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ALKALINE PHOSPHATASE IN PLASMA MEMBRANES FROM HUMAN LIVER

by

AVIJIT CHAKRABARTTY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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IN

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Supervisor

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DATE ..... May 13, 1985.

To my mother

and father.

ABSTRACT

To determine whether the properties of alkaline phosphatase in human liver are altered by releasing the enzyme from its native environment, we studied the membrane-bound and purified forms, and the enzyme released by applying phosphatidylinositol specific phospholipase-C. The bound enzyme had the lowest affinities, for eight substrates and the competitive inhibitor phenylphosphonate. The K_i for inorganic phosphate was lower with the bound enzyme than with the purified and released forms, whereas the values for uncompetitive inhibitors were the same with all three. Phenylglyoxal reacted with essential residues of arginine at similar rates with the bound and purified enzymes. Essential cations were readily removed and replaced in the bound and released forms but not from the purified form. The bound enzyme was insensitive to changes in Mg^{2+} concentration; however, when the enzyme was purified, Mg^{2+} caused activation. The isolated membranes contained a high concentration of bound Mg^{2+} which could be removed by extensive dialysis. During removal of Mg^{2+}, a concomitant loss of alkaline phosphatase activity resulted, but replacement of Mg^{2+} restored the activity to its original value. Arrhenius plots of the bound enzyme revealed two breaks, with activation energy above the second break similar to that of the purified enzyme. Activity of the bound enzyme increased when the membrane was perturbed by butanol and assayed below 30°C.

Native molecular weights of membrane-bound enzyme released by butanol and nonionic detergents were more than twice that of the purified dimeric enzyme. Alkaline phosphatase released by phosphatidylinositol specific phospholipase-C was of both high and low molecular

weights. The high molecular weight enzyme is made up of a single protein, of the same molecular size as monomeric alkaline phosphatase.

In conclusion, the active site and inhibitor binding sites are equally exposed in membrane-bound and purified alkaline phosphatases but the former is less efficient at phosphohydrolysis when substrate concentrations are low. The membrane-bound enzyme probably has a lower binding affinity for Zn^{2+} and is completely saturated and activated by Mg^{2+} *in situ*. The enzyme has a tetrameric quaternary structure in the plasma membrane but dissociates into dimers when solubilized and purified. Alkaline phosphatase released by phospholipase-C displayed properties intermediate between the membrane-bound and purified forms; this may be a consequence of the presence of both tetrameric and dimeric enzymes in the preparation.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
CHAPS	3-[(3-chloroamidopropyl)dimethylammonio]-1-propanesulfonate
cyclic AMP	adenosine 3':5'-cyclic phosphate
EDTA	ethylene diamine tetra-acetic acid
Mes	2(N-morpholino)ethanesulfonate
Mg^{2+}-ATPase	Mg^{2+}-stimulated adenosine triphosphatase
M_r	relative molecular weight
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NP40	Nonidet P-40
SDS	sodium dodecyl sulfate
Tris	2-amino-2-hydroxy-methylpropane-1,3-diol

CHAPTER ONE

REVIEW OF PREVIOUS WORK

A. HISTORICAL ASPECTS

The understanding and demonstration that a group of nonspecific enzymes, the alkaline phosphatases (orthophosphoric-monoester phosphohydrolase, EC 3·1·3·1), hydrolyze phosphomonoesters occurred over the first twenty years of this century [1-3]. Dr. R.H.A. Plimmer elucidated the nonspecific nature of the enzyme and coined the term "phosphatase" [2]. The mammalian enzymes were first detected in 1912, in intestinal mucosa and kidney [1-3]. The phosphatases from mammalian bone, blood, liver, and placenta were discovered, in that order, over the next 23 years [4,5,6,7].

The original researchers of alkaline phosphatase would measure enzyme activity by quantitation of the inorganic phosphate produced; these assays required incubation periods of several days because of the relative insensitivity of the available methods for phosphate determination [8]. Fortunately, in 1933, Aaron Bodansky described an improved assay for the enzyme in which the incubation time was reduced to one hour [9], making it the method of choice to measure phosphatase activity.

Early attempts to purify the enzyme involved incubation of aqueous homogenates of tissue at room temperature to allow endogenous enzymes to release the insoluble alkaline phosphatase [2]. In 1950, Dr. Robert Morton suggested the solubilization of alkaline phosphate by n-butyl alcohol [10]; this procedure allowed complete solubilization within one hour and is still used in most purification protocols for the mammalian

alkaline phosphatases.

The idea that the alkaline phosphatase present in various tissues, although catalyzing the same reaction, are structurally different was introduced by Oscar Bodansky (younger brother of Aaron Bodansky) in 1937 [11]. Although this idea has been verified using many different techniques, recent questions still arise regarding the classification of certain alkaline phosphatases.

B. GENETICS OF THE HUMAN ALKALINE PHOSPHATASES

Oscar Bodansky's hypothesis about structural differences among the alkaline phosphatases of various tissues was not reinforced until the 1950's when empirical techniques such as fractional precipitation, electrophoresis, chromatography, and protein denaturants were used to detect differences [12-14]. Starch gel electrophoresis proved to be the most popular of the mentioned techniques in differentiating the various enzymes. Electrophoresis of enzymes from liver, bone, or kidney reveal a fast moving alkaline phosphatase band, whereas the placental enzyme has intermediate mobility and the intestinal enzyme has the slowest mobility [15]. Rates of thermal denaturation at 56°C indicated that alkaline phosphatases originating from liver, bone, or kidney were relatively heat labile, the intestinal enzyme had intermediate stability and the placental enzyme was stable [13]. Similar results were obtained when urea was used as the denaturant [16]. These early experiments revealed structural differences which separated the human alkaline phosphatases into three types: liver/bone/kidney type, intestinal type,

and placental type. The nature of the differences between the enzymes could not be discerned because the techniques employed are influenced by many factors such as molecular size, shape, charge, amino acid sequence, the degree of folding, and noncovalent intramolecular bonding.

Experiments investigating specific structural properties helped explain the differences observed with the empirical techniques. The native molecular weight of placental alkaline phosphatase was 116 k to 125 k, whereas the other types were 140 k to 170 k [15]. Unlike the intestinal enzyme, the liver/bone/kidney type and the placental type contain sialic acid [17]. Alkaline phosphatases from human liver, bone, and kidney are immunologically similar. Antibodies of placental or intestinal alkaline phosphatase display partial cross-reactivity but will not react with the enzymes from liver, bone, or kidney [15].

Dr. W.H. Fishman, a pioneer in alkaline phosphatase research, discovered that the three forms of alkaline phosphatase were differentially inhibited by the amino acids phenylalanine and homoarginine [18, 19]. Phenylalanine is a strong uncompetitive inhibitor of the intestinal and placental enzymes, while the enzymes from liver, bone, or kidney are not affected; homoarginine inhibits the liver/bone/kidney enzyme but not the intestinal or placental enzymes. Recently, Mulivor, Plotkin and Harris reported the specific uncompetitive inhibition of the placental enzyme by phenylalanylglycylglycine; using this new inhibitor along with homoarginine and phenylalanine, they devised a very efficient method for categorizing unknown alkaline phosphatases into one of the three groups [20].

To understand the significance of the heterogeneity, the mechanism by which these differences appeared, had to be determined. The possible explanations of how these differences arose are: identical alkaline phosphatase proteins produced in the various tissues may have been modified after initial synthesis (i.e., post-translational modification); the different alkaline phosphatases represent allelic variants of one gene; the various alkaline phosphatases are different proteins coded for by different genes. This laboratory attempted to determine the cause of the structural differences between the alkaline phosphatases by comparison of the primary structures of each of the five enzyme forms by a peptide mapping procedure. The peptide maps of the enzyme originating from liver, bone, and kidney were remarkably similar; whereas, the maps for intestinal and placental alkaline phosphatase were markedly different from each other and from those of the other tissues [21]. From these observations, it was concluded that the alkaline phosphatases from liver, bone, and kidney are coded for by a single structural gene and two separate genes code for the placental and intestinal enzymes. Structural differences among the liver, bone, and kidney enzymes from post-translational modification do arise (see Chapter 1-C).

Even though the human alkaline phosphatases are constituents of a multilocus enzyme system, the amino acid residues which are essential for activity are the same for the different gene products [22]. Furthermore, the sequence of amino acids around the active site of the placental and bacterial alkaline phosphatases is identical [23]. This conservation of structure, amongst the alkaline phosphatases, infers a common ancestoral origin for these enzymes.

By comparing the number and types of the alkaline phosphatases present in organisms appearing early in the evolutionary process with those appearing later, the genetic evolution of these proteins was mapped out. The alkaline phosphatases have a tremendous distribution in nature; the enzyme has been detected in bacteria, algae, protozoa, fungi, and vertebrates [23]. Of the alkaline phosphatases from bacteria, most is known about the E. coli enzyme. Although structural variation may occur, only one structural gene codes for the enzyme in E. coli [24]. The appearance of variants among this bacterial alkaline phosphatase is probably due to "translational ambiguities" [25]. The alkaline phosphatases from algae, protozoa, and fungi also appear to be coded for by one structural gene [23]. The multilocus enzyme system, for alkaline phosphatase, first developed in fish, which contain two structural genes for the enzyme [23]. The products of the two genes appear to be similar to the tissue unspecific alkaline phosphatase (referred to above as the liver/bone/kidney enzyme) and the intestinal enzyme found, in humans. The two gene system has been preserved in amphibians, reptiles, birds, and mammals (except in the higher primates) [26]. The gene coding for placental alkaline phosphatase, a recent product of evolution, is present in humans, chimpanzees and orangutans but not in gorillas and lower primates [27].

Another finding relating to the recent appearance in evolution of the gene for placental alkaline phosphatase was the discovery of allelic polymorphism with this isoenzyme [15]. There are three common autosomal alleles (frequency = 97.5%) and at least eighteen rarer ones (frequency = 2.5%) for the placental alkaline phosphatase locus [28]. The three

common alleles, referred to as $P1^1$, $P1^2$, and $P1^3$ account for the six common phenotypes: 1; 2; 3; 2-1; 3-1; 3-2; thirty-nine other less common phenotypes have also been reported [28]. Individuals who are heterozygous for the placental enzyme locus will produce both allelic forms of the enzyme and because the enzyme is composed of two subunits (see Chapter 1-C), a heterodimer will also be found [29]. Therefore, a heterozygous individual expressing the $P1^1$ and $P1^2$ alleles will produce the type 1, 2, and 2-1 form of the enzyme. The frequencies of the three common alleles are dependent on the ethnic origin of the population surveyed. $P1^3$ is relatively frequent in oriental individuals; $P1^2$ allele is predominant in caucasian populations; $P1^1$ allele has the highest absolute frequency, among all the alleles, in all ethnic populations [28].

In summary, there are three established genes for the human alkaline phosphatases. The gene for the placental enzyme displays tremendous allelic polymorphism. The enzymes from liver, bone, and kidney are coded for by a single gene but post-translational modification does occur (see Chapter 1-C). Recent evidence suggests that the enzymes from fetal intestine [30] and testes [31] are products of two new genes.

C. PHYSICAL AND CHEMICAL STRUCTURE

The structural properties of the phosphatases from placenta and liver were the first to be investigated. The native molecular weight of the purified placental enzyme is 116 k, and in guanidine hydrochloride the molecular weight is 58 k, indicating a dimeric conformation of the native enzyme [32]. The dimeric placental enzyme is stable between pH

4.7 and pH 10.3, but the dimer dissociates rapidly at pH 2.3 and slowly at pH 10.5 [33,34]. The pH dependent dissociation was accompanied by a loss in enzyme activity indicating the essential requirement of a dimeric conformation to maintain the catalytic function of the enzyme. Using various techniques, the native molecular weight of alkaline phosphatase from human liver is between 130 k and 180 k [35-38] and is dimeric [39]. The subunit molecular weight is in the range of 68 k to 72 k [35-38]. Thus, both alkaline phosphatase from human liver and placenta are dimeric, but the liver enzyme is composed of larger subunits. The enzymes originating from human intestine, kidney, and bone resemble the liver form with regards to native and subunit molecular weights [40].

The composition of amino acids of alkaline phosphatases from humans, animals, and bacteria are remarkably similar [23]. These enzymes are especially rich in the acidic amino acids, aspartate, and glutamate [23]. The sequence of amino acids around the active site of the enzyme from numerous sources, is Asp-Ser-Ala; the serine residue becomes phosphorylated during catalysis [41]. The other amino acids that are important for the catalytic function of the enzyme include histidine, lysine, arginine, and cysteine [42-45].

Another essential component for the catalytic function of all alkaline phosphatases is Zn^{2+} [23]. Each subunit of E. coli alkaline phosphatase contains three metal ion-binding sites designated as A, B, and C, in descending order of metal ion affinity [46]. Site A is composed of three histidyl residues and is 3.9 Å from site B, which is composed of one histidyl residue and a number of carboxyl functions; site C is composed of unidentified oxygen ligands and is 4.9 Å from B

and about 7 Å from A [46]. Purified preparations of E. coli alkaline phosphatase contain 2.7 to 3.5 g·atoms of Zn^{2+} and 1.0 to 1.8 g·atoms of Mg^{2+} [47] per enzyme dimer. The dimer must contain two g·atoms of Zn^{2+} to display catalytic function but four g·atoms are required for maximum activity [47]. Zinc ions are able to fill sites A and B, whereas Mg^{2+} can be bound by sites B and C [47]. In the native state, the A sites are filled with Zn^{2+} and C sites with Mg^{2+}; the occupants of the B sites are less consistent. Zinc ions bound by site A co-ordinate the conversion of the phosphoenzyme intermediate to a noncovalent phosphate–enzyme complex (see Chapter 1-D) [47]. The roles of the metals in sites B and C are not well understood. Although this information is derived from the enzyme from E. coli, it is not unreasonable to expect the human enzymes to behave in a similar fashion, considering the similarities in the active site region.

Magnesium ions, unlike Zn^{2+}, are not essential for activity, but they do enhance the activity of mammalian alkaline phosphatases and the presence of Mg^{2+} facilitates the incorporation of Zn^{2+} into alkaline phosphatases which have been depleted of metal ions [47]. The human alkaline phosphatases vary in their response to Mg^{2+}. The placental enzyme is least enhanced by Mg^{2+}, whereas the tissue unspecific alkaline phosphatases are almost inactive in the total absence of Mg^{2+} [23]. The mechanism by which Mg^{2+} enhances enzyme activity is not known, but the ions produce shifts in the circular dichroism spectra of placental alkaline phosphatase, indicating a slight separation of subunits which may increase access to the active site [34].

Besides metal ions, the other nonprotein component of alkaline phosphatase is the carbohydrate moiety. The carbohydrate chains are composed of galactose, mannose, fucose, galactosamine, and glucosamine [48], and except for the enzyme from adult intestine, N-acetyl neuraminic acid [49]. Human liver, kidney, and bone alkaline phosphatase differ in carbohydrate content, but are identical in all other respects [40].

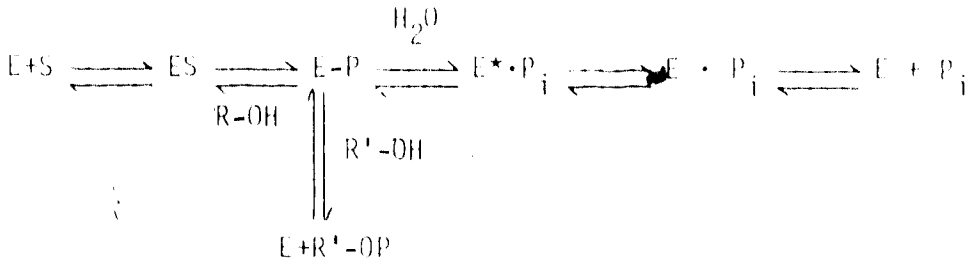
D. MECHANISM OF CATALYSIS

Alkaline phosphatases are able to hydrolyze a variety of phosphomonoesters as follows:

$$R\text{-}O\text{-}\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}\text{-}O^- + H_2O \rightleftharpoons R\text{-}OH + HPO_4^{2-}$$

The nature of the leaving group (R-OH) does not influence the rate of the phosphohydrolysis but does determine the relative affinity the enzyme has for the substrate [23]. As a general rule, phosphoesters with leaving groups which are hydrophobic and not bulky are usually the best substrates. Phosphodiesters, such as NAD and cyclic AMP, are not hydrolyzed by these enzymes [23]. Inorganic pyrophosphate and adenine nucleotides are good substrates if no Mg^{2+} is complexed to them [50]. The other nonphosphoester substrates include small linear polyphosphates ($n<8$) and esters of phosphoramidic acid and phosphorothioates [23].

The current proposal for the reaction mechanism of alkaline phosphatase is as follows:



The enzyme noncovalently binds the substrate, rapidly cleaves the phosphoester bond, releasing the leaving group, and becomes phosphorylated at the active serine residue [51]. If this reaction is conducted at low pH, then the phosphorylated enzyme is stabilized and after the initial burst, no further phosphoesterase activity occurs [52]. At alkaline pH, the phosphorylated enzyme intermediate isomerizes and the active serine residue is dephosphorylated, leaving the isomerized enzyme noncovalently complexed with HPO_4^{2-}. Restoration of the original conformation of the enzyme determines the rate of the overall reaction. The rate determining step may be bypassed and the reaction velocity increased, if phosphoacceptors other than water are present (e.g., ethylaminoethanol and diethanolamine) [51]. The acceptors react with the phosphorylated intermediate, and the resulting transphosphorylation reaction prevents the isomerization of the enzyme and eliminates the formation of the $E^*\cdot P_i$ complex.

The possibility that alkaline phosphatase operates as an allosteric enzyme is a current controversy. Under certain conditions, negative co-operativity in phosphate binding has been demonstrated [53]. This has been disputed by workers who believe that the curved Scatchard plots obtained by equilibrium dialysis experiments arise not from co-operativity but from residual phosphate contamination in the enzyme

preparations [55].

The observation of the migration of metal ions from one subunit to the other when inorganic phosphate is introduced to the system is additional evidence, indicating communication between subunits [54].

E. LOCATION OF ALKALINE PHOSPHATASE IN HUMAN TISSUES

Alkaline phosphatase is located on the endothelial and Kupffer cells lining the blood sinusoids of human liver [56]. The surface of the bile canaliculi, the central hepatic veins, and the portal veins are also rich in alkaline phosphatase, but special fixation procedures must be used to preserve the enzyme activity at these locations [57]. The outer membrane of the chondrocytes in calcifying cartilage and the osteoblasts are sites of bone alkaline phosphatase [23]. Kidney alkaline phosphatase is localized in the microvilli of the renal tubular cells [23]. In neutrophils, alkaline phosphatase is found on the plasma membrane and in unique vesicles termed "phosphasomes" [58].

The brush border of mucosal cells in the small intestine contains the intestinal enzyme [59]. Examination of these tissues by electron histochemistry also reveals enzyme activity in the golgi apparatus where it is probably in transit to the apical membrane; lysozomal granules, the site of enzyme degradation, also contains alkaline phosphatase activity [59]. The enzyme is attached onto the intestinal brush border by a 3000 dalton foot [60]. Placental alkaline phosphatase is located on the plasma membrane of the syncytiotrophoblast [61]. Most of the enzyme molecule, including the active site, is on the extracellular side of the membrane; a 2000 dalton foot is buried in the membrane [62].

F. INDUCTION OF ALKALINE PHOSPHATASE IN CELLS

Induction, in this case, is defined as an increase in alkaline phosphatase protein in cell or organ culture upon addition of a specific compound. Many compounds are able to induce alkaline phosphatase [62], the ones discussed here include: bile, cyclic-AMP, glucocorticoids, and 1,25-dihydroxyvitamin D_3.

The development of cholestasis by ligation of the bile ducts in rats causes an increase in alkaline phosphatase activity, of hepatic origin, in the serum [63]. The content of alkaline phosphatase in the small intestine of mice also increases after ligation of the bile ducts [64]. Further experiments demonstrated that the increase in bile acids, after ligation, is the stimulus which promotes the de novo synthesis of the hepatic enzyme and the subsequent spillage into serum [65]. Glycosylation, as well as protein synthesis, are needed for maximum induction [66]. This induction was also observed in cultured hepatocytes which were exposed to bile acids [67]. Bile-duct ligation and exposure of cells to bile acids also increases the content of intracellular cyclic-AMP, and inhibitors of adenylate cyclase inhibit the induction of alkaline phosphatase by bile acids; this suggests that cyclic-AMP is the mediator of bile acid induction [68].

Direct induction of alkaline phosphatase by cyclic-AMP has been demonstrated in many cell lines [62]. Preventing the degradation of cyclic-AMP, by phosphodiesterase inhibitors, enhances the induction by this hormonal messenger [69]. Inhibiting RNA and protein synthesis blocks the induction [69] which occurs when cyclic-AMP stimulates the production of mRNA which codes for the alkaline phosphatase protein [70].

The newly synthesized enzyme requires a functioning glycosylation system for the intercalation of the enzyme into the plasma membrane, consequently, inhibitors of glycosylation prevents the expression of the induced activity [71].

The administration of glucocorticoids will increase alkaline phosphatase activity in animals [72] and humans [73]. The steroids greatly increases production of placental alkaline phosphatase in tumour cells with low basal levels of the enzyme but not in cells which produce the enzyme in a constitutive fashion [74]. Few cell lines which produce the tissue unspecific enzyme respond to these inducers [75]. The induction is dependent on protein and RNA synthesis and on the growth phase of the cell culture [76]. The mechanism of this induction is not well understood, but the participation of the glucocorticoids in the assembly of the dolichol-linked oligosaccharides during protein glycosylation may play a part [77].

1,25-dihydroxyvitamin D_3 increases the alkaline phosphatase activity in selected regions of the small intestine in rats [78] and chicks [79]. This vitamin D metabolite also induces alkaline phosphatase activity in cultured osteoblast-like cells, and the amount of enzyme produced depends on the dose [80]. The magnitude of the response to the vitamin depends on the maturity of the osteoblastic cells, with the younger ones being more responsive [81]. The induction is dependent on protein and RNA synthesis, and is mediated by the binding of the vitamin D metabolite to a cytoplasmic receptor which activates the enzyme genome and promotes the de novo synthesis of alkaline phosphatase [82].

G. ALKALINE PHOSPHATASE IN DIAGNOSTIC ENZYMOLOGY

Increased levels of alkaline phosphatase in the serum of a patient, who is not pregnant, usually indicate the presence of hepatic or bone disease [23]. The particular enzyme which is elevated in the serum is determined by the site of the disease. The greatest elevation of alkaline phosphatase originating from liver occurs in hepatic diseases which involved cholestasis (obstruction of bile flow) and results in the induction of the enzyme in liver cells (see Chapter 1-F). The cholestasis may occur in the bile ducts outside of the liver (e.g., primary biliary cirrhosis, cholangitis) or in the bile canaliculi within the liver (e.g., by drugs, viral infections, or infiltration by malignant cells) [83]. Primary carcinoma of the liver and secondary infiltration (causing cholestasis) will also elevate serum alkaline phosphatase of hepatic origin [23].

Bone diseases in which the activity of the osteoblasts (cells involved in bone formation) is increased will raise the serum level of alkaline phosphatase from bone. Patients suffering from Paget's disease (osteitis deformans) usually present the highest elevations of the bone enzyme [83]. The increased osteoblastic activity seen in patients with rickets and osteomalacia accounts for the high levels of the enzyme in their serum [23]. Osteoscarcomas which are osteogenic (bone-forming) will raise serum levels of the enzyme, but in osteolytic (bone-resorbing) osteosarcomas, serum levels are normal or only slightly elevated [83].

Hypophosphatasia, a genetic disorder, can be diagnosed by a low serum alkaline phosphatase level and increased phosphoethanolamine and pyrophosphate in the serum and urine [84]. The disorder may be inherited

as an autosomal recessive or autosomal dominant trait, and the onset may occur in infantile, juvenile or adult life [84]. In the infantile disease, the tissue unspecific gene is not expressed, but there is some compensatory expression of the intestinal gene [85].

The discovery of an alkaline phosphatase indistinguishable from the placental enzyme in the serum of a patient with an oat cell carcinoma of the lung stimulated great interest in this enzyme as a tumour marker [86]. This new enzyme was named the Regan isoenzyme after the patient. The purified Regan enzyme is identical to the placental enzyme with regard to subunit size, N-terminal sequence, and two dimensional peptide maps [87]. This enzyme has since been detected in carcinomas of the pancreas, lung, breast, colon, lymph nodes, kidney, stomach, and bladder [88]. The Regan enzyme is most prevalent in the serum of patients with germ cell tumours of the testes, and ovarian and uterine cancer [88].

Another onco-alkaline phosphatase, discovered in a patient (Nagao) with lung cancer, is similar to but not identical with the placental enzyme [89]. The Nagao enzyme has been reported to be identical to the rare D-variant allele of placental alkaline phosphatase [90], but this has been refuted [91].

H. PHYSIOLOGICAL FUNCTIONS OF ALKALINE PHOSPHATASE

The presence of alkaline phosphatase in organisms at each stage of evolutionary development implies an essential role of the enzyme in some fundamental biological process. The lack of this enzyme in humans from birth (infantile hypophosphatasia) presents severe clinical problems and

is often fatal. Many suggestions of the role of alkaline phosphatase have been reported [23], but none have received universal support. Three proposed roles which show the greatest promise are discussed below.

Dr. Robert Robison, in 1923, speculated that alkaline phosphatase participates in calcification of human bone by liberating free phosphate from phosphoesters and the local increase in phosphate concentration led to the deposition of calcium phosphate salts on the organic bone matrix [92]. Although Robison's explanation of how alkaline phosphatase initiates calcification is not possible [93], his belief in the participation of the enzyme in calcification has been substantiated. The most convincing evidence of the participation of alkaline phosphatase in calcification of bone arose from the pathologic investigation of the infantile hypophosphatasia [84,94]. Patients who have inherited this disease do not produce alkaline phosphatase in their bones, liver, kidneys and neutrophils, and majority of their skeleton is osteoid (organic bone matrix which has not been calcified) [84]. In the developing teeth of rats, alkaline phosphatase was concentrated around areas of calcification [95]. The matrix-vesicles of epiphyseal cartilage, the initial sites of calcification, are rich in alkaline phosphatase [96]. With the demonstration of pyrophosphate hydrolysis by alkaline phosphatase from matrix-vesicles [96], a new explanation of how the enzyme participates in calcification was forwarded. Pyrophosphate inhibits calcification and the role of alkaline phosphatase may be to remove the pyrophosphate and allow the calcification to continue [97]. Additional evidence was the presence of excess pyrophosphate in the serum and urine of patients with hypophosphatasia [84]. Critics of this

theory argue the concentration of Ca^{2+} and inorganic phosphate in epiphyseal cartilage is high enough to essentially inactivate the phosphohydrolase activity of the bone enzyme [98]. With the discovery of specific pyrophosphatases in calcifying costal cartilage in rats and scapula cartilage in pigs [99], the theory was weakened further. Although the role of alkaline phosphatase in removing pyrophosphate from the calcification site is still controversial, the participation of alkaline phosphatase in the calcification process is generally accepted.

The alkaline phosphatases from liver, intestine, kidney, and placenta are associated with microvilli; the site of extensive active and passive transport. From this observation, the possible participation of alkaline phosphatase in transporting solutes across membranes was presented [23]. The affinity these enzymes have for inorganic phosphate makes the ion an obvious candidate as the transported solute. Early experiments demonstrated that the rate of synthesis of the E. coli enzyme is inversely proportional to the phosphate concentration in the growth media [100], and strains of E coli which cannot produce alkaline phosphatase will not grow in phosphate-poor media [101]. Similarly, rats feed a phosphate-poor diet will induce the de novo synthesis of the enzymes from kidney and intestine, and simultaneously increase the rate of phosphate transport at these sites [103]. The addition of alkaline phosphatase antiserum to segments of rat intestine greatly inhibited phosphate transport [103]. NAD and NADH inhibit alkaline phosphatase and phosphate transport in the kidneys of rats [104], but other inhibitors of alkaline phosphatase do not inhibit phosphate transport at these and other sites [105,106]. Using phosphatidylinositol specific phospho-

lipase-C, an enzyme which selectively removes alkaline phosphatase from various membranes [10], Yusufi et al. reported identical rates of phosphate transport in renal brush border membrane vesicles that were depleted of alkaline phosphatase and in untreated membrane vesicles [108]. The experiment of Yusufi et al. provides the best evidence against the direct involvement of alkaline phosphatase in phosphate transport, assuming that the in vitro measurement of phosphate transport is an accurate representation of the in vivo system.

A third possible role for alkaline phosphatase is participation in reversible phosphorylation reactions which regulate many metabolic processes. The intrinsic protein-phosphatase from ox brain cortex and alkaline phosphatases are both inhibited by EDTA and activated by Mg^{2+} [109]. Placental alkaline phosphatase dephosphorylates ^{32}P-labelled histones, protamine, glycogen synthetase, casein, and phosvitin but not phosphorylase a [110]. Similarly, the E. coli enzyme is able to dephosphorylate glycogen synthase D, histones, and phosphorylase kinase but not phosphorylase a ; the dephosphorylation of glycogen synthase D is inhibited by glycogen [111]. Alkaline phosphatase from E. coli, calf intestine, and bovine liver appear to selectively dephosphorylate the phosphotyrosine residues in phosphorylated proteins [112]. Evidence against the participation of alkaline phosphatase in reversible phosphorylation has not been reported; this proposed role shows the greatest promise.

I. PURPOSE OF THIS STUDY

Most research on alkaline phosphatase is conducted to establish physiological and biochemical roles for this enzyme. Previous studies of highly purified preparations have yielded much information about the physical, chemical, and kinetic features of the enzyme, and many suggestions of possible roles have been reported (see Chapter 1-H); but, none have received universal support. The enzyme is found in the plasma membrane of most cells and may have different properties in this hydrophobic environment. Thus, the present study was undertaken to assess the effect of the plasma membrane on some properties of the enzyme. A protocol for the isolation of plasma membranes from normal human liver, obtained at necropsy, was established. The alkaline phosphatase in these membranes was extensively studied with respect to certain physical, chemical, and kinetic parameters, and these properties were compared with those of the solubilized and purified enzyme from this same tissue. Evaluation of the information obtained will, hopefully, contribute to a better understanding of the role(s) for human alkaline phosphatase.

CHAPTER TWO

CATALYTIC PROPERTIES OF MEMBRANE-BOUND ALKALINE PHOSPHATASE

A. INTRODUCTION

Most comparisons of the properties of soluble and membrane-bound alkaline phosphatase from animal tissues have shown only minor differences [98,113-116]. These include different Michaelis constants for substrates and cation requirements of membrane and cytosolic forms of rat hepatic alkaline phosphatase [113], and minor differences in inhibitor constants of the purified and membrane-bound enzyme from chick bone [98].

In the present study, I sought to determine whether the properties of alkaline phosphatase from human liver are different when the enzyme is in its native environment from when it is in purified soluble form. In characterizing the membrane-bound enzyme, I considered substrate and inhibitor specificities, sensitivity to covalent modifications, cation requirements, and the influence of the state of the membrane on enzyme activity. To determine whether differences between the bound and free enzymes arise from removal of the alkaline phosphatase from the membrane, or from other steps in purification, I also studied the enzyme released from membranes by phosphatidylinositol-specific phospholipase-C [107].

B. MATERIALS AND METHODS

1. Chemicals

Phospholipase-C (B. cereus) type V, p-nitrophenyl-phosphate, naphthol AS-MX phosphate, β-naphthylphosphate, β-glycerophosphate, phospho-L-serine, phospho-DL-threonine, phospho-DL-tyrosine, pyridoxal phosphate, pyrophosphate, homoarginine, phenylglyoxal, phenylalanine, phenylalanylglycylglycine, tetramisole, and NADH were purchased from Sigma Chemical Co. (St Louis, MO); phenylphosphonic acid from Aldrich Chemical Co. (Milwaukee, WN); and Percoll from Pharmacia Fine Chemicals AB (Uppsala, Sweden). All other chemicals were of highest reagent grade (Fisher Scientific, Fairlawn, NJ).

2. Preparation of Plasma Membranes

Samples of liver from patients with no known disease affecting hepatic function were obtained at necropsy within 12 h of death and were stored at -25°C in 10% (v/v) glycerol with 0.15 mol/l NaCl until use.

The membranes were isolated by the method outlined by Neville [117], with some modifications. The samples were thawed, and aliquots of 6 g were minced in a Sorvall Omni-mixer in 30 ml of cold 0.25 mol/l sucrose, and 5.0 mmol/L sodium bicarbonate (pH 7.5) for 15 sec. The mince was then subjected to three strokes in a glass Potter-Elvehejm homogenizer with a Teflon piston. The homogenate, consisting mainly of free intact cells, were sonicated (Model W-375, Heat Systems Ultrasonics) for 2.5 min at setting 5, then centrifuged for 20 min at 27,000 xg. The upper layer of the resulting pellet was suspended and was centrifuged at 27,000 xg for 20 min; the centrifugation was repeated until the

suspension was no longer separated in two layers.

The final suspension was layered onto a solution of 15 Percoll and 0.25 mol/l sucrose (starting density, 1.051 g/ml and centrifuged at 28,000 xg for 1 h. The ultracentrifuge tube was fractionated, and the membrane-enriched fractions were identified. Succinate dehydrogenase, NADPH cytochrome-C reductase, and 5'-nucleotidase were assayed [8-10] as markers for mitochondria, microsomes, and plasma membranes, respectively (Table 1).

3. Preparation of Enzymes and Assays

Alkaline phosphatase was purified to homogeneity, as judged by gel electrophoresis on sodium dodecyl sulfate as described previously [121]. Phosphatidylinositol-specific phospholipase-C was obtained by purification from a commercial preparation of non-specific phospholipase-C [122]. Phospholipase-C (8 µg protein) was added to liver membrane fractions (4 mg protein) suspended in 4 ml of 0.25 mol/l sucrose and 50 mmol/l Tris, pH 7.6. The mixture was incubated at 30°C for 1 h, then centrifuged at 100,000 xg for 20 min. The supernatant fluid containing the released alkaline phosphatase was stored at 4°C until used.

Activities of the purified and membrane-bound alkaline phosphatases were determined in medium containing 10 mmol/l p-nitrophenylphosphate, 50 mmol/l sodium bicarbonate, 1.5 mmol/l magnesium chloride, and 0.25 mol/l sucrose (to maintain iso-osmotic conditions), pH 9.0 at 30°C. Purified enzyme (50 µl) or membrane suspension (5 µg protein) was added to 1 ml of assay medium, and the increase in absorbance at 404 nm was monitored in a spectrophotometer (Varian Model 2200). Enzyme activity was expressed as micromoles of p-nitrophenol released/min. When sub-

strates other than p-nitrophenylphosphate were tested, incubation was for 15 min at 30 C, the reaction was stopped with trichloroacetic acid, the inorganic phosphate produced was determined [123] and activity was expressed as micromoles of P_i released/min. Mg^{2+} concentration in membrane preparations and detergent extracts was determined by atomic absorption spectrophotometry (GBC Model 902), according to the procedure outlined in the GBC manual.

4. Phenylglyoxal Reaction with Arginine Residues

To determine the reaction of phenylglyoxal with arginine residues, 4 mU of purified or membrane-bound alkaline phosphatase was incubated at 30°C in 0.4 ml of a solution containing 0.125 mol/l sucrose, 62.5 mmol/l sodium bicarbonate, and 0 to 10 mmol/l phenylglyoxal, at pH 8.3. Activity was monitored for 36 min. (The concentration of phenylglyoxal was kept low to reduce perturbation of the membranes.) Second-order rate constants were determined from the slopes of the graph of pseudo-first-order rate constants vs phenylglyoxal concentration.

C. RESULTS

1. Preliminary Characterization of Plasma Membranes

The degree of plasma membrane purification is given by the enrichment of enzyme markers specific for various organelles; these are calculated from specific activities determined before and after purification (Table 1). For alkaline phosphatase, this enrichment was 15-fold.

Experiments investigating the properties of alkaline phosphatase bound to plasma membranes did not solubilize the enzyme; centrifugation

TABLE I

ENRICHMENT OF MARKER ENZYMES: CRITERIA OF PURITY OF FINAL MEMBRANE PREPARATION

Organelle	Marker Enzyme	Enrichment[a]
Microsomes	NADPH-cytochrome C reductase	1.8±0.27
Mitochondria	Succinate-cytochrome C reductase	0.77±0.053
Plasma membranes	Alkaline phosphatase	15±6.1
Plasma membranes	5'-Nucleotidase	7.4±2.2

[a] The enzyme's specific activity (U/mg protein) in the final membrane fraction, divided by its specific activity in the whole-liver homogenate. Mean±SD (n=5).

of the membrane suspension after concluding the experiment revealed 5 solubilization of the enzyme. Treatment of plasma membranes with phospholipase-C released variable amounts of the bound alkaline phosphatase (Fig. 1), dependent on the amount of phospholipase-C protein added; the unsolubilized activity was recovered in the pellet. Measurement of phosphohydrolytic activity as a function of pH confirmed that activities in the purified and enriched-plasma membrane fractions were from alkaline phosphatase. Both the purified and the membrane-bound enzymes were most active in the alkaline region and no difference in pH profile was observed. The profile obtained with gene-specific inhibitors (homoarginine, phenylalanine, and phenylalanylglycylglycine) confirmed that the purified enzyme and the membrane-bound activity were the same isoenzyme, products of the gene for tissue unspecific alkaline phosphatase [21,124].

2. Investigation of Active and Inhibition Sites

The bound enzyme showed no altered preference for any of the substrates examined (Table II), but the K_m values were 2.3 ± 0.91 (SD)-fold higher than the values for the purified enzyme. The release enzyme had K_m values intermediate to those of the other two forms. In the inhibition experiments (Table III), the apparent K_i for phenylphosphonic acid was 2.4-fold higher for the membrane-bound alkaline phosphatase; this is close to the substrate K_m ratios of the phosphoester substrates. The constant for inorganic vanadate was not very different for the purified and membrane-bound activities. However, the constant for inorganic phosphate was 1.78-fold lower for the bound enzyme than for the purified form, the reverse of the K_m ratios of the substrates. The phosphate K_i

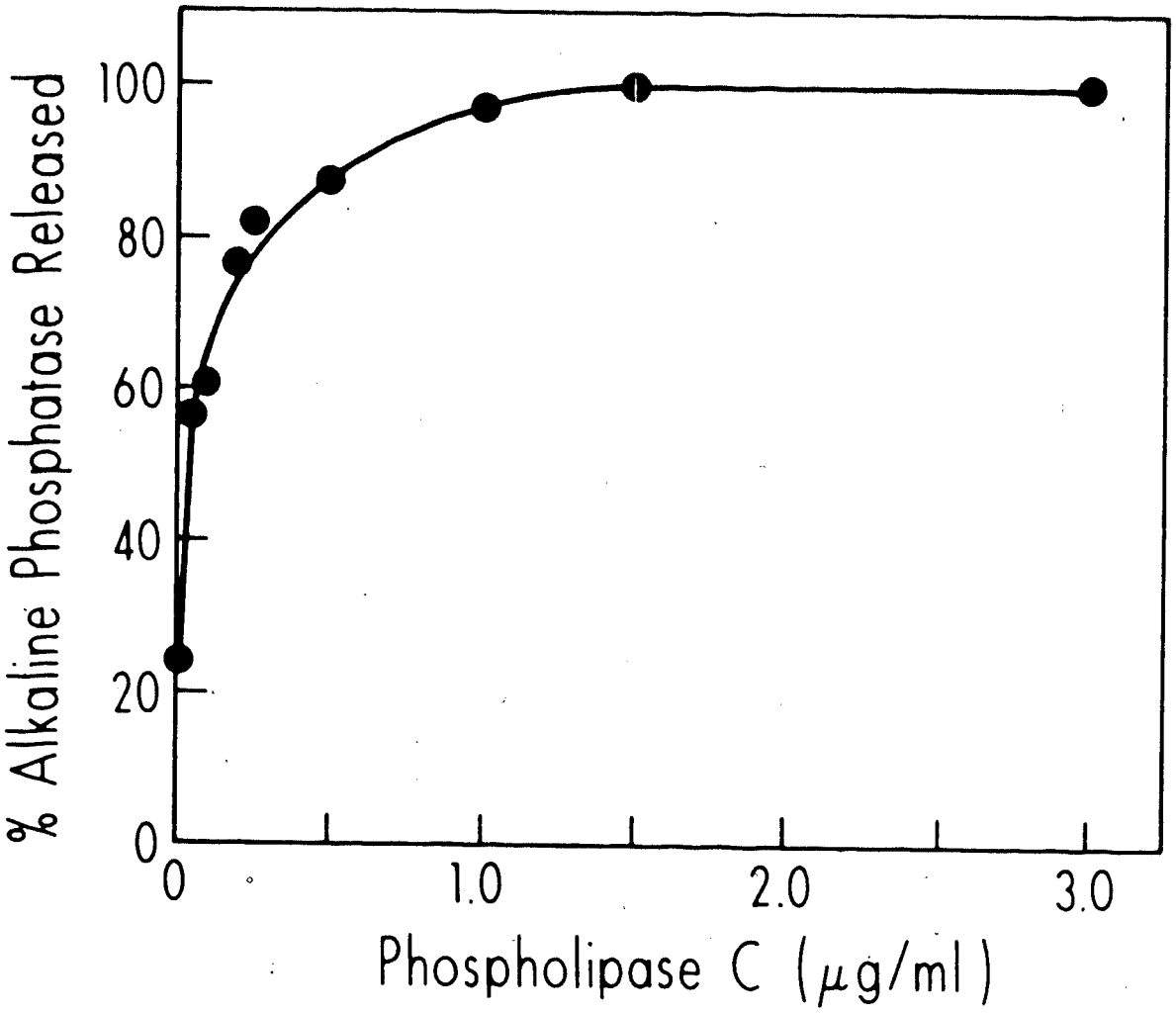


Figure 1. Release of alkaline phosphatase by phospholipase-C. Aliquots of isolated membranes in 0.25 mol/l sucrose and 10 mmol/l Tris (pH 7.6) were incubated with various amounts of phospholipase-C for 30 min at 30°C and then centrifuged for 10 min at 100,000 xg. Alkaline phosphatase activity in the supernatant fluid was expressed as a percentage of the activity in the sample before centrifugation.

for the released enzyme was similar to that for the purified form, and the uncompetitive inhibitors were equally effective against both.

Decay in activity resulting from phenylglyoxal reaction with arginine residues in the enzyme demonstrated similar rates of inactivation for both the bound (Fig. 2) and the soluble forms, the second-order rate constants being 0.044 $(mol/l)^{-1}(s)^{-1}$ and 0.035 $(mol/l)^{-1}(s)^{-1}$, respectively.

3. Interaction of Metal Ions

Incubation of membrane preparations, released enzyme, and purified alkaline phosphatase in an iso-osmotic EDTA solution reduced activity by 50% in 8, 4, and 60 min, respectively (Fig. 3). When the EDTA treatment was continued until only 10% of the original activity remained (105 min for the purified enzyme), the addition of 0.30 mmol/l $ZnCl_2$ restored activity to 70% of its original activity in the bound and released forms (Fig. 4), but this and other concentrations had no appreciable effect on the purified enzyme. Compared with the membrane-bound enzyme, the released form appeared less sensitive to inhibition by high concentrations of Zn^{2+}. When membrane preparations were treated with EDTA for 5, 20, 30, 45 and 60 min, Zn^{2+} reconstitution restored a constant percentage of activity, independent of EDTA incubation time (data not shown).

Mg^{2+} increased the activity of purified alkaline phosphatase 5-fold and the phospholipase-C released enzyme by 1.2-fold, but had little effect on the membrane-bound form (Fig. 5). The Mg^{2+} concentration in the membrane preparations was 75 nmol/mg of membrane protein. Dialysis of the membrane preparation resulted in coincident rates of removal of Mg^{2+}

TABLE II

SUBSTRATE SPECIFICITIES OF PURIFIED AND MEMBRANE-BOUND ALKALINE PHOSPHATASE, AND THE FORM RELEASED BY PHOSPHOLIPASE-C

K_m values for acid-stable phosphoesters were determined from double reciprocal plots. For the acid-labile phosphoesters (phospho-DL-threonine, phospho-DL-tyrosine, and pyrophosphate), apparent K_m values were determined from Dixon plots, using p-nitrophenylphosphate as the competitive substrate; n.d., not determined.

Substrate	K_m or apparent K_m (mmol/l)			K_m ratio[a]
	Purified enzyme	Released enzyme	Membrane-bound enzyme	
p-nitrophenyl phosphate[b]	0.088 ±0.006	0.15 ±0.022	0.39 ±0.018	4.4
β-Naphthyl phosphate	0.026	0.059	0.078	3.0
β-Glycerophosphate	1.5	2.2	3.0	2.0
Phospho-L-serine	0.38	n.d.	0.68	1.8
Pyridoxal phosphate	0.20	n.d.	0.37	1.8
Phospho-DL-threonine	0.39	n.d.	0.90	2.3
Pyrophosphate	0.12	n.d.	0.21	1.7
Phospho-DL-tyrosine	0.40	n.d.	0.72	1.8

[a] K_m (bound enzyme)/K_m (purified enzyme)

[b] values are means±SD (n=5)

TABLE III

INHIBITION SPECIFICITIES OF PURIFIED AND MEMBRANE-BOUND ALKALINE PHOSPHATASE, AND THE FORM RELEASED BY PHOSPHOLIPASE-C

K_i values for the competitive inhibitors were determined from Dixon plots. The K_{ii} for each uncompetitive inhibitor was determined from double reciprocal plots, using five concentrations of both the substrate and the inhibitor. p-Nitrophenylphosphate was the substrate; n.d., not determined.

Inhibitor	Inhibitor constant (mmol/l)		
	Purified enzyme	Released enzyme	Membrane-bound enzyme
Competitive			
Phosphate	0.96	1.09	0.54
Vanadate	0.044	n.d.	0.065
Phenylphosphonate	0.25	n.d.	0.60
Uncompetitive			
Homoarginine	0.91	n.d.	0.87
Tetramisole	0.012	n.d.	0.015
NADH	0.45	n.d.	0.37

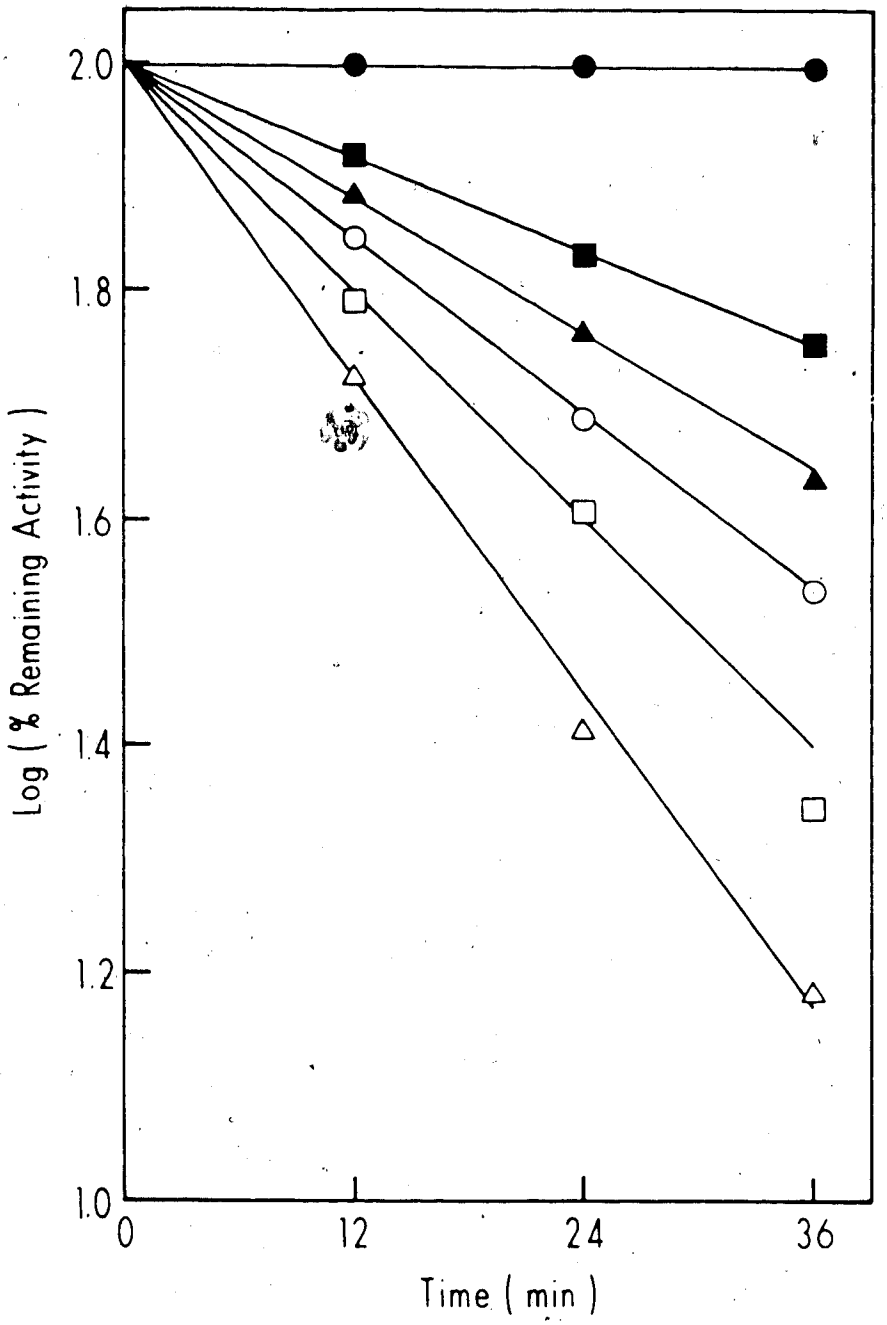


Figure 2. Activity decay curve of membrane-bound alkaline phosphatase by reaction with phenylglyoxal. Membrane suspension were incubated in 0.125 mol/l sucrose, 62.5 mmol/l $NaHCO_3$ (pH 8.3), and phenylglyoxal in a concentration (mmol/l) of: ●, 0; ■, 2; ▲, 4; ○, 6; □, 8; or △, 10.

and loss in alkaline phosphatase activity (Fig. 6). If 5 mmol/l Mg^{2+} was added to the dialyzed membrane preparation, then enzyme activity returned to the original level. Dialysis of Nonidet P-40 solubilized enzyme resulted in faster rates of Mg^{2+} removal and loss in activity, but addition of 5 mmol/l Mg^{2+} did not restore all of the original activity.

4. Membrane Fluidity and Enzyme Activity

Arrhenius plots of the membrane-bound and purified enzyme (Fig. 7) showed the former to have a major and a minor break, at 15.7°C and 26.2°C, respectively. The activation energy of the reaction it catalyzed at temperatures above the second break was 10.98 kcal/mol, very similar to that obtained with the purified enzyme (11.71 kcal/mol), indicating that both forms are equally efficient catalysts at higher temperatures. Measurement of the activity after the application of either butanol or pentanol, on the assumption that their perturbant action would increase fluidity of the membrane, showed that the membrane-bound form responded to changes in fluidity by increases in activity only at lower temperatures (Fig. 8). Repetition of the experiment with purified enzyme revealed no changes in activity, and incubation of membrane fractions in the butanol solutions showed no solubilization of alkaline phosphatase. Direct inhibition of enzyme activity prevented the use of benzyl alcohol.

D. DISCUSSION

Substrate and inhibitor studies (Tables II and III) show reduced binding affinity of phosphoesters and phosphoester analogs, but not of

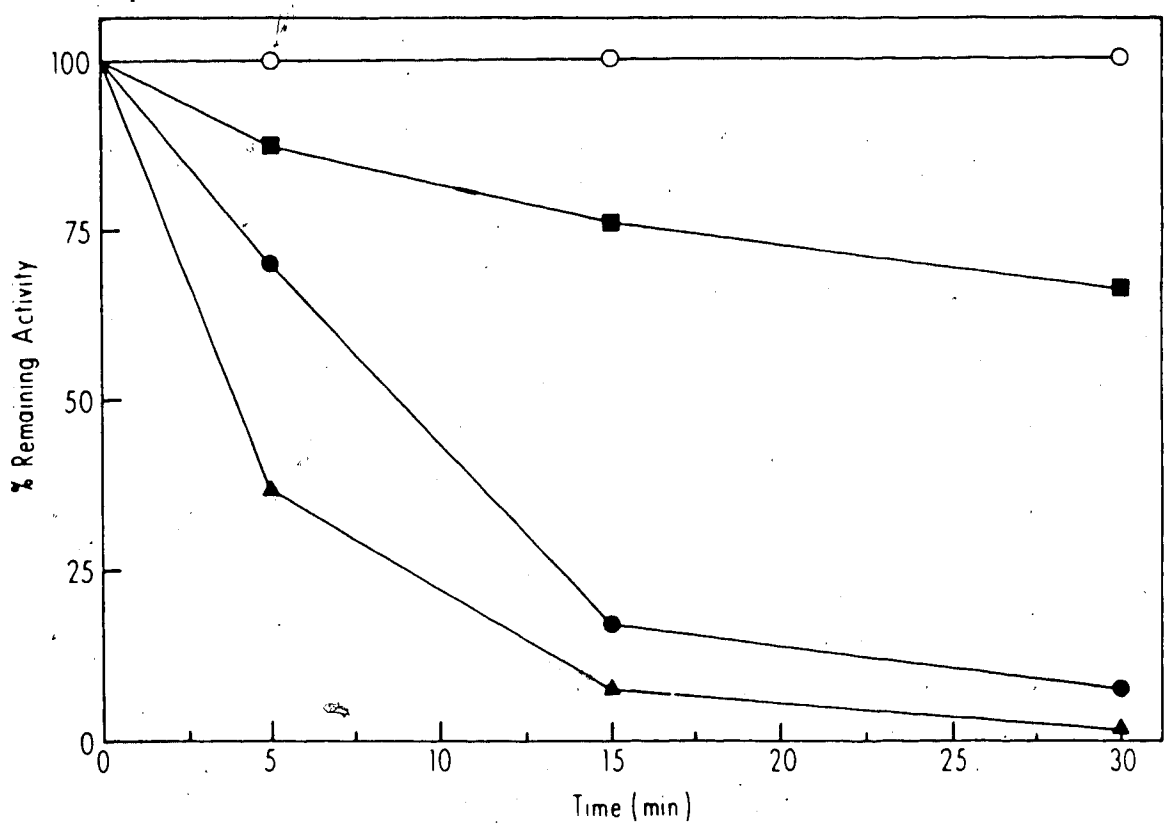


Figure 3. Time course of alkaline phosphatase inactivation by EDTA. Alkaline phosphatase activity in solutions containing 1.0 mmol/l EDTA, 0.25 mol/l sucrose, and 10 mmol/l Tris (pH 7.2), with various enzyme forms, was monitored as: ●, membrane-bound enzyme; ▲, phospholipase-C-released enzyme; ■, purified enzyme; ○, control containing no EDTA (the same curve was obtained using all three enzyme forms).

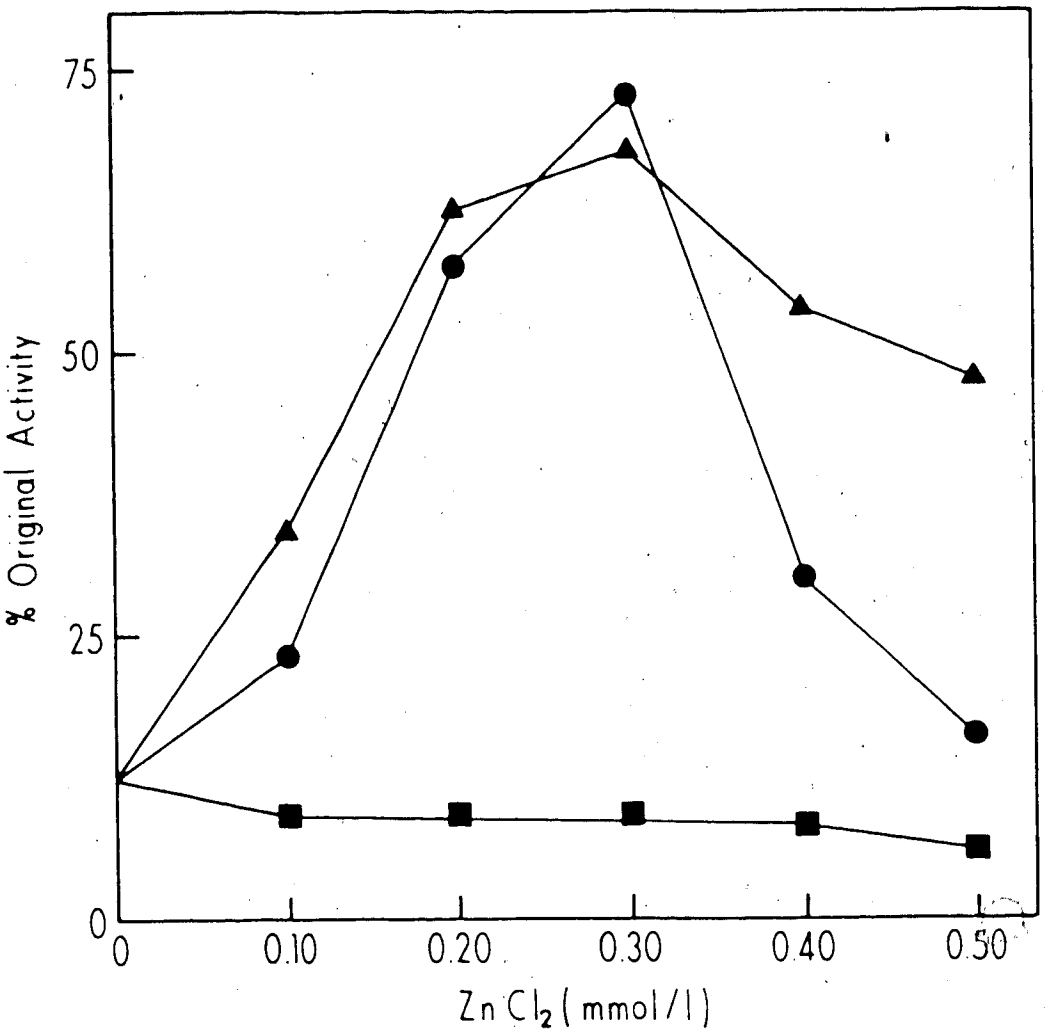


Figure 4. Reactivation of EDTA-treated alkaline phosphatase by Zn^{2+}. EDTA-treated enzyme was assayed in standard assay medium supplemented with 0 to 0.5 mmol/l $ZnCl_2$. ●, membrane-bound enzyme; ▲, phospholipase-C-released enzyme; ■, purified enzyme.

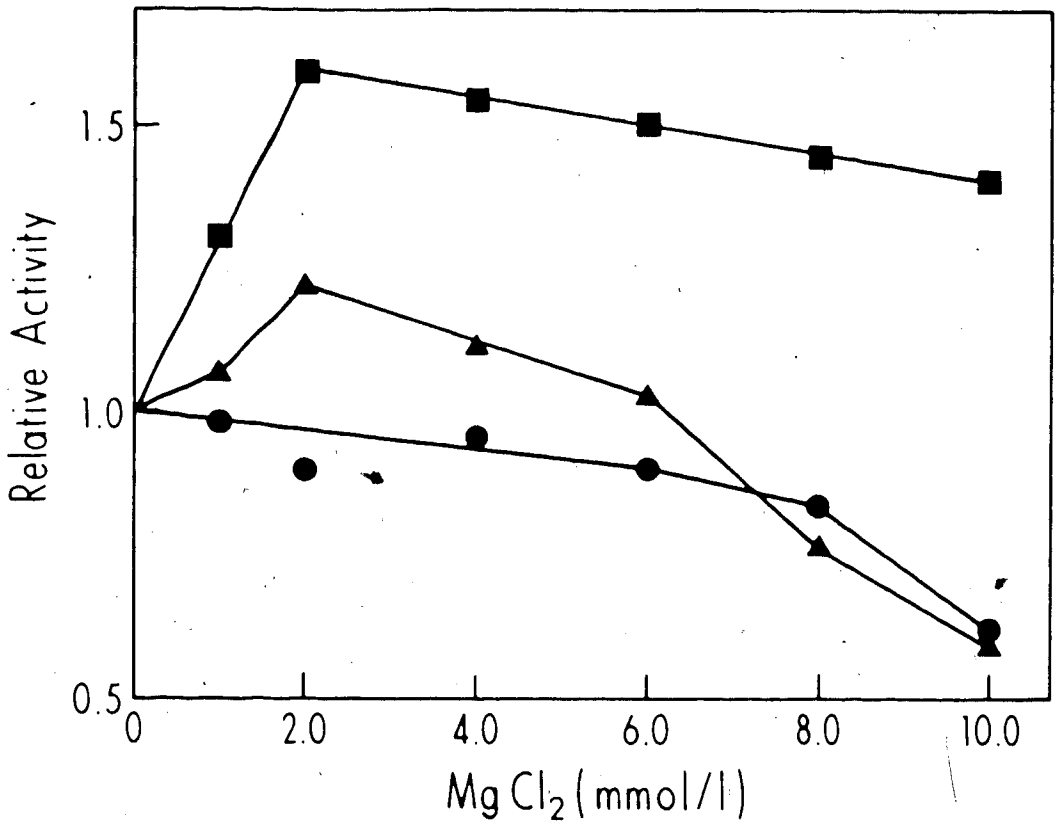


Figure 5. Effect of Mg^{2+} on alkaline phosphatase activity. Various enzyme forms were assayed in medium containing different concentrations of $MgCl_2$. ●, membrane-bound enzyme; ■, purified enzyme; ▲, phospholipase-C-released enzyme.

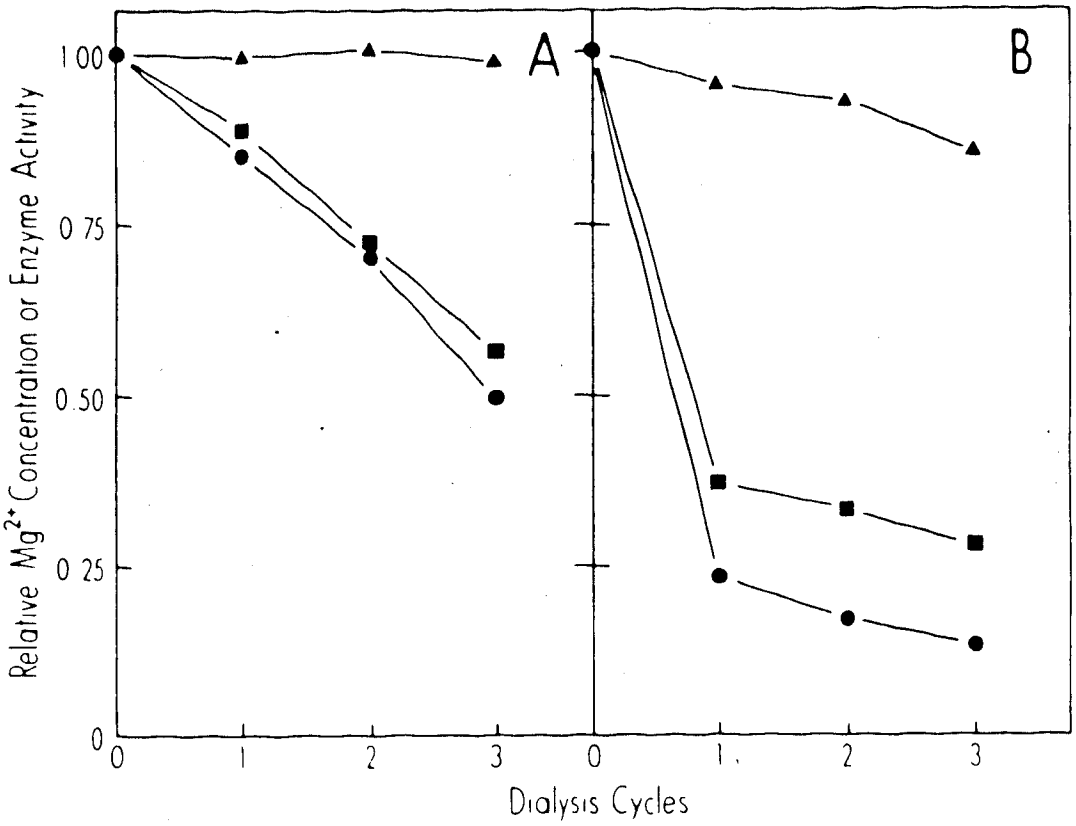


Figure 6. Loss of endogenous Mg^{2+} and alkaline phosphatase activity by dialysis. A: plasma membranes in 0.25 mol/l sucrose and 10 mmol/l Tris, pH 7.6 (10 ml) were dialyzed at 4°C against 1 litre of the same buffer for 72 h; the buffer was replaced every 24 h. 1 ml aliquot of sample was withdrawn every time the buffer was replaced and the Mg^{2+} concentration and enzyme activity was measured. Exogenous $MgCl_2$ was then added to the aliquot to a concentration of 5 mmol/l and after 30 min at 30°C, the enzyme activity was measured again. B: same as A except 12.5 mmol/l NP40 was added to sample and buffer: ■, Mg^{2+} concentration; ●, enzyme activity; ▲, enzyme activity after addition of $MgCl_2$.

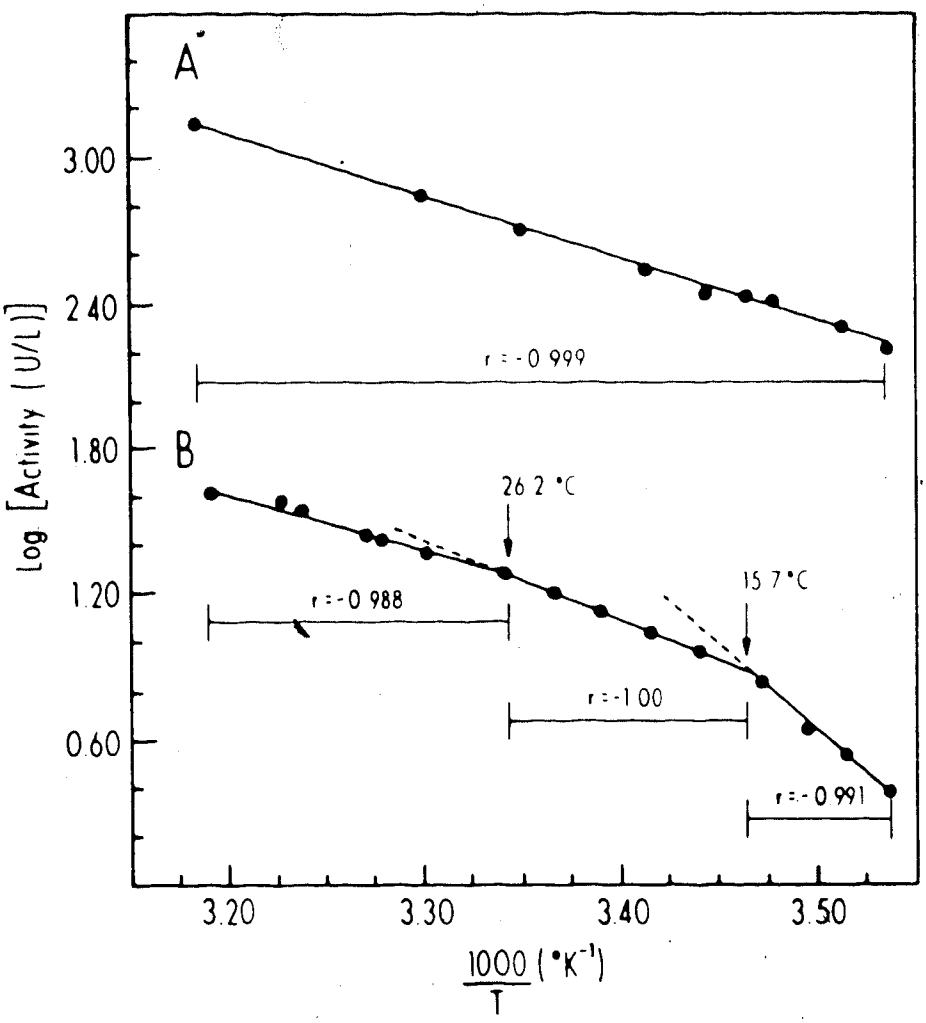


Figure 7. Arrhenius plots of alkaline phosphatase activity. Curve A represents purified alkaline phosphatase, and the activation energy (E) is 11.71 kcal/mol. Curve B represents membrane-bound alkaline phosphatase; E = 10.98 kcal/mol at temperatures above 26.2°C; E = 14.98 kcal/mol at temperature between 15.7°C and 26.2°C; E = 32.17 kcal/mol at temperatures below 15.7°C; r, coefficient of correlation.

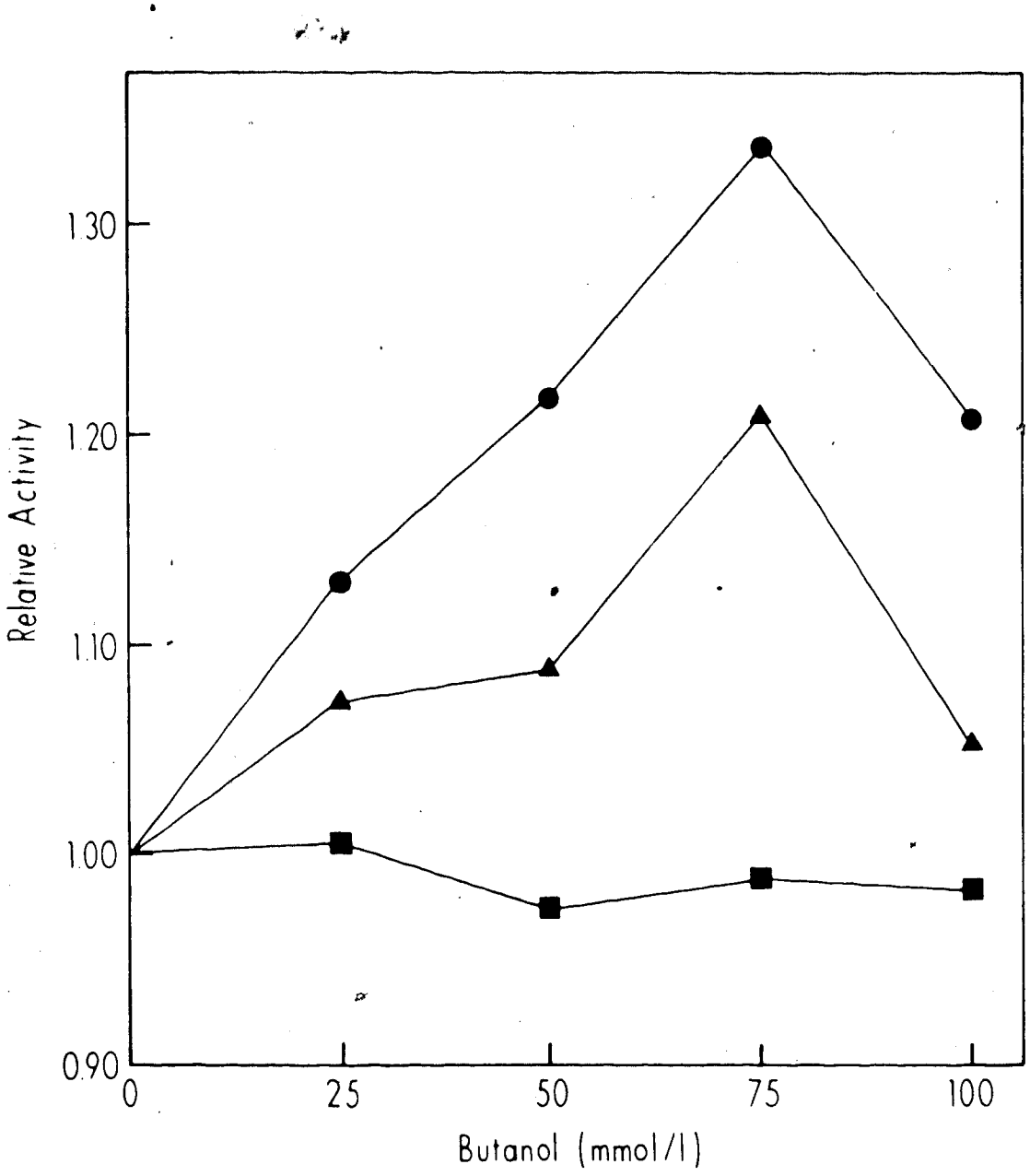


Figure 8. Effect of membrane perturbation by low concentrations of butanol on membrane-bound alkaline phosphatase activity. Membrane fractions were assayed in medium supplemented with various concentrations of butanol, at 14°C (●), 22°C (▲), and 30°C (■).

the inhibitory small molecules (phosphate and vanadate), when the enzyme is membrane-bound. My results show that NADH, like the other uncompetitive inhibitors tetramisole and homoarginine, can regulate the activity of human liver alkaline phosphatase and that membrane attachment does not hinder this. Others have found that NAD and NADH regulate phosphate transport and alkaline phosphatase activity [104, 125]. An essential arginine residue similar to that in alkaline phosphatase from pig kidney [43] was detectable in the human liver enzyme (Fig. 2). As access to this residue by phenylglyoxal was not limited when the enzyme was membrane-bound, I conclude that the active site, and perhaps regulatory centers, are equally exposed in the membrane-bound and purified enzymes but that the former is less efficient at phosphohydrolysis when substrate concentrations are low.

Zinc cations, which are essential for alkaline phosphatase activity, facilitates phosphohydrolysis by inducing conformational changes. There are three Zn^{2+}-binding sites per monomer of E. coli enzyme, and the sites vary in their affinit for Zn^{2+} and other metal ions (see Chapter 1-C). In this study (Figs. 3 & 4), removal and replacement of zinc ions of human liver alkaline phosphatase were more efficient when the enzyme was bound to membranes or released by phospholipase-C than when it was purified. This indicates that the bound and released enzyme forms bind Zn^{2+} less tightly than the purified form. Also, purification induces certain structural changes or removes protective components from the enzyme which does not occur when selective removal by phospholipase-C is employed.

The enhancement of alkaline phosphatase activity by Mg^{2+} has been

reported as early as 1931 [126]. The ions are thought to bind reversibly to the enzyme, causing a slight separation of the subunits which increases the accessibility to the active site and thus improves the catalytic ability of the enzyme (see Chapter 1-C). My findings show that exogenous Mg^{2+} does not enhance the alkaline phosphatase activity of isolated plasma membranes because the enzyme is completely saturated and activated by Mg^{2+} *in situ* (Figs. 5 & 6). Removal of endogenous Mg^{2+}, by dialysis, from isolated membranes results in a coincident loss of activity. The rate of Mg^{2+} removal is higher if the enzyme has been removed from the membrane, indicating that Mg^{2+} is less available for removal when the enzyme is membrane-bound.

Arrhenius plots of alkaline phosphatase activity from renal brush-border membranes of pigs and dogs revealed one break at 12.4°C [127] and 26°C [128], respectively. I noticed two breaks in the Arrhenius plot of membrane-bound alkaline phosphatase from human hepatocytes (Fig. 7); the second break, although minor, was affirmed by statistical analysis and by similarities between the activation energies of the bound and free enzymes. The breaks in the Arrhenius plot are due to interactions with membrane components and do not arise from conformational changes within the enzyme because the plot obtained with the purified enzyme was linear. Although alkaline phosphatase, like phosphodiesterase I and Mg^{2+}-ATPase [129], has prominent breaks in its Arrhenius plots, the enzyme is probably quite loosely constrained in the membrane and its active center may protrude from the bilayer.

This investigation has revealed certain unique properties of membrane-bound alkaline phosphatase; these disappear during purification.

Also, the use of phospholipase-C to release the enzyme displays a technique by which alkaline phosphatase can be obtained in a form which has properties intermediate between the membrane-bound and purified states.

CHAPTER THREE

MOLECULAR SIZE OF ALKALINE PHOSPHATASE IN PLASMA MEMBRANES OF HUMAN LIVER CELLS

A. INTRODUCTION

Certain chemical and kinetic differences between membrane-bound and purified alkaline phosphatase were outlined in Chapter 2. Determining if these differences arise from binding of the enzyme to the plasma membrane or from a physical difference between the membrane-bound and purified forms, the molecular size of alkaline phosphatase in the plasma membrane of human liver cells was investigated. Various detergents, butanol, and phosphatidylinositol specific phospholipase-C were used to release the enzyme from the plasma membrane and the molecular size of the different released forms was determined.

B. MATERIALS AND METHODS

1. Chemicals

Phospholipase-C (Bacillus cereus) type III, p-nitrophenylphosphate, naphthol AS-MX phosphate, NP40, and the molecular-weight markers for chromatography (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, and albumin) were purchased from Sigma Chemical Co. (St Louis, MO); Triton X-100, sodium dodecyl sulfate, and CHAPS from Bio-rad Laboratories (Richmond, CA); octyl-β-D-glucopyranoside from Calbiochem-Behring Corp. (La Jolla, CA); ethylaminoethanol from Aldrich Chemical Co.

(Milwaukee, WI); Sepharose-6B, and the molecular-weight markers for electrophoresis (thyroglobulin, ferritin, catalase, lactate dehydrogenase, and bovine serum albumin) from Pharmacia Fine Chemicals AB (Uppsala, Sweden); 2.5–27 polyacrylamide gradient gels from Isolab (Akron, OH). All other chemicals were of highest reagent grade (Fisher Scientific, Fairlawn, NJ).

2. Plasma Membrane and Enzyme Preparation, and Assays

Procedures for the isolation of plasma membranes and purification of alkaline phosphatase and phosphatidylinositol specific phospholipase-C are described in Chapter 2-B. Alkaline phosphatase was assayed in medium containing 10 mmol/l p-nitrophenylphosphate and 1.0 mol/l ethylaminoethanol, pH 10.3 at 30°C; the increase in absorbance at 404 nm was monitored in a spectrophotometer (Varian Model 2200). Units of enzyme activity were expressed as micromoles of p-nitrophenol released/min. Protein concentrations were determined using the Markwell modification of the Lowry procedure [130].

3. Solubilization of Alkaline Phosphatase

Isolated plasma membranes (1 ml) containing 0.5 U of alkaline phosphatase activity, in 125 mmol/l sucrose, 50 mmol/l KCl, 25 mmol/l Tris–HCl, 1.0 mmol/l $MgCl_2$, 0.1 mmol/l $ZnCl_2$, and 0–125 mmol/l detergent (pH 7.6) were incubated for 1 h at 4°C with continuous gentle mixing followed by centrifugation at 100,000 xg for 10 min to recover the released enzyme. The detergents used included: CHAPS (zwitter-ionic), SDS (ionic), NP40, Triton X-100, β-octylglucoside (non-ionic).

When phosphatidylinositol specific phospholipase-C was used as the solubilizing agent, 2 µg of phospholipase-C protein was added to isolated plasma membranes (containing 0.5 U of alkaline phosphatase activity) suspended in 1.0 ml of 0.25 mol/l sucrose and 50 mmol/l Tris–HCl, pH 7.6. The preparation was incubated for 1 h at 30°C followed by centrifugation at 100,000 xg for 10 min to recover the released enzyme.

Butanol treatment involved adding 1 ml of the solvent to 1 ml of membranes suspended in 0.25 mol/l sucrose and 10 mmol/l Tris–HCl, pH 7.5; gentle mixing for 1 min was followed by centrifugation for 10 min at 3500 xg. The bottom (aqueous) layer of the centrifuged preparation was divided into two aliquots and CHAPS detergent was added to one of the aliquots to a final concentration of 25 mmol/l.

4. Molecular Weight Determinations

Molecular weights were determined for samples (100 µL, 0.05 U) of both solubilized alkaline phosphatase and the purified enzyme by electrophoresis in gradient polyacrylamide gels for 24 h at 150 V in 0.09 mol/l Tris and 0.08 mol/l borate buffer, pH 8.3 [35]. Molecular-weight markers were added to the sample before electrophoresis. After electrophoresis, the gels were stained for activity in naphthol AS-MX phosphate (1 mg/ml) in 1.0 mol/l ethylaminoethanol, pH 10.3, and viewed under UV light until band(s) of alkaline phosphatase became detectable [38]. Then the gels were stained for protein using a rapid Coomassie blue R-250 stain [131].

The molecular weight of alkaline phosphatase solubilized by 17 mmol/l Nonidet P40 was determined using chromatography on Sepharose-6B equilibrated in 50 mmol/l Tris–HCl and 1.7 mmol/l Nonidet P40, pH 7.6. A Pharmacia K 16/100 column was employed, the total bed volume was 175 ml

and a flow rate of 8 ml/h was used. Molecular weights were calculated from a standard curve of Log M_r vs K_{av}, using molecular-weight markers. Markers and samples were run in triplicate.

5. Purification and SDS-polyacrylamide-gel-electrophoresis of high M_r alkaline phosphatase

Two millilitres of plasma membranes, solubilized by NP40 (12.5 mmol/l), containing 1.0 unit of alkaline phosphatase activity, was applied to a 7 ml phosphonic acid–Sepharose column [12], equilibrated in 10 mmol/l Mes, 6.25 mmol/l Nonidet P40, 1.0 mmol/l $MgCl_2$, 0.1 mmol/l $ZnCl_2$, pH 6.0. After washing the column with 100 mmol/l NaCl in equilibration buffer, the alkaline phosphatase was selectively eluted with β-naphthyl phosphate (25 mmol/l) in equilibration buffer. Fractions containing enzyme activity were pooled and three aliquots of 200 µL were subjected to gradient gel electrophoresis and the resulting high M_r-weight band of alkaline phosphatase was cut out. The three gel slices were suspended in 1.0 ml of 10 mmol/l Tris, pH 7.6, and homogenized in a Potter-Elvehjem homogenizer with a Teflon piston; lyophilization of the homogenate followed. The lyophilized powder was chemically iodinated with Na ^{125}I (specific activity, 17 Ci/mg), using Chloramine T [132] and dialyzed against 10% methanol in deionized water to remove any free ^{125}I. The resulting radio-labelled gel slurry was subjected to discontinuous SDS-polyacrylamide-gel-electrophoresis [133]. After electrophoresis, the protein bands in the gel were electrophoretically transferred onto a nitrocellulose sheet [134] using a Trans blot cell (Bio-rad Laboratories). The nitrocellulose sheet was autoradiographed [135] on X-ray film (Kodak XAR-5).

C. RESULTS

Various detergents were employed in solubilizing alkaline phosphatase from plasma membranes (Fig. 9). NP40 and Triton X-100 were most efficient at solubilization; higher concentrations of CHAPS and β-octylglucoside were necessary to completely solubilize the enzyme. Gradient gel electrophoresis revealed that only the nonionic detergents NP40, Triton X-100, and β-octylglucoside liberated alkaline phosphatase of homogenous M_r (Table IV); CHAPS and SDS-solubilized enzyme ran as a smear on top of the gradient gels. Butanol treatment released alkaline phosphatase from membranes but these enzymes entered the gel only in the presence of CHAPS detergent and had a M_r similar to the form solubilized by the nonionic detergents (Table IV). Phosphatidylinositol specific phospholipase-C released alkaline phosphatase species of both high and low molecular weights, their values being 350 k and 173 k, respectively (Plate 1).

Precipitation of the enzyme released by detergent and phospholipase-C in 75% acetone converted the high M_r enzyme to the low M_r form. The high M_r form in NP40 was stable from pH 5.0 to 10.0 for 24 h, but some conversion to the low M_r form occurred after heating the preparation at 40°C for 24 h (Plate 1). Removal of detergent by gel-permeation chromatography rendered the enzyme unable to enter the electrophoretic gels, but when the detergent was replaced, the high M_r species returned (Plate 2). Neither the purified enzyme nor the detergent-solubilized form treated with acetone changed their M_r in the presence of detergent (Plate 2).

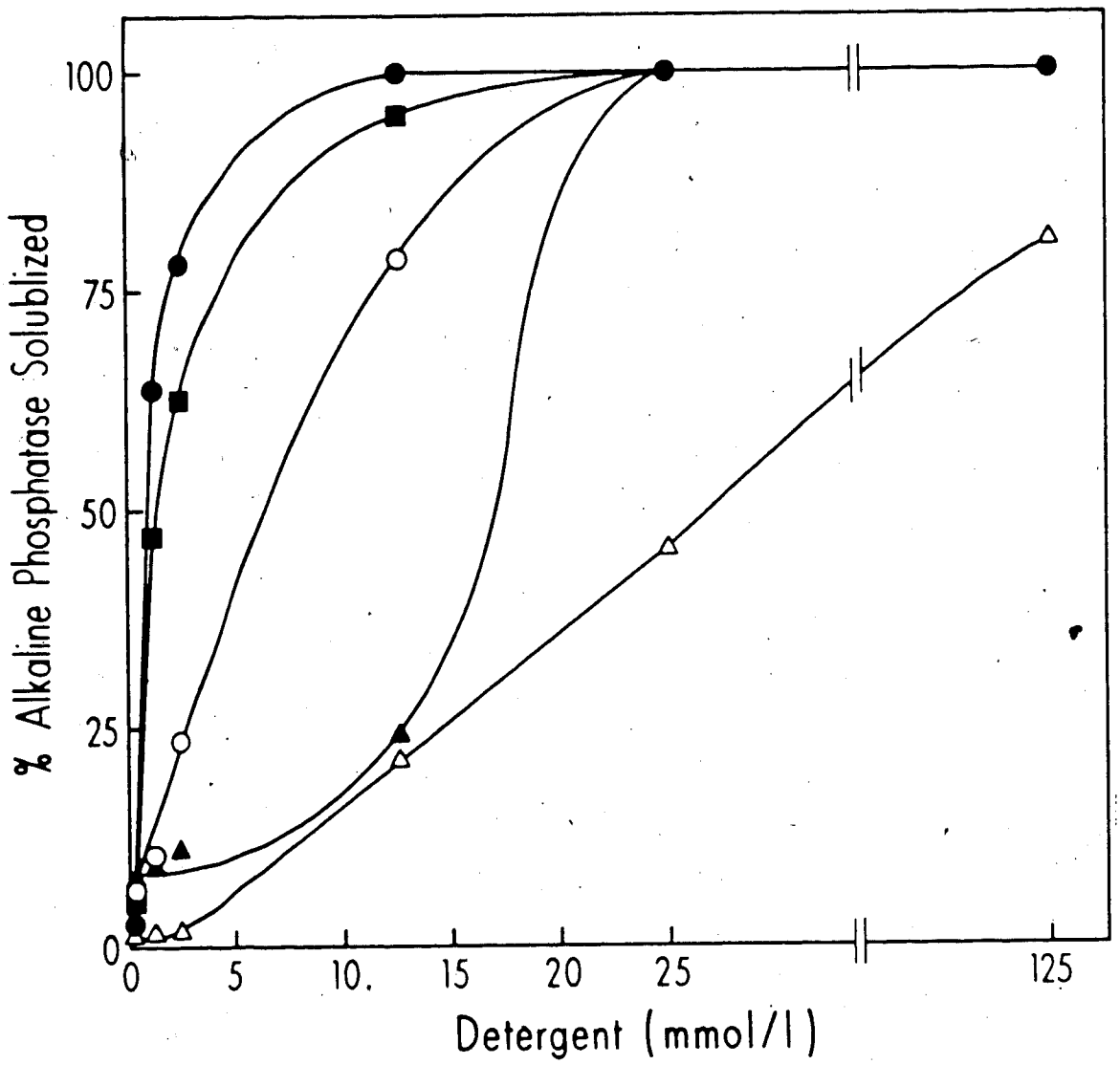


Figure 9. Solubilization of alkaline phosphatase by detergents. Membrane preparations were solubilized (1 h at 4°C) with various detergents: ●, NP40; ■, Triton X-100; ○, CHAPS; ▲, -octyl-glucoside; △, SDS. After solubilization, the samples were centrifuged at 100,000 xg for 10 min. Alkaline phosphatase activity in the supernatant was expressed as a percentage of the activity in the suspension before centrifugation.

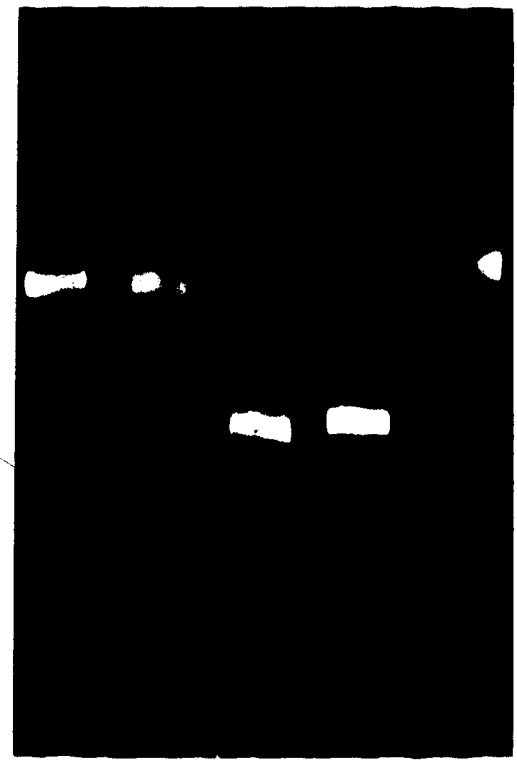
TABLE IV

RELATIVE MOLECULAR WEIGHTS OF PURIFIED AND DETERGENT SOLUBILIZED ALKALINE PHOSPHATASE

$\underline{M}_r$ were determined by gradient polyacrylamide (2.5–27%) gel electrophoresis. Alkaline phosphatase was located by staining for activity with naphthol AS-MX phosphate. $\underline{M}_r$ values given as mean ± SD (n=5).

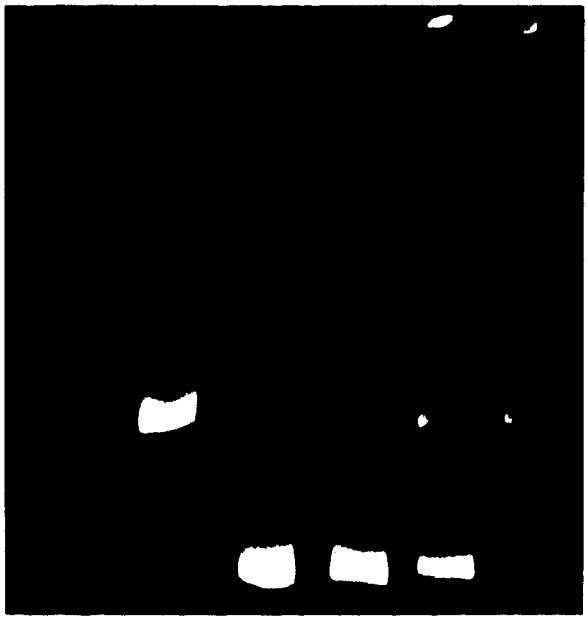
Form of Enzyme	$\underline{M}_r \times 10^{-3}$
Purified	176±13
Released by:	
Nonidet P40	411±38
Triton X-100	417±37
β-Octylglucoside	567±40
Butanol*	380±32
Phospholipase-C	350 & 173

* Required 25 mmol/l CHAPS to enter gradient gels



a b c d e

Plate 1. Electrophoresis of various alkaline phosphatase forms on gradient polyacrylamide gels. Gel a, NP40 solubilized enzyme; b, phospholipase-C solubilized enzyme; c, phospholipase-C solubilized enzyme precipitated in 75% acetone, and redissolved in electrophoretic buffer; d, purified enzyme preparation; e, NP40 solubilized enzyme heated for 24 h at 40 C. Activity staining followed electrophoresis.



a b c d e f

Plate 2. Electrophoresis of various alkaline phosphatase forms on gradient polyacrylamide gels. Gel a, NP40 solubilized enzyme after detergent removal by [illegible] graphy; b, sample from gel a plus 12.5 mmol l NP40; c, purified enzyme; d, purified enzyme plus 12.5 mmol l NP40; e, purified enzyme plus NP40 solubilized enzyme; f, NP40 solubilized enzyme. Activity staining followed electrophoresis.

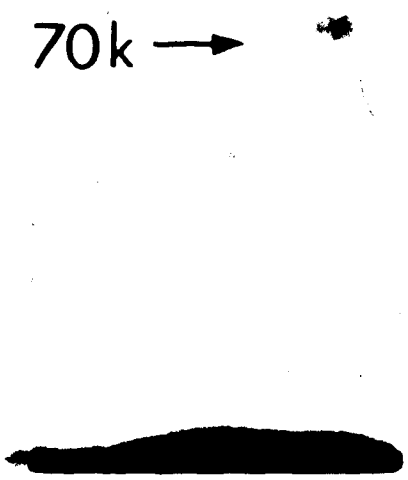


Plate 3. Autoradiography of purified high $\underline{M}_r$ enzyme after SDS-electrophoresis. Numbers on the left margin represent $\underline{M}_r$.

Molecular weights were confirmed using gel-permeation chromatography on a calibrated Sepharose-6B column, for the purified enzyme and for membrane-bound alkaline phosphatase solubilized in NP40 $\underline{M}_r$ values were 198 k and 476 k, respectively.

Electrophoresis, under denaturing conditions, of the purified high $\underline{M}_r$ species of alkaline phosphatase revealed only one protein band of $\underline{M}_r$ 70 k (Plate 3).

D. DISCUSSION

The native molecular weight of alkaline phosphatase purified from human liver has been reported in the range of 130 k to 180 k, and in most instances the native enzyme has been described as a dimer [35-38]. The molecular weight of 176 k reported here is within this range, but the molecular weights of the enzyme released by butanol and nonionic detergents were more than two-fold higher (Table IV). I believe that the high molecular weight species represents a tetrameric conformation. Heating at 40°C or acetone treatment resulted in dissociation of the complex (Plate 1). This enzyme complex, unlike the dimeric form, requires the presence of detergents to remain soluble (Plate 2), and is composed of one type of protein ($\underline{M}_r$ 70 k) (Plate 3) which is the same molecular size as the monomer of alkaline phosphatase from human liver [35-38].

It is unlikely that the species of high molecular weight resulted solely from an association of the dimeric enzyme and detergent, because neither the purified nor the acetone-treated enzyme had any affinity for detergent molecules (Plate 2). An enzyme dimer-

detergent complex of a size similar to the alkaline phosphatase solubilized by n-octylglucoside would require the enzyme to bind more than twice its weight in detergent molecules. The presence of the high molecular weight alkaline phosphatase in the (detergent free) phospholipase-C released preparation obviates the possibility of enzyme-detergent complexes. The amount of detergent bound to the high M_r enzyme most likely accounts for the difference in the molecular weights of the enzyme solubilized by detergents and the enzyme released by phospholipase-C.

Alkaline phosphatases of high molecular mass have been identified in the serum of patients with liver disease and in normal hepatic bile [136]. These high M_r alkaline phosphatases are thought to be multi-enzyme complexes which also comprise 5'-nucleotidase, γ-glutamyltransferase, and leucine-aminopeptidase, and probably represent fragments of the liver-cell membranes [137,138]. The addition of Triton X-100 to these complexes liberates alkaline phosphatase of M_r 410 k [137], similar to the detergent-solubilized form reported here.

There are several reports in the literature suggesting the presence of tetrameric alkaline phosphatase. The E. coli enzyme dimer can form tetramers at pH 8.3 in the presence of excess Zn^{2+} [139]. Alkaline phosphatase from Pseudomonas aeruginosa has been reported to contain minor amounts of tetramer [140]. The enzymes from human placenta, pig kidney, chick kidney, and chick epiphyseal cartilage have been reported to be purified in a tetrameric state [141-144].

These studies show that alkaline phosphatase operates as a tetramer in the plasma membrane. Detergents are required to keep the tetrameric enzyme soluble outside of its membrane environment. Acetone precipita-

tion probably disrupts the noncovalent and hydrophobic forces, which stabilizes the tetramer, causing dissociation; solubilization of the acetone pellet results in dimeric alkaline phosphatase which is soluble in aqueous media. The tetrameric conformation of the membrane-bound enzyme may explain some of the differences between the properties of the membrane-bound enzyme and the purified form, discussed in Chapter 2.

CHAPTER FOUR

GENERAL CONCLUSIONS

The primary goal of this study was to determine whether the properties of alkaline phosphatase was altered by releasing the enzyme from its native environment. Differences have been found but, how does this information contribute to a better understanding of the role(s) for alkaline phosphatase?

The membrane-bound enzyme has similar affinities for phosphomonoesters (substrates) and inorganic phosphate (competitive inhibitor) at pH 9.0. Decreasing the pH to physiological levels will increase the affinities for phosphomonoesters and inorganic phosphate to the same extent [50]. The physiological concentration of inorganic phosphate is much higher than that of phosphomonoesters; consequently, alkaline phosphatase is probably saturated with inorganic phosphate *in vivo*. Thus, the ability of the enzyme to nonspecifically hydrolyze phosphomonoesters is probably not physiologically important. The enzyme may act as a phosphohydrolase of a specific substrate or group of substrates (e.g., phosphoproteins) if it can bind the substrate with high affinity or if the substrate has a high local concentration.

The demonstration that alkaline phosphatase is a tetramer in the plasma membrane is a noteworthy finding in this study. To determine whether the differences in chemical and kinetic properties between the membrane-bound and purified enzymes arise as a result of the tetrameric structure of the enzyme, the latter must be purified and studied. The tetrameric conformation will probably need stabilization before purification, this may be achieved by chemically crosslinking the tetramer.

The possibility of crosslinking other proteins to alkaline phosphatase will be reduced if the procedure is performed while the tetramer is bound to the membrane matrix. The crosslinked tetramer may require detergents to remain soluble outside of the plasma membrane. The detergent chosen should: preserve activity, be compatible with purification procedures, and have a high critical micellar concentration to facilitate its removal. The zwitterionic detergent, CHAPS, fulfills these requirements.

Applying the information gathered in this study and other previous work, a conceptual model of alkaline phosphatase in the plasma membrane can be constructed (Fig. 10). The hydrophobic tail attaching the enzyme to the plasma membrane is probably of M_r 2-3K similar to those of the placental and intestinal enzymes and based on the signal hypothesis, this would comprise approximately the first 20 amino acids of the c-terminal. The binding of nonionic detergents protects the hydrophobic region of the tetramer outside the membrane environment and acetone treatment will dissociate it, and may remove the bound detergent. The acetone treated enzyme does not require detergents to remain soluble; thus, the hydrophobic tail may have been internalized into the core of enzyme.

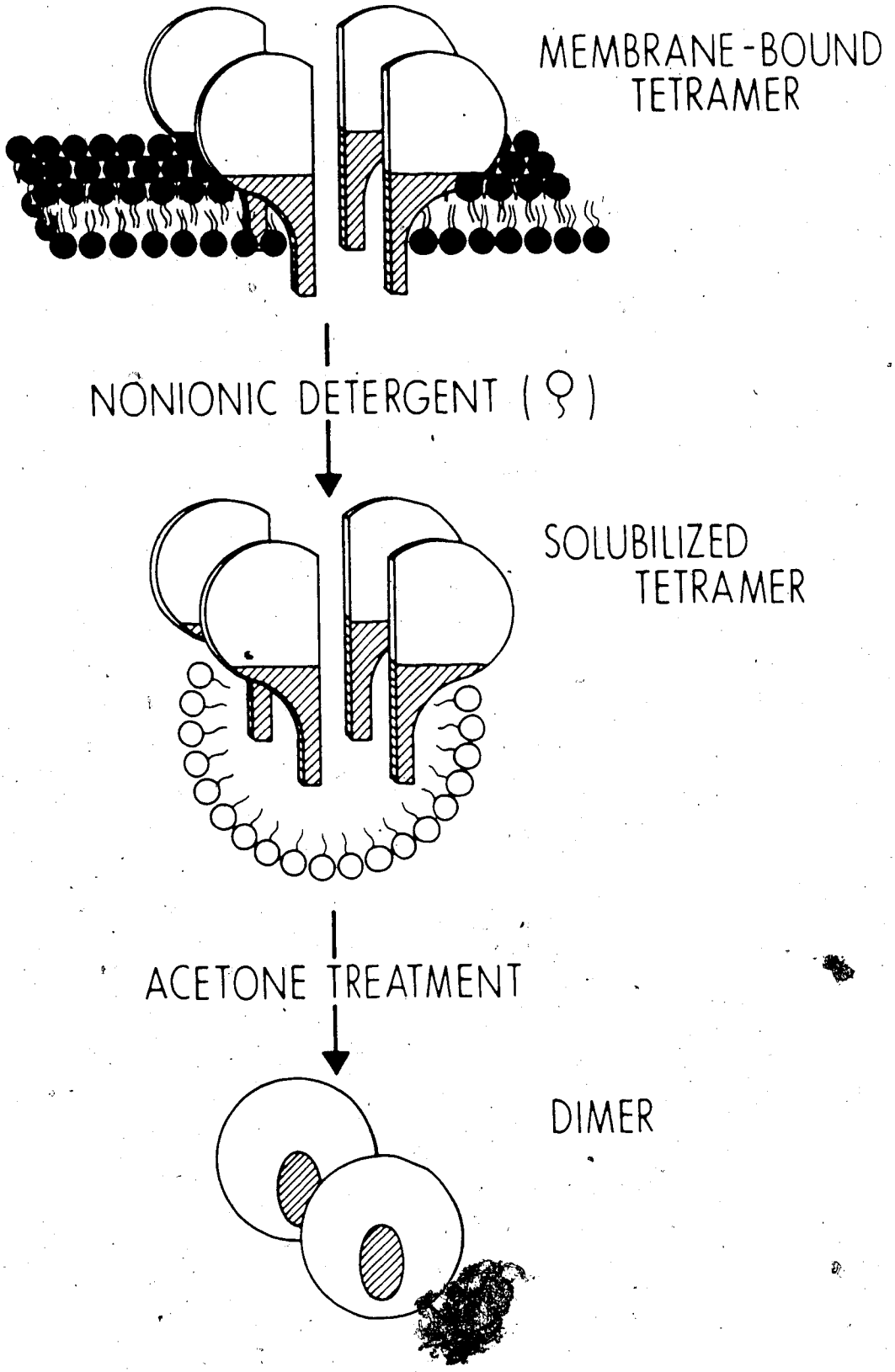


Figure 10. Conceptual model of membrane-bound and soluble alkaline phosphatases.

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