mu\text{mole G-6-P}}{\mu\text{mole phenol}}\right) \times 100$. The rate of formation of glucose-6-phosphate curve C, is linear up to about 20 minutes at which time the degree of hydrolysis, as measured by the phenol liberated, is approximately 9% of the total substrate added at the start of the reaction. After 50 minutes, the glucose-6-phosphate reaches a maximum value and thereafter (up to about 90 minutes) it remains constant. The inorganic phosphate formed, curve B, is linear with time up to about 10% hydrolysis of the substrate.

Morton found that glucose-6-phosphate is the major product in the transferase reaction with glucose (67). In the experiment reported here, Figure 9, a major portion of the ester(s) formed (µmole phenol less µmole inorganic phosphate = glucose phosphate) could not be accounted by the glucose-6-phosphate formed when measured enzymatically. This has also been observed with other experiments presented

Figure 9. The hydrolytic and phosphotransferase activities of intestinal alkaline phosphatase in relation to reaction time.

Curve A, μmoles of phenol per reaction vessel.

Curve B, μmoles of inorganic phosphate (P_i) per reaction vessel.

Curve C, μmoles of glucose-6-phosphate (G-6-P) per reaction vessel.

Curve D, G-6-P formed as % phosphate transfer.

The reaction mixture (2.0 ml) at pH 9.25 contained 1.5 M glucose, .042 M ($NaHCO_3$-Na_2CO_3) 1.5×10^{-3}M $MgCl_2$, 19.8 μg enzyme protein, and 2.5×10^{-2}M substrate.

Following a 15 minute temperature equilibration of the reaction vessel at 38°C, the reaction was commenced by the addition of substrate. The reaction was stopped by heating the tube for 30 seconds in a boiling water bath.

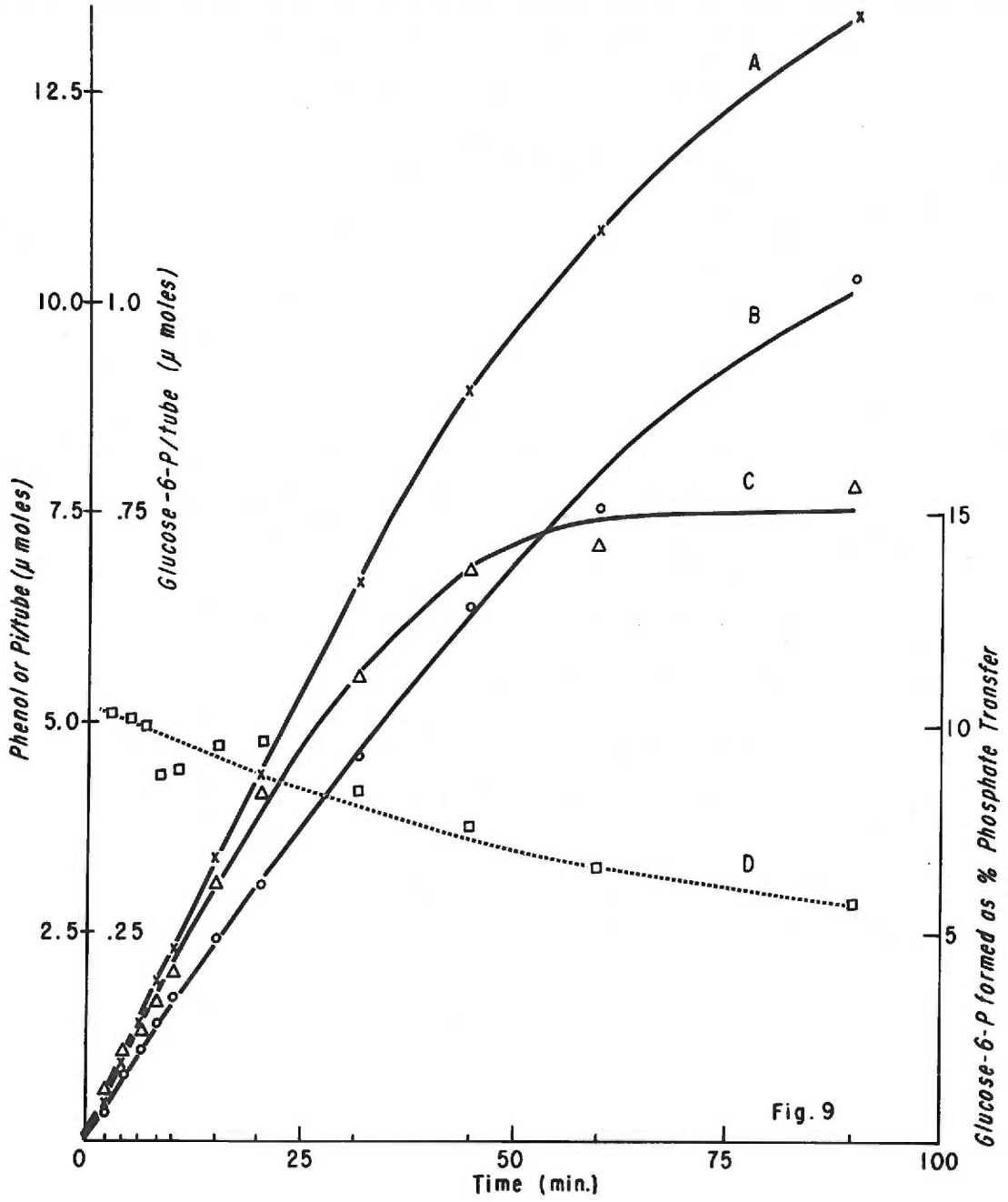

12.5
10.0
7.5
5.0
2.5
1.0
.75
.25
A
B
C
D
Phenol or Pi/tube (μ moles)
Glucose-6-P/tube (μ moles)
Glucose-6-P formed as % Phosphate Transfer
15
10
5
0
25
50
75
100
Time (min.)

Fig. 9

TABLE VII

Hydrolytic and Phosphotransferase Activities of Alkaline Phosphatase in the Presence of Varying Concentrations of Glucose

Reaction mixture (2.0 ml) contained different concentrations of buffered glucose at pH 9.8. The total concentration of $NaHCO_3$-Na_2CO_3 buffer being .04 to .06 M for the 2.7 to 0.3 M glucose. Magnesium chloride 1.5×10^{-3}M, enzyme protein 21.1 µg, and 2.5×10^{-2}M phenyl phosphate.

The reaction mixture was equilibrated at 38°C for 15 minutes before commencing the reaction with the substrate. Reaction time was 6 minutes at 38°C. The enzyme reaction was stopped by heating the tube for 30 seconds in a boiling water bath.

TABLE VII

Hydrolytic and Phosphotransferase Activities of Alkaline Phosphatase in the Presence of Varying Concentrations of Glucose

Glucose concentration (M)	µmoles phenol	µmoles P_i	µmole G-6-P	µmole phenol less µmole P_i	Phosphate transfer % (based on G-6-P formed)
0.30	5.230	4.998	0.230	0.232	4.40
0.75	4.470	3.481	0.325	0.989	7.27
1.05	4.164	3.034	0.343	1.130	8.24
1.20	3.955	2.823	0.361	1.130	9.13
1.50	3.834	2.461	0.368	1.370	9.60
2.10	2.765	1.871	0.296	0.894	10.7
2.70	2.143	1.391	0.224	0.752	10.4
None	4.240	4.130	-	-	-

later. No attempt was made to identify or measure the non-glucose-6-phosphate ester(s) synthesized by phosphatase.

The effect of glucose concentration on hydrolytic and phospho-transferase reaction of phosphatase is presented in Table VII. With increasing concentration of glucose up to 1.5 M an increase of glucose-6-phosphate was observed. A further increase in glucose from 1.5 to 2.7 M there was a decrease on the rate of glucose-6-phosphate synthesis. The percentage phosphate transfer increased with increasing glucose concentration. There was an initial increase on the rate of phenol production with glucose concentration up to .75 M and thereafter the increase of glucose concentration inhibited the rate of phenol liberation. The rate of formation of inorganic phosphate was increased from 4.13 µmole (reaction vessel contained no glucose) to 5.00 µmole in the presence of 0.3 M glucose. At higher concentrations of glucose a constant decline of the rate of inorganic phosphate formation occurred.

The effect of varying the substrate concentration on the ratio of substrate to initial velocity ($\frac{S}{V}$), for phenol, inorganic phosphate and glucose-6-phosphate is shown in Figure 10. The rate of phenol liberation in this case includes both hydrolysis and transfer reactions. The rate of glucose-6-phosphate synthesis is a result of transfer reaction, and the rate of inorganic phosphate formation may be considered as the hydrolysis reaction. Such plots, Figure 10, showed that within the narrow range of substrate employed (5.0×10^{-3} to 3.75×10^{-2} M) the Michaelis equation was followed when rates of phenol, inorganic phosphate, and glucose-6-phosphate were measured

Figure 10. Plots of $(\frac{S}{v})$ against (S) for phenol, inorganic phosphate, and glucose-6-phosphate, formed by intestinal alkaline phosphatase in the presence of phenyl phosphate and glucose.

These plots were derived from data presented in Table IX.

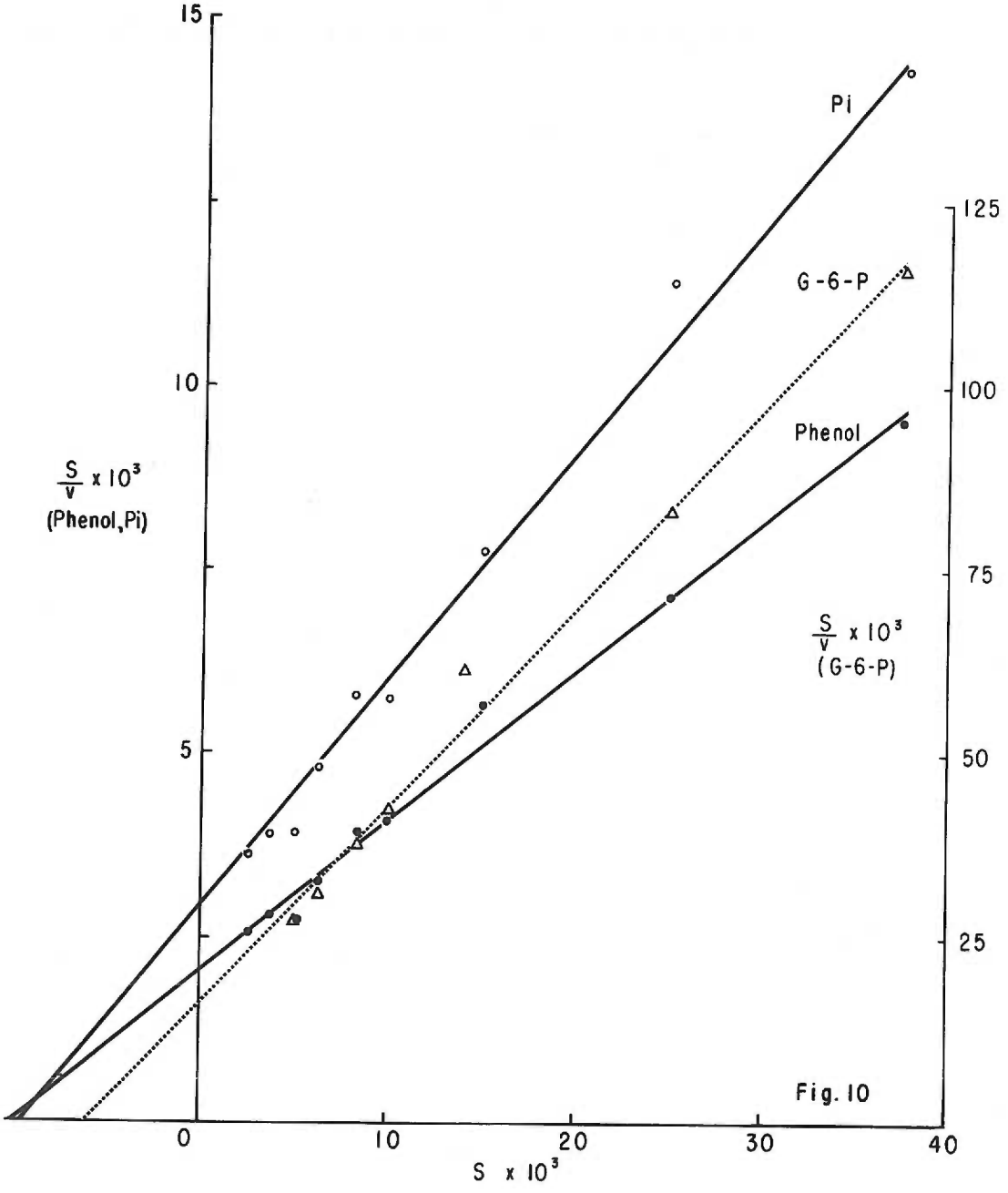

15
10
5
0
10
20
30
40
125
100
75
50
25
Pi
G-6-P
Phenol
S/V x 10³ (Phenol, Pi)
S/V x 10³ (G-6-P)
S x 10³

Fig. 10

TABLE VIII

A. Protective Effect of Glucose on the Enzyme

Reaction mixture (2.0 ml) at pH 10.0 contained 1.2 M glucose, .045 M buffer ($NaHCO_3$-Na_2CO_3), 1.5×10^{-3}M $MgCl_2$, 18.1 µg enzyme protein, the indicated concentration of the inhibitor, and 3.75×10^{-2}M phenyl phosphate.

Experiment 1. To the reaction mixture containing the buffered-acceptor, $MgCl_2$, and the enzyme, the inhibitor was added and the mixture incubated at 38°C for 20 minutes. The reaction was commenced by addition of substrate, and stopped, after a 5 minute reaction period, by heating it in a boiling water bath for 30 seconds.

Experiment 2. To 0.2 ml of .075 M ($NaHCO_3$-Na_2CO_3) pH 10.0 18.1 µg enzyme were added. Following the addition of the inhibitor the mixture was left at room temperature for 20 minutes. The buffered acceptor solution (1.5 ml of 1.6 M glucose pH 10.0 and 0.1 ml of .03 M $MgCl_2$) were then added and after temperature equilibration at 38°C for 10 minutes, the reaction was commenced by adding the substrate. After 5 minutes reaction period, the reaction was stopped as in Experiment 1.

B. Synthesis of Glucose-6-Phosphate from Inorganic Phosphate and Glucose in Presence of Alkaline Phosphatase; and its Inhibition Cysteine and Iodine

The reaction mixture (1.1 ml) at pH 8.7 contained 180 µg enzyme protein, the inhibitor at the concentration indicated, Na_2HPO_4 0.114 M, and .068 M glucose. In this experiment the enzyme and then the inhibitor were added to 0.20 ml of .075 M $NaHCO_3$-Na_2CO_3 pH 9.1 making a total volume of 0.60 ml.

The solution was incubated at 38°C for 20 minutes and then the reaction was commenced by addition of .125 µmole Na_2HPO_4 and .75 µmole glucose (temperature equilibrated). The resulted pH of the mixture was 8.7. Following 2 hours of reaction time at same temperature the enzymic activity was arrested by heating the mixture for 30 seconds in a boiling water bath.

TABLE VIII

A. Protective Effect of Glucose on the Enzyme

Inhibitor concentration (M)	µmoles phenol		µmole G-6-P	
	Experiment 1.	Experiment 2.	Experiment 1.	Experiment 2.
None	3.801	4.051	.358	.358
Cysteine				
0.8×10^{-6}	3.494	3.137	.344	.318
2.0×10^{-6}	3.533	2.872	.344	.288
Iodine				
2.5×10^{-6}	3.456	1.368	.339	.158
5×10^{-6}	3.456	0.252	.339	.065

B. Synthesis of Glucose-6-Phosphate from Inorganic Phosphate and Glucose in Presence of Alkaline Phosphatase; and its Inhibition Cysteine and Iodine

Inhibitor concentration (M)	µmole G-6-P per tube
None	.098
	.096
Cysteine	
1.45×10^{-4}	.034
	.034
2.9×10^{-4}	.029
Iodine	
2.27×10^{-4}	.044
	.044
4.54×10^{-4}	.054

at pH 10. The kinetic parameters, for each component (inorganic phosphate, glucose-6-phosphate, and phenol) were calculated and are presented in Table X.

b) Inhibition of Phosphotransferase Activity by Iodine and Cysteine

The degree of inhibition of phosphatase by cysteine and iodine at concentrations of 10^{-6}M, was considerably reduced in the presence of glucose when the inhibitors were added to the reaction vessel containing the buffered-acceptor solution and the enzyme. This protective effect of glucose on the phosphatase as shown by measuring the rate of phenol liberation and glucose-6-phosphate formation, is presented in Table VIII-A. It is clearly shown that the rate of formation of phenol and glucose-6-phosphate (experiment 2 of Table VIII-A) was considerably reduced when the inhibitors were added to the enzyme-buffer mixture before the addition of glucose. Only a slight decrease in the rate of phenol and glucose-6-phosphate formation was observed (experiment 1), when the inhibitors were added to enzyme-buffered-acceptor mixture. Such a protection of the enzyme by glucose against cysteine and iodine, might suggest a possible interaction of glucose with the enzyme at sites to which the inhibitors combine. Interactions of protein with glucose and with other small nonelectrolytes other than water have been reported (42).

Formation of phosphate esters from inorganic phosphate and sugars by phosphate have been reported by others (39,54,67). The synthesis of glucose-6-phosphate by phosphatase from inorganic phosphate and glucose with intestinal alkaline phosphatase and its

TABLE IX

Relationship of Hydrolytic and Transferase Activities of Alkaline Phosphatase with Substrate Concentration, in Presence and Absence of Inhibitors

The reaction mixture (2.0 ml) at pH 10.0 contained 1.2 M glucose, .04 M $NaHCO_3$-Na_2CO_3, 1.5×10^{-3} M $MgCl_2$, 18.0 µg enzyme protein and the indicated concentrations of inhibitor and substrate.

Experimental conditions were the same as in experiment 2 of Table VIII-A, except that the reaction was stopped by adding perchloric acid to vessels containing 3.57×10^{-3} and 2.5×10^{-3} M substrate.

The phenol, inorganic phosphate, and glucose-6-phosphate were calculated based on initial rates and expressed as µmoles of each product per 5 minutes.

TABLE IX

		Substrate concentration (M) x 10^3								
		37.5	25	15	10	8.33	6.25	5	3.57	2.5
No Inhibitor	Phenol	3.926	3.494	2.650	2.447	2.149	1.930	1.848	1.282	0.975
	P_i	2.629	2.191	1.941	1.742	1.446	1.302	1.280	0.908	0.690
	G-6-P	0.324	0.301	0.244	0.238	0.220	0.201	0.185	-	-
% Phosphate Transfer		8.25	8.61	9.21	9.73	10.24	10.4	10.0	-	-
Iodine										
1.25×10^{-6}M	Phenol	2.536	2.222	1.758	1.772	1.652	1.509	1.294	0.998	0.755
	P_i	1.864	1.534	1.227	1.197	1.025	0.932	0.796	0.685	0.502
	G-6-P	0.222	0.207	0.170	0.163	0.183	-	-	-	-
% Phosphate Transfer		8.75	9.32	9.67	9.20	10.8	-	-	-	-
2.5×10^{-6}M	Phenol	1.368	1.329	1.042	1.037	0.909	0.819	0.752	0.620	0.460
	P_i	1.079	0.959	0.784	0.703	0.617	0.557	0.500	0.442	0.302
	G-6-P	0.158	0.152	-	0.126	0.120	0.111	0.099	-	-
% Phosphate Transfer		11.5	11.4	-	12.2	13.1	13.6	13.2	-	-
Cysteine										
2.125×10^{-6}M	Phenol	2.872	2.540	2.488	2.130	1.880	1.699	1.561	1.104	0.862
	P_i	2.092	1.737	1.547	1.417	1.233	1.059	0.924	0.731	0.599
	G-6-P	0.270	0.260	0.218	0.210	0.185	0.160	-	-	-
% Phosphate Transfer		9.40	10.2	8.76	9.86	9.84	9.42	-	-	-
3.4×10^{-6}M	Phenol	2.762	2.384	2.060	1.827	1.628	1.395	1.287	0.932	0.734
	P_i	1.916	1.744	1.399	1.285	0.998	0.912	0.798	0.678	0.495
	G-6-P	0.250	0.230	0.212	0.183	0.170	0.158	0.148	-	-
% Phosphate Transfer		9.05	9.65	10.3	10.0	10.4	11.3	11.5	-	-

inhibition by iodine and cysteine is shown in Table VIII-B.

The effect of the concentration of phenyl phosphate (donor) on the initial velocity of phenol, inorganic phosphate, and glucose-6-phosphate formation by phosphatase in the presence and absence of inhibitors is shown in Table IX. Within the range of substrate concentration ($5x10^{-3}$ to $3.75x10^{-2}$M) examined, the rate of glucose-6-phosphate synthesis (in the absence of inhibitors) increased with increasing substrate concentration. The percentage phosphate transfer increased only slightly with decreasing substrate concentration. It appears that the hydrolytic and phosphotransferase activities of phosphatase, with and without the inhibitors, were influenced by the concentration of phenyl phosphate in a similar manner. Those observations were also true when cysteine and iodine at different concentrations were present in the system.

c) Inhibition Kinetics. Figures 7-a and 7-b, revealed that, according to Ackermann and Potter's test for reversible and irreversible inhibitors (1) the cysteine was a reversible inhibitor while iodine behaved (possibly) as an irreversible inhibitor. Sizer, however, demonstrated that the inhibition by iodine could be reversed by Na_2S and partially by dialysis (80).

For the inhibition kinetics to be followed, reversible inhibition was assumed for both inhibitors which means that the activity of the enzyme is regained by removing the inhibitor through dialysis or by other chemical means. This then implies that there is an equilibrium between the inhibitor and the enzyme, and the constant Ki denotes the reciprocal of the enzyme-inhibitor affinity.

The type of inhibition produced by iodine and cysteine with respect to the hydrolytic and transferase activities of phosphatase, acting on phenyl phosphate as a donor substrate and under the experimental conditions employed, is graphically shown in Figures 11 to 14.

The equation describing the linear relationship of $(\frac{S}{v})$ vs (S) for phenol, inorganic phosphate and glucose-6-phosphate, was derived by the method of least squares (18). The apparent kinetic parameters shown in Table X, were calculated from the constants of the equation derived from the experimental data given in Table IX. These apparent kinetic parameters, which are considered here for comparative purposes only, describe the changes produced by cysteine and iodine on the Km and Vmax of the enzyme. The plots presented in Figures 11 to 14 were constructed according to the general equation $\frac{S}{v} = \frac{S + Kp}{Vp}$, where Vp and Kp are equal to Vmax and Km respectively, in the absence of inhibitor. In the presence of inhibitor, however, these kinetic parameters are altered depending on the type of inhibition produced. A detail derivation of equations pertaining to each type of inhibition is given by Dixon and Webb in their textbook (24).

Before describing the type of inhibition produced by cysteine and iodine on the hydrolytic and transferase actions of phosphatase, the following assumptions were made:

(a) Phenol liberation is a function of both hydrolytic and transferase activities of phosphatase,

(b) Inorganic phosphate formed is related to the hydrolytic activity of the enzyme,

(c) The glucose-6-phosphate formed is an index of the transferase action of the enzyme, and that this product (glucose-6-phosphate) is not hydrolysed by phosphatase within its initial rate of formation.

Figure 10 revealed that the phenol and inorganic phosphate formation in the absence of inhibitor differed only with respect to Vmax while the Km was almost the same (see Table X). The presence of iodine however, Figure 11, influenced both Vp and Kp of phenol and inorganic phosphate formation. For the formation of phenol (hydrolytic and transferase activities), in the presence of 1.25×10^{-6}M iodine, the substrate concentration giving half the maximum velocity (Kp) became 6.95×10^{-3}M (Table X) as compared to 10×10^{-3}M in the absence of iodine, and the maximum velocity in excess substrate (Vp) changed from 4.90 in the absence of iodine to 2.93 in the presence of iodine. For the formation of inorganic phosphate (hydrolytic activity) however, the Kp in the presence of iodine (1.25×10^{-6}M) was 9.14×10^{-3}M and did not alter significantly in comparison to the Km obtained without the presence of inhibitor (Table X). The same influence was observed with the 2.5×10^{-6}M iodine (Table X).

The inhibition produced by iodine on the rate of phenol, inorganic phosphate and glucose-6-phosphate formation ($\frac{S}{v}$ vs S) by intestinal alkaline phosphatase is shown in Figure 13-a to 13-c. With respect to inorganic phosphate formation (Figure 13-b) the inhibitor (iodine) affected only the Vmax while the Km remained almost the same. This then implies either a purely or partially

Figure 11. Effect of iodine on the formation of phenol and inorganic phosphate by the action of intestinal alkaline phosphatase on phenyl phosphate and in the presence of glucose.

Values of $(\frac{S}{v})$ and (S) were calculated from data presented in Table IX.

Plots representing phenol (● - ●) and inorganic phosphate (▲ ---- ▲) were obtained in the presence of 1.25×10^{-6}M iodine; and those representing phenol (O - O) and inorganic phosphate (Δ ---- Δ), were obtained in the presence of 2.5×10^{-6}M iodine.

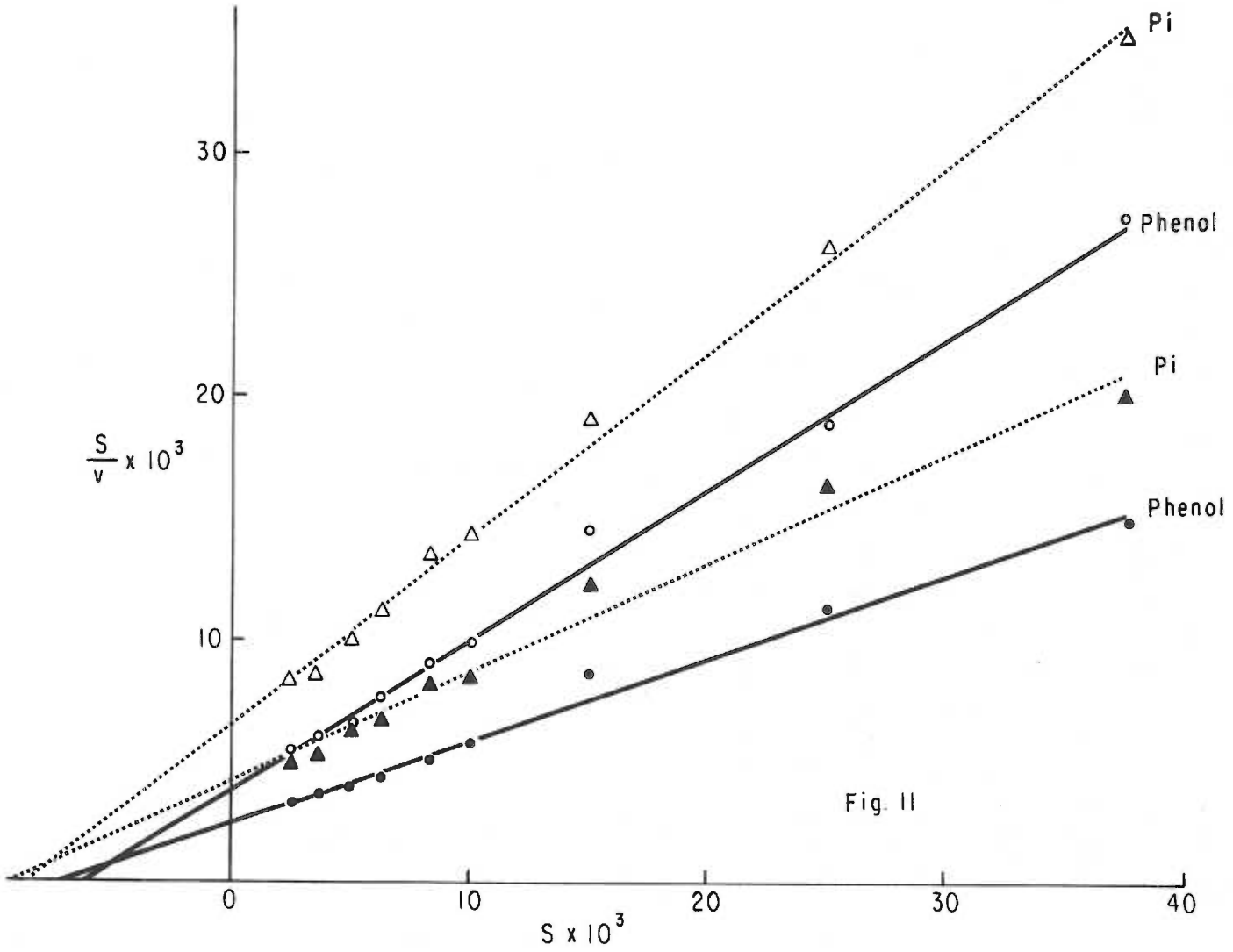

Pi
Phenol
Pi
Phenol
30
20
10
0
10
20
30
40
$\frac{S}{v} \times 10^3$
$S \times 10^3$

Fig. II

non-competitive type inhibition. According to Dixon (24) for the purely non-competitive type,

$$Vp = \frac{V}{1 + \frac{i}{Ki}}$$

and for the partially non-competitive type,

$$Vp = \frac{\frac{V' i}{V + Ki}}{1 + \frac{i}{Ki}}$$

where V = maximum velocity in excess substrate

V' = velocity in presence of excess inhibitor and substrate (page 27)

i = inhibitor concentration

Ki = inhibitor constant

In both types of inhibition mentioned above Kp = Km.

The type of inhibition produced by iodine when phenol (Figure 13-a) and glucose-6-phosphate (Figure 13-c) were plotted could not be clarified with the available data.

Both kinetic parameters Vp and Kp (Table X) have been altered by iodine, in the case of phenol (hydrolytic acid transferase action) and glucose-6-phosphate (transferase action) while the K_p in the case of inorganic phosphate (hydrolytic action) remained the same (Kp = Km). It might logically be assumed then that the hydrolytic activity of phosphatase was affected by iodine in a different way when compared to the transferase activity (glucose-6-phosphate).

The type of inhibition produced by cysteine is shown in Figure 12,

Figure 12. Effect of cysteine on the formation of phenol and inorganic phosphate by the action of intestinal alkaline phosphatase on phenyl phosphate and in the presence of glucose.

Calculated values of $(\frac{S}{v})$ against (S) were derived from the data of Table IX.

Plots representing phenol (● - ●) and inorganic phosphate (▲----▲) and those representing phenol (O - O) and inorganic phosphate (Δ----Δ), were obtained in the presence of 2.12×10^{-6}M and 3.4×10^{-6}M cysteine respectively.

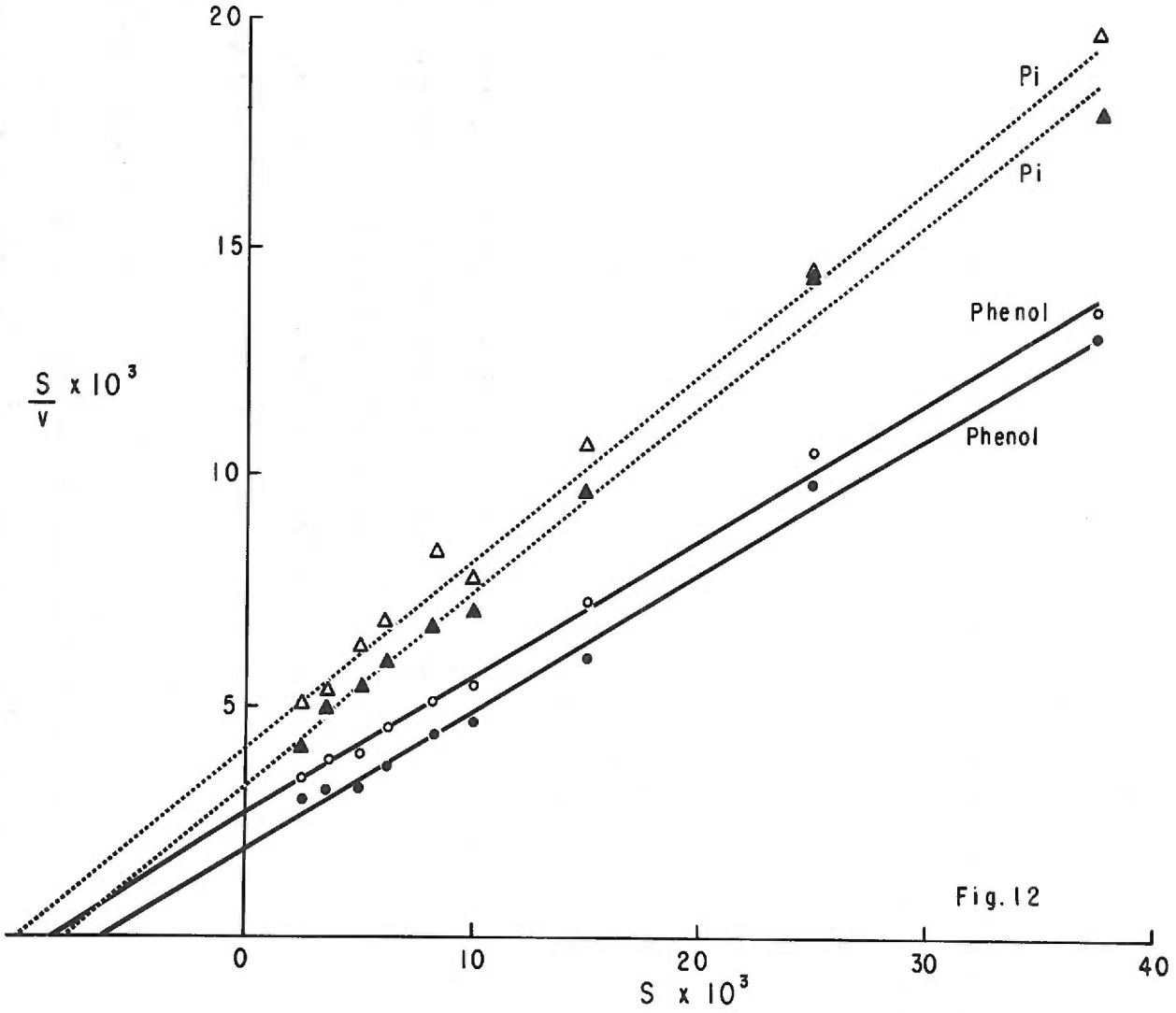


Fig. 12

Figure 13-a, Phenol

Plot A, no inhibitor present.

Plot B, in presence of 1.25×10^{-6}M I_2

Plot C, in presence of 2.5×10^{-6}M I_2

Figure 13-b, Inorganic Phosphate

Plot A, no inhibitor present.

Plot B, in presence of 1.25×10^{-6}M I_2

Plot C, in presence of 2.5×10^{-6}M I_2

Figure 13-c, Glucose-6-Phosphate

Plot A, no inhibitor present.

Plot B, in presence of 2.5×10^{-6}M I_2

Figures 13-a, 13-b, and 13-c. Plots of $(\frac{S}{v})$ against (S) were derived from data of Table IX for phenol, inorganic phosphate, and glucose-6-phosphate respectively.

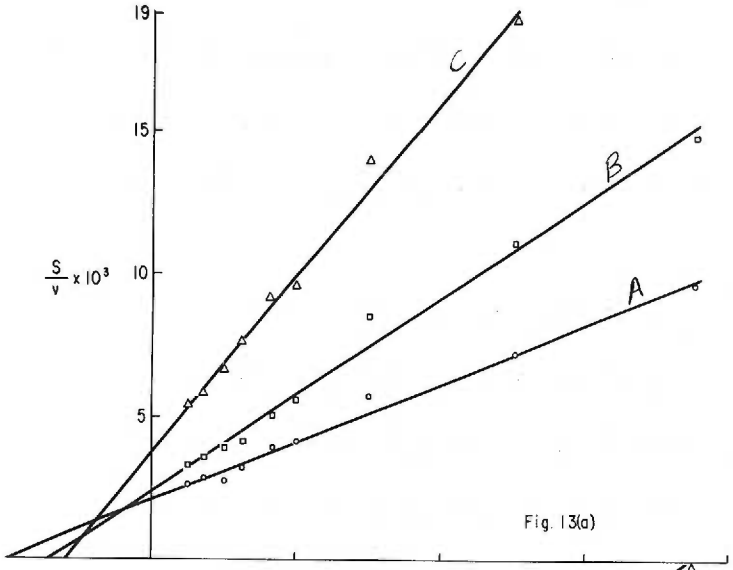


Fig. 13(a)

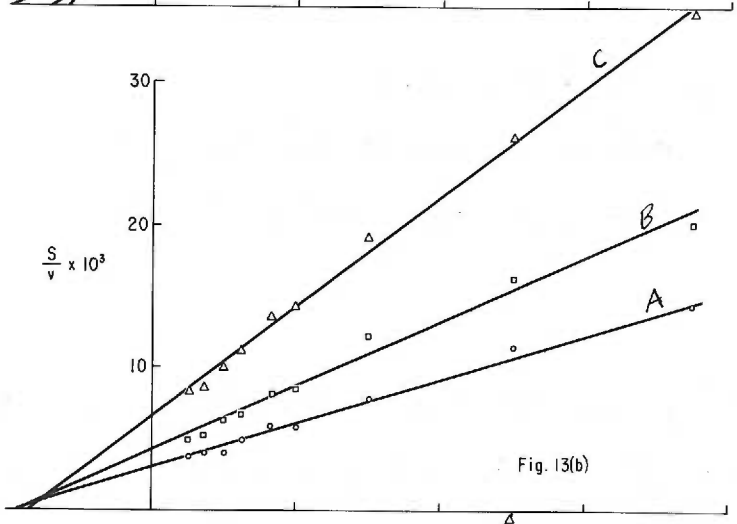


Fig. 13(b)

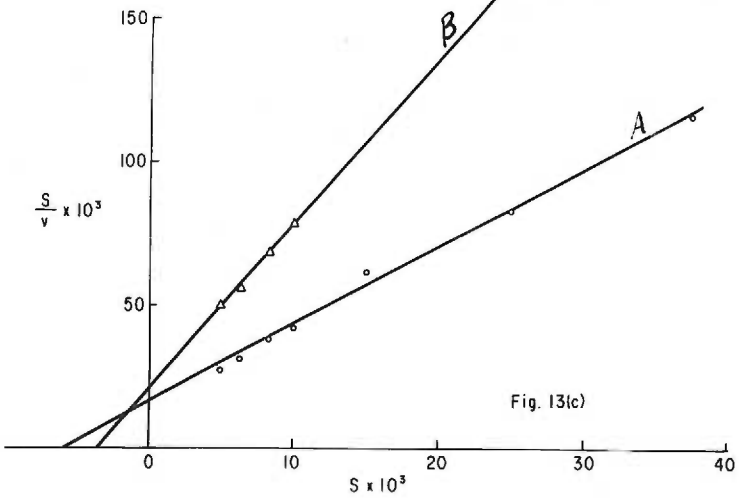


Fig. 13(c)

Figure 14-a. Phenol plot ($\frac{S}{v}$ vs S) in absence of inhibitor (A), and in presence of 3.4×10^{-6}M cysteine (B).

Figure 14-b. Inorganic phosphate plot ($\frac{S}{v}$ vs S) in absence of inhibitor (A), and in presence of cysteine 2.12×10^{-6}M for (B), and 3.4×10^{-6}M for (C).

Figure 14-c. Glucose-6-phosphate plot ($\frac{S}{v}$ vs S) in absence of inhibitor (A), in presence of 2.12×10^{-6}M (B), and 3.4×10^{-6}M (C) cysteine.

Figures 14-a, 14-b, and 14-c. Plots of ($\frac{S}{v}$) against (S) were derived from data of Table IX.

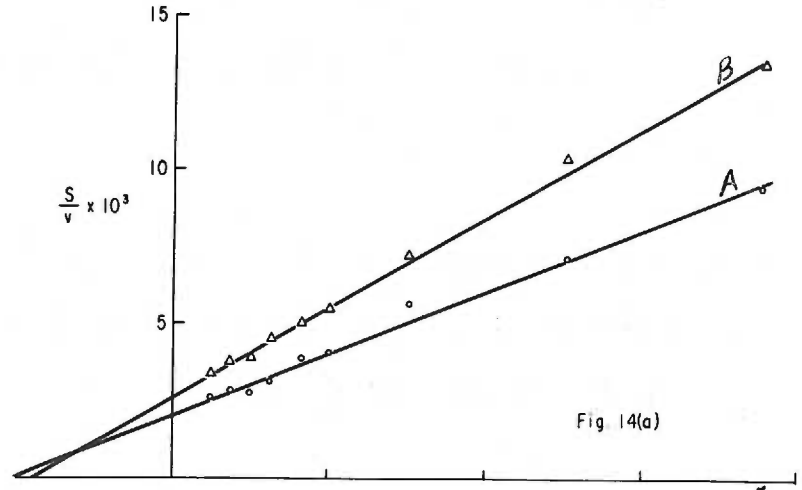


Fig. 14(a)

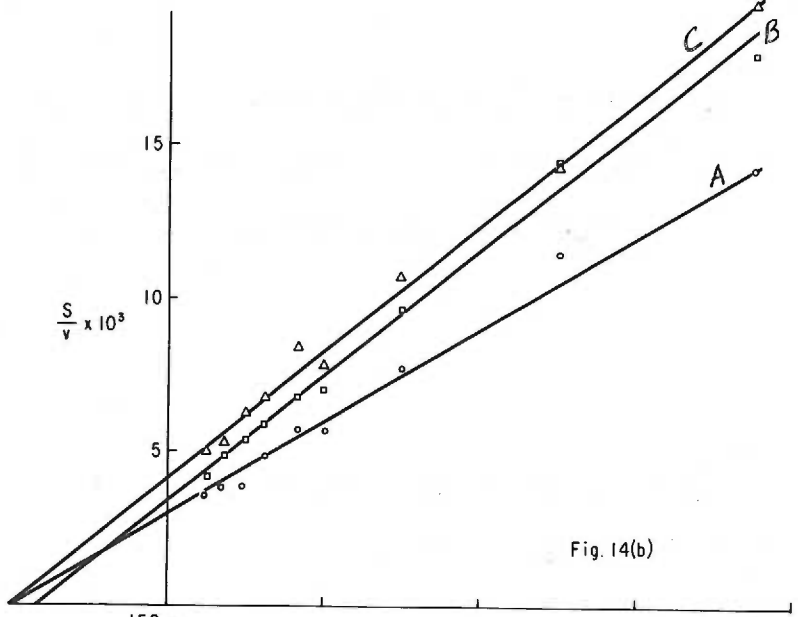


Fig. 14(b)

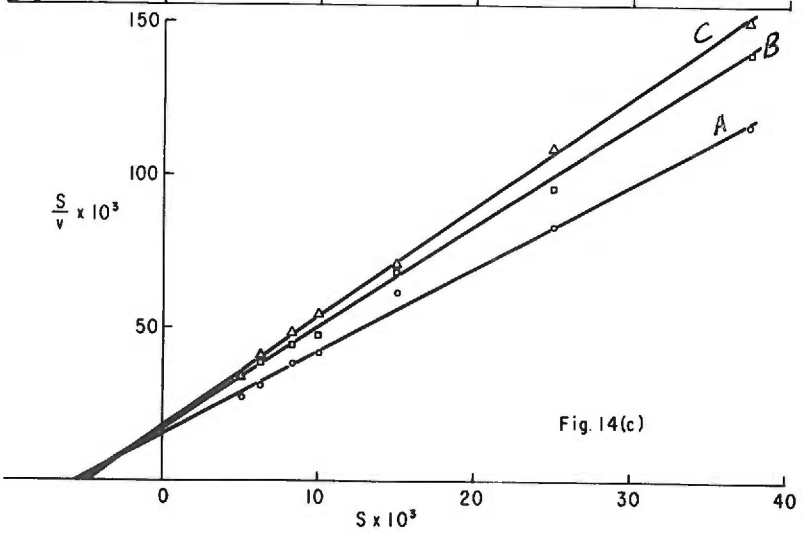


Fig. 14(c)

TABLE X

Comparison of Apparent Kinetic Parameters in Presence and Absence of Cysteine and Iodine

(computed from $\frac{S}{v}$ vs S plots).

TABLE X

Comparison of Apparent Kinetic Parameters in Presence and Absence of Cysteine and Iodine

	Phenol	P_i	G-6-P
No Inhibitor			
Km	10.0×10^{-3}	9.41×10^{-3}	5.97×10^{-3}
Vmax	4.90	3.14	.371
1.25×10^{-6}M I_2			
Kp	6.95×10^{-3}	9.14×10^{-3}	4.46×10^{-3}
Vp	2.93	2.22	.245
2.5×10^{-6}M I_2			
Kp	5.95×10^{-3}	8.4×10^{-3}	3.48×10^{-3}
Vp	1.59	1.30	.171
2.125×10^{-6}M CySH			
Kp	6.3×10^{-3}	8.4×10^{-3}	5.56×10^{-3}
Vp	3.32	2.48	.311
3.4×10^{-6}M CySH			
Kp	8.78×10^{-3}	10.1×10^{-3}	5.05×10^{-3}
Vp	3.34	2.43	.281

where the phenol and the inorganic phosphate were plotted for the two concentrations of cysteine, and in Figure 14-a to 14-c, where phenol, inorganic phosphate, glucose-6-phosphate were compared to the control (plot A).

The plots of Figure 12 for phenol in the presence of 2.12×10^{-6}M and 3.4×10^{-6}M cysteine respectively showed that this inhibitor altered only the Kp of the system and Vp was the same for both concentrations of the cysteine employed. This was also true with the inorganic phosphate plots (Figure 12). The effect of inhibitor then, comparing the 2.12×10^{-6}M against the 3.4×10^{-6}M cysteine (Figure 12), was to induce a change in Kp but not in Vp. This might suggest a competitive type inhibition. When the rate of phenol formation in the presence of cysteine (plot B of Figure 14-a) was compared to that in the absence of cysteine (plot A of Figure 14-a), it showed a considerable change in Vp as well as in Kp. This was also true with inorganic phosphate when compared to the plot in the absence of cysteine (Figure 14-b). Such a change in Vp, between the inhibited and non-inhibited systems but not between the systems containing two different concentrations of cysteine (Table X), could not be explained. Whether the inhibition produced by cysteine with respect to phenol and inorganic phosphate formation was competitive in nature or some other type could not be decided from the plots shown in Figure 12 and Figures 14-a and 14-b.

The rate of glucose-6-phosphate synthesis in the presence (plot B and C) and in the absence (plot A) of cysteine is shown in Figure 14-c. From these plots and the apparent kinetic parameters

presented in Table X the type of inhibition induced by cysteine on the transferase action of phosphatase seemed to be non-competitive.

DISCUSSION

Purification and Kinetic Properties of Intestinal Alkaline Phosphatase

The phosphatase preparation used in these studies was purified from the commercially available "purified" alkaline phosphatase (calf intestinal mucosa), by acetone fractional precipitation according to Morton (60). This preparation which was nine times the purity of the starting material (Table I) had a specific activity of about 5,500 units* per mg. protein nitrogen.

Morton (60), and Schramm and Ambruster (77) have reported specific activities on their phosphatase preparations of 83,500 and 46,000 units per mg. nitrogen respectively. A phosphatase preparation was reported by Portmann with a specific activity of 400,000 units per mg. nitrogen (72). Ahmed et al., in a recent communication reported specific activities of 450 and 480 King-Armstrong units per mg. nitrogen (equivalent to about 10,000 units per mg. nitrogen) for intestinal phosphatase purified by autolysis and butanol extraction respectively.

The low specific activity of the phosphatase preparation used in these experiments might be due to a decline in specific activity of the original source of the enzyme. A decline in the specific activity of phosphatase from that obtained immediately after purification was observed by Morton (67).

Paper electrophoresis of the acetone preparation revealed two components and only one component carried the phosphatase activity

* The enzyme unit has been defined by Morton, as that amount of enzyme which liberates 1 µg. inorganic phosphate per minute at 38°C, using b-glycerophosphate as the substrate, and under optimal conditions (58).

(Figure 2-c). Although the preparation obtained by continuous flow electrophoresis consisted of only one component as detected by paper electrophoresis (Figure 2-b), its specific activity was lower than that of the acetone preparation.

A higher degree of purification of the enzyme could have been obtained if the flow electrophoresis technique was used following the acetone fractional precipitation method, and if the enzyme was extracted directly from the intestinal mucosa.

The complex kinetic behavior of alkaline phosphatase towards phenyl phosphate as the substrate can be seen from the results shown on Figures 4 and 5. The change of optimum pH with substrate concentration (Figure 4-a), the dependence of optimum and narrow substrate concentration with pH (Figure 5-a), and the change of pKm with pH (Figure 5-b), all are in agreement with Morton's findings (66). The non-linear relationship of Lineweaver-Burk plots for all pH values (8.8 to 10) observed by Morton using a wider range of substrate concentration (10^{-2} to 2.5×10^{-5}M) and phosphatase preparation of higher purity could not be demonstrated for pH values of 8.3 and 8.9 to 9.4 (Figure 4-b). The pKm values as determined from the reciprocal plots of $\frac{1}{v}$ vs $\frac{1}{S}$ (Table III) for pH 8.3 and 8.9 to 9.4, were in close agreement with those determined from Michaelis-Menten plots (Figure 5-a).

A Km value of 8.6×10^{-4}M for calf intestinal alkaline phosphatase at pH 10.0 using phenyl phosphate has been reported by Morton (65, 66); a value of 4.0×10^{-3}M by Motzok and Branion (70) using the commercially purified enzyme, and a value of 2×10^{-4}M by Zittle and

Bingham at pH 9.7 (90).

In the present studies, the Km at pH 10.0 (Table III) was found equal to 2.95×10^{-3}M. An apparent Km value of 4.8×10^{-4}M was calculated from the plot of $\frac{S}{Umax}$ vs S (Figure 5-c). The pKm values (Table III) calculated from Michaelis-Menten plots (Figure 5-a) for each pH were lower than the ones reported by Morton (66).

The use of Na_2CO_3-$NaHCO_3$ buffer has been critized by Morton (62) and Zittle and Della Monica (88,89). This buffer gives lower activities and acts as a competitive inhibitor of phosphatase when compared to ethanolamine-HCl buffer.

In the present studies the Na_2CO_3-$NaHCO_3$ buffer was used because ethanolamine or other hydroxyl organic compounds might interfere with the phosphotransferase studies according to Morton (67).

The pKm-pH plot shown in Figure 5-b, revealed a discontinuity at about pH 9.2. A change of slope from zero (in terms of whole numbers), for pH values below 9.0, to -1 unit, for pH values above 9.3 was observed. Dixon has described this change of slope from 0 to -1 unit as follows:

$$1.\quad R{\cdot}P^{2-} + Mg^{+}{\cdot}E \rightarrow R{\cdot}P^{2-}{\cdot}Mg^{+}{\cdot}E$$

$$2.\quad R{\cdot}P^{2-} + E \rightarrow R{\cdot}P^{-}{\cdot}E$$

At pH below 9.0, alkaline phosphatase combines with its substrate by an electrostatic attraction as shown in equation 1; since there is no alteration of the total charge, a zero slope results. At pH above 9.3, equation 2, the enzyme combines with its substrate and

forms a non-ionized link. At this pH region there is a charge change of -1 on "desubstration" and a -1 unit slope results (23).

The discontinuity in the slope of the line (Figure 5-b) may be due to an ionizing group in the enzyme concerned with the combination of phenyl phosphate. It was suggested by Morton that the pK of the phenolic hydroxyl of a tyrosine residue, which was essential for enzymic activity, might be responsible for the discontinuity observed at pH 9.2, although the possibility that it is due to the ϵ-amino group of lysine could not be excluded. The exclusion of a possible -SH group or a substrate ionizing group has been discussed by Morton (66). The pKm-pH plot shown in Figure 5-b, revealed the same findings as those of Morton's (66).

The isolation of phosphorylserine by acid-hydrolysis of alkaline phosphatase previously incubated with P^{32}-labeled inorganic phosphate has been reported and the suggestion was made that an intermediate enzyme-phosphate (covalent linkage) is formed during the reaction of this enzyme with a phosphate donor compound (2,27). Koshland and Erwin, working on phosphogluco-mutase, indicated that the amino acid sequence of the active site for this enzyme was the same as that of chymotrypsin, namely; Asp,Ser,Gly,Glu,Ala,Val. They suggested that the common sequence of amino acids around serine is involved in making the otherwise inert ($-CH_2OH$) side chain reactive to the phosphoryl group (47).

The above mentioned findings and suggestions then, could not exclude the serine side chain ($-CH_2OH$) as a possible residue in the phosphatase molecule having a pK value close to the discontinuity

observed at pH 9.2 in the pKm-pH plot (Figure 5-b).

Inhibition of Alkaline Phosphatase by Cysteine and Iodine

Several investigators have reported on the inhibition of phosphatase by cysteine and iodine, using concentrations of these inhibitors in the range of 10^{-4} to 10^{-3}M (65,75,79,80).

In the present studies, however, strong inhibitory effect was observed with cysteine and iodine at concentrations in the range of 10^{-6}M (see Table V). The possibility that the intestinal alkaline phosphatase preparation used in these studies had been altered during the purification procedure was excluded by testing the effect of cysteine and iodine on the original source of the enzyme. It was found that the commercially available "purified" alkaline phosphatase was also strongly inhibited by cysteine; this compound at a concentration of $8x10^{-6}$M produced 80 percent inhibition. With iodine however, at a concentration of $2.5x10^{-5}$M, a 50 percent inhibition was produced. This reduction in the degree of inhibition of the original enzyme by iodine could be attributed to the more impure state of the enzyme.

The mechanism of inhibition of alkaline phosphatase by iodine has not been previously elucidated. Sizer suggested that the tyrosine residue in the enzyme molecule was iodinated by iodine (80). Morton made the hypothesis based on the inhibition of phosphatase by iodine, that certain tyrosine groups of intestinal phosphatase may be directly involved in the formation of an enzyme-substrate complex (65). This hypothesis was supported by his finding, from a pKm-pH plot, of a discontinuity at pH 9.2 which he considered to represent

the pK of the tyrosine residue (phenolic hydroxyl group) in the phosphatase molecule. Such a discontinuity at pH 9.2 in the pKm-pH plot was also shown in Figure 5-b.

Iodine is known to (a) substitute tyrosine groups, (b) oxidize -SH groups to disulfide bonds, (c) destructively oxidize the indol ring of tryptophan, and (d) to react with histidine residue forming a labile N-iodo compound which later rearranges into the more stable C-iodo compound (32).

Alkaline phosphatase is not inhibited by compounds which react with thiol groups (23,66); these groups however, may be "masked" toward most -SH reagents (32). Tryptophan was excluded by Sizer who indicated that the ultraviolet absorption spectrum of phosphatase, attributable to the tryptophan and tyrosine residues, shifts to higher wave-lengths, just as does the absorption spectrum of tyrosine, but not of tryptophan, upon the addition of strong oxidants (80). The imidazolium group of histidine could be excluded as a possible functional group of phosphatase, since its pK value in the protein molecule is 5.6 to 7.0 (24). This value did not correspond to the discontinuity observed at pH 9.2 on the pKm-pH plot (Figure 5-b) and therefore histidine was not considered as a possible functional group for phosphatase (66).

Electrophilic substitution of phenol by aqueous iodine solution has been shown to be mediated through a cation, either the iodinium ion (I^+) or the hypoiodous acidium ion (IOH_2^+), which is much more powerful than the molecular iodine or the tri-iodide ion (38).

The observed difference in the degree of inhibition of phosphatase

when iodine was added to the reaction mixture prior to enzyme addition (curve B of Figure 6) in comparison to curve D where the inhibitor was added to the enzyme, can not be well explained. It is possible that at pH 9.8 the rate of formation of iodinium ion (I^+) from the added I_2KI solution was greater when the phenoxide ion (tyrosine residue of the enzyme) was already present in the reaction mixture. Information about the rate by which phosphatase is inhibited by iodine (added to the buffer before, or after the enzyme) prior to assaying the enzymic activity, might clarify the above mentioned difference in inhibition.

Assuming that: (a) the tyrosine residue of phosphatase is a functional grouping for its hydrolytic action, (b) the iodine inhibits phosphatase activity by iodination of the tyrosine, and (c) that cysteine affects another functional group, then the inhibition produced by cysteine and iodine should be additive when both inhibitors were present in the reaction mixture containing the enzyme. Such an additive effect of inhibitors was demonstrated by the results shown in Table IV. In addition to this, the cysteine (at the concentrations employed) did not reverse the inhibition induced by iodine.

The inhibition of phosphatase by iodine at different pH values revealed a considerable increase in the percent inhibition at pH values greater than 9.5 (Table V). The pK values of the phenolic hydroxyl (tyrosine) group in proteins range from 9.8 to 10.4 (24). The occurrence of phenoxide ions therefore, is greater at the higher pH values. This then might be a suggestive explanation for the increased inhibition observed at the higher pH values.

The mechanism of inhibition of phosphatase by sulfhydryl compounds is quite complex. Cysteine for example, depending on its concentration and pH of the reaction vessel, can inhibit or stimulate alkaline phosphatase (Table V). Although the reduction potentials for the cysteine and thioglycolic acid are the same, cysteine is a more potent inhibitor of phosphatase than thioglycolic acid or mercaptoethanol (compare Table V with Table VI). The inhibition of phosphatase by cysteine at concentrations of about 10^{-6}M excludes the possibility that the inhibition is due to metal chelation (73).

The increase of inhibition by cysteine with increasing pH or with increasing concentration of cysteine at one particular pH (Table V) points out that the substance responsible for inhibition might be some ionic species whose relative concentration increases with increasing pH. A comparison of the degree of inhibition produced by cysteine, thioglycolic acid, and mercaptoethanol, at pH 10.2 (Table V and VI) also suggests that the decreasing potency of these inhibitors might be due to the relative occurrence of RS^- species. The occurrence of RS^- species at pH 7.4 for cysteine is 6 percent and for thioglycolic acid is zero (15). The pK value for the -SH group of cysteine has been reported as 8.53 and 10.03 for reaction (1) and reaction (2), respectively (15).

$$HSRNH_3^+ \rightleftharpoons {}^-SRNH_3^+ \qquad (1)$$

$$HSRNH_2 \rightleftharpoons {}^-SRNH_2 \qquad (2)$$

The pK values for thioglycolic acid and mercaptoethanol are probably higher and an estimate of 10.7 has been given (16) for the aliphatic

mercaptans.

It was found by Morton that the efficiency of phosphorylation of glucose by microsomes from the intestinal mucosa was the same as that obtained with partially purified and purified phosphatase preparations. From these findings and other properties of the purified alkaline phosphatase (with respect to its transferase action) he established that phosphotransferase activity was undoubtedly a property of the enzyme itself (67).

The results of Table VII showed that the rate of formation of glucose-6-phosphate increased with increasing concentration of glucose up to a limiting concentration, beyond which further increase of glucose concentration caused a fall in the rate. A similar behavior of the rate of formation of product versus substrate concentration is observed with many enzymes (see Figure 5-a, for example). This suggests that glucose is adsorbed probably at a specific site of the enzyme and that this site is saturated when glucose reaches a limiting concentration at which the maximum rate of glucose-6-phosphate is obtained. The decline in the rate of phenol liberation with increasing concentration of glucose may be due to a direct competition of glucose with water and also to the lowering of the concentration of water molecules as suggested by Morton (67). These observations may suggest that the transferase activity is the result of the competition between the glucose molecules and water molecules for site(s) at the enzyme surface (67).

The protective effect of glucose on the enzyme against the inhibitors shown in Table VIII-A does support the suggestion that

glucose is adsorbed at a specific site of the phosphatase molecule. The interaction of glucose with proteins has been reported by Mohammad, et al. (56). These workers established that the amino groups were involved in the "browning" reaction of proteins with glucose. They also suggested that tyrosine, tryptophan, and histidine groups, are involved in the interaction of protein with glucose. The possible interaction of glucose with the phenolic hydroxyl of tyrosine residue and therefore the slight inhibitory effect of iodine on the enzyme (Table VIII-A) supports the previously discussed suggestion of the reaction of iodine with the tyrosine residue of phosphatase.

From kinetic studies and from the nature of the products synthesized with different acceptors, Morton described (59) the hydrolytic and transferase actions of phosphatase as follows:

1. Formation of an enzyme-substrate (donor) complex.
2. Cleavage of the substrate between the phosphorus and oxygen bond with the formation of a "phosphorylated" enzyme.
3. Cleavage of the "phosphorylated" enzyme by water (hydrolysis) or an acceptor (transfer) forming either inorganic phosphate or a new phosphate ester.

According to this mechanism, the enzyme molecule has two different sites on its surface which are necessary for its activity. One site for binding the substrate and a second site (water site) for binding either water or the acceptor.

Vescia and Chance suggested from inhibition studies on acid phosphatase, that there are two distinct sites on the enzyme surface where water and glucose, respectively, are bound (85).

It was shown in Figure 10, that the phosphotransferase activity of phosphatase reduced only the apparent maximum velocity of inorganic phosphate (hydrolytic action) as compared to that of phenol (hydrolytic and transferase actions) formation, and the apparent Michaelis constant (Km) remained the same for both inorganic phosphate and phenol. This then might suggest that the transferase activity of phosphatase affected the rate of phenol and inorganic phosphate formation in a non-competitive manner. The apparent Km obtained when glucose-6-phosphate formation was plotted according to Lineweaver-Burk (Figure 10 and Table X) may be erroneous and it was used only for comparative purposes for the inhibition studies.

The results obtained with the inhibitors (Figure 11 to 14 and Table X) showed that with iodine the hydrolytic action (P_i) of phosphatase was affected in a different way when compared to the transferase activity (G-6-P) of this enzyme. The type of inhibition produced by cysteine on the transferase action of phosphatase seemed to be non-competitive. With respect to the rate of phenol and inorganic phosphate formation the type of inhibition by cysteine could not be clarified.

The observations mentioned suggest that there are two sites in the phosphatase molecule where water and glucose respectively are adsorbed. This is in accord with the opinion of Koshland (46) and is in disagreement with the assumption made by Morton (62) that glucose and water are simply competing for the same site.

SUMMARY AND CONCLUSIONS

1. The kinetics of hydrolysis of phenyl phosphate by alkaline phosphatase, purified from the commercially available "purified" alkaline phosphatase of calf intestinal mucosa, have been reinvestigated.
2. A nine-fold purification of phosphatase was obtained by acetone fractional precipitation of the original source of the enzyme. Paper electrophoresis of the phosphatase preparation showed two components and only one carried the phosphatase activity.
3. The optimum pH for the hydrolysis of phenyl phosphate by phosphatase was found to change to higher values with increasing the substrate concentration. The optimum substrate concentration also varied with pH. The intestinal enzyme had a narrow substrate concentration over which optimal activity was obtained. Considerable inhibition of the rate of hydrolysis was observed with higher concentrations of phenyl phosphate
4. The pKm values calculated from Michaelis-Menten plots and also from Lineweaver-Burk plots for pH 8.3 and 8.9 to 9.6, decreased with increasing pH (9.2 to 10.1). The Dixon plot of pKm against pH revealed a change of slope from -0.16 (for pH values below 9.0), to -1.2 for pH values above 9.3, with a discontinuity at about pH 9.2. Possible interpretations of the pKm-pH plot were discussed.
5. The mechanism of inhibition of the hydrolytic activity of phosphatase by iodine, cysteine, thioglycolic acid, and mercaptoethanol, has been investigated and discussed.

6. A strong inhibitory effect was noted with cysteine and iodine at concentrations in the range of 10^{-6}M. Plots of enzyme activity against enzyme concentration (Ackerman and Potter test) revealed that the inhibition produced by iodine was irreversible and by cysteine was reversible.

7. Iodine and cysteine, under the experimental conditions employed, could not be classified as non-competitive or competitive inhibitors according to the Dixon plot (1/V vs inhibitor concentration). A mixed type of inhibition has been suggested based on Lineweaver-Burk plots.

8. An additive inhibitory effect of cysteine and iodine was demonstrated when both inhibitors were added to the reaction mixture. It was assumed that iodine and cysteine acted on distinct functional groups of the enzyme.

9. The increase of inhibition by cysteine with increasing pH or with increasing concentration of cysteine at one particular pH indicated that the substance responsible for inhibition might be some ionic species whose relative concentration changes with pH. A comparison of the degree of inhibition produced by cysteine, thioglycolic acid, and mercaptoethanol at pH 10.2 indicated that the decreasing potency of these inhibitions might be due to the relative occurrence of RS^- species. The inhibition of phosphatase by cysteine at concentrations of about 10^{-6}M excluded the possibility that the inhibition was due to metal chelation.

10. The synthesis of glucose-6-phosphate from phenyl phosphate or inorganic phosphate by phosphatase was shown to be inhibited by cysteine and iodine.

11. The degree of inhibition of phosphatase by cysteine and iodine was considerably reduced in the presence of glucose when the inhibitors were added to reaction vessels containing the buffered-glucose solution and the enzyme. This protective effect of glucose on the enzyme supports the suggestion that glucose is adsorbed at a specific site in the phosphatase molecule. The possible interaction of glucose with the phenolic hydroxyl of tyrosine residue of phosphatase has been considered.

12. Lineweaver-Burk plots ($\frac{S}{V}$ vs S) revealed that with iodine the hydrolytic action (P_i) of phosphatase was affected in a different way when compared to the transferase activity (G-6-P) of this enzyme. The type of inhibition produced by cysteine on the transferase action of phosphatase seemed to be non-competitive. With respect to the rate of phenol and inorganic phosphate formation the type of inhibition by cysteine could not be clarified.

13. It was suggested that there are two sites in the phosphatase molecule where water and glucose, respectively, are adsorbed.

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