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PURIFICATION AND PROPERTIES OF HUMAN ALKALINE PHOSPHATASES

by

© LORNE EDWIN SEARGEANT

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

PURIFICATION AND PROPERTIES OF HUMAN ALKALINE PHOSPHATASES

submitted by LORNE EDWIN SEARGEANT

in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in MEDICAL SCIENCES

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Supervisor

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Date June 4, 1979

to Marietta,

whose encouragement has

made this work possible

ABSTRACT

A general procedure for the purification of alkaline phosphatase from human tissues using affinity chromatography on a phosphonic acid-Sepharose derivative was developed. The enzymes from human liver, kidney, intestine, placenta and the serum of a patient with Paget's disease of bone were purified to apparent homogeneity as judged by several criteria.

Some catalytic properties of alkaline phosphatase purified from human liver were studied. Under *in vitro* conditions the enzyme catalysed the hydrolysis of a number of phosphomonoesters which have been suggested to be possible physiological substrates. The V_{max} values for the hydrolysis of most substrates were very similar and the lowest K_m values were obtained at near-neutral pH. A calcium-sensitive ATPase activity could not be demonstrated, even in the presence of a calcium-dependent regulator protein. Ca^{2+} and Mg^{2+} ions abolished the ATPase and pyrophosphatase activities of liver alkaline phosphatase by formation of metal-substrate complexes. Phosphodiesters and phosphonic acids were not substrates of the enzyme although the latter compounds were inhibitors.

The inhibition of liver alkaline phosphatase by phosphate (K_i = 35 μM) suggested that the enzyme activity *in vivo* may be largely inhibited. Vanadate was a potent competitive inhibitor of alkaline phosphatases from several sources (K_i values were less than 1 μM). The inhibition at physiological concentrations of vanadate, and the reversal of this inhibition by compounds such as L-epinephrine indicated that alkaline phosphatase may be regulated by vanadate.

The removal of sialic acid residues from liver alkaline phosphatase by neuraminidase treatment caused the isoelectric point to change from 4.0 to 6.5, but had no influence on the specific activity of the enzyme, the K_m values for six substrates, or the inhibition by L-homoarginine. The neuraminidase-treated enzyme was inactivated by heating at 56°C at the same rate as the native enzyme, but was inactivated by SDS more rapidly than the native enzyme.

The amino acid compositions from 13 reports of alkaline phosphatase from mammalian and bacterial sources were compared. With the exception of the enzyme from human intestine, the results suggested that a high degree of compositional similarity was present. Apparent subunit molecular weight values, determined by SDS-PAGE for human alkaline phosphatases from liver, kidney, intestine, and Paget's serum were similar (92000-96000). The corresponding value for the enzyme from placenta was 74000.

Maps of the radioiodinated peptides of alkaline phosphatase purified from human liver, kidney and Paget's serum were highly similar whereas the corresponding maps of the enzyme purified from intestine or placenta were different from each other and from the maps for the enzymes from the other tissues. These results and the data from heat inactivation and differential inhibition studies strongly suggest that three different structural genes code for the protein moiety of the alkaline phosphatase present in these tissues.

The general purification protocol and the procedure for mapping the tryptic peptides from alkaline phosphatase radioiodinated within polyacrylamide gels appear to be useful for the characterization and structural identification of tumor-associated alkaline phosphatases.

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LIST OF ABBREVIATIONS

ADP	adenosine 5'-pyrophosphate
AMP	adenosine 5'-phosphate
ATP	adenosine 5'-triphosphate
(Ca)-ATPase	Ca^{2+}-stimulated adenosine triphosphatase
(Ca, Mg)-ATPase	Ca^{2+}- and Mg^{2+}-stimulated adenosine triphosphatase
(Mg)-ATPase	Mg^{2+}-stimulated adenosine triphosphatase
(Na, K)-ATPase	Na^{+}- and K^{+} -stimulated adenosine triphosphatase
Buffer mixture A	25 mM Tes + 25 mM Tris + 25 mM MAP
cyclic AMP	adenosine 3':5'-cyclic phosphate
DEAE-cellulose	diethylaminoethyl-cellulose
DEAE-Sephadex	diethylaminoethyl-Sephadex
MAP	2-amino-2-methylpropan-1-ol
Mes	2(N-morpholino)ethanesulfonic acid
p-NPP	para-nitrophenylphosphate
PAGE	polyacrylamide-gel electrophoresis
phosphonic acid-Sepharose derivative	tyraminyldiazobenzylphosphonic acid-Sepharose
PRPP	5-phosphoribosyl-1-pyrophosphate
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Tes	N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid
Tris	2-amino-2-hydroxy-methylpropane-1,3-diol

CHAPTER ONE

THE ALKALINE PHOSPHATASE PROTEIN

Alkaline phosphatases (EC 3.1.3.1) are a group of apparently non-specific phosphatases that are widely distributed in mammalian tissues and whose physiological role(s) are not known. Despite intensive investigation since 1929 when the pathological significance of an elevated serum alkaline phosphatase was first recognized (Kay, 1929), a great deal remains to be learned about the molecular and catalytic properties of the enzymes. Several reviews on the subject are available (Brière, 1979; Sussman, 1978; Fishman, 1974; Moss, 1974; Fernley, 1971) and therefore I will limit my discussion to topics that relate specifically to my research.

A. CATALYTIC PROPERTIES

Reaction catalyzed

Alkaline phosphatase catalyzes the transfer of the phosphoryl group from phosphomonoesters to water (phosphohydrolase activity) or other hydroxyl-containing acceptor molecules (phosphotransferase activity). A large amount of information has accumulated concerning this reaction in E. coli (Reid & Wilson, 1971a) and in mammalian tissues (Fernley, 1971; Fishman, 1974). The phosphotransferase activity has been largely ignored presumably because acceptor molecules with high affinity for the enzyme have not been found. However it is possible that under physiological conditions the enzyme may have significant phosphotransferase activity and be responsible for the synthesis of biologically important phosphomonoesters.

Placental alkaline phosphatase contains phosphoprotein phosphatase activity towards some phosphorylated proteins (Huang et al, 1976). Phosphorylation and dephosphorylation as a means of metabolic regulation has received a great deal of attention in recent years (Rubin & Rosen, 1975). Thus tissue alkaline phosphatases may have some metabolic regulatory function as phosphoprotein phosphatases as well.

Substrate specificity

Alkaline phosphatases from different mammalian tissues show significant differences in kinetic behaviour (including pH optima, magnesium activation, heat stability, sensitivity to inhibitors and substrate specificity). Strict comparison of the available data for the enzyme from various tissues is impossible because assay conditions are so variable.

Alkaline phosphatase catalyses the hydrolysis of several types of phosphomonoesters (Fishman, 1974 and references therein). The enzyme cleaves the phosphoryl group from compounds containing P-O-C, P-F, P-O-P, P-S or P-N bonds. Derivatives of phosphonic acids containing the more stable P-C bond are not hydrolysed. The lack of availability of pure enzyme preparations, especially from human tissues, has hampered the determination of substrate specificity because of the presence of contaminating enzyme activities. Our contribution to this area as well as further discussion can be found in Chapters IV & V of this thesis.

One of the most characteristic features of alkaline phosphatases in general is the increase of K_m and V_{max} values with increasing pH (Fernley, 1971). Therefore at low substrate concentration the efficiency of hydrolysis is greater at neutral pH than at more alkaline pH. Wachstein & Meisel (1957) have demonstrated histochemically that

human liver alkaline phosphatase is catalytically active at pH 7.4. However the physiological significance of alkaline phosphatase activity has not been established.

Mechanism of enzymic action

Alkaline phosphatase can be phosphorylated at a serine residue (see section B below) and evidence exists that the catalytic mechanism involves the formation of phosphoryl-enzyme (Fernley, 1971; Reid & Wilson, 1971a). Recent stereochemical evidence using a chiral [^{16}O, ^{17}O, ^{18}O] phosphoryl group supports the idea that the reaction is a 'double-displacement' process (Jones et al, 1978). The rate-limiting step in catalysis at alkaline pH involves conformational changes rather than dephosphorylation of the phosphoryl-enzyme (Halford et al, 1969; Reid & Wilson, 1971b).

The detailed reaction mechanism of alkaline phosphatase is controversial. Lazdunski et al, (1971) have proposed a flip-flop mechanism for E. coli alkaline phosphatase involving extreme negative co-operativity with effective half-of-sites reactivity. Several mammalian alkaline phosphatases have also been reported to contain non-equivalent sites (Fernley, 1973; Chappelet-Tordo et al, 1974; Cathala et al, 1975b; Malik & Butterworth, 1977). However Bloch & Schlesinger (1973) have presented strong evidence that tightly-bound phosphate may account for the apparent negative co-operativity seen in other studies. The studies of Waight et al, (1977) do not support a half-site, flip-flop mechanism for E. coli alkaline phosphatase and Whitaker & Moss (1976) were unable to demonstrate that the two sites of placental alkaline phosphatase had different affinities for phosphate. However studies on E. coli alkaline phosphatase using ^{31}P NMR and ^{113}Cd NMR

(Chlebowski et al, 1977) are consistent only with the existence of negative cooperativity. Resolution of this conflict must await further investigation

B. STRUCTURE AND MOLECULAR PROPERTIES

Molecular weight and subunit analysis

Mammalian alkaline phosphatases are generally considered to be dimeric zinc-containing glycoproteins. A number of molecular forms of the enzyme are known and are discussed in section C below. In recent years the enzyme from a number of sources has been purified and partially characterized. Some of the more recently reported molecular weight values are listed in Table I. Although most investigators report a dimeric structure with very similar or identical subunits, Gerbitz et al (1977) and Wachsmuth & Hiwada (1974) favour a tetrameric structure with subunit molecular weight values of 39000 and 32000 - 35000 for the enzyme from human liver and pig kidney respectively. No molecular data is available for alkaline phosphatase derived from bone.

Amino acid composition

The amino acid analyses of alkaline phosphatases from a number of sources are given in Table II. There is a remarkable similarity among the reported values for the enzyme from mammalian tissues or Escherichia coli (see chapter 7 for a detailed comparison). Whitaker et al (1976) have found that the amino acid composition of the active-site peptide from human placental alkaline phosphatase was similar to that from calf intestine and E. coli. Serine was the only amino acid that was labelled with ^{32}P orthophosphate in human placental alkaline phosphatase which

TABLE I. Molecular weight values for alkaline phosphatase preparations

Source	Native enzyme MW	Native enzyme Method*	Subunit MW	Subunit Method*	Reference
Human placenta	127000	a	64000	b	Badger & Sussman, 1976
	130000	c	65000	b	Hirano et al, 1977
	119000	b,d	64000	b	Holmgren et al, 1977
Human liver	180000	c	–		Sugiura et al, 1975
	146000	c	76700	b	Trepanier et al, 1976
	135800	c	–		Korngold, 1976
	130000	e			
	135000	a	69000	b	Badger & Sussman, 1976
	156000	a	80000	e	Latner & Hodson, 1976
	160000	e			
	136000	d	35000	d	Gerbitz et al, 1977
			32000	b	
	175000	c	–		Komoda & Sakagishi, 1978
Rat liver	154000	d	71200	b	Ohkubo et al, 1974
	155000	b			
Human intestine	130000	e	–		Korngold, 1976
	80000	e			
	170000	c	86000	b	Hirano et al, 1977
Bovine intestine	140000	d	69000	b	Fosset et al, 1974
	138000	c	66000	c	
			60000	d	
Rat intestine	157000	c	64000	b	Malik & Butterworth, 1976
	160000	f	79000	b	
			92000	b	
Pig intestine	135000	d	64000	b	Colbeau & Maroux, 1978
	125000	c			
Human kidney	180000	c	–		Sugiura et al, 1976
	150000	e	–		Korngold, 1977
	120000	e	–		
	80000	e			
Pig kidney	153000	a,c,d	79000	b	Wachsmuth & Hiwada, 1974
			39000	d	
Bovine kidney	172000	c,d	87000	b,d	Cathala et al, 1975a
E. coli	80000	d	40000	d	Applebury & Coleman, 1969

* Molecular weights were determined by:

a Sedimentation-velocity measurements
b Sodium dodecyl sulphate polyacrylamide-gel electrophoresis
c Gel-permeation chromatography
d Sedimentation-equilibrium ultracentrifugation
e Electrophoresis in polyacrylamide-gradient gels
f Density-gradient centrifugation

TABLE II. Amino acid composition of alkaline phosphatases

Number of residues (moles/1000 moles of amino acids)*

	Human liver			Human placenta					Intestine		Kidney		E. coli
									human	calf	calf	pig	
	a	b	c	d	e	f	g	h	i	j	k	l	m
Lys	57	66	59	45	50	44	43	50	16	48	65	55	58
His	38	46	33	29	27	26	24	30	7	29	54	43	23
Arg	42	42	40	62	57	56	65	52	15	93	39	57	31
Asp	121	120	115	104	96	102	103	104	82	116	108	98	111
Thr	75	69	61	65	65	70	73	67	119	68	60	65	86
Ser	62	58	72	49	61	57	62	60	120	43	51	54	51
Glu	98	96	100	106	104	99	108	109	107	94	89	95	103
Pro	49	53	51	55	57	56	68	61	113	56	63	50	49
Gly	92	90	98	94	96	107	96	99	93	82	86	89	105
Ala	91	92	96	116	100	115	111	111	80	109	96	101	146
Val	72	70	57	66	77	64	46	65	59	80	74	76	53
Met	24	10	-**	21	23	22	25	22	12	23	33	30	16
Ile	31	36	57	36	34	29	24	33	22	27	38	47	38
Leu	82	79	81	82	84	83	80	78	83	67	75	73	87
Tyr	35	32	35	34	34	36	37	29	35	37	41	40	24
Phe	31	30	34	37	35	35	35	30	37	28	28	27	19
Cys***	-	12	-	14	-	-	6	-	15	9	20	-	9
Trp***	-	-	-	-	-	-	27	-	58	12	5	-	10

* Data taken from: (a,f) Badger & Sussman, 1976; (b) Latner & Hodson, 1976; (c) Gerbitz et al, 1977; (d) Harkness, 1968a; (e) Sussman & Gottlieb, 1969; (g,i) Hirano et al, 1977; (h) Holmgren et al, 1977; (j) Fosset et al, 1974; (k) Cathala et al, 1975a; (l) Wachsmuth & Hiwada, 1974; (m) Lazdunski & Lazdunski, 1967.

** Methionine content not reported. An estimate of 10 moles/1000 moles of amino acids was used in the calculation of the relative content of amino acids based on the data of Latner & Hodson, 1976.

*** Not included in summation to 1000.

agrees with the results for the enzyme from calf intestine (Engstrom, 1961) and E. coli (Schwartz & Lipmann, 1961).

Carbohydrate composition

Carbohydrate determinations have been reported for human alkaline phosphatase from placenta (Ghosh et al, 1974; Holmgren & Stigbrand, 1976; Hirano et al, 1977), liver (Gerbitz et al, 1977; Komoda & Sakagishi, 1978) and intestine (Hirano et al, 1977; Komoda & Sakagishi, 1978). Reports of the total carbohydrate content of human alkaline phosphatases range from 2% - 20% which is within the same range reported for other mammalian alkaline phosphatases (Dorai & Bachhawat, 1977; Malik & Butterworth, 1976; Cathala et al, 1975a; Fosset et al, 1974; Wachsmuth & Hiwada, 1974). The sugar chains are 'complex' and contain galactose, mannose, fucose, N-acetylneuraminic acid (with the exception of the intestinal enzyme), galactosamine and glucosamine (Ghosh et al, 1974; Hirano et al, 1977; Komoda & Sakagishi, 1978). The difference in carbohydrate composition of the enzyme from different tissues has not yet been established. The values reported by various investigators show large discrepancies possibly due to the use of different analytical methods or to the purity of some of the enzyme preparations. In addition, it is known that the enzyme from some tissues such as pig kidney (Hiwada & Wachsmuth, 1974b) exhibits microheterogeneity which is probably due to variable degrees of glycosylation.

Metal-ion composition

It is known that alkaline phosphatases require both zinc and magnesium ions for maximum activity: zinc is essential and magnesium

is stimulatory. Zinc is more tightly bound to the pig kidney enzyme than magnesium. [Dissociation constants for zinc and magnesium are 8×10^{-13} and 4×10^{-7} respectively (Ackermann & Ahlers, 1976b).] The zinc content of a number of alkaline phosphatases has been determined and the results are listed in Table III.

TABLE III. Zinc content of alkaline phosphatases

Source	Zinc content*	MW used in calculation	Reference
Human placenta	2.5	125000	Harkness, 1968a
Human liver	3.5	220000	Komoda & Sakagishi, 1976a
Human intestine	3.7	140000	Komoda & Sakagishi, 1976b
Calf intestine	4.0	140000	Fosset et al, 1974
Pig kidney	2.0	155000	Hiwada & Wachsmuth, 1974a
Calf kidney	4.5	172000	Cathala et al, 1975a
Cow's milk	4.9	170000	Linden & Alais, 1976
E. coli	4.0	89000	Bosron et al, 1975

* Zinc content is expressed as g atoms/mole of protein. The value listed is the mean value in those cases where the content was expressed as a range.

Magnesium ions cannot replace zinc at the zinc-site to give an active enzyme and zinc-binding at the magnesium-site causes an inhibition (Cathala et al, 1975b; Bosron et al, 1977; PetitClerc & Fecteau, 1977; Linden & Alais, 1978). E. coli alkaline phosphatase binds a total of 4 zinc as well as 2 magnesium ions (Bosron et al, 1977). These investigators have presented evidence that magnesium regulates the expression of catalytic activity by zinc. PetitClerc & Fecteau (1977) have also proposed a similar regulatory mechanism

for the enzyme from rat placenta.

Isoelectric point

Alkaline phosphatases are acidic proteins. The isoelectric point of the enzyme from a number of tissues including liver, bone, intestine, kidney and placenta ranges from 3.9 to 4.7 (Latner et al, 1970; Trepanier et al, 1976; Angellis et al, 1976; Sugiura et al, 1976; Gerbitz et al, 1977; Holmgren et al, 1977). Most tissues give more than one enzymically-active band after electrofocusing. Khattab & Pfleiderer (1976) report the presence of 15 forms of human kidney alkaline phosphatase after electrofocusing. Such heterogeneity is probably accounted for by variable sialic acid content but it is also likely that loss of bound metal ions from the enzyme by chelation with the synthetic ampholytes or electrolysis would result in enzyme forms differing in their isoelectric points.

C. MULTIPLE-MOLECULAR FORMS

It is well known that alkaline phosphatases from various tissues differ in molecular properties such as heat and urea stability, response to chemical inhibitors, immunologic reactions and electrophoretic mobility. The enzyme from placenta also exhibits genetically-determined forms. In addition to the enzyme forms present in normal tissues, enzyme forms associated with development as well as malignant and benign tumors have been described.

Heat and urea inactivation

It is well established that alkaline phosphatases from various tissues exhibits different stabilities toward heat or urea treatment (reviewed by Brière, 1979 and Fishman, 1974). Fennelly et al (1969)

have shown that inactivation by heat or urea is correlated. The enzyme from bone shows the greatest inactivation and that from placenta the least while other tissue alkaline phosphatases give intermediate values.

Differential chemical inhibition

Numerous studies have been carried out to demonstrate organ-specific inhibition of alkaline phosphatases using uncompetitive inhibitors such as L-phenylalanine, L-tryptophan, L-homoarginine, L-leucine, Levamisole and imidazole (reviewed by Fishman, 1974). Mulivor, Plotkin & Harris (1978) have performed a systematic study using 5 inhibitors including L-phenylalanyl-glycyl-glycine and L-leucyl-glycyl-glycine and were unable to demonstrate any differences in the enzymes from liver, bone, or kidney. Alkaline phosphatase from intestine and placenta could be differentiated from each other and from the liver/bone/kidney group of enzymes.

Immunological studies

General agreement on the immunological identity of tissue alkaline phosphatases has not been reached. Early reports (Schlamowitz and Bodansky, 1959) demonstrated cross-reactivity between antiserum to bone alkaline phosphatase and the enzymes from liver, kidney and intestine. Boyer (1963) identified three groups of enzymes. One class included liver, bone, spleen and the major kidney enzyme. Intestinal and placental alkaline phosphatase formed the other two classes and showed partial cross-reactivity with each other and with a minor kidney component. Cross-reactivity between antisera to intestinal and placenta alkaline phosphatase has also been demonstrated (Fishman et al, 1968a; Korngold, 1970; Lehmann, 1975a; Hirano et al, 1977). Sussman et al (1968)

demonstrated that antiserum to an impure liver alkaline phosphatase preparation cross-reacted with alkaline phosphatase from neutrophils or kidney whereas antiserum to a liver alkaline phosphatase preparation of higher purity did not cross-react. They concluded that there are at least three antigenic types of alkaline phosphatases: one derived from liver, one from placenta, and one or more from other organs. Pankovich et al (1972) demonstrated the presence of four major antigenically distinct enzyme forms in liver, bone, intestine and placenta. Tissue from bone, intestine or placenta appeared to contain more than one enzyme form. Lehmann (1975b) found that antiserum to liver alkaline phosphatase also reacted with kidney alkaline phosphatase, partially with bone alkaline phosphatase, and not with placental or intestinal alkaline phosphatase. Khattab and Pfleiderer (1976) reported that antiserum to intestinal alkaline phosphatase cross-reacts with placental alkaline phosphatase but not with alkaline phosphatases derived from 10 other tissues including bone and liver. They reported that kidney contains a minor component of intestinal-like alkaline phosphatase and a major form that reacted with antisera to liver or kidney alkaline phosphatase. Abdullah and Gowland (1977) found three immunologic classes of alkaline phosphatase: one enzyme form was detected in liver, another form in placenta, while extracts from kidney and intestine both contained two enzyme forms, one of which reacted with liver alkaline phosphatase antiserum and another which reacted with antiserum to intestinal or placental alkaline phosphatase. Most recently Miki et al (1978) have found that alkaline phosphatase derived from 18 fetal tissues can be divided into two classes. The 'universal type' was present in fetal liver, spleen, adrenal gland,

kidney, lung, heart, pancreas, thymus and bone. This class reacted with antiserum to purified adult liver alkaline phosphatase and was similar in a number of other properties to adult liver alkaline phosphatase. The other class of alkaline phosphatase was present only in fetal intestine and meconium, reacted with antiserum to purified adult intestinal alkaline phosphatase, and was similar in a number of other properties to alkaline phosphatase from adult intestine.

There is probably no single explanation that could account for the apparent discrepancies in these immunological studies of alkaline phosphatase. Perhaps the most important factor is the purity of the alkaline phosphatase preparation used to produce the antiserum. In those studies in which highly purified enzyme was used to produce antibodies and subsequently tested against the purified enzyme from various sources cross-reactivity was not found (Sussman et al, 1968). However purification of the enzyme may result in the loss of minor components and therefore in an incomplete assessment of the enzyme forms actually present in the tissue. It is possible that some of the cross-reactivity can be attributed to common antigenic determinants of different enzyme proteins. Indeed the production of tissue-specific antiserum by adsorption with the enzyme from a different tissue (Pankovich et al, 1972) suggests that the antiserum contains both tissue-specific and non-specific antibodies. Another possibility is that the tissues synthesize more than one form of alkaline phosphatase or accumulate the enzyme from the circulation. This has been suggested as a possible explanation for the presence of intestine-like alkaline phosphatase in 25% of human livers (Korngold, 1976) and in human

kidney (Korngold, 1977). A more thorough discussion of the usefulness and possible inaccuracy in the immunologic analysis of alkaline phosphatase is available (Sussman, 1978).

Electrophoresis

Characterization of the alkaline phosphatases present in serum based on their electrophoretic behaviour has been the subject of numerous reports (reviewed by Fishman, 1974). Electrophoretic studies in combination with other physical and biochemical methods have been helpful in identification of the origin of serum alkaline phosphatase in pathological states. The electrophoretic mobility of intestinal alkaline phosphatase is unchanged upon treatment with neuraminidase (Moss et al, 1966) whereas the mobilities of other tissue alkaline phosphatases are decreased. The native enzymes from liver, bone, or kidney have different electrophoretic mobilities while the neuraminidase-treated enzymes are almost indistinguishable by electrophoresis (Miki et al, 1978). Butterworth and Moss (1966) have shown that much of the microheterogeneity of alkaline phosphatase found in kidney extracts can be attributed to variable amounts of sialic acid. Differences in sialic acid content may well account for at least some of the molecular differences seen in the enzyme forms separated by electrophoresis. Ion exchange chromatography has also been used extensively to demonstrate differences in the net molecular charge of the various enzyme forms.

Developmental forms

Multiple-molecular enzyme forms have been described that appear to be associated with development. Whereas the alkaline phosphatase

activity of most fetal organs appears to remain relatively constant during fetal development, the enzyme activity of fetal intestine and fetal thymus increases (Miki et al, 1978; Pataryas and Christodoulou, 1970). Fetal intestinal alkaline phosphatase shows greater electrophoretic mobility than the adult form (Miki et al, 1978; Mulivor, Hannig & Harris, 1978) and the fetal enzyme is retarded by neuraminidase treatment whereas the adult form is not. Mulivor et al (1978) report that the fetal enzyme shows greater electrophoretic mobility then the adult form even after extensive neuraminidase treatment. Adult and fetal intestinal alkaline phosphatase differed in heat stability and reactivity with Concanavalin A (Higashino et al, 1977) but could not be distinguished by chemical inhibition (Higashino et al, 1977; Mulivor, Hannig et al, 1978). It is not known if the difference between fetal and adult enzyme forms is due to the expression of a different gene locus or to post-translational modification.

Developmental forms of human placental alkaline phosphatase have been described by Fishman et al (1976). Alkaline phosphatase present in 6-10 week placenta was heat-sensitive, inhibited by L-homoarginine and migrated as 2 bands upon cellulose-acetate electrophoresis. The fast-moving band lacked all known antigenic determinants and the slower band possessed antigenic sites characteristic of liver or bone alkaline phosphatase. Alkaline phosphatase from term placenta first appeared at 11-13 weeks and was the only form present after 14 weeks of trophoblast development. These developmental forms could represent the expression of separate genes or possibly a metabolic modification of the enzyme.

Multiple enzyme forms have also been described that appear to be

associated with the large increase in alkaline phosphatase activity which occurs upon differentiation of the microvillar surface of the duodenum in the mouse (Moog et al, 1969) and chick (Moog, 1950; Overton and Shoup, 1964). In the mouse the change of enzyme activity is associated with the appearance of a variant enzyme form having different catalytic, electrophoretic, chromatographic, and immunologic properties (Moog et al, 1966; Moog et al, 1969). In the chick the increase in activity is associated with a change of alkaline phosphatase present in a large phosphatase complex to low molecular weight forms (Chang and Moog, 1972a; 1972b).

Genetic forms

Alkaline phosphatases from liver or from full-term placenta have been shown to be products of different structural genes (Badger & Sussman, 1976). It is not known whether the enzyme forms from other tissues are products of other structural genes or represent modifications of the liver and placental isoenzymes. In addition electrophoretic variants of placental alkaline phosphatase have been described that are determined by a number of alleles at a single autosomal locus (Robson & Harris, 1967; Donald & Robson, 1974). Three alleles, F, S, and I account for the six relatively common electrophoretic phenotypes which are determined by the genotype of the fetus (Boyer, 1961; Robson & Harris, 1965). Approximately 2% of human placentae exhibit phenotypes which appear to be due to combination of a rare allele with one of the common alleles. Evidence for six rare alleles at an autosomal locus has been provided (Robson & Harris, 1967). Gene frequencies of these alleles show marked variations for various population groups (Robson & Harris, 1967; Beckman et al, 1967; Beckman and Johannson,

1967; Beckman & Beckman, 1969).

The appearance of intestinal alkaline phosphatase in serum is associated with genetic factors. The intestinal enzyme is found more commonly in the serum of individuals of blood group type B and O and in individuals who are ABH blood group secretors (Arfors et al, 1963; Beckman, 1964; Shreffler, 1965; Bamford et al, 1965; Evans, 1965). Komoda and Sakagishi (1978) have recently demonstrated that purified alkaline phosphatase from adult intestine, but not liver or fetal intestine, is associated with blood group substances.

Tumor-associated alkaline phosphatases

Several different alkaline phosphatases have been described that are present in the sera or tumor extracts of some patients with various carcinomas. These oncoalkaline phosphatases can be classified as either having characteristics of alkaline phosphatase from full-term placenta or as not having these characteristics.

The placental-like alkaline phosphatases, 'Regan isoenzyme' (Fishman et al, 1968b) and 'Nagao isoenzyme' (Nakayama et al, 1970), are the ones most commonly found. They are similar to placental alkaline phosphatase in a number of properties such as heat inactivation, L-phenylalanine sensitivity and immunologic reaction. The Nagao enzyme and the rare D-variant placental enzymes (Boyer, 1961) are sensitive to L-leucine inhibition and appear to be closely related (Inglis et al, 1973). Benham et al (1978) have found that the electrophoretic mobilities of the placental-like tumor alkaline phosphatases were not identical to the mobilities of the common placental alkaline phosphatase phenotypes and have suggested the possibility that tumor alkaline phosphatase may represent the

expression of rare alleles at the alkaline phosphatase locus.

A number of tumor-associated alkaline phosphatases have been described that can be distinguished from the alkaline phosphatase from term placenta. The 'Regan variant' (Warnock & Reisman, 1969), found mostly in hepatocarcinoma tissue had properties of both placental and liver alkaline phosphatases. The Regan variant was similar to the placental enzyme in immunologic reaction and sensitivity to L-phenylalanine, but was similar to the liver enzyme in heat-stability and some kinetic parameters (Higashino et al, 1972). It has also been reported that some hepatocarcinoma tissues contain a fetal intestinal-like alkaline phosphatase in addition to the Regan variant and normal liver enzyme (Higashino et al, 1977). Korngold (1976) has suggested that the Regan variant enzyme may be identical to the intestine-like liver alkaline phosphatase that is found in small amount in some normal human livers.

Another tumor-associated alkaline phosphatase has been described (Ehrmeyer et al, 1978) that is sensitive to L-homoarginine and has the same electrophoretic mobility as the fastest of the alkaline phosphatases extracted from approximately 10-week placenta. This enzyme may be identical to the early placental-type alkaline phosphatase found in some human neoplasms (Fishman et al, 1976).

The identity of most of these tumor-associated alkaline phosphatases has not yet been conclusively established (Sussman, 1978) although Greene & Sussman (1973) have presented evidence that the peptide structures of placental and one tumor-associated alkaline phosphatase were similar. It is generally accepted that activation of embryonic genes is a genuine manifestation of neoplasia (Fishman &

Singer, 1975) and that synthesis of placental-like alkaline phosphatase by malignant cells of non-trophoblastic origin may represent derepression of the placental alkaline phosphatase structural gene locus in these cells (Fishman et al, 1968a).

Sussman (1978) has recently stressed the need for basing identification of ectopically-produced alkaline phosphatases on their structural properties. Chapter VII of this thesis gives a comparison of the peptide-fingerprints of the enzyme from several tissues and describes a general procedure for the structural classification of alkaline phosphatases.

D. PURPOSE OF THIS INVESTIGATION

The aims of this study can be grouped into three broad categories:

1. To develop a general procedure for the purification of alkaline phosphatase from various sources in order to facilitate comparative studies.

2. To obtain a better knowledge of the catalytic properties of human alkaline phosphatases. With the exception of the enzyme from placenta, substrate specificity studies have not been carried out on 'pure' alkaline phosphatases from human sources. A thorough study of the *in vitro* hydrolytic activity of alkaline phosphatase should contribute to the elucidation of the physiological roles of the enzyme.

3. To elucidate the molecular differences which occur among the enzyme forms found in various tissues. It is not known whether some multiple-molecular enzyme forms are caused by post-translational changes or differences in primary structure. A knowledge of the nature

of the various molecular forms of alkaline phosphatase should lead to a more thorough understanding of the inter-relationships of tissue alkaline phosphatases and the alterations in serum alkaline phosphatase which occurs in pathological conditions.

Approach

Highly purified alkaline phosphatase preparations were prerequisite to the contemplated studies. Our first efforts were therefore directed to affinity purification of liver alkaline phosphatase and subsequently to alkaline phosphatase from kidney, bone, small intestinal mucosa, and placenta. Development of enzyme purification procedures accounts for a considerable amount of the experimental effort.

Substrate specificity studies were carried out using selected phosphoesters, some of which have been proposed as possible physiological substrates. In addition to the influence of pH, metal ions, and inhibitors, the effect of sialic acid residues on substrate specificity was evaluated by comparison of native and neuraminidase-treated enzyme.

To assess the structural relatedness of the enzyme from different tissues subunit molecular weights were compared by SDS-PAGE and two-dimensional maps of the peptides produced by tryptic hydrolysis of ^{125}I-labelled enzyme were compared.

CHAPTER TWO

MATERIALS AND GENERAL METHODS

MATERIALS

Chemical and biochemicals were from Fisher Scientific Co. or Sigma Chemical Co., unless otherwise stated, and were generally of the highest purity available. All compounds used as substrates for alkaline phosphatase were tested for phosphate contamination (see Chapter IV) and were found to contain less than 1% (mol/mol) of inorganic phosphate.

Sodium pyrophosphate, L-arginine and malachite green were from BDH Chemicals. Phenylphosphonic acid and cyanogen bromide were from Aldrich Chemical Co. L-Phenylalanine, L-homoarginine, ouabain and cyclic AMP were from Calbiochem-Behring Corp. ^{125}I-sodium iodide was from Edmonton Pharmaceutical Center. p-Nitrophenylphenylphosphonate was from Regis Chemical Co.

3-Aminobenzylphosphonic acid was synthesized as described by Kosolapoff (1948).

4-Aminobenzylphosphonic acid was prepared by hydrolysis of diethyl-p-aminobenzylphosphonic acid (Aldrich Chemical Co.). The diethyl ester was refluxed for 8 h in concentrated HCl. After adjustment to pH 4 with NaOH, the product was collected by centrifugation. The fine crystals were washed with water and ethanol before drying.

Chemicals used for the preparation of polyacrylamide gels were from Bio-Rad Laboratories. Ampholytes used for isoelectric focusing were from LKB. Concanavalin A-Sepharose, Sepharose 4B, Sephadex G-25, Sephadex G-200 and DEAE-Sephadex were from Pharmacia Fine Chemicals. DEAE-cellulose (DE 52) was from Whatman Biochemicals Ltd.

Neuraminidase from Clostridium perfringens (6 μmol/min/mg) was from Worthington Biochemical Corp. Trypsin (bovine pancreas, treated to inactivate chymotrypsin), purified alkaline phosphatases (bovine intestine and E. coli), phosphorylase a (rabbit muscle), bovine albumin, glutamate dehydrogenase (bovine liver) and aldolase (rabbit muscle) were from Sigma Chemical Co. Partially purified alkaline phosphatase from human placenta (19 μmol/min/mg) was obtained from Calbiochem-Behring Corp. Endoglycosidase D was from Miles Laboratories. Calcium-dependent regulator protein (bovine brain) was a generous gift from Dr. T. Vanaman.

Unless otherwise stated, the pH of all buffers was adjusted with HCl or NaOH solutions.

GENERAL METHODS

Alkaline phosphatase assays

Unless otherwise stated alkaline phosphatase was assayed as described by Bowers & McComb (1966) at 30°C in a Beckman Acta CIII spectrophotometer equipped with scale expansion and a temperature-controlled cuvette holder. The assay medium contained 10 mM p-NPP and 1.5 mM $MgCl_2$ in 0.8 M MAP buffer (pH 10.3). The rate of the reaction was monitored continuously at 404 nm and the calculations were based on a molar extinction coefficient of 16700 (1 cm lightpath) for p-nitrophenol (Halford, 1970). In order to obtain linear reaction-rate curves, the reaction was initiated with substrate after a 10 min preincubation of the enzyme solution in 0.8 M MAP buffer (pH 10.3) that contained 1.5 mM $MgCl_2$. One unit of enzymic activity corresponds to one μmol of substrate hydrolysed/min.

Protein concentration

Protein concentrations were determined by the method of Schacterle

& Pollack (1973) with bovine serum albumin as standard. Samples for protein determination were dialysed against 50 mM sodium phosphate buffer, pH 7.6, to avoid interference from the buffers. Alternatively, protein concentrations were estimated by measurement of the absorbance at 280 nm (assuming an $E^{1\%}_{1\ cm}$ of 10) or at 230 nm and 260 nm as described by Kalb & Bernlohr (1977).

Polyacrylamide-gel electrophoresis (PAGE)

Analytical disc electrophoresis was run in 7.0% (w/v) acrylamide gels in Tris-borate buffer, pH 9.5, by the method of Green et al (1972). Alkaline phosphatase samples (0.003 units of enzyme activity or 1 - 5 µg of protein) that contained 10% (v/v) glycerol were applied to the top of cylindrical gels (0.5 cm x 6.5 cm) and electrophoresed at 2.0 mA/gel for 45 min. Enzyme activity was located as described below and protein was stained with Coomassie Brilliant Blue G (Diezel et al, 1972).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in gels with acrylamide concentration from 4% to 7.5% (w/v) as described by Weber & Osborn (1969) except that 0.05 M phosphate buffer (rather than 0.1 M) was used. Alkaline phosphatase samples (1 - 5 µg) were dialysed against 0.01 M phosphate buffer containing 0.1% (w/v) SDS and 0.1% (v/v) 2-mercaptoethanol and denatured by incubation at 100°C for 2 - 5 min in buffer containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. If alkaline phosphatase samples were incubated at room temperature rather than 100°C, they were not fully denatured, and a slow-moving enzymically-active band could be demonstrated. The standards used for the determination of apparent subunit molecular weight values were: phosphorylase a (rabbit muscle), 100000; albumin (bovine serum), 68000; glutamate dehydrogenase (bovine

liver), 53000; and aldolase (rabbit muscle), 40000. Protein was stained with Coomassie Brilliant Blue R (Fairbanks et al, 1971).

Isoelectric focusing on polyacrylamide gels

Electrofocusing was carried out on a MRA electrofocusing apparatus (Metaloglass Inc., Boston, Ma) at 0°C over the range from pH 3 to pH 10 in cylindrical gels (3 mm x 93 mm) which contained 4% (w/v) acrylamide, 2% (w/v) Ampholine, 10% (v/v) glycerol and 0.25 mM $ZnCl_2$. The catholyte was 0.02 M NaOH and the anolyte was 0.01 M H_3PO_4 which contained 0.25 mM $ZnCl_2$. Gels were prefocused for 1 h (maximum of 0.5 mA/gel or 400 V) and alkaline phosphatase samples (0.003 units of enzyme activity or up to 5 µg protein) that contained 10% (v/v) glycerol in a total volume of 75 µl or less were layered on the top of the gel beneath the catholyte solution. The samples were electrofocused for 16 h at 400 V. Enzymic activity was located as described below and protein was stained using Coomassie Brilliant Blue R (Allen et al, 1974).

The pH gradients were measured using a Radiometer model 26 pH meter after elution of 3 mm gel segments for 2 h with deaerated 10 mM KCl.

Localization of enzymic activity in polyacrylamide gels

Alkaline phosphatase activity was detected in polyacrylamide gels after PAGE, SDS-PAGE or isoelectric focusing by immersion of the gels in a solution which contained 6 mM naphthol AS-MX phosphate, 1.5 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ in 0.8 M MAP buffer (pH 10.3). Fluorescent bands developed in less than 10 min and were photographed under ultraviolet light with a Polaroid camera. Alternatively the enzymic activity was detected as described by Smith et al (1968). The gels were placed in a solution that contained 2-naphthyl phosphate (2 mg/ml) and Fast Blue BB salt (1 mg/ml) in 60 mM borate buffer (pH 9.7) and incubated in

the dark until the bands were sufficiently developed. The gels could then be stored indefinitely in 7% (v/v) acetic acid.

Neuraminidase treatments

Removal of sialic acid residues with neuraminidase was generally carried out at 30°C in 10 mM Mes buffer (pH 6.0), containing 30 mM NaCl, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.02 mM $ZnCl_2$ and 0.02% (w/v) sodium azide. The amount of alkaline phosphatase protein was 25 times greater than that of neuraminidase. The progress of the desialylation was monitored by isoelectric focusing in polyacrylamide gels and was judged to be complete when the pattern of the enzyme activity bands became constant. For the experiments described in Chapter VI the conditions for neuraminidase treatment were different and are described in the Methods section for that chapter.

CHAPTER THREE

PURIFICATION OF HUMAN ALKALINE PHOSPHATASES

Comparative studies on the molecular properties of alkaline phosphatase from human tissues have been severely hampered by the lack of pure enzyme preparations and the small amounts of protein obtained. Procedures for the purification of placental alkaline phosphatase have been available for several years (Harkness, 1968a) and more recently procedures have been published for the purification of the enzyme from small-intestinal mucosa (Sugiura et al, 1975; Komoda & Sakagishi, 1976b), kidney (Sugiura et al, 1976) and liver (Latner & Hodson, 1976; Badger & Sussman, 1976; Gerbitz et al, 1977). However, the reported recoveries are low and the degree of purity of the intestinal and kidney preparations have not been conclusively established.

In this chapter a general procedure for the purification of alkaline phosphatase from human tissues is described.

METHODS

Synthesis of tyraminyldiazobenzylphosphonic acid coupled to Sepharose (phosphonic acid-Sepharose derivative)

The procedure for the preparation of the phosphonic acid-Sepharose derivative is described in detail in sections 1 to 3 below and represented schematically in Figure 1.

1. Diazotization of p-aminobenzylphosphonic acid. Immediately before use p-aminobenzylphosphonic acid (0.2 g) was dissolved in 1 M HCl (10 ml) and chilled to 0°C. Sodium nitrite solution (490 mg in 1 ml) was added to the stirred solution at 0°C over 1 minute and the mixture incubated at 0°C for 8 min.

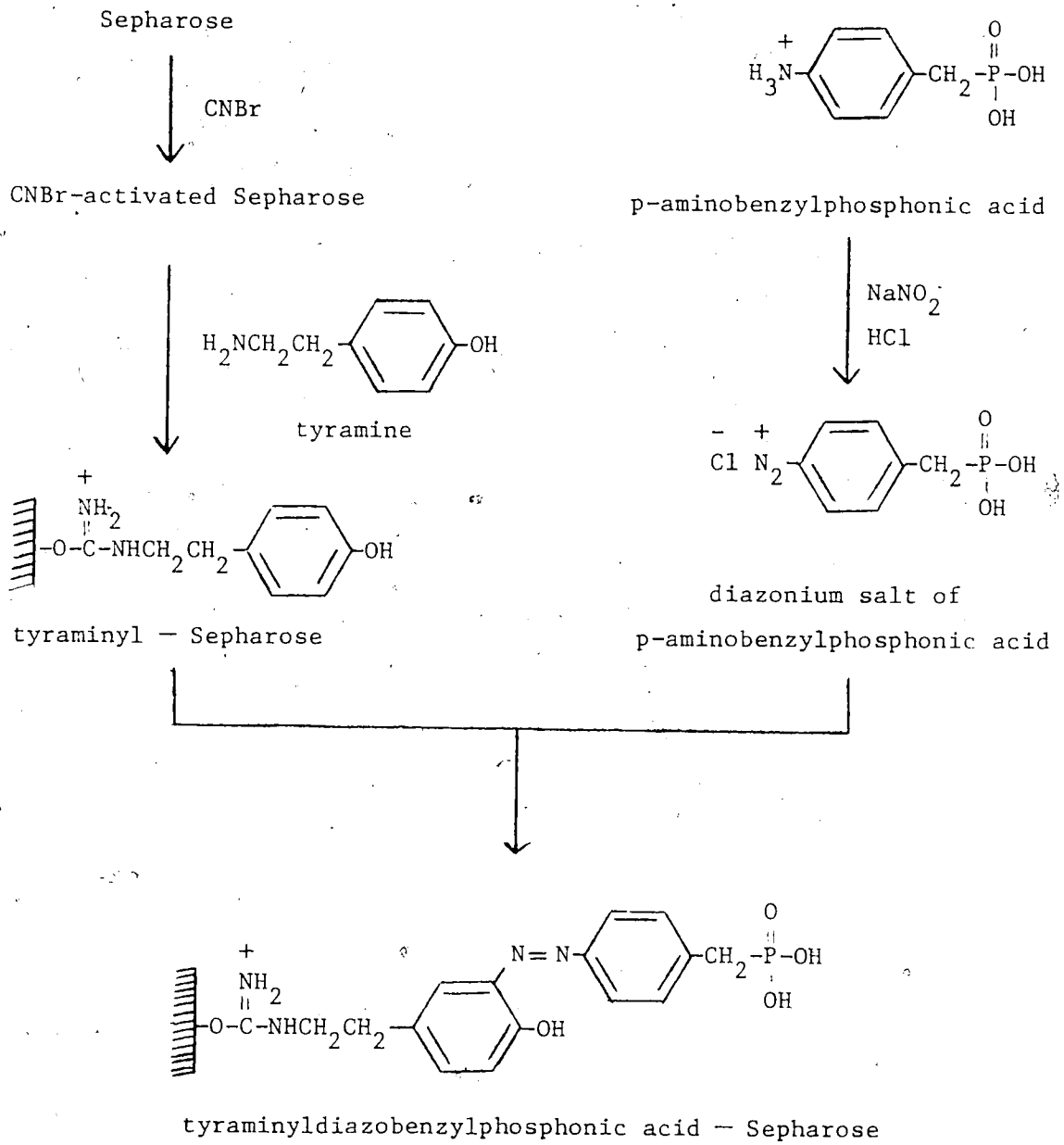


Fig. 1. Synthesis of tyraminyldiazobenzylphosphonic acid-Sepharose

The product is referred to in the text as the phosphonic acid-Sepharose derivative. The Sepharose matrix is indicated by the hatched line.

2. Preparation of tyraminyl-Sepharose. Tyramine-HCl (1.3 g) was dissolved in 0.1 M Na_2CO_3 (pH 10.0) which contained 40% (v/v) N,N-dimethylformamide and added to 25 ml of Sepharose 4B that was freshly activated with CNBr as described by Cuatrecasas (1970). The reaction mixture was mixed gently for 12 h at 4°C. The tyraminyl-Sepharose was then filtered and washed with 0.1 M Na_2CO_3 buffer (pH 10.0) with and without 40% N,N-dimethylformamide.

3. Coupling of diazotized p-aminobenzylphosphonic acid to tyraminyl-Sepharose. Freshly diazotized p-aminobenzylphosphonic acid (see section 1 above) was added with gentle stirring to tyraminyl-Sepharose (see section 2 above) suspended in 25 ml of 0.1 M Na_2CO_3 (pH 10.0) at 0°C. The pH was immediately adjusted to 9.3 with NaOH and the mixture incubated with gentle mixing for 4 h at 4°C.

4. Preparation for use. The phosphonic acid-Sepharose derivative was washed with 600 ml of 0.1 M Na_2CO_3 (pH 10.0) followed by the same volume of 0.1 M acetate buffer, 0.5 M NaCl (pH 4.0) and then with 0.1 M Tris-HCl buffer, 0.5 M NaCl (pH 8.0). The gel was equilibrated with 10 mM Mes buffer (pH 6.0) which contained 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$ and kept at 4°C until required. Under these conditions the gel was stable for more than 2 years. After use the gel was regenerated by repeating the washing procedure.

Quantity of ligand bound to Sepharose

The amount of the phosphonic acid bound to Sepharose was estimated by digestion of the gel followed by phosphate analysis (Chen et al, 1956). The gel typically contained 10-15 μmol of phosphorus per ml of packed gel.

RESULTS

The purification of human alkaline phosphatases is described under the headings of (1) purification of liver alkaline phosphatase (Method A), (2) development of an improved procedure for the purification of liver alkaline phosphatase (Method B), and (3) purification of alkaline phosphatases from other tissues. An evaluation of the purity of the enzyme preparations is found at the end of this section.

1. Purification of liver alkaline phosphatase (Method A)

The preparation of the enzyme was followed in all steps by the measurement of specific activity (μmol of p-NPP hydrolyzed/min per mg of protein). All procedures were carried out at 4°C unless stated otherwise.

Enzyme extraction

Human liver, obtained at autopsy within 12 h of death from specimens that appeared 'normal', was stripped of most vessels and the outer membrane and sliced into cubes (2.5 cm). After washing with 0.9% NaCl to remove blood, the tissue was frozen at -70°C until required. A sample (500 g) was thawed and homogenized in a Waring Blendor in 1 litre of 10 mM Tris-HCl buffer (pH 7.6) which contained 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$, for 5 min at low speed followed by 2 min at high speed. This homogenate was treated with 1-butanol to release the membrane-bound enzyme as described by Morton (1950). Butanol (750 ml) cooled to -20°C was added over a period of 30 min to the stirred suspension. The mixture was stirred for a further 30 min and then centrifuged at 9000 g for 30 min at 4°C. One litre of aqueous layer was recovered.

Acetone fractionation

Acetone (430 ml), chilled to -20°C, was added to the stirred aqueous solution at 0°C to give a final concentration of 30% (v/v) acetone. The mixture was gently stirred for an additional 15 min, and then centrifuged at 9000 g for 20 min. The supernatant (1400 ml), which contained the alkaline phosphatase activity, was made 50% (v/v) in acetone by the slow addition, with stirring, of 550 ml of acetone. After centrifugation at 9000 g for 20 min, the pellet was recovered and suspended in 60 ml of 100 mM Tris-HCl buffer (pH 7.6) which contained 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.02 mM $ZnCl_2$. The mixture was centrifuged at 9000 g for 20 min to remove undissolved residue.

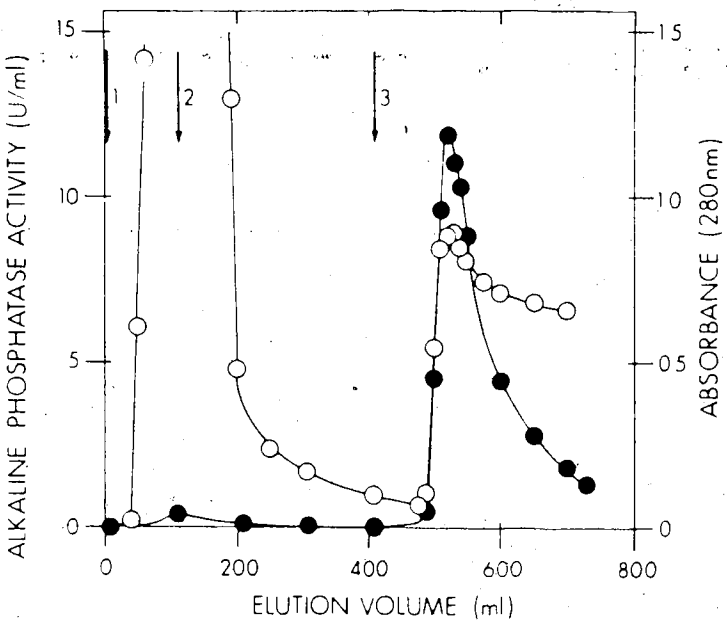
Chromatography on Concanavalin A-Sepharose

The material from the dissolved acetone pellet was applied to a column (2 cm diameter x 13 cm) of Concanavalin A-Sepharose equilibrated at 4°C with same buffer in which the acetone pellet was suspended. The column was washed with the column buffer until the absorbance at 280 nm began to fall and was then eluted with 500 ml of the same buffer containing mannose in a linear gradient from 0 to 0.1 M at a flow rate of 85 ml/h. A large amount of protein was not retained by the column and alkaline phosphatase was partially separated from some glycoproteins that were more tightly bound to the column (Figure 2). The specific activity was increased 17.5 times by this procedure.

Chromatography on DEAE-cellulose

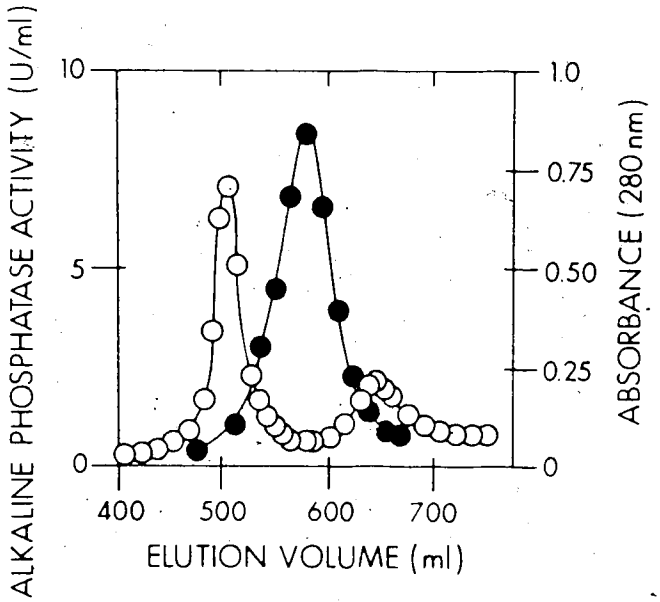
The fractions with the highest specific activity from the Concanavalin A-Sepharose column were pooled and concentrated by ultrafiltration using a PM-10 membrane in an Amicon stirred cell (Amicon Corp., Lexington, Ma). The concentrate was equilibrated with 10 mM Tris-HCl buffer (pH 7.6)

Fig. 2. Chromatography of alkaline phosphatase from liver on Concanavalin A-Sepharose



Enzymic activity (●) and absorbance at 280 nm (o) were monitored throughout a linear gradient of mannose from 0 to 0.1 M in 100 mM Tris-HCl buffer (pH 7.6) which contained 100 mM NaCl and 1 mM each of $MgCl_2$, $CaCl_2$ and $MnCl_2$. The start of the sample, buffer wash, and mannose gradient are indicated by 1, 2 and 3 respectively.

Fig. 3. Chromatography of alkaline phosphatase from liver on DEAE-cellulose



Enzymic activity (●) and absorbance at 280 nm (o) were monitored throughout a linear gradient of NaCl from 0 to 0.2 M in 10 mM Tris-HCl buffer (pH 7.6) which contained 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. The gradient was started after a buffer wash of 320 ml subsequent to sample application.

which contained 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$, by repeated dilution and concentration in the cell. This material was applied to a column (1.5 cm diameter x 90 cm) of DEAE-cellulose equilibrated with the same buffer at 4°C. The column was washed with 2 column volumes of buffer and eluted with 1 litre of buffer which contained NaCl in a linear gradient from 0 to 0.2 M at a flow rate of 40 ml/h. Alkaline phosphatase was separated from a large amount of protein that was not bound by the column and partially separated from protein impurities that were eluted by the NaCl gradient (Figure 3). This step resulted in an increase in specific activity of 11.6 times.

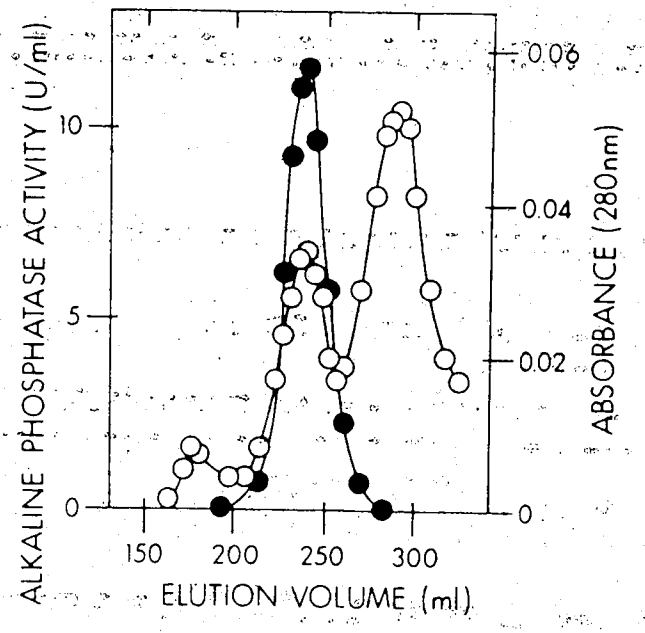
Gel-permeation chromatography

The fractions with the highest specific activity from the DEAE-cellulose column were concentrated to 5 ml in an Amicon stirred cell with a PM-10 membrane and applied to a column (2.5 cm diameter x 90 cm) of Sephadex G-200 equilibrated with 100 mM Tris-HCl buffer (pH 7.6) which contained 100 mM NaCl, 0.1 mM $MgCl_2$, and 0.02 mM $ZnCl_2$. A 3-fold increase in specific activity was obtained and the enzyme was partially resolved from both smaller and larger molecular weight proteins (Figure 4).

Chromatography on DEAE-Sephadex

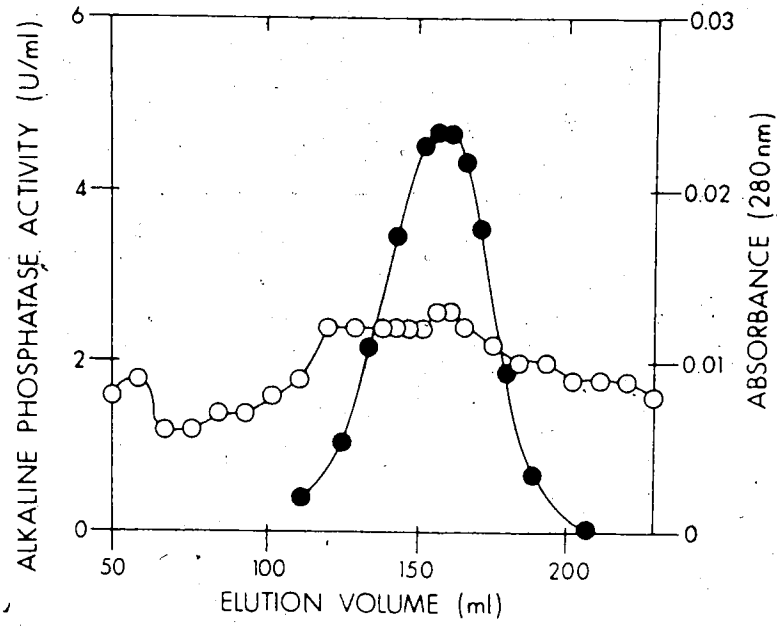
The most active fractions from the peak that contained the alkaline phosphatase from the Sephadex G-200 column were pooled and equilibrated with 10 mM Mes buffer (pH 6.0) which contained 100 mM NaCl, 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$ by repeated concentration and dilution in an Amicon stirred cell with a PM-10 membrane. The material was applied to a column (1.5 cm diameter x 25 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer at 4°C. The column was washed with 1 column volume of buffer and eluted with 200 ml of buffer that contained NaCl in a linear

Fig. 4. Gel filtration of alkaline phosphatase from liver on Sephadex G-200



Enzymic activity (•) and absorbance at 280 nm (o) were monitored during elution of the proteins with 100 mM Tris-HCl buffer (pH 7.6) which contained 100 mM NaCl, 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$ from a column of Sephadex G-200 equilibrated with the same buffer.

Fig. 5. Chromatography of alkaline phosphatase from liver on DEAE-Sephadex



Enzymic activity (•) and absorbance at 280 nm (o) were monitored throughout a linear gradient of NaCl from 0.1 to 0.2 M in 10 mM Mes buffer (pH 6.0) which contained 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. The gradient was started after a buffer wash (40 ml) subsequent to sample application.

gradient from 0.1 to 0.2 M at a flow rate of 20 ml/h (Figure 5). The highest-activity fractions were pooled so that 50% of the eluted activity was recovered. The pooled fractions were again concentrated by ultrafiltration and dialyzed for 24 h against 200 ml of 10 mM Tris-HCl buffer (pH 7.6) which contained 100 mM NaCl, 0.1 mM $MgCl_2$, 0.02 mM $ZnCl_2$, 10% (v/v) glycerol and 0.02% sodium azide, and stored at 4°C.

Summary of the purification of alkaline phosphatase from liver by Method A

Table IV is a summary of a typical purification of alkaline phosphatase from human liver by Method A. The recovered enzyme activity represented 6% of the original activity and had a specific activity of 650 units/mg. [Protein was determined colorimetrically by the method of Schacterle & Pollack (1973)]. Approximately 0.25 mg of alkaline phosphatase protein was obtained from 500 g wet weight of liver tissue. This represents a purification of 2200 times over that of the butanol-treated homogenate.

TABLE IV. Purification of human liver alkaline phosphatase by Method A

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor
Butanol-treated homogenate	7200	2110	0.3	100	1
30-50% Acetone	3650	1410	0.4	67	1.3
Concanavalin A-Sepharose	160	1110	6.8	53	23
DEAE-cellulose	4.5	356	80	17	260
Sephadex G-200	1.2	279	230	13	780
DEAE-Sephadex	0.21	136	650	6	2200

2. Development of an improved procedure for the purification of liver alkaline phosphatase

(Method B)

Although 'pure' alkaline phosphatase from liver was obtained using Method A (see section 4 below), the yield was only 6% and thus a more efficient purification procedure was required. In this section the biospecific elution of liver alkaline phosphatase from the phosphonic acid-Sepharose derivative is described. This affinity chromatographic purification of the enzyme has resulted in a considerably improved yield.

Conditions for adsorption and elution of alkaline phosphatase

The optimal conditions for chromatography of alkaline phosphatase were determined using a column (0.9 cm diameter x 16 cm) of the phosphonic acid-Sepharose derivative equilibrated at 4°C with 10 mM Mes buffer (pH 6.0) which contained 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. An aliquot (25 ml) of impure alkaline phosphatase (0.7 units/mg) obtained after the acetone-fractionation step (see section 1 above) was applied to the column and washed with 2 column volumes of buffer. Almost all of the applied protein and alkaline phosphatase were retained by the column. Conditions were therefore sought that would selectively elute the enzyme and are described below.

Ten column volumes of a linear gradient of 0-0.1 M phosphate (pH 6.0) failed to elute either alkaline phosphatase or other adsorbed protein. Less than 30% of the applied phosphatase activity was eluted with ten column volumes of a linear gradient of 0.1-1.0 M NaCl (pH 6.0). Hydrophobic interactions were not responsible for the adsorption of protein since forty column volumes of a linear gradient of 0-50% (v/v) ethylene glycol (pH 6.0) failed to elute a significant amount of enzyme activity or protein. However, quantitative elution of both

the adsorbed protein and alkaline phosphatase activity was obtained with 50 mM Tris-HCl (pH 9.0).

Alkaline phosphatase was selectively eluted with 2-naphthylphosphate at pH 6.0 (Figure 6). The enzyme was recovered in 89% yield in a volume of 240 ml. After concentration and dialysis to remove 2-naphthylphosphate, the specific activity was found to be 300 units/mg. This represented a 400-fold purification of the enzyme.

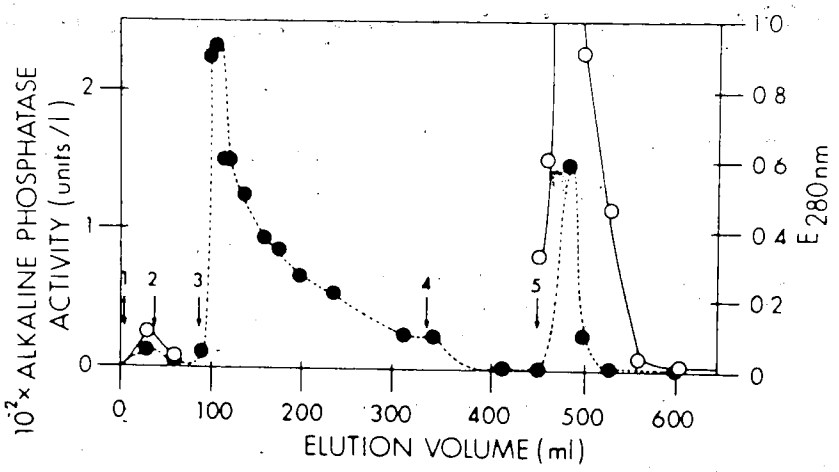


Fig. 6. Substrate elution of liver alkaline phosphatase from the phosphonic acid-Sepharose derivative

The arrows indicate the start of the following: (1) sample application; (2) buffer wash; (3) elution with 25 mM 2-naphthylphosphate in 10 mM Mes buffer (pH 6.0); (4) buffer wash; and (5) regeneration with 50 mM Tris-HCl buffer (pH 9.0). Enzyme activity is represented by (•) and the absorbance at 280 nm by (o). The substrate, 2-naphthylphosphate, absorbs strongly at 280 nm and interferes with the detection of protein. Essentially all of the protein impurities were present in the pH 9.0 eluate.

Other substrates and phosphonic acids also eluted the alkaline phosphatase activity and selected profiles are shown in Figure 7. The best eluant, 2-naphthylphosphate, had the highest affinity for the enzyme: the K_m values determined at pH 9.0 for 2-naphthylphosphate and 2-phosphoglycerate were 4 μM and 78 μM respectively (see Chapter IV), and the K_i value at pH 9.0 for the competitive inhibitor, phenylphosphonic acid, was 75 μM (see Chapter V). A combination of 10% (v/v) dimethylformamide and 180 mM NaCl eluted the enzyme

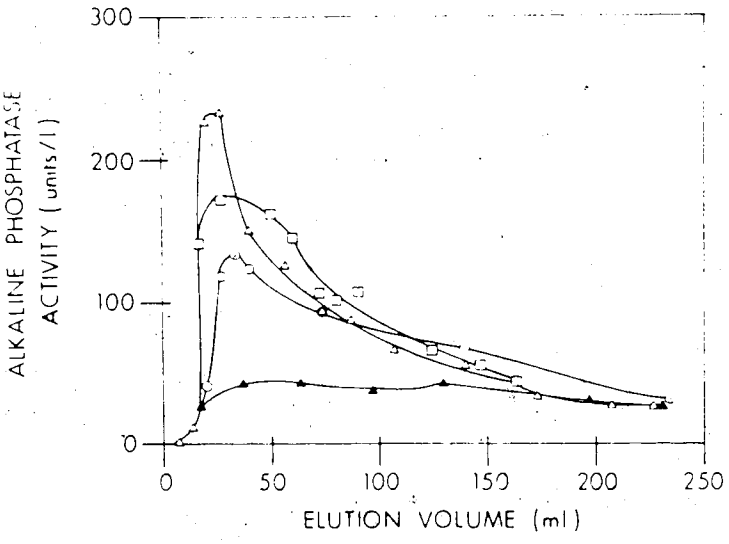


Fig. 7. Elution of human liver alkaline phosphatase from the phosphonic acid-Sepharose derivative

Samples containing 35 mg of protein and 23 units of alkaline phosphatase were applied to a column (0.9 cm diameter x 16 cm) of the phosphonic acid-Sepharose derivative equilibrated with 10 mM Mes buffer (pH 6.0) which contained 200 mM NaCl, 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. The eluants used were in 10 mM Mes buffer adjusted to a final pH of 6.0 and were as follows; (Δ) 25 mM 2-naphthylphosphate, (□) 10% (v/v) dimethylformamide plus 180 mM NaCl, (o) 100 mM 2-phosphoglycerate, and (▲) 100 mM phenylphosphonic acid.

relatively well (Figure 7) but the eluate had a lower specific activity than that obtained by elution with 2-naphthylphosphate. Dimethylformamide at 10% (v/v) considerably sharpened the profile of enzyme activity eluted with 25 mM 2-naphthylphosphate, but again the specificity obtained with substrate alone was lost.

When insoluble p-aminobenzylphosphonic acid was suspended in Sephadex G-25 (30 µmol/ml of packed gel) and an impure solution of alkaline phosphatase added, the enzyme was tightly bound and could be eluted under the condition described above.

Use of the phosphonic acid-Sepharose derivative in preparation of alkaline phosphatase from human liver

The initial homogenization, butanol treatment, and acetone fractionation were done as described for Method A (section 1 of this chapter). The material from the 30-50% acetone pellet was then resolubilized in 10 mM Mes buffer (pH 6.0) which contained 50 mM NaCl, 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$ and applied to a column of DEAE-Sephadex (2.5 cm diameter x 40 cm) equilibrated with the same buffer at 4°C. The column was washed with 1 column volume of buffer and eluted with 2000 ml of buffer that contained NaCl in a linear gradient from 50 to 300 mM at a flow rate of 90 ml/h. The alkaline phosphatase fraction was then equilibrated and chromatographed on a column of Concanavalin A-Sepharose (0.9 cm diameter x 25 cm) as described in section 1 of this chapter.

The fractions which contained alkaline phosphatase were adjusted to pH 6.0 with 100 mM Mes buffer (pH 5.0) and applied to a column (0.9 cm diameter x 9 cm) of the phosphonic acid-Sepharose derivative equilibrated at 4°C with 10 mM Mes buffer (pH 6.0) which contained 100 mM NaCl, 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. The column was washed

with two column volumes of buffer and eluted with 25 mM 2-naphthyl-phosphate in 10 mM Mes buffer (pH 6.0) which also contained 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. The fractions which contained alkaline phosphatase activity were pooled and N,N-dimethylformamide was added to a final concentration of 10% (v/v) to prevent the precipitation of 2-naphthol formed by the hydrolysis of 2-naphthylphosphate. The pool was concentrated and chromatographed on Sephadex G-200 as described in section 1. The enzyme preparation was stored at 4°C in the presence of 10% (v/v) glycerol.

Table V is a summary of a typical purification of liver alkaline phosphatase by Method B. The recovery for this preparation was 22% but higher recoveries can be expected because of the unusually large loss which occurred here at the acetone fractionation step. Approximately 1.1 mg of alkaline phosphatase protein was obtained with a specific activity of 1300 units/mg (protein estimated by the absorbance at 280 nm assuming an $E^{1\%}_{1\ cm}$ of 10).

TABLE V. Purification of human liver alkaline phosphatase by Method B

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)*	Yield (%)	Purification factor
Butanol-treated homogenate	22,600	6800	0.3	100	1
30-50% acetone fractionation	4,900	4200	0.9	62	3
DEAE-Sephadex	1,300	4100	3.2	60	11
Concanavalin A-Sepharose	104	2600	25.5	39	85
Phosphonic acid-Sepharose**		1700		26	
Sephadex G-200	1.13	1500	1300	22	4300

* Protein was estimated by the absorbance at 280 nm assuming an $E^{1\%}_{1\ cm}$ of 10.

** The presence of 2-naphthylphosphate prevented quantitative determination of protein at this step.

3. Purification of alkaline phosphatases from other tissues

Alkaline phosphatases from small-intestinal mucosa, kidney, placenta and serum from a patient with Paget's disease of bone (Osteitis deformans) were purified using slight modifications of Method B (see section 2 above). The preliminary steps in these purifications were designed to decrease the total protein content to amounts that could be applied to the phosphonic acid-Sepharose derivative without overloading the column. The purified enzymes were then obtained by substrate elution. A brief description of the procedures used for purification of the enzyme from these sources is given in this section.

Alkaline phosphatase from small-intestinal mucosa

Small-intestinal mucosa (70 ml), was prepared by gentle scraping of washed small intestine obtained at autopsy 3 hours after death. The purification procedure included butanol treatment of the homogenate, acetone fractionation and chromatography on DEAE-cellulose, phosphonic acid-Sepharose and Sephadex G-200. These steps were carried out as described in sections 1 and 2 above except that 0.1% (v/v) Triton X-100 was present in the buffers used in the chromatographic procedures. When Triton X-100 was not included, there were large losses of enzyme activity at each step. Most of the enzyme appeared in the void volume of the Sephadex G-200 column and thus had a high apparent molecular weight, probably due to aggregation of the enzyme. The purified enzyme was obtained in 17% yield. The purity of the preparation is assessed in section 4 below.

Alkaline phosphatase from kidney

Alkaline phosphatase from kidney (250 g) was prepared as described above for the enzyme from intestine. Triton X-100 (0.1% v/v) was included in the buffers used for chromatography on DEAE-cellulose and on the phosphonic acid-Sepharose derivative, but not in the buffer used for Sephadex G-200 gel filtration. The enzyme was rechromatographed, in the absence of Triton, on the phosphonic acid-Sepharose and on Sephadex G-200. The purified enzyme was obtained in 5% yield and had a specific activity of 840 units/mg. The purity of the enzyme preparation is assessed in section 4 below.

Alkaline phosphatase from the serum of a patient with Paget's disease of bone (Osteitis deformans)

The procedure used for purification of alkaline phosphatase from Paget's serum (50 ml) was identical to the procedure described above for the enzyme from kidney. The serum alkaline phosphatase was approximately 3000 units/l (normal range, 20-90 units/l) and was classified as 'bone-type' alkaline phosphatase by heat-stability and electrophoretic studies. The purified enzyme was obtained in 26% yield and had a specific activity of 890 units/mg. The purity of the enzyme preparation is assessed in section 4 below.

Alkaline phosphatase from placenta

Partially purified alkaline phosphatase from placenta (1000 units, specific activity 19.4 units/mg) was obtained commercially (see Materials, Chapter II). The purification procedure was the same as that described for liver alkaline phosphatase (Method B, section 2 of this chapter) except that chromatography on Concanavalin A-Sepharose was omitted and the buffer used for ion-exchange chromatography on DEAE-Sephadex was 50 mM Tris-HCl (pH 8.6) which contained 50 mM NaCl, 0.1 mM $MgCl_2$ and

0.02 mM $ZnCl_2$. The purified enzyme was obtained in 48% yield and had a specific activity of 790 units/mg. The purity of the enzyme preparation is assessed in section 4 below.

4. Evaluation of the purity of human alkaline phosphatase preparations

Alkaline phosphatase preparations from liver, Paget's serum, intestine, kidney and placenta were shown by several criteria to be greater than 95% pure.

The purity of liver alkaline phosphatase was demonstrated by PAGE, isoelectric focusing of native and desialylated enzyme in polyacrylamide gels and SDS-PAGE (Plate I). A single protein-stained band was observed after PAGE which was coincident with the band stained for enzyme activity (Plate I, a-c). Similarly, the coincident changes in the isoelectric points of the enzyme activity and protein-stained bands upon neuraminidase treatment (Plate I, d and e) show the purity of the preparation. The results of SDS-PAGE are shown in Plate I, f-j. A single inactive protein-stained band was obtained (gel i) although incomplete denaturation (see Chapter II) resulted in an additional enzymically-active protein band (gels f-h, j).

The results of SDS-PAGE of the enzyme purified from intestine, Paget's serum, kidney and placenta are shown in Plate II. As observed for the enzyme from liver (Plate I, i) enzymically-active bands could be demonstrated when samples for SDS-PAGE were prepared by incubation at 25°C in 1% SDS and 1% 2-mercaptoethanol (10 mM phosphate buffer, pH 7.2). Prolonged incubation of samples for SDS-PAGE at 25°C resulted in a decrease in intensity of the slower, active band and a corresponding increase in intensity of the faster, inactive band. Fully denatured alkaline phosphatase purified from intestine, Paget's serum or placenta gave a single protein band on SDS-PAGE whereas the fully

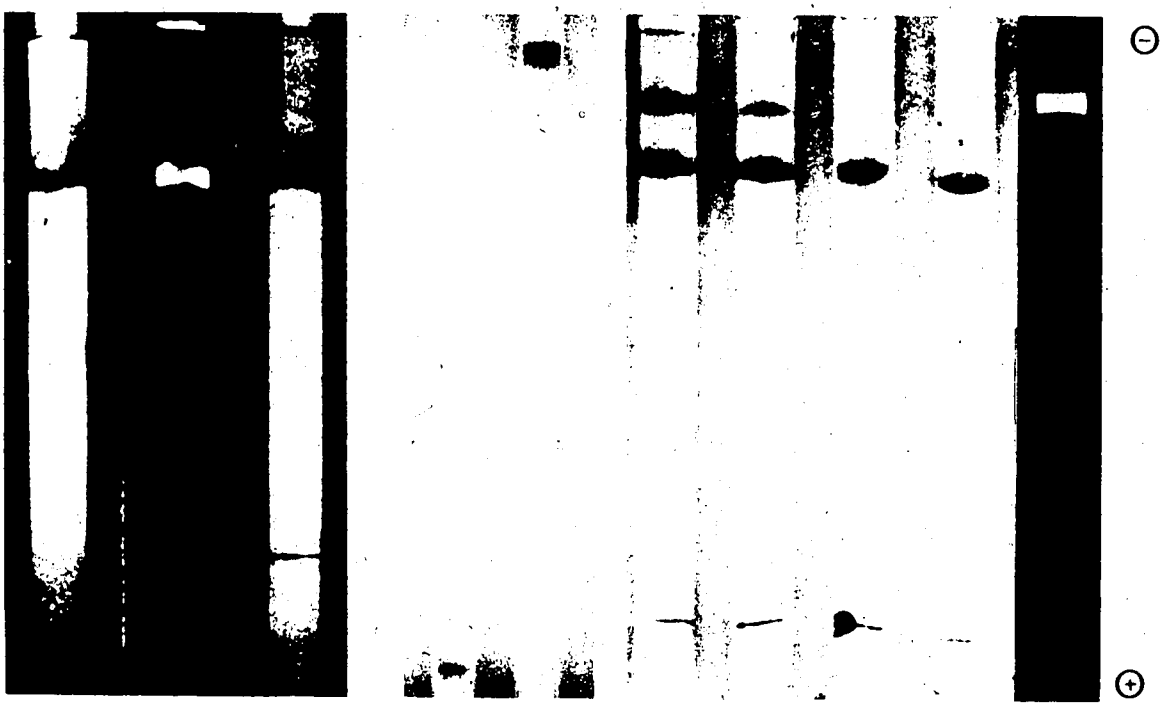


PLATE I. Criteria for purity of alkaline phosphatase from human liver

Gels a-c, PAGE of liver alkaline phosphatase; gel a was stained for protein and gels b and c were stained for fluorescent and colorimetric localization of the enzyme activity respectively.

Gels d and e, isoelectric focusing in polyacrylamide gels (pH gradient 3-10) and subsequent protein staining of liver alkaline phosphatase and neuraminidase-treated liver alkaline phosphatase respectively.

Gels f-i, SDS-PAGE of liver alkaline phosphatase; gels f, g and h, protein-stained bands obtained when the enzyme was incubated at 37°C in 1% SDS and 1% 2-mercaptoethanol for 0.5, 2 and 24 h respectively, followed by SDS-PAGE in 7.5% polyacrylamide gels. Gel i, protein-stained band when neuraminidase-treated liver alkaline phosphatase was similarly treated for 2 h. Gel j, enzyme activity band obtained when the enzyme was treated as in gel f.

Experimental details are outlined in Chapter II.

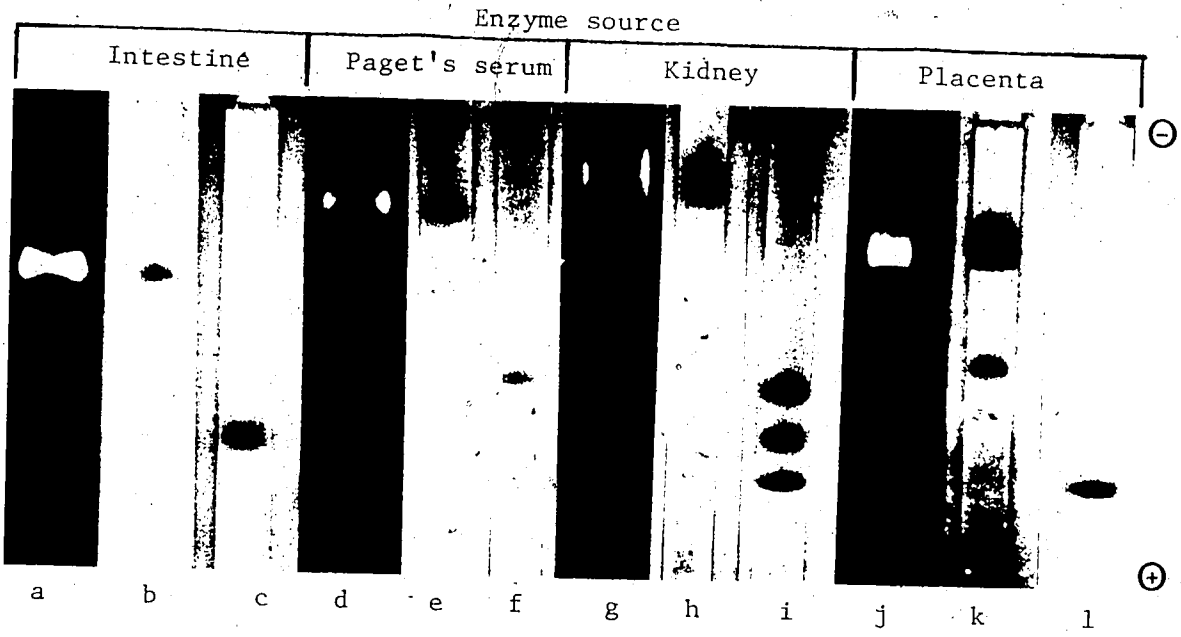


PLATE II. Purity of alkaline phosphatases from human tissues

Alkaline phosphatases purified from small-intestinal mucosa, Paget's serum, kidney or placenta were subjected to SDS-PAGE under denaturing and non-denaturing conditions as described in Chapter II. When samples were prepared under non-denaturing conditions an enzymically active band which corresponded to a protein-stained band was observed. When samples were prepared under denaturing conditions a single protein-stained band was observed for the enzyme from intestine, Paget's serum and placenta and 3 bands were observed for the enzyme from kidney. Gels a, d, g and j, samples prepared under non-denaturing conditions and the gels stained for enzyme activity and subsequently for protein (b, e, h, and k respectively). Gels c, f, i and l, samples prepared under denaturing conditions and the gels stained for protein.

denatured enzyme from kidney gave 3 protein bands (Plate II; c, f, i, l). A different kidney preparation (see Plate III, Chapter VII) gave a single band upon SDS-PAGE with the same mobility as the intermediate band in the preparation shown here. However, the results of the peptide-mapping studies (Chapter VII) suggest that the two lower molecular weight forms may have been derived from degradation of the larger molecular weight form.

Additional evidence for the purity of the enzyme from liver, kidney and Paget's serum was also obtained from the peptide-mapping studies (Chapter VII). The peptide maps of the enzyme from these sources showed an almost identical relative distribution of peptides separated on thin-layer cellulose plates by electrophoresis and chromatography. This evidence proves that an identical or almost identical protein was purified from each of these sources. The association of alkaline phosphatase activity with the protein band after separation by PAGE with or without SDS, or by isoelectric focusing of native and desialylated enzyme is strong evidence that the purified protein was in fact alkaline phosphatase.

DISCUSSION

The development of a biospecific chromatographic procedure has facilitated the purification of human alkaline phosphatases. Table VI lists the yields and specific activities for enzyme preparations from five human tissues. The enzymes appeared to be pure proteins as judged by several criteria. It is difficult to compare the specific activities with the values reported by other investigators because assay conditions and protein methods vary considerably. However the

TABLE VI. Purification of human alkaline phosphatases

Enzyme source	Total activity (units)		Yield	Specific activity (units/mg)		Purification
	initial	final	(%)	initial	final	factor
Intestine	330	55	17	-	-	-
Kidney	1450	76	5	.3	840	3100
Placenta	980	470	48	19.4	790	40
Paget's serum	150	38	26	0.04	890	22400
Liver	6800	1500	22	0.3	1300	4300

specific activities obtained are either higher or among the highest values reported (see references to Tables I and II in Chapter I). The specific activity of the enzyme from intestine was not determined because Triton X-100 interfered with the protein methods used. All attempts to remove Triton led to substantial loss of the enzyme protein. It is possible that the apparent partial degradation of the enzyme from kidney (see Results) may have resulted in a falsely low value for the specific activity of that enzyme.

The reasons for the high non-specific adsorption of proteins to the phosphonic acid-Sepharose derivative are not known. The gel contains both positive and negative charges and has a high degree of hydrophobicity. Complete elution of alkaline phosphatase by alkaline buffers and partial elution by 1.0 M NaCl suggest that ionic interactions are involved, but the severe tailing may mean that hydrophobic interactions are also important. Ethylene glycol at 50% (v/v) did not elute the protein and hence hydrophobic interaction is not the major adsorption factor. Since alkaline phosphatase was retained by p-aminobenzylphosphonic acid suspended in a column of Sephadex G-25, it is unlikely

that the isourea and azo portions of the phosphonic acid-Sepharose derivative (Figure 1) play a significant role in the adsorption of the enzyme. Yon (1977) has described the non-selective binding of membrane proteins to a gel containing hydrophobic as well as positively and negatively charged groups, and was able to elute with biospecific ligands. Elution of alkaline phosphatase from the phosphonic acid-Sepharose derivative was achieved using 2-naphthylphosphate, a substrate exhibiting a relatively high affinity for the enzyme (see Chapter IV). Since this purification protocol was completed it has been found that alkaline phosphatase can also be selectively eluted with 1 mM vanadate at pH 6.0. Since vanadate is a potent competitive inhibitor (K_i less than 1 μM) of alkaline phosphatases (see Chapter V), it appears that the elution of alkaline phosphatase depends on biospecific interactions.

Biospecific chromatographic procedures for purification of alkaline phosphatase have been reported previously. Dean et al (1971) used cellulose-phosphate to retain the phosphatases from bacterial sources. Brenna et al (1975) have described affinity chromatography of alkaline phosphatase from bovine intestine on arsanilic acid derivatives of agarose, and Landt et al (1978) have recently investigated several phosphonic acid derivatives of agarose for the adsorption and elution of alkaline phosphatase from bovine intestine. My attempts to purify liver alkaline phosphatase using cellulose-phosphate or arsanilic acid derivatives of agarose were unsuccessful because the enzyme was poorly retained by the columns. Similarly a number of other derivatives of agarose were prepared that did not provide adequate retention of the enzyme. These derivatives included 3-aminopropylphosphonic acid, 3-aminophenylphosphonic acid, 5'-AMP and L-homoarginine coupled to agarose derivatives which contained 'spacer-arms' of various lengths.

Landt et al (1978) observer an 11-fold purification of alkaline phosphatase from bovine intestine using L-histidyldiazobenzylphosphonic acid-agarose. In the chromatogram shown in Figure 6, human liver alkaline phosphatase was purified 400-fold by chromatography on tyraminyldiazobenzylphosphonic acid-Sepharose. When the column was included as the penultimate step in the purification to homogeneity of the enzyme, a 50-fold purification was obtained in 67% yield (Table V).

Because of the high non-specific adsorption of protein to the phosphonic acid-Sepharose derivative, ion-exchange chromatography has been used to decrease the total amount of protein. For preliminary purification of the enzyme from liver, chromatography on Concanavalin A-Sepharose was effective, however this step was not used for the purification of the enzyme from other sources because of low recoveries.

The purification procedure reported here provides a relatively simple and efficient general procedure for purification of alkaline phosphatase from human tissues.

CHAPTER FOUR

PHOSPHOESTER SPECIFICITY OF PURIFIED HUMAN LIVER ALKALINE PHOSPHATASE

Despite extensive investigation, the physiological role(s) of mammalian alkaline phosphatases (EC 3.1.3.1) have not been defined. Substrates whose hydrolysis by alkaline phosphatase may be of physiological significance include AMP (Fox & Marchant, 1976; Sattin & Rall, 1970), ATP (Haussler et al, 1970), phosphoribosylpyrophosphate (Fox & Marchant, 1974), pyridoxal phosphate (Lumeng & Li, 1975), carbamyl phosphate (Jernigan & Kraus, 1975), phosphocholine (Pekarthy et al, 1972; Paddon & Vance, 1977) and inorganic pyrophosphate (Russell et al, 1971). It has been reported that intestinal alkaline phosphatase may be a (Ca)-ATPase (Haussler et al, 1970; Russell et al, 1972), and that purification of the enzyme resulted in loss of the calcium sensitivity (Haussler et al, 1970). Recently Gopinath & Vincenzi (1977) have shown that certain calcium-binding proteins can activate the (Ca)-ATPase of human erythrocyte membranes. Also, Vittur & De Bernard (1973) have demonstrated that alkaline phosphatase from calf scapula cartilage is activated by Ca^{2+} ions and is associated with a calcium-binding glycoprotein.

In order to assess the possible <u>in vivo</u> contribution of liver alkaline phosphatase to the hydrolysis of these compounds, it is desirable to have some knowledge of the substrate specificity and kinetic parameters of the purified enzyme. Some catalytic properties of purified alkaline phosphatase from kidney (Hiwada & Wachsmuth, 1974a), intestine (Chappelet-Tordo et al, 1974), and placenta (Harkness, 1968b) have been reported. Previous studies carried out with partially

purified preparations of human liver alkaline phosphatase (Eaton & Moss, 1967; Komoda & Sakagishi, 1976a) would not yield reliable results since other phosphatase activities, both specific and non-specific, could be present.

The hydrolytic specificity of human liver alkaline phosphatase purified to apparent homogeneity (Trépanier et al, 1976) is described in this chapter. In addition, the influence of calcium and magnesium ions on the ATPase and pyrophosphatase activities are described as well as the effect of calcium-dependent regulator protein on the (Ca)-ATPase activity of the enzyme.

METHODS

Buffers

Unless otherwise stated a mixed buffer solution was used in order to provide adequate buffering capacity over the range from pH 7-11. The buffer solution (Buffer mixture A) contained 25 mM MAP, 25 mM Tes and 25 mM Tris. The pH was adjusted with HCl or NaOH as required. Other additions are as stated in the text.

Enzyme preparation

Human liver alkaline phosphatase was purified as described in Chapter III. The enzyme appeared to be homogeneous on polyacrylamide and sodium dodecylsulfate-polyacrylamide gel electrophoresis and had a specific activity of 1300 units/mg. (Protein was determined by the absorbance at 280 nm assuming an $E^{1\%}_{1\ cm}$ of 10.)

Enzyme assays

Alkaline phosphatase activity was measured at 30°C either by direct spectrophotometric measurement of hydrolysis of p-nitrophenylphosphate (Chapter II), or by the release of phosphate from the substrate. The assay mixture was routinely preincubated for a minimum of 10 min in the absence of substrate in order to avoid non-linear reaction rates. Assays were linear with respect to time and amount of enzyme present over the range of substrate concentrations utilized. Substrates were tested for phosphate contamination and contained less than 1% (molar ratio) of inorganic phosphate.

(Ca)-ATPase activity was measured at 30°C as the difference between simultaneous assays of (Mg)-ATPase and of (Ca + Mg)-ATPase as described by Russell et al (1972). Alternatively the (Mg)-ATPase activity was assayed in a system which contained 40 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 1 mM ATP and 0.5 mM EGTA. (Ca + Mg)-ATPase was determined in the same medium with the additional presence of 1 mM $CaCl_2$. ATPase incubations were at 30°C for 30 min. Inorganic phosphate was determined by the method of Lebel et al (1978).

Phosphate determinations

For most assays phosphate was determined by a modification of the method of Anner & Moosmayer (1975) which is sensitive to submicromolar phosphate concentrations. The enzymic reaction (1 ml) was stopped by the addition of 0.8 ml of an ice-cold solution containing 0.6 M trichloroacetic acid, 0.4 M H_2SO_4 and 0.025 M sodium molybdate. The mixtures were kept at 0°C until the addition of 0.2 ml of a solution containing 18.5 mg of malachite green in 100 ml of 1% (w/v) polyvinyl alcohol. After 60 min at room temperature the absorbance at 623 nm was read in a Beckman Acta CIII spectrophotometer.

In the presence of the acid-labile substrates, phosphoarginine or phosphoribosylpyrophosphate, phosphate was determined by a modification of the method of Ohnishi et al (1975). The incubation mixture (1 ml) was chilled to 0°C and 1 ml of a solution containing 9 mM ammonium molybdate, 9 mM Na_4EDTA, 0.14 M hydroxylamine, 0.8 mM polyvinylpyrrolidone and 0.073 M H_2SO_4 was added. After 30 sec 0.5 ml of 6.47 M NaOH was added. The absorbance at 720 nm was read after incubation at room temperature for 30 minutes.

When ATP was present at concentrations greater than 0.5 mM, phosphate was determined by the method of LeBel el al (1978) because high blank values interfered with the analysis by the method of Anner & Moosmayer (1975).

Blanks were included for each substrate concentration in order to correct for any non-enzymic hydrolysis.

Kinetic parameters

K_m and V_{max} values were obtained from linear double-reciprocal plots (Lineweaver & Burk, 1934) using a minimum of 5 substrate concentrations. Magnesium inhibited the hydrolysis of certain substrates that form magnesium complexes. Kinetic parameters for these substrates were determined at 5 µM $MgCl_2$. At magnesium chloride concentrations less than 5 µM, enzyme assays were non-reproducible. Substrate concentrations were generally chosen to span the range from the K_m value to several times this value but substrate inhibition limited the range for some substrates. Also substrate concentrations less than 5 µM were not included because excessive hydrolysis was necessary in order to produce sufficient phosphate for analysis.

All experiments were repeated at least once with good agreement.

Mixed-substrate experiments

Inhibition of p-nitrophenylphosphatase activity by phosphomonoesters was done at 30°C at pH 9.0 in the presence of 1.5 mM $MgCl_2$. The hydrolysis of p-nitrophenylphosphate, at concentrations of 15 μM and 30 μM, was monitored continuously at 404 nm at 5 concentrations of the alternate substrate. The alternate substrate concentrations ranged from 0-500 μM except for 2-naphthylphosphate which varied from 0-100 μM. The alternate substrates behaved as competitive inhibitors and K_i values were obtained from Dixon (1953) plots.

Concentrations of free components and complex species

The concentrations of the free components and the complex species present in the ATPase assays were calculated by computer according to the method of Storer & Cornish-Bowden (1976). The assays contained buffer mixture A at pH 9.0, supplemented with 0.1 M NaCl. ATP and $MgCl_2$ concentrations ranged up to 10 mM. At pH 9 the concentration of $HATP^{3-}$ and $MgHATP^{-}$ were always less than 1% of the ATP^{4-} concentration and were not considered further. The association constants (K_{ass}) used in the calculations are listed in Table VII. The values for Ca_2ATP and $CaMgATP$ were assumed to be similar to the value for Mg_2ATP.

TABLE VII. Association constants for various ATP complexes

Reaction	K_{ass} (M^{-1})	Reference
$ATP^{4-} + Mg^{2+} \rightleftharpoons MgATP^{2-}$	1.1×10^4	Walaas, 1958
$HATP^{3-} + Mg^{2+} \rightleftharpoons MgHATP^{-}$	5.42×10^2	Phillips et al, 1966
$ATP^{4-} + Ca^{2+} \rightleftharpoons CaATP^{2-}$	5.89×10^3	Walaas, 1958
$MgATP^{2-} + Mg^{2+} \rightleftharpoons Mg_2ATP$	4.0×10^1	Noat et al, 1970
$ATP^{4-} + H^{+} \rightleftharpoons HATP^{3-}$	1.09×10^7	Phillips et al, 1966
$ATP^{4-} + Na^{+} \rightleftharpoons NaATP^{3-}$	1.5×10^1	O'Sullivan & Perrin, 1964

RESULTS

Influence of pH on kinetic parameters

The increasing K_m and V_{max} values with increasing pH reported for pure alkaline phosphatases from pig kidney (Hiwada & Wachsmuth, 1974a; Ahlers, 1975) and bovine intestine (Chappelet-Tordo et al, 1974) have been confirmed with the human liver enzyme. Figure 8 shows the dramatic increase in apparent K_m values for p-nitrophenylphosphate and 2-phosphoglycerate over the range pH 8 to pH 10. The K_m values between pH 7 and pH 8 appeared to be less than 5 μM. V_{max} values also increased dramatically over the range from pH 7 to pH 10, but values for p-nitrophenylphosphate and 2-phosphoglycerate were similar at each pH despite different K_m values.

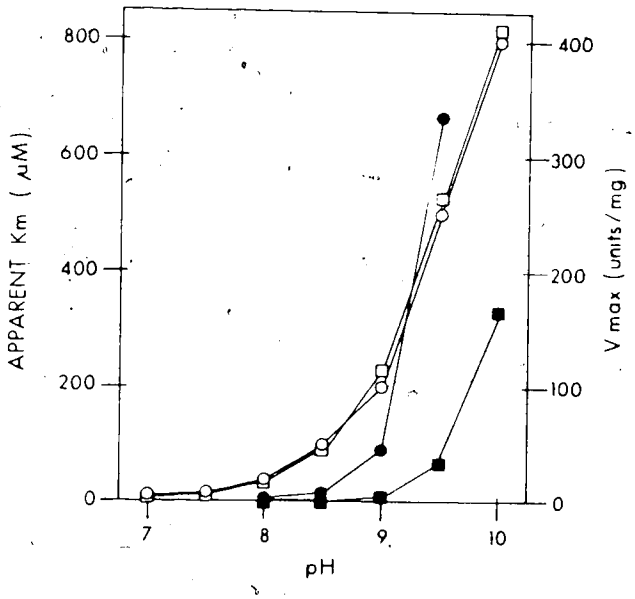


Fig. 8. Influence of pH on the kinetic parameters of human liver alkaline phosphatase

Enzyme activity was measured at each pH by the amount of phosphate released. The open symbols represent the variation of V_{max} with pH for 2-phosphoglycerate (o) and p-NPP (□), and the filled symbols represent the variation of K_m with pH for 2-phosphoglycerate (●) and p-NPP (■).

Substrate specificity

Pure human liver alkaline phosphatase catalyzed the hydrolysis of almost all the phosphomonesters tested (Table VIII). The similarity of the V_{max} values for most substrates together with the data from Figure 8 suggest that the rate-limiting step for hydrolysis is independent of the substrate utilized. Magnesium ions inhibited the hydrolysis of some substrates, especially inorganic pyrophosphate and ATP, so the K_m and V_{max} were determined at 5 μM $MgCl_2$. At pH 9.0 inorganic pyrophosphate and ATP were hydrolysed at approximately half the rate of p-nitrophenylphosphate but at pH 7.4 the rates were 91% and 84% respectively of the rate of p-nitrophenylphosphate hydrolysis.

No hydrolysis of dipalmitoylphosphatidate at pH 9.0 was detected. The phosphatidate was used as a sonicated dispersion at a final concentration of 0.25 mM. In addition, there was no inhibition of p-nitrophenylphosphatase activity by dipalmitoylphosphatidate suggesting that the compound was not accessible to the active site of the enzyme.

Carbamylphosphate and phosphocreatine were found to be competitive inhibitors of the p-nitrophenylphosphatase activity with K_i values of 25 μM and 75 μM respectively. Because of their lability no attempt was made to determine phosphate release by alkaline phosphatase but it is likely that these compounds are substrates. Phosphoarginine, which contains a P-N bond similar to that present in phosphocreatine, was hydrolysed by the enzyme (Table VIII).

TABLE VIII. Substrate specificity of human liver alkaline phosphatase[a]

Substrate	pH 9.0			pH 7.4	
	K_m (μM)	K_i (μM)[b]	V_{max}[c]	K_m (μM)	V_{max}[c]
p-NPP	9		100	2[d]	100[d]
2-Phosphoglycerate	78, 87[d]	95	114, 127[d]		
2-Naphthylphosphate	4	6	109		
Glucose-6-phosphate	270	350	95		
Ribose-5-phosphate	156	300	93		
PRPP	21[e]	28[e]	100		
Phospho-L-arginine	96[e]	83[e]	51		
Phosphoenolpyruvate	86	105	108		
Pyridoxyl-5'-phosphate	65	–	76		
Phosphocholine	185	263	106		
Phosphoethanolamine	213	400	100		
AMP	48	75	90		
ADP	15[d]		89[d]	1[d]	91[d]
ATP	39[d]		50[d]	3[d]	84[d]
Inorganic pyrophosphate	65[d]		46[d]	3[d]	91[d]
Carbamylphosphate		25			
Phosphocreatine		75			
Dipalmitoylphosphatidate					
p-Nitrophenylphenylphosphonate	no hydrolysis detected				
3',5'-cyclic AMP					

a The rate of hydrolysis was measured at 30°C in buffer mixture A containing 1.5 mM $MgCl_2$ (unless otherwise stated).

b K_i values were obtained from mixed-substrate experiments in which the inhibition of p-NPP hydrolysis was measured.

c V_{max} values are expressed relative to the V_{max} obtained using p-NPP as substrate under the same conditions.

d Enzyme assays were performed at a $MgCl_2$ concentration of 5 μM.

e Values are corrected for the purity of the substrate stated by the supplier.

Phosphodiesters do not appear to be substrates for liver alkaline phosphatase as there was no detectable production of p-nitrophenol from 1 mM p-nitrophenylphenylphosphonate in 100 mM Tris-Cl buffer (pH 8.0) although very weak inhibition of p-nitrophenylphosphatase activity was found. Also cyclic AMP at a concentration of 0.5 mM had no influence on hydrolysis of p-nitrophenylphosphate at pH 9.0 (Table VIII).

The K_m values, in agreement with the trend shown in Figure 8 are considerably lower at pH 7.4 than at pH 9.0. K_m values at pH 9.0 varied from 4 μM to 270 μM (Table VIII). The aromatic substrates had the lowest K_m values but there was no obvious correlation between K_m values and characteristics of the substrate such as size, hydrophobicity or other functional groups.

Influence of Ca^{2+} and Mg^{2+} ions on hydrolysis of ATP and pyrophosphate

Calcium and magnesium ions inhibited the hydrolysis of inorganic pyrophosphate and ATP. Ca^{2+} had no effect on the hydrolysis of other substrates whereas Mg^{2+} had a stimulatory effect. At pH 9.0 the ATPase and pyrophosphatase activities were undetectable when sufficient $CaCl_2$ or $MgCl_2$ was added to ensure that ATP and pyrophosphate were present only as their metal complexes. Pyrophosphatase activity was inhibited to a greater extent by $MgCl_2$ than by $CaCl_2$ (Figure 9).

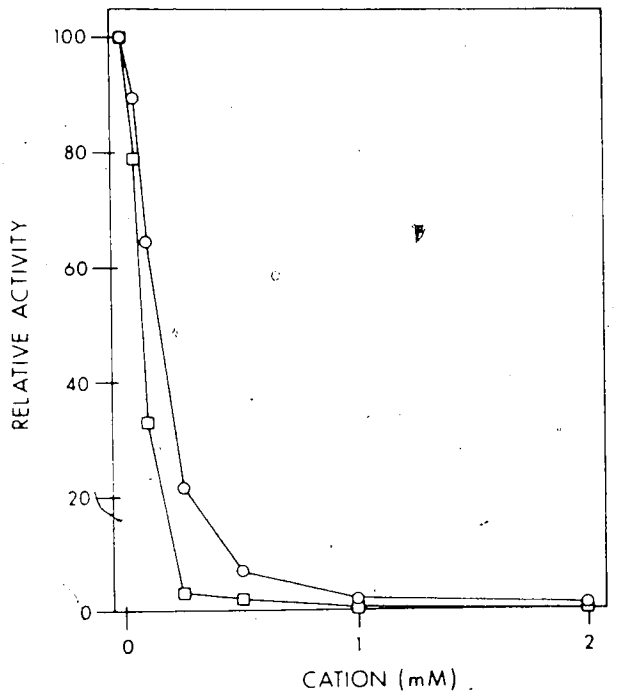


Fig. 9. Inhibition of the pyrophosphatase activity of liver alkaline phosphatase

Activities were measured at pH 9.0 in the presence of 0.1 mM pyrophosphate and added calcium (o) or magnesium (□) ions. The buffer also contained 5 μM $MgCl_2$ in order to stabilize the enzyme activity.

Since pyrophosphate forms more stable complexes with Mg^{2+} than with Ca^{2+} (Sillen & Martell, 1971), the data suggests that pyrophosphate-metal complexes may not be substrates. ATPase activity paralleled the concentration of free ATP [Figures 10(b), 11(b)]. Similar plots were obtained for 7 total ATP concentrations between 0.25 mM ATP and 10 mM ATP. ATPase activity was highest at 5 μM $MgCl_2$ without further additions of $MgCl_2$ or $CaCl_2$ [Figures 10(a), 11(a)].

Additional evidence that free ATP^{4-} is the actual substrate species is the agreement between the K_m values for free ATP obtained at low (5 μM) and at high (10 mM) $MgCl_2$ concentrations. At 5 μM $MgCl_2$ the K_m value at pH 9.0 for ATP was 39 μM (Table VIII). A K_m value of 95 μM was determined when the free Mg^{2+} concentration was 1 mM or greater and the calculated values for free ATP concentrations

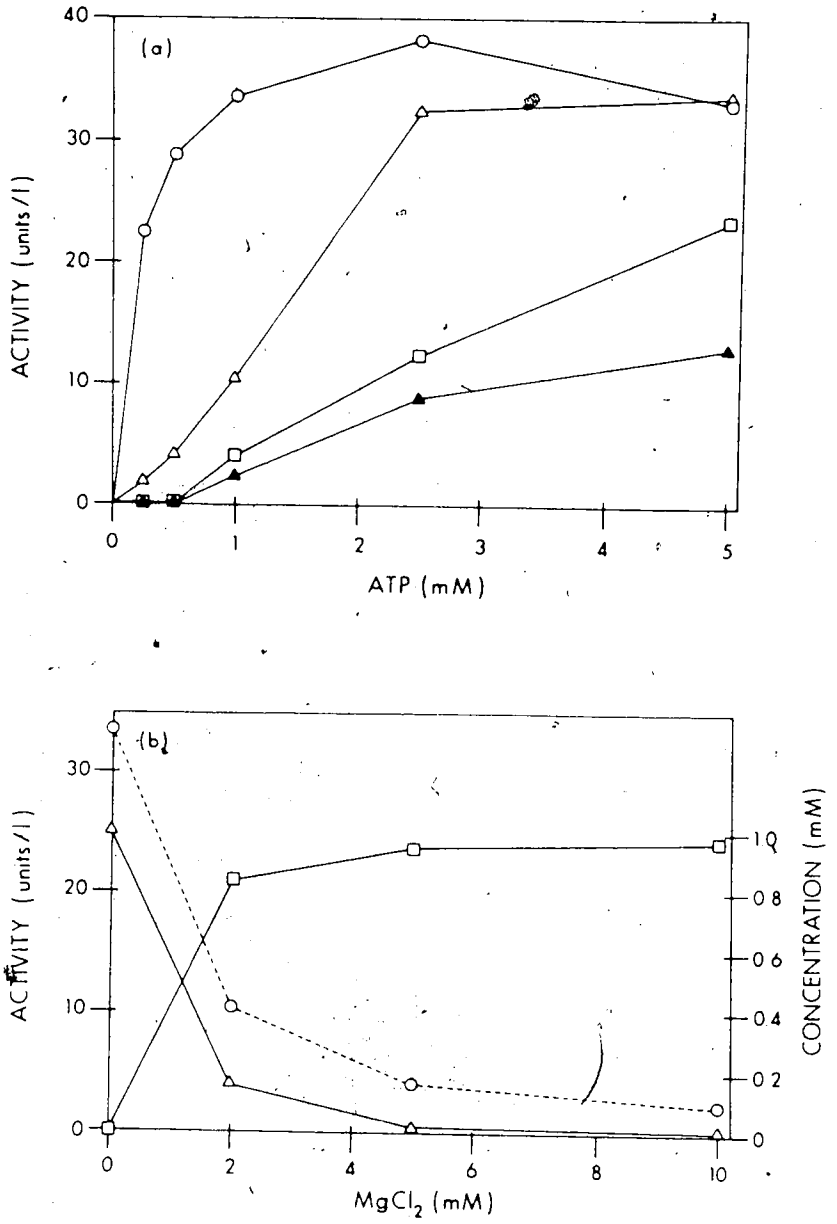


Fig. 10. Inhibition of the ATPase activity of human liver alkaline phosphatase by $MgCl_2$ at pH 9.0

(a) ATPase activities were measured at the following concentrations of $MgCl_2$: o, 5 μM; Δ, 2 mM; □, 5 mM; ▲, 10 mM.

(b) Relationship between ATPase activity (o) and the concentration of ATP^{4-} (Δ) or $MgATP^{2-}$ (□) at a total ATP concentration of 1 mM.

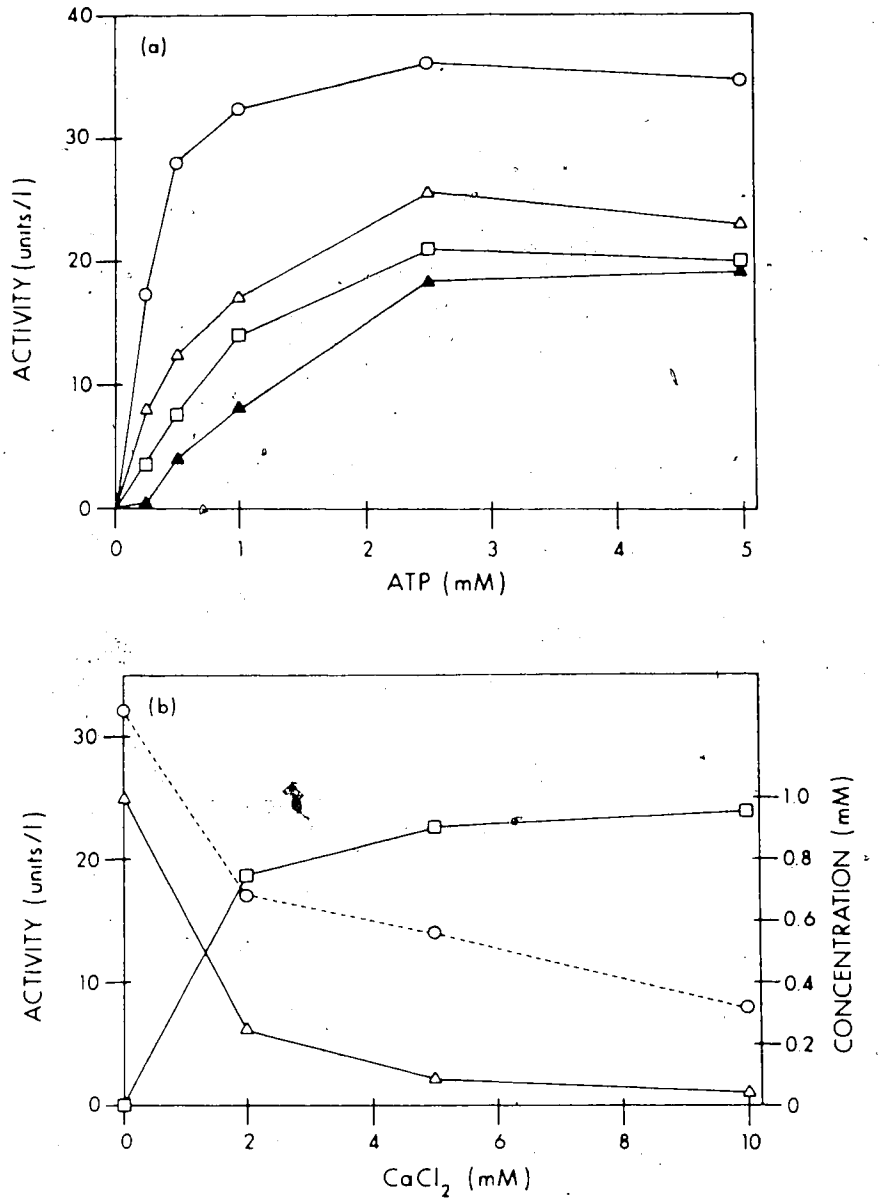


Fig. 11. Inhibition of the ATPase activity of human liver alkaline phosphatase by $CaCl_2$ at pH 9.0

(a) ATPase activities were measured in the presence of 5 μM $MgCl_2$ and the following concentrations of $CaCl_2$: o, 0 mM; Δ, 2 mM; □, 5 mM; ▲, 10 mM.

(b) Relationship between ATPase activity (o) and the concentration of ATP^{4-} (Δ) or $CaATP^{2-}$ (□) at a total ATP concentration of 1 mM.

were used for the substrate species. The association constant for $MgATP^{2-}$ used in the calculation of free ATP concentration was among the lowest values reported (Phillips et al, 1966). Closer agreement would be obtained if a higher stability constant had been used. (Substrate K_m values do not appear to be influenced significantly by magnesium ions: as shown in Table VIII, the K_m values for 2-phosphoglycerate determined at 1.5 mM $MgCl_2$ or 5 μM $MgCl_2$ were in good agreement.)

Evidence that $MgATP^{2-}$ has a very low affinity for alkaline phosphatase was derived from experiments in which the inhibition of p-nitrophenylphosphatase activity by ATP^{4-} or $MgATP^{2-}$ was assessed. The concentration of ATP that produced 50% inhibition of activity at a free Mg^{2+} concentration of 0.1 mM produced less than 10% inhibition when Mg^{2+} was present in a large excess (100 mM). In the latter experiment a correction was made for the decreased velocity as a result of Mg^{2+} binding to p-nitrophenylphosphate.

At pH 7.4, the interpretation is more complex because a larger number of complexes must be considered (Storer & Cornish-Bowden, 1976), and because the substrate K_m values are at least an order of magnitude lower (Table VIII, Figure 8). Figure 12 shows the inhibition at pH 7.4 of pyrophosphatase and ATPase activities by $MgCl_2$ at substrate concentrations of 25 μM. Since the K_m values are well below this concentration, the activities should represent maximal velocities. At 5 μM $MgCl_2$ the activities with the 4 substrates shown were similar. However, as the Mg^{2+} concentration is increased the activities decrease in relation to the strength with which the substrates bind Mg^{2+}. If the true substrate is the uncomplexed species, then the

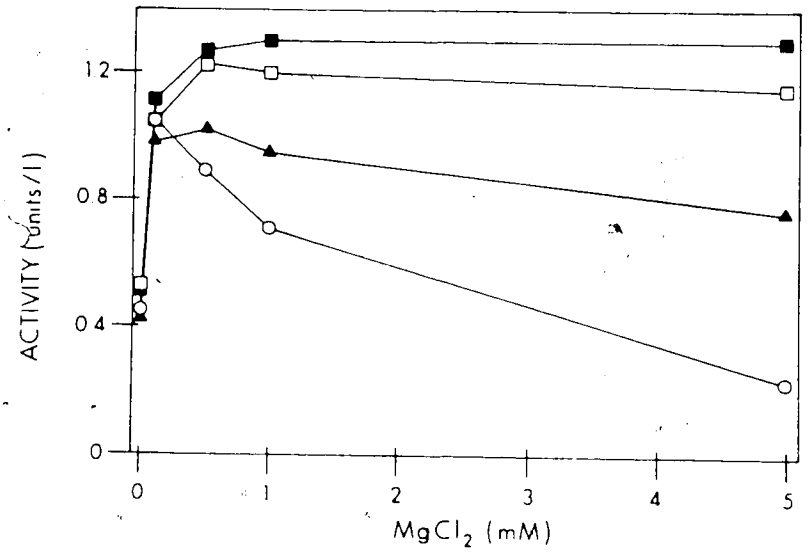


Fig. 12. Influence of $MgCl_2$ on the hydrolysis of various substrates by human liver alkaline phosphatase at pH 7.4

Enzyme activity was measured in the presence of 25 μM each of p-NPP (■), ADP (□), ATP (▲) or pyrophosphate (o) at concentrations of $MgCl_2$ from 5 μM to 5 mM.

inhibition is explained by the reduction of the true substrate concentration to non-saturating levels. At ATP concentrations of 0.25 mM, inhibition at pH 7.4 by $MgCl_2$ or $CaCl_2$ at concentrations up to 10 mM, is not observed. This may be due to the fact that free ATP has not been reduced to non-saturating concentrations.

All these experiments at pH 7.4 and pH 9:0 suggest that the calcium and magnesium complexes of pyrophosphate and ATP are neither substrates nor inhibitors of liver alkaline phosphatase. The concentrations of free Mg^{2+}, free Ca^{2+}, Mg_2ATP, Ca_2ATP and MgCaATP were also calculated (data not shown) but the changes in these species do not account for the observed changes in enzyme activity.

(Ca)-ATPase activity. Influence of calcium-dependent regulator protein

At concentrations of 5 μM $MgCl_2$ and 0.25 mM ATP, $CaCl_2$ (5 mM) activated the ATPase activity of alkaline phosphatase at pH 7.4 by a factor of 2. Inhibition by Ca^{2+} ions was not observed, probably because free ATP was present at saturating concentrations. Since ATP binds both Mg^{2+} and Ca^{2+} ions, the addition of $CaCl_2$ would cause an increase in the free Mg^{2+} ion concentration and possibly account for the increase in enzyme activity. When 5 mM $MgCl_2$ was added in the absence of added $CaCl_2$, the activity was activated by a factor of 3 and subsequent addition of $CaCl_2$ did not cause a further increase in ATPase activity.

EGTA is used in some (Ca)-ATPase assays in order to bind calcium ions and obtain the ATPase activity that is independent of calcium. Incubation of alkaline phosphatase for 5 min with 0.5 mM EGTA and 1 mM $MgCl_2$ resulted in 78% inhibition of the ATPase activity. The inhibition after a 60 min incubation was 92%. This effect could be abolished by incubation with 1 mM $CaCl_2$ simultaneously with the EGTA or by preincubation with substrate. This ability of substrate to protect the enzyme against a metal complexing agent has been reported for the enzyme from pig kidney (Ackermann & Ahlers, 1976a). It is known that EGTA strongly binds zinc ions (Sillen & Martell, 1971) and therefore could inhibit alkaline phosphatase by chelation of enzyme-bound zinc.

Additional experiments carried out as described in 'Methods' at $MgCl_2$ concentrations up to 5 mM, and $CaCl_2$ concentrations up to 1 mM confirmed that under our experimental conditions purified liver alkaline phosphatase does not possess a calcium-ion stimulated ATPase activity.

Incubation of alkaline phosphatase with 1 μM calcium-dependent regulator protein and calcium ions up to 1 mM did not influence the ATPase activity of the enzyme. Calcium-binding protein has recently been shown to be an activator of the (Ca)-ATPase activity of human erythrocytes (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977).

DISCUSSION

The lack of substrate specificity of pure liver alkaline phosphatase is in agreement with earlier findings for the enzyme from other sources (Fernley, 1971; Fishman, 1974). Under *in vitro* conditions the purified enzyme hydrolyses a number of phosphomonoesters which have been suggested as possible physiological substrates.

The pH 'optimum' of alkaline phosphatase obtained from various sources is reported to be dependent on substrate concentration (Fernley, 1971; Fishman, 1974; Chen, 1976; Van Belle, 1976). This statement would appear to be incorrect in that it is customary to measure the pH optimum with the enzyme saturated with substrate at each pH (Lehninger, 1975). When this is done the highest activity is always obtained at alkaline pH. However it is apparent that the lowest K_m values (less than 5 μM) are obtained near neutral pH, therefore low concentrations of substrate are hydrolysed more efficiently at physiological pH than at the pH optimum where K_m values are in the millimolar range (Van Belle, 1976). The similar V_{max} values found for the various substrates are in accord with the mechanism proposed for *E. coli* alkaline phosphatase in which the rate-determining step occurs after the phosphorylation of the enzyme (Trentham & Gutfreund, 1968; Reid & Wilson, 1971b). Similar findings

have been reported for pig kidney alkaline phosphatase (Hiwada & Wachsmuth, 1974a), bovine intestinal alkaline phosphatase (Chappelet-Tordo et al, 1974) and human placental alkaline phosphatase (Harkness, 1968b).

The physiological significance of the phosphomonoesterase acti ity of liver alkaline phosphatase remains unclear. If the enzyme has same specificity in its native membranous environment then it is possible that activities commonly ascribed to other phosphatases could be due, at least in part, to alkaline phosphatase. In this regard alkaline phosphatase may contribute to the regulation of purine ribonucleotide catabolism by hydrolysis of AMP and PRPP (Sattin & Rall, 1970; Fox & Marchant, 1974). Alkaline phosphatase may also be implicated in hormonal regulation in that the product of AMP hydrolysis, adenosine, has been reported to be an activator of adenyl cyclase (Sattin & Rall, 1970). Liver alkaline phosphatase may also be one of the tissue phosphatases responsible for the rapid removal of carbamyl phosphate from blood when it is administered as an antisickling agent (Jernigan & Kraus, 1975). The hydrolysis of pyridoxal phosphate by the human liver enzyme supports the suggestion that alkaline phosphatase is important in the regulation of the tissue content of this coenzyme (Lumeng & Li, 1975).

Alkaline phosphatase is generally considered to be a tightly-bound component of the plasma membrane (Fishman, 1974; Chen, 1976) so that some substrate specificity might well be conferred on the enzyme by virtue of its natural environment. There are some suggestions that alkaline phosphatase may be an ecto-enzyme but the orientation of the enzyme in the membrane remains controversial (Fishman, 1974). Another enzyme, glucose-6-phosphatase, shows high specificity for glucose-6-

phosphate when the enzyme is present in untreated microsomes, and broad specificity when the microsomes are disrupted (Arion, Carlson et al, 1972; Arion, Wallin et al, 1972). To explain this phenomenon Arion et al (1975) have proposed a model in which a specific transport system functions to shuttle glucose-6-phosphate from the cytoplasm to glucose-6-phosphatase bound to the luminal surface of the endoplasmic reticulum. It is possible that a similar mechanism could influence the specificity of alkaline phosphatase. Alkaline phosphatase is known to possess phosphotransferase activity (Fishman, 1974) although the physiological importance of this activity has not been established. We have not attempted to evaluate the phosphotransferase activity of pure liver alkaline phosphatase, but it seems possible that this activity may be of physiological importance. The hydrolytic rates at neutral pH are significantly lower than at more alkaline pH and the hydrolytic activity would appear to be largely inhibited by phosphate (see chapter V). Thus it may be that the enzyme is responsible for important synthetic reactions rather than the hydrolytic reactions with which it is generally associated.

The inhibition of ATP and pyrophosphate hydrolysis by calcium and magnesium ions is consistent with the suggestion that metal-substrate complexes are not substrates of alkaline phosphatase (Fernley, 1971). A number of important regulatory reactions involve transphosphorylations using Mg-ATP or Mg-ADP as substrates. The concentrations of these complexes remain relatively constant in cultured cells (Fodge & Rubin 1973) and depends on the availability of Mg^{2+} ions and the nucleotide species. It is possible that hydrolysis of ATP, but not Mg-ATP by alkaline phosphatase represents another mechanism whereby the enzyme could regulate metabolic processes.

We were unable to demonstrate the calcium-sensitive ATPase activity in purified liver alkaline phosphatase that has been reported for intestinal alkaline phosphatases (Haussler et al, 1970; Russell et al, 1972). In addition, we were unable to demonstrate any influence of calcium ions when calcium-dependent regulator protein was included in the assay system. Calcium-dependent regulator protein has recently been shown to be an activator of the (Ca)-ATPase present in the erythrocyte plasma membrane (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977). We conclude either that under our experimental conditions liver alkaline phosphatase is not a (Ca)-ATPase or that purification of the enzyme has resulted in loss of this activity.

The presence of alkaline phosphatase would appear to be a potential source of error in (Ca)-ATPase assays. (Ca)-ATPase activity is calculated as the ATPase activity in the presence of Ca^{2+} and Mg^{2+} ions less the ATPase activity when only Mg^{2+} is added. Our results show that the ATPase activity of alkaline phosphatase is altered by changes in the concentration of these ions. In addition, subtraction of the ATPase activity in the presence of Mg^{2+} would appear to be an inappropriate correction for the ATPase activity of alkaline phosphatase since it appears that free ATP is the actual substrate of alkaline phosphatase. This error may not be large when sufficient free ATP is present to saturate alkaline phosphatase, but a significant error may be introduced at the low levels of ATP used in some assays using ^{32}P ATP (Shigekawa et al, 1978). Another source of error in (Ca)-ATPase assays can occur when EGTA is used to bind free Ca^{2+}. As shown here EGTA can inhibit alkaline phosphatase especially when the concentration of EGTA is greater than the concentration of Ca^{2+} or when substrate

is not present before the addition of EGTA. Thus it appears that precautions must be taken in order to ensure that alkaline phosphatase does not interfere with (Ca)-ATPase assays.

CHAPTER FIVE

INHIBITION OF HUMAN ALKALINE PHOSPHATASES

Various phosphonic acids have been shown to be competitive inhibitors of alkaline phosphatase from E. coli (Halford et al, 1969; Williams et al, 1973) and from calf intestine (Kochman et al, 1964). The enzyme from calf intestine was also inhibited competitively by inorganic arsenate (Morton, 1955). An assessment of the inhibition of alkaline phosphatase from human tissues may prove useful in the development of affinity purification procedures (see Chapter III), and therefore the inhibition constants for several inhibitors of alkaline phosphatase from human liver are reported here.

During studies on the ATPase activity of alkaline phosphatase purified from human liver (see Chapter IV), unusual reaction-rate curves were observed when the ATP used was from horse muscle and had not been treated to remove compounds containing vanadium. Cantley et al (1977) and Quist & Hokin (1978) have identified orthovanadate (VO_4^{3-}) as an inhibitor present in horse muscle ATP that accounts for the potent inhibition of (Na, K)-ATPase observed by several investigators (Josephson & Cantley, 1977; Hudgins & Bond, 1977; Beauge & Glynn, 1978). Vanadate has previously been shown to be a competitive inhibitor of alkaline phosphatase from E. coli (Lopez et al, 1976) and acid phosphatase from human liver or wheat germ (Van Etten et al, 1974). The present report demonstrates that the ATPase activity of alkaline phosphatase from human liver is inhibited by a contaminant in ATP preparations from horse muscle that have not been treated to remove compounds containing vanadium. Vanadate at physiological concentrations is shown to be a potent competitive inhibitor of the p-nitrophenyl-

phosphatase activity of alkaline phosphatase from several human tissues.

METHODS

Enzyme activity with p-NPP as substrate was measured at 30°C in buffer mixture A supplemented with 1.5 mM $MgCl_2$, as described in Chapter IV. Substrate concentrations ranged from 5 to 100 µM (pH 7.4) or 25 to 1000 µM (pH 9.0). When the influence of L-epinephrine on vanadate inhibition was studied, p-nitrophenylphosphatase activity was monitored by the release of phosphate (Anner & Moosmayer, 1975).

ATPase activity was measured at pH 9.0 as described above by the amount of phosphate released (LeBel et al, 1978). Since magnesium ions inhibited the ATPase activity (see Chapter IV), the $MgCl_2$ concentration was kept at 5 µM.

Inhibition constants were determined from double-reciprocal plots (Lineweaver & Burk, 1934) or Dixon (1953) plots using a minimum of five substrate or inhibitor concentrations respectively.

RESULTS AND DISCUSSION

Inhibition of alkaline phosphatase from human liver

Several phosphonic acids and p-arsanilic acid inhibited alkaline phosphatase from human liver (Table IX). Although double-reciprocal plots showed mixed-type inhibition for some amino-substituted inhibitors, the inhibition was predominantly competitive. The low K_i values for the aromatic phosphonic acids were similar to the K_m values for substrates (see Chapter IV) which suggests that a specific interaction with the enzyme occurs.

The uncompetitive inhibition by L-homoarginine was almost ten times greater than the inhibition by L-arginine (Table IX). Since L-arginine

TABLE IX. Inhibition of the p-nitrophenylphosphatase activity of alkaline phosphatase from human liver at pH 9.0

Inhibitor	K_i (mM)	Type of inhibition
3-Aminopropylphosphonic acid	8.0	mixed-type
Phenylphosphonic acid	0.075	competitive
3-Aminophenylphosphonic acid	0.025	mixed-type
4-Aminobenzylphosphonic acid	0.55	mixed-type
p-Arsanilic acid	0.035	competitive
L-Homoarginine	0.71	uncompetitive
L-arginine	5.6	uncompetitive
Phospho-L-arginine	0.083	competitive
Inorganic phosphate	0.090	competitive
Inorganic vanadate	0.0009	competitive
Ouabain	-	none detected

has only one less CH_2 residue than L-homoarginine, this suggests that the inhibition is dependent on the length of the inhibitory molecule. However, phospho-L-arginine was a competitive inhibitor of the p-nitrophenylphosphatase activity and the inhibition was almost ten times greater than the inhibition by L-homoarginine (Table IX). Phospho-L-arginine was also a substrate with a K_m value of 96 µM (see Chapter IV) which was similar to its K_i value of 83 µM (Table IX).

Alkaline phosphatase from liver was inhibited competitively by phosphate (Table IX). The K_i value at pH 9.0 was 90 µM whereas the corresponding value at pH 7.4 was 35 µM. At the phosphate concentrations found in liver tissue [approximately 4 mM (Erecinska et al, 1977)] it seems probable that if the enzyme is accessible, the hydrolytic activity would be largely inhibited. Recently the importance of phosphate as a contributor to the regulatory mechanisms of cellular

metabolism has been emphasized (Erecinska et al, 1977; Barankiewicz et al, 1977) and thus alkaline phosphatase may play a fundamental role in regulation by maintenance of the intracellular phosphate concentration at the expense of phosphomonoesters.

Inhibition by vanadate

Inorganic vanadate was a potent competitive inhibitor of alkaline phosphatase from liver (Figure 13, Table IX). At pH 9.0 the inhibition constant was less than 1 μM, which is approximately 2 orders of magnitude lower than the K_i value for inorganic phosphate under the same conditions (Table IX). Similar results were obtained at pH 7.4 where the K_i values for vanadate and phosphate were found to be 0.6 μM and 35 μM respectively. This is in contrast with the enzyme from E. coli where the inhibitor constants were of similar magnitude (Lopez et al, 1976).

Inhibition by vanadate was reversible. Addition of L-epinephrine (1 mM) to the reaction mixture abolished the inhibition by vanadate and restored full p-nitrophenylphosphatase activity. Similar findings have been reported by others for the reversal of the vanadate inhibition of (Na, K)-ATPase (Cantley et al, 1977) and dynein ATPase (Kobayashi et al, 1978; Gibbons et al, 1978). L-epinephrine is known to form complexes with vanadate (Kustin et al, 1974) so it is probable that the reversal of inhibition is due to complexing of vanadate. The extent of inhibition of alkaline phosphatase from liver was independent of the order of addition of inhibitor and substrate and of the time that enzyme and inhibitor were preincubated before the addition of substrate.

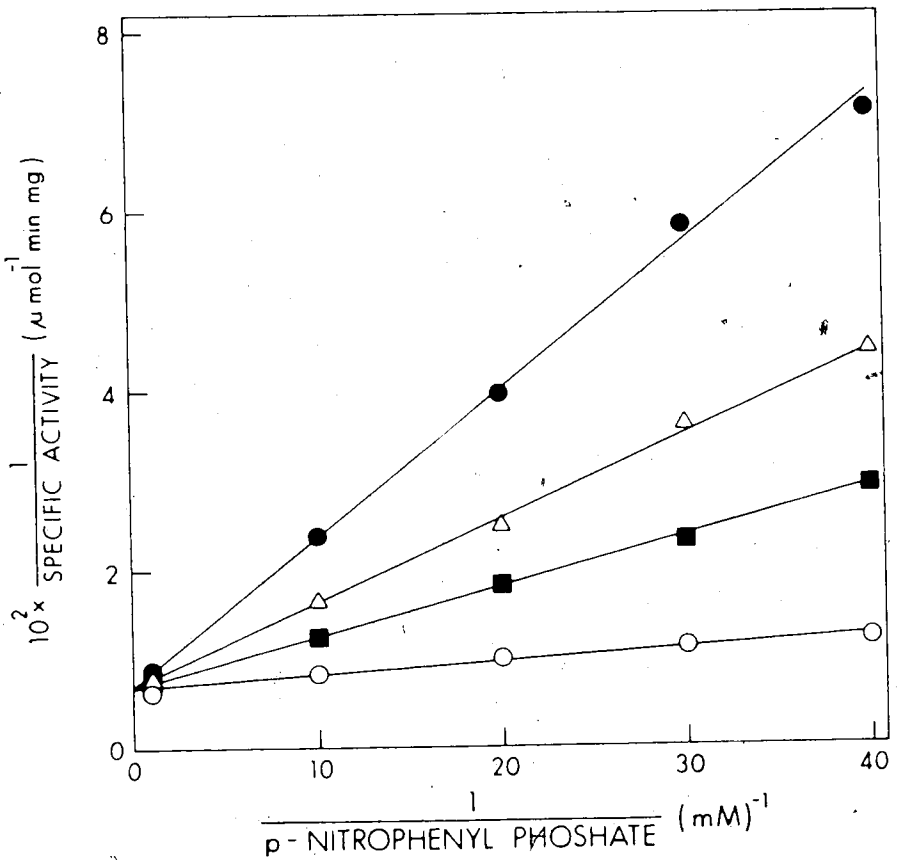


Fig. 13. Inhibition of alkaline phosphatase activity by vanadate

Double-reciprocal plot for alkaline phosphatase from human liver (40 ng/ml) at pH 9.0. Orthovanadate concentrations were: o, none; ■, 2.5 μM; Δ, 5 μM; and ●, 10 μM. Similar plots were obtained with the enzyme from human intestine or kidney.

Vanadate and phosphate competed for the same binding-site on alkaline phosphatase from liver. The series of parallel lines obtained from the Yonetani-Theorell plot (Yonetani & Theorell, 1964) shown in Figure 14 for the enzyme from human liver suggests that the two inhibitors, vanadate and phosphate, were bound in a mutually exclusive fashion. Similar findings have been reported for alkaline phosphatase from E. coli (Lopez et al, 1976).

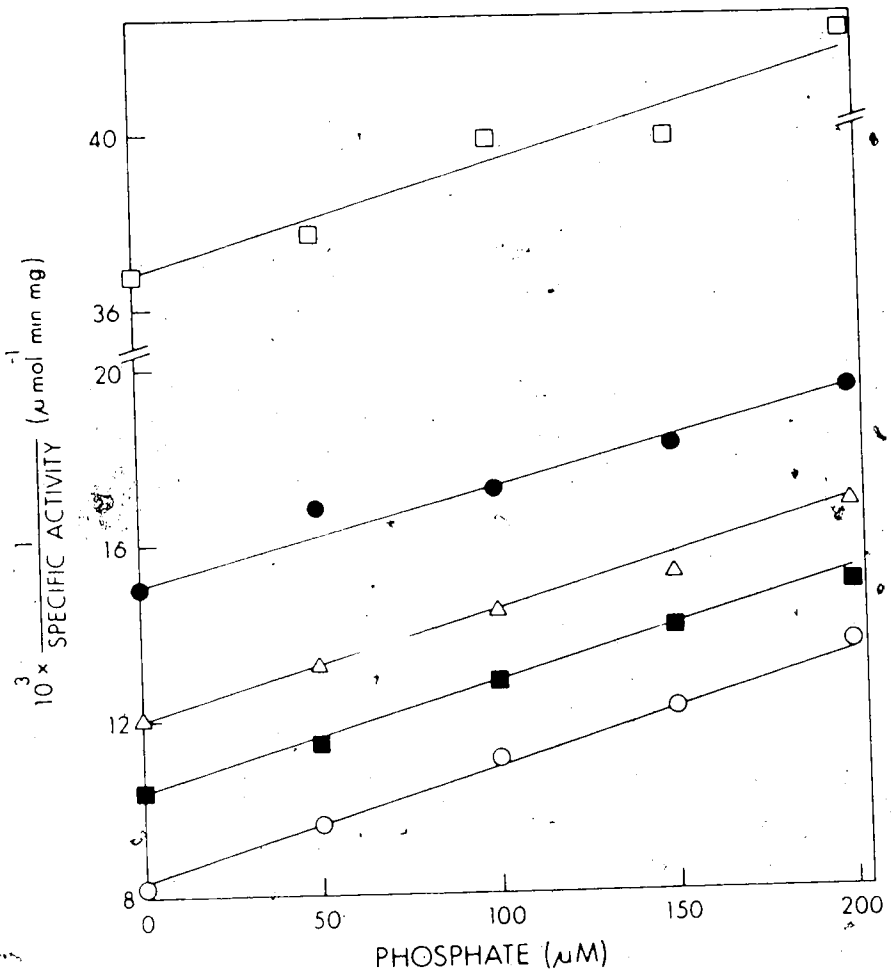


Fig. 14. Yonetani-Theorell plot showing the effect of varying concentrations of vanadate and phosphate on the alkaline phosphatase activity from human liver

Enzyme activity was measured at pH 9.0 at 25 μM p-NPP and an enzyme concentration of 40 ng/ml. Vanadate concentrations were: o, none; ■, 0.25 μM; Δ, 0.5 μM; ●, 1 μM; and □, 5 μM.

In order to determine if traces of vanadate in ATP preparations were responsible for the unusual reaction-rate curves for ATP hydrolysis by alkaline phosphatase from liver, ATPase activity was measured using vanadium-free ATP to which vanadate was added in a molar ratio of 1:1000. As shown in Figure 15, the addition of vanadate appeared to account for

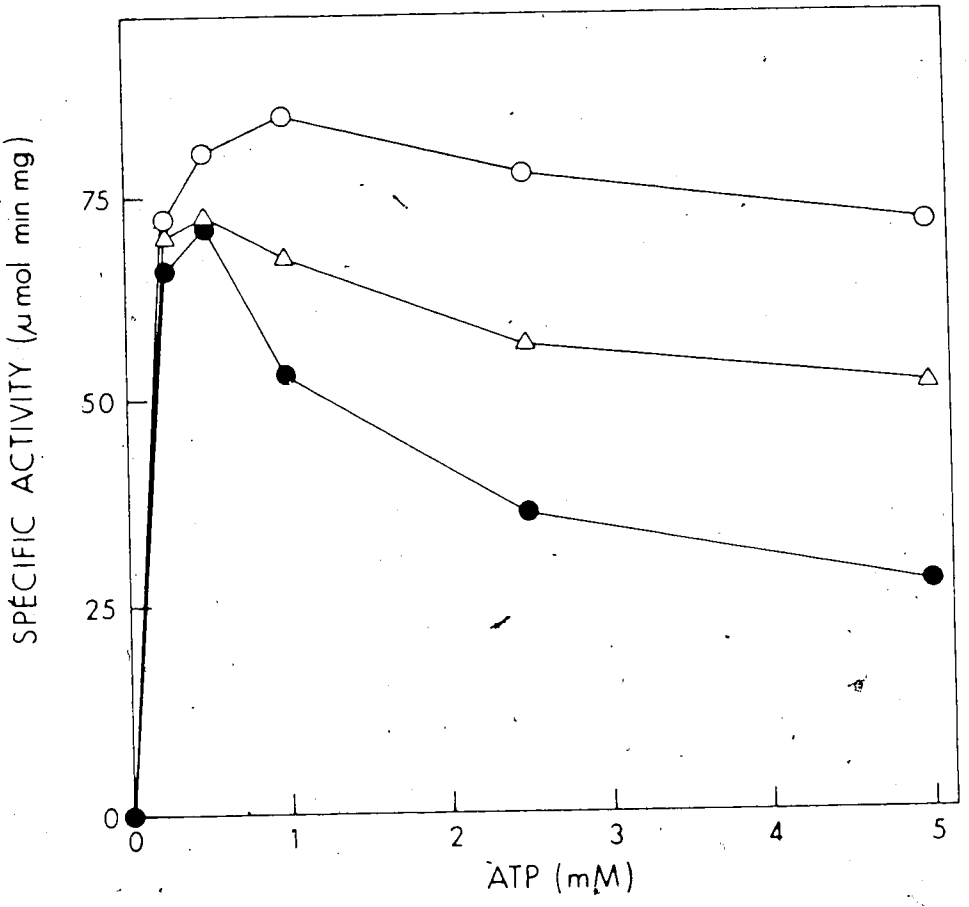


Fig. 15. The effect of increasing substrate concentration on the ATPase activity of alkaline phosphatase from human liver

Enzyme activity was measured at pH 9.0 at an enzyme concentration of 40 ng/ml. O, ATP from horse muscle that had been treated to remove vanadium; Δ, vanadium-free ATP to which vanadate had been added in a molar ratio of 1:1000; ●, ATP extracted from horse muscle.

at least part of the inhibitory effect observed when ATP was used that was not treated to remove vanadium. The supplier stated that this ATP contains 5-40 ppm of vanadium (molar ratio up to 1:2000). Since less inhibition was observed when vanadate was added to vanadium-free ATP in a molar ratio of 1:1000, the inhibition cannot be attributed solely to the presence of vanadate. Quist & Hokin (1978) have found two

(Na, K)-ATPase inhibitors in horse muscle ATP, vanadate and a dithioerythritol-dependent inhibitor. It is thus possible that the ATPase activity of alkaline phosphatase may also have been influenced by an inhibitor other than vanadate. In addition, the shape of the curves (Figure 15) may be very dependent on small changes in the free magnesium concentration. Although very similar concentrations of free magnesium would be expected at each concentration of ATP, the absolute concentrations were low (total magnesium was 5 μM). If vanadate binds magnesium appreciable (published data not available), the inhibitory effect could be due to further lowering of the free magnesium concentration by complexing to vanadate.

Because vanadate was such a potent inhibitor of alkaline phosphatase from liver, the influence of vanadate on the enzyme from other tissues was evaluated. As shown in Table X, the K_i values for the inhibition by vanadate of alkaline phosphatase from liver, small intestine and kidney were all less than 1 μM. These values are of the same order of magnitude reported by Cantley et al (1977) for the (Na, K)-ATPase from striated muscle. Since tissue concentrations of vanadate are in the near-micromolar range, with concentrations of 20-30 μg/kg dry weight

TABLE X. Inhibition by vanadate of alkaline phosphatase from human liver, intestine and kidney

Enzyme source	K_i (μM)
Liver	0.9
Small intestine	0.5
Kidney	0.6

in adult human liver, spleen, pancreas and prostate gland (Underwood, 1962), it appears that the phosphohydrolytic activity of alkaline phosphatase in vivo may be influenced by vanadate.

With the exception of alkaline phosphatase it appears that (Na, K)-ATPase and dynein ATPase are the only ATPases that are inhibited by vanadate ions. (Ca)-ATPase from sarcoplasmic reticulum, actomyosin ATPase and the F_1 ATPase from mitochondria were all relatively insensitive to the metal (Josephson & Cantley, 1977; Quist & Hokin, 1978). Intestinal alkaline phosphatase has been suggested to be a (Ca)-ATPase (Haussler et al, 1970; Russell et al, 1972), but since intestinal alkaline phosphatase is inhibited by vanadate and (Ca)-ATPase is apparently not, it appears that the two enzyme activities are distinct. It is possible, however, that the intestinal enzyme acquired sensitivity to vanadate as a result of the process of purification. Vanadate ions do not inhibit the ATPases in a strictly competitive manner (Josephson & Cantley, 1977; Kobayashi et al, 1978) as observed for alkaline phosphatases (Figure 13). Cantley et al (1978) have found two vanadate-binding sites on the (Na, K)-ATPase from dog kidney and suggested that vanadate may be a physiological regulator of that enzyme.

The more potent inhibition of alkaline phosphatase by vanadate than by phosphate suggests that the former compound through hydration or chelation can resemble a transition-state analogue of phosphate in the mechanism that involves a phosphoryl-enzyme intermediate. Lopez et al (1976) point out that one of the transition states may be a trigonal bipyramidal species, and vanadate can resemble this type of structure (Van Etten et al, 1974; Lopez et al, 1976).

CHAPTER SIX

STUDIES OF NEURAMINIDASE-TREATED ALKALINE PHOSPHATASE FROM HUMAN LIVER

Certain of the different molecular forms of alkaline phosphatase may be attributed to differences in sialic acid content (Hiwada & Wachsmuth, 1974b; Mulivor, Plotkin & Harris, 1978). It is well established that all human alkaline phosphatases, with the exception of the enzyme from intestine, show decreased electrophoretic mobility after treatment with neuraminidase (Fishman, 1974). However, relatively little data is available regarding the role(s) of sialic acid and the influence of the removal of sialic acid residues on the properties of the enzyme. Komoda & Sakagishi (1978) have suggested that a possible role of sialic acid residues may be to protect the active conformation of the enzyme and to maintain the three-dimensional structure of the enzyme. The present investigation was carried out to determine the influence of sialic acid residues on (1) some kinetic properties of the purified enzyme from liver and on (2) the inactivation of the enzyme by heating or by incubation in solutions containing SDS.

METHODS

Treatment with neuraminidase

The removal of sialic acid residues by incubation with neuraminidase was done essentially as described (Mori & Hollands, 1971; Badger & Sussman, 1976). Alkaline phosphatase from human liver (100 µg in 1 ml) was dialyzed against 10 mM acetate buffer (pH 5.0) which contained 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. Neuraminidase (Worthington: NEUA, 6 µmol/min/mg) from Clostridium perfringens was dissolved in the same buffer, and 6 µl of the solution, which contained 0.3 µg of neuraminidase was added to the

alkaline phosphatase solution. An additional 0.3 µg was added 4 h later. The mixture was then dialyzed against the same buffer for 48 h at 22°C with two changes of buffer, followed by dialysis at 4°C against 100 mM Tris-HCl buffer (pH 7.6) which contained 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. Two controls were treated identically except that in one the addition of neuraminidase was omitted and in the other 1 ml of buffer was substituted for the alkaline phosphatase preparation.

The completeness of the treatment with neuraminidase was evaluated by isoelectric focusing in polyacrylamide gels as described in Chapter II. The pH gradient in the gels was relatively linear and extended from pH 3.9 to pH 8.1.

Kinetic parameters

Michaelis constants were determined from double-reciprocal plots (Lineweaver & Burk, 1934) using a minimum of five substrate concentrations. The hydrolysis of substrates at pH 9.0 was followed by phosphate analysis (Anner & Moosmayer, 1975) and was linear up to 20% hydrolysis of the substrate. The inhibition of the p-nitrophenyl phosphatase activity by L-homoarginine was measured as described above at three concentrations of L-homoarginine from 0.5 to 2.0 mM. Corrections for non-enzymic hydrolysis were made at each substrate concentration by subtraction of phosphate present in substrate solutions to which alkaline phosphatase was not added.

Heat inactivation

Alkaline phosphatase (0.12 µg) purified from human liver in 0.1 ml of buffer (0.1 M Tris-HCl, 100 mM NaCl, 0.1 mM $MgCl_2$, 0.02 mM $ZnCl_2$, pH 7.6), was placed in thin-walled glass tubes (8 mm x 75 mm) and incubated at 56.0°C in a waterbath for timed intervals up to 30 minutes. The samples were immediately chilled to 0°C and diluted to 1.0 ml with 0.8 M MAP buffer (pH 10.3) which contained 1.5 mM $MgCl_2$. The enzyme activity

was measured as described in Chapter II and was expressed as a percentage of the activity of samples that were incubated at 30°C.

SDS inactivation

Alkaline phosphatase (0.2 µg) purified from human liver in 1.0 ml of buffer (10 mM Tris-HCl, 1% (w/v) SDS, 1.5 mM $MgCl_2$, pH 7.5), was incubated at 30°C for timed intervals up to 4 hours. Alkaline phosphatase activity was then measured at pH 7.5 in order to avoid any changes in SDS binding that could occur by changing the pH. The rate of reaction was monitored kinetically at 404 nm following the addition of p-NPP (final concentration = 0.1 mM) to the incubation mixture. Enzyme activity was expressed as a percentage of the activity of samples that did not contain SDS.

RESULTS

Treatment with neuraminidase

Alkaline phosphatase from human liver that was treated with neuraminidase as described above and subjected to isoelectric focusing on polyacrylamide gels was mainly present in a band at pH 6.5. This band accounted for more than 90% of the recovered enzyme activity. In addition, there were several minor bands of enzyme activity (less than 8% of the recovered activity) with isoelectric points between 5.8 and 6.5. Alkaline phosphatase that was not treated with neuraminidase focused as a single band of enzyme activity with an isoelectric point of 4.0, but the enzyme appeared to be largely inactivated probably because of the loss of bound metal ions.

In order to determine if the minor bands were due to incomplete desialylation, the preparation was treated again with neuraminidase as described in the Methods section except that 5 times as much neuraminidase

was used. The minor bands of enzyme activity were still present on electrofocused gels after this extensive treatment with neuraminidase, and thus these bands were not partially desialylated alkaline phosphatase.

Kinetic parameters

Treatment with neuraminidase did not significantly alter the specific activity of alkaline phosphatase from human liver. Neuraminidase-treated alkaline phosphatase had a specific activity of 1172 units/mg, and the control alkaline phosphatase preparation had a specific activity of 1205 units/mg. These specific activities were similar to that of the untreated enzyme and showed that neither the dialysis procedure, nor the removal of sialic acid residues resulted in a significant loss of enzyme activity.

A comparison of the K_m values for six substrates of alkaline phosphatase is given in Table XI. The K_m values determined for neuraminidase-treated alkaline phosphatase were considerably higher than those determined for untreated alkaline phosphatase. However, they were very similar to the values for the control alkaline phosphatase preparation, thus it is likely that dialysis at pH 5.0 rather than removal of sialic acid residues was responsible for the change in K_m values.

L-homoarginine was found to be an uncompetitive inhibitor of neuraminidase-treated alkaline phosphatase with a K_i value of 0.7 mM. This value is in agreement with the K_i values determined for the untreated and control alkaline phosphatase preparations which were 0.7 mM and 0.6 mM respectively.

TABLE XI. K_m values for native and neuraminidase-treated alkaline phosphatase from human liver*

Substrate	K_m		
	Untreated enzyme**	Enzyme treated with neuraminidase	Control enzyme***
p-NPP	9	20	24
2-Phosphoglycerate	78	267	286
AMP	48	120	152
Glucose-6-phosphate	270	1053	909
Phosphocholine	185	526	500
Ribose-5-phosphate	156	435	556

* Enzyme activities were measured in buffer mixture A at pH 9.0 in the presence of 1.5 mM $MgCl_2$.

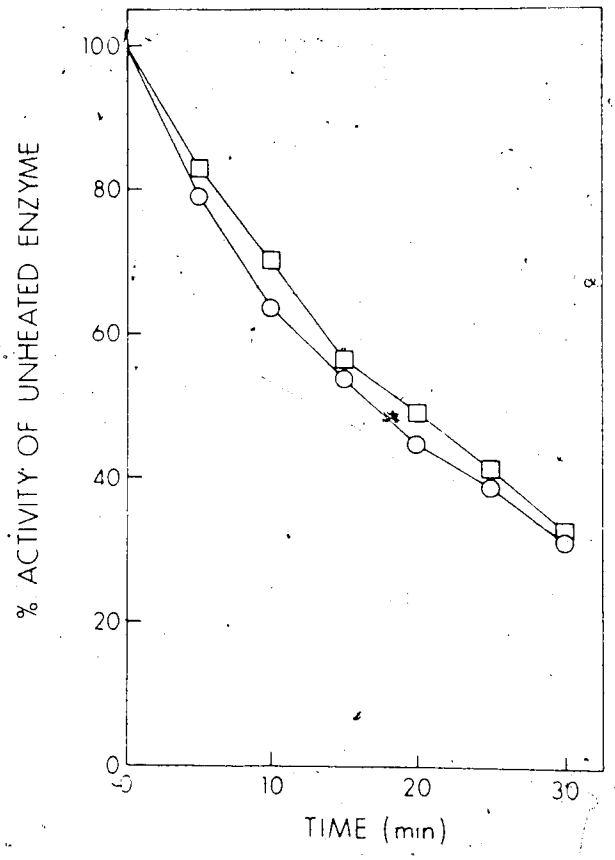
** K_m values for untreated enzyme were taken from Table VIII, (Chapter IV).

*** The control alkaline phosphatase preparation was subjected to the same conditions as the neuraminidase-treated enzyme except that the addition of neuraminidase was omitted.

Heat inactivation

Very similar heat inactivation curves were obtained when the neuraminidase-treated and control alkaline phosphatase preparations were incubated at 56.0°C (Figure 16). Although the neuraminidase-treated preparation appeared to be slightly more sensitive than the control preparation toward heat inactivation, the differences were too small to suggest that sialic acid residues stabilized the structure of the enzyme.

Fig. 16. Heat inactivation of native and neuraminidase-treated alkaline phosphatase from human liver



Samples that contained native or neuraminidase-treated alkaline phosphatase were incubated simultaneously at 56.0°C. Native alkaline phosphatase had been treated in the same way as neuraminidase-treated enzyme except that neuraminidase was not added. Enzyme assays and experimental details are given in the Methods section. —, native alkaline phosphatase; O, alkaline phosphatase treated with neuraminidase.

Inactivation by SDS

Neuraminidase-treated alkaline phosphatase was inactivated by 1% SDS at pH 7.5 (30°C) more rapidly than native alkaline phosphatase (Figure 17). After incubation for 1 h, the enzyme activity remaining in neuraminidase-treated and native alkaline phosphatase was 25% and 52% respectively of the enzyme activity of the preparations incubated in the absence of SDS.

Additional evidence that neuraminidase-treated alkaline phosphatase was inactivated by SDS more rapidly than native alkaline phosphatase is

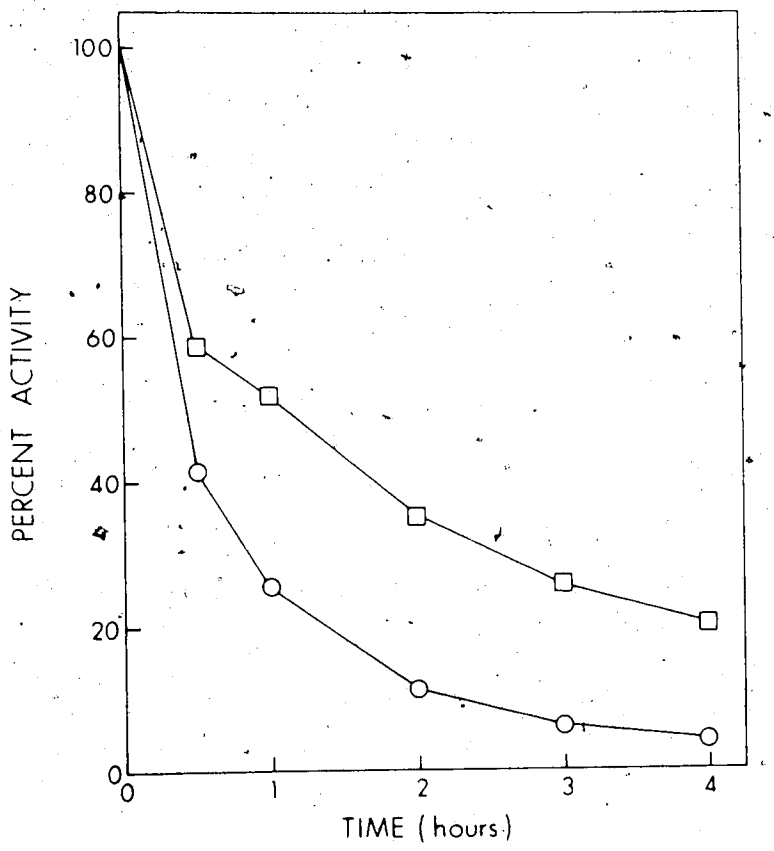


Fig. 17. Inactivation of alkaline phosphatase from human liver by SDS

Samples that contained native or neuraminidase-treated alkaline phosphatase were incubated in 1% SDS at pH 7.5 (30°C). Both enzyme preparations had been treated similarly except that neuraminidase was not added to the native enzyme. Enzyme activity was measured at pH 7.5 and expressed relative to that of samples incubated in the absence of SDS. Enzyme assays and experimental details are given in the Methods section. □, native alkaline phosphatase; O, alkaline phosphatase treated with neuraminidase.

presented in Plate I, gels g and i (Chapter III). Samples were  ated in 1% (w/v) SDS for 2 h at 37°C and subjected to SDS-PAGE. The neuraminidase-treated enzyme was fully inactivated and showed a single protein-stained band, whereas the native enzyme was not fully inactivated and one of the two protein-stained bands was associated with enzyme activity.

DISCUSSION

It is surprising that the properties of alkaline phosphatase treated with neuraminidase were so similar to those of the native enzyme. The removal of the negatively-charged sialic acid residues from the enzyme resulted in a change of the isoelectric point from 4.0 to 6.5 and would be expected to be associated with significant conformational changes of the enzyme. Conformational changes would likely affect the kinetic properties and the stability of the enzyme to treatments such as heating or incubation in solutions containing SDS. However, the neuraminidase-treated enzyme was similar to the native enzyme in kinetic properties and heat stability and differed only in the rate of inactivation by SDS.

Komoda & Sakagishi (1976a) have suggested that sialic acid residues are involved in subunit interaction since they found that native alkaline phosphatase from human liver, but not the neuraminidase-treated enzyme, was inhibited by high concentrations of substrate. However our results do not support this suggestion since the specific activity determined at high substrate concentration (10 mM pNPP) was not altered by treatment with neuraminidase. Although the K_m values for neuraminidase-treated enzyme were similar to the values for the control alkaline phosphatase, they were higher than the K_m values for

untreated alkaline phosphatase. This suggests that the higher K_m values are probably not related to the removal of sialic acid residues but rather reflect changes in the enzyme that occurred during dialysis at pH 5.0. Dialysis may have resulted in the loss of some metal ions bound to the enzyme and consequently in weaker binding of substrates. The increase in the K_m values might have been attributed to the loss of sialic acid residues had the control preparation not been included. Thus it appears that sialic acid residues have little influence on the substrate specificity of the enzyme.

The inhibition of alkaline phosphatase from human liver by L-homoarginine was not influenced by the removal of sialic acid residues, in agreement with the results of Mulivor, Plotkin & Harris, 1978. Since the K_m values were also unchanged, this suggests that the carbohydrate-containing domains of the enzyme may be distant from the active site and from the binding site for L-homoarginine.

Sialic acid removal did not influence the heat stability of alkaline phosphatase from human liver. Similar findings have been reported for the enzyme from rat liver (Righetti & Kaplan, 1971) and human liver (Komoda & Sakagishi, 1978). The latter investigators stated that the heat stability of neuraminidase-treated alkaline phosphatase was lower than the native enzyme in the presence of 0.5% SDS. However the results reported here show that neuraminidase-treated alkaline phosphatase was more rapidly inactivated by SDS than was the native enzyme. Therefore their results may be due to SDS inactivation rather than heat denaturation. Alkaline phosphatases from bacterial and mammalian sources are remarkably stable to SDS denaturation (Mather & Keenan, 1974). The more rapid inactivation by SDS of alkaline phosphatase treated with neuraminidase may be due to increased binding

of SDS to the enzyme. Sialic acid and SDS molecules are negatively charged and thus the native enzyme may repel SDS because of its high negative charge (isoelectric point = 4.0).

The minor bands of alkaline phosphatase activity on focused gels may represent forms of the enzyme that do not contain a full complement of Zn^{2+} and Mg^{2+} ions. Loss of metal ions may have occurred during dialysis at pH 5.0. It is unlikely that these bands were due to incomplete desialylation since the bands were not altered when the treatment was repeated using 5 times more neuraminidase. Some commercial neuraminidase preparations may contain proteolytic activity (Chien et al, 1975). However, if the minor bands were caused by proteolysis, then further treatment would be expected to produce an increase in the amount and number of minor bands. Thus contamination of neuraminidase with proteases cannot account for the presence of the minor bands.

The studies reported here suggest that the sialic acid residues of alkaline phosphatase from human liver do not stabilize the structure of the enzyme appreciably or influence the catalytic activity of the enzyme. It is probable that sialic acid residues are important in the orientation and interactions of the enzyme within the membrane but further studies are required to establish such a role.

CHAPTER SEVEN

COMPARISON OF THE MULTIPLE-MOLECULAR FORMS OF ALKALINE PHOSPHATASE

Present evidence suggests that the protein moieties of human alkaline phosphatases are coded by at least three structural genes. One codes for alkaline phosphatase from placenta, another for alkaline phosphatase from intestine and at least one for the enzyme from other tissues. Alleles have been demonstrated only for alkaline phosphatase from placenta. Studies of hypophosphatasia, an inherited disease, suggest that the enzymes from intestine and placenta are different than the enzymes from other tissues, since the disease is characterized by a deficiency of alkaline phosphatase in liver, bone and kidney but not in intestine or placenta (Mulivor, Mennuti et al, 1978). A variety of biochemical methods have been used to discriminate between the various forms of the enzyme (see Chapter I) and the results are consistent with the hypothesis that there are three main categories of human alkaline phosphatases.

Sussman (1978) has stressed the need to classify alkaline phosphatases on the basis of structure. Structural evidence has been presented to show that the enzymes from liver and placenta are products of different genes (Badger & Sussman, 1976), but structural data for the enzymes from other tissues has not been available. Classification on the basis of structure would be valuable in order to identify the enzyme forms associated with development, various tumors, and cultured cell lines. This information may prove useful in the study of the regulatory mechanisms responsible for the production of oncoalkaline phosphatases.

In an effort to assess the degree of homology of alkaline phosphatase from different sources, the published amino acid compositions were compared. Further studies were carried out on the enzymes purified from five human tissues. The enzymes were characterized by chemical inhibition, heat inactivation, subunit molecular weight, and peptide maps. The results were consistent with the hypothesis that three structural genes code for human alkaline phosphatases.

METHODS

Chemical inhibition

Alkaline phosphatase preparations were diluted to approximately 1000 units/l and equilibrated with 0.8 M MAP and 1.5 mM $MgCl_2$ (pH 10.3). Samples (50 µl) were assayed by the standard procedure described in Chapter II in the absence of added inhibitors, or in the presence of 10.0 mM L-homoarginine or 2.5 mM L-phenylalanine. Inhibition was expressed as the percentage of the enzyme activity in the absence of the inhibitor.

Heat inactivation

Alkaline phosphatase preparations were diluted to approximately 1000 units/l and equilibrated with 0.8 M MAP and 1.5 mM $MgCl_2$ (pH 9.0). An aliquot (100 µl) was added to 1.0 ml of the same buffer at 56.0°C or 22°C and 50 µl was removed at 5 min intervals for measurement of enzyme activity as described in Chapter II. Enzyme activity was expressed as the percentage of the activity of samples incubated at 22°C. There was no significant change in the enzyme activity of the preparations incubated at 22°C.

Maps of radiolabelled peptides

Alkaline phosphatases from human tissues were purified as described in Chapter III, and subjected to SDS-PAGE as described in Chapter II. Each gel slice (approximately 1 mm thick) containing the stained band (1-5 µg protein) was washed extensively with 25% (v/v) 2-propanol and then with 10% (v/v) methanol and dried under a stream of nitrogen. The protein within the gel slice was then radioiodinated with ^{125}I by a modification of the chloramine T method (Greenwood et al, 1963) as described by Elder et al (1977). The following solutions were added to each gel slice: 20 µl of 0.5 M sodium phosphate buffer (pH 7.5), 300 µCi of ^{125}I in 5 µl, and 5 µl of chloramine T (1 mg/ml). The reaction was stopped after 45 min by the addition of 1 ml of sodium bisulfite (1 mg/ml). After 15 min the gel slice was placed in a siliconized culture tube (16 x 100 mm) and washed with several changes of 10% methanol over a period of approximately 24 hours. The slice was dried under a stream of nitrogen and incubated with 0.5 ml of 50 µg/ml trypsin in 50 mM NH_4HCO_3 buffer (pH 8.0) for 16 h at 37°C. The supernatant was then lyophilized. Each tryptic digest was analysed by thin-layer electrophoresis followed by thin-layer chromatography in a second dimension. The lyophilized radioactive residue was dissolved in 20 µl of solution A (acetic acid: formic acid: water, 15:5:80) and approximately 1 x 10^6 cpm (1-5 µl) was spotted onto a 20 cm x 20 cm cellulose-coated plate (Eastman Kodak). The plate was moistened with solution A and subjected to electrophoresis at 1000 V for 1 h at 8°C. The plate was dried and chromatographed using a solvent mixture which contained butanol: pyridine: acetic acid: water (65:50:10:40). Radioactivity was detected by autoradiography using Kodak XR-1 X-ray film with an intensifying screen (Dupont, Cronex par speed). Exposure was for 4 h at -70°C, or as required.

RESULTS

Comparison of the amino acid composition of alkaline phosphatases from mammalian tissues and E. coli

To compare the relatedness of alkaline phosphatase from different sources, the amino acid compositions (Table II, Chapter I) were compared by calculation of the 'difference index' as described by Metzger et al (1968). The difference index for two proteins with the same amino acid composition would be zero, and two proteins with no amino acid in common would have a difference index of 100. Based on the distribution of the difference index for 630 pairs (36 different proteins) Metzger et al (1968) found that approximately 1% of the protein pairs had a difference index less than 10, and approximately 95% of the protein pairs had a difference index between 10 and 40.

Table XII gives the difference index for the amino acid compositions of 78 pairs of alkaline phosphatase derived from 7 different sources. The amino acid compositions of alkaline phosphatases from mammalian and bacterial sources (except human intestine) were strikingly similar as evidenced by difference indices from 3.1 to 13.6. Only one report was available for the amino acid composition of the enzyme from human intestine and higher difference indicies (17.4-23.5) were obtained when its amino acid composition was compared to that of the enzymes from the other sources. In contrast, the amino acid composition of the enzyme from bovine intestine was more similar to the enzymes from all the other sources than was the enzyme from human intestine. Difference indicies for the enzyme from bovine intestine ranged from 7.0 to 13.6. This suggests that alkaline phosphatase from human intestine has an amino acid composition that is considerably different

TABLE XII. Comparison of the amino acid composition of alkaline phosphatases from various sources*

Source	Difference index** a	b	c	d	e	f	g	h	i	j	k	l
a Human liver												
b Human liver	3.3											
c Human liver	5.7	5.0										
d Human placenta	7.2	7.2	7.9									
e Human placenta	5.6	7.1	7.2	3.7								
f Human placenta	6.7	7.9	8.0	3.1	3.9							
g Human placenta	8.3	10.4	9.5	4.9	5.5	4.4						
h Human placenta	6.2	6.2	7.4	3.5	3.6	3.6	4.9					
i Human intestine	18.3	19.7	18.4	18.8	17.5	18.0	17.4	18.2				
j Bovine intestine	8.6	9.4	11.7	7.0	7.6	7.7	8.8	7.8	23.5			
k Bovine kidney	6.7	5.5	8.5	8.9	8.2	9.8	11.1	8.2	22.0	9.4		
l Pig kidney	6.2	6.4	8.0	6.6	5.0	7.2	9.5	6.7	21.9	7.6	5.1	
m *E. coli*	9.7	10.4	9.7	9.2	10.3	8.8	10.8	8.9	20.5	13.6	12.4	12.0

* Amino acid composition data are listed in Table II (see Chapter I) and were obtained from the following sources: a and f, Badger & Sussman (1976); b, Latner & Hodson (1976); c, Gerbitz et al (1977); d, Harkness (1968a); e, Sussman & Gottlieb (1969); g and i, Hirano et al (1977); h, Holmgren et al (1977); j, Fosset et al (1974); k, Cathala et al (1975a); l, Wachsmuth & Hiwada (1974); m, Lazdunski & Lazdunski (1967).

** The difference index for the various pairs was calculated as described by Metzger et al (1968). (Proteins with the same amino acid composition have a difference index of zero. Based on the data from 630 pairs, Metzger et al (1968) found that approximately 1% of the protein pairs had a difference index less than 10 and approximately 95% of the protein pairs had a difference index between 10 and 40.)

than that of the enzyme from all the other sources, or possibly that the enzyme preparation was not pure.

The 3 reports of the amino acid composition of the alkaline phosphatase from human liver were in excellent agreement with difference indicies from 3.3 to 5.7. Similarly the 5 reports for the enzyme from human placenta were in agreement (difference indices from 3.1 to 5.5). The amino acid composition of alkaline phosphatase from bovine or pig kidney were also similar (difference index, 5.1).

Despite the similarity of the amino acid content of alkaline phosphatases from all the various sources (except human intestine), some differences were evident. When the amino acid composition of the enzyme from human liver, human placenta, bovine and pig kidney, and bovine intestine were compared with each other, the difference indices ranged from 5.5 to 11.7. The difference indices for alkaline phosphatase from these sources and the enzyme from E. coli ranged from 8.8 to 13.6. These values indicated that mammalian alkaline phosphatases were more similar to each other than to the bacterial enzyme. Since the differences among the mammalian enzymes were small, further classification on the basis of composition was not attempted. A better comparison might be obtained if the amino acid compositions of the tissue enzymes from a single animal source were available.

Characterization of alkaline phosphatases purified from human tissues

Alkaline phosphatases from liver, kidney, placenta, intestine, and serum from a patient with Paget's disease of bone were purified as described in Chapter III and characterized by chemical inhibition, heat inactivation at 56.0°C, subunit molecular weight determination, and peptide fingerprinting.

Chemical inhibition

L-homoarginine (10.0 mM) inhibited the purified enzymes from liver, kidney and Paget's serum by 75-78%, whereas the enzymes from intestine and placenta were inhibited by less than 10% (Table XIII). In contrast, L-phenylalanine (2.5 mM) inhibited the enzymes from liver, kidney and Paget's serum by less than 10%, and the enzymes from intestine and placenta by approximately 45%. These results are in agreement with the results of Mulivor, Plotkin & Harris (1978) who found that the enzymes from liver, kidney and bone responded almost identically to each of these inhibitors. The sensitivity of the enzymes from placenta and intestine to L-phenylalanine but not L-homoarginine, and the sensitivity of the other enzymes to L-homoarginine but not L-phenylalanine is evidence that the purified enzymes were 'typical' of the enzymes generally found in those tissues. Differential inhibition by L-phenylalanine and L-homoarginine has been widely used to assist in the identification of the tissue alkaline phosphatases present in serum (Fishman, 1974).

Heat inactivation

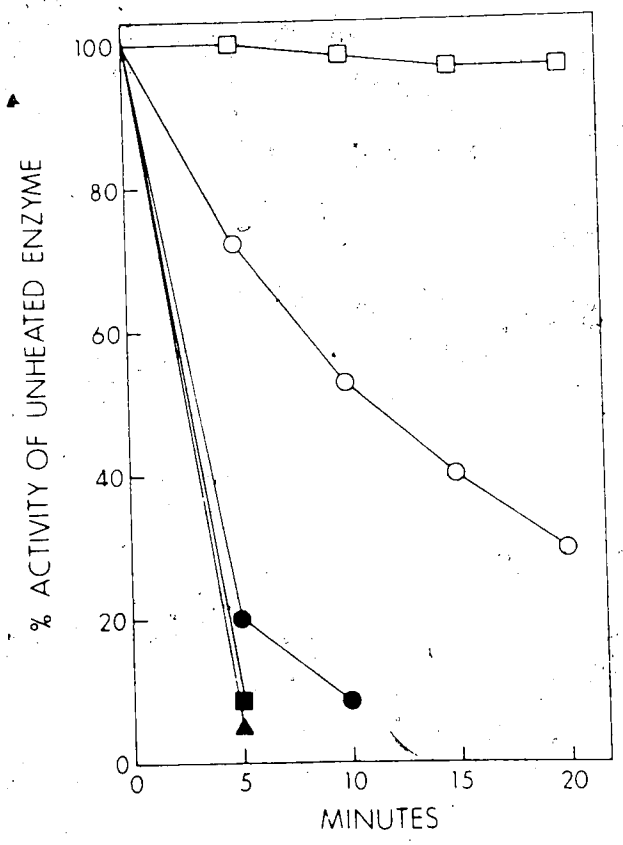
As shown in Figure 18, alkaline phosphatase from different tissues was inactivated at different rates by heating at 56.0°C. After incubation for 10 min at 56.0°C, the enzyme from placenta had retained full activity, the enzyme from intestine had lost almost 50% of its original activity, and the enzymes from liver, kidney and Paget's serum had lost more than 90% of their original activities. Thus, under the conditions used here, alkaline phosphatase from intestine was easily distinguished from the enzymes from liver, kidney and Paget's serum, as well as from the enzyme from placenta. Fishman (1974) found that alkaline phosphatase from intestine and liver were inactivated at

TABLE XIII. Differential inhibition of purified human alkaline phosphatases at pH 10.3*

Enzyme source	Percent inhibition	
	L-Phenylalanine (2.5 mM)	L-Homoarginine (10.0 mM)
Liver	2	78
Kidney	9	75
Paget's serum	4	78
Intestine	47	8
Placenta	43	6

* Enzyme activity was measured as described in the Methods section of this chapter.

Fig. 18. Heat inactivation of purified human alkaline phosphatases



Samples that contained alkaline phosphatase in 0.8 M MAP and 1.5 mM $MgCl_2$ (pH 9.0) were incubated at 56.0°C. Enzyme activity was expressed as a percentage of the activity of samples incubated at 22°C (unheated enzyme). Enzyme assays and experimental details are given in the Methods section of this Chapter. Alkaline phosphatase purified from: □, placenta; O, intestine; ●, liver; ■, kidney and ▲, Paget's serum.

similar rates. Many investigators have shown that the enzyme from bone is more heat-labile than the enzymes from other tissues (reviewed by Brière, 1979). Since alkaline phosphatase purified from Paget's serum was more heat labile than the enzyme from kidney or liver, it appears to represent 'bone-type' alkaline phosphatase.

Subunit molecular weight determination

The enzymes purified from liver, kidney, intestine, placenta and Paget's serum were subjected to SDS-PAGE on 4% (w/v) polyacrylamide gels and subsequent subunit molecular weight determination as described in Chapter II. The electrophoretic mobilities of alkaline phosphatase subunits from Paget's serum, liver or intestine were almost identical (Plate III, b-d), with apparent subunit molecular weights of 92000-95000 (Table XIV). One kidney alkaline phosphatase preparation (gel f)

TABLE XIV. Apparent subunit molecular weight values of human alkaline phosphatases*

Enzyme source	Apparent subunit molecular weight
Liver	92400
Paget's serum	94800
Kidney	95800, 71000, 53500**
Intestine	92400
Placenta	74000

* The apparent subunit molecular weights were determined by SDS-PAGE on 4% (w/v) polyacrylamide gels as described in Chapter II.

** The two lower molecular weight forms may represent partially degraded kidney alkaline phosphatase. See the text for discussion.

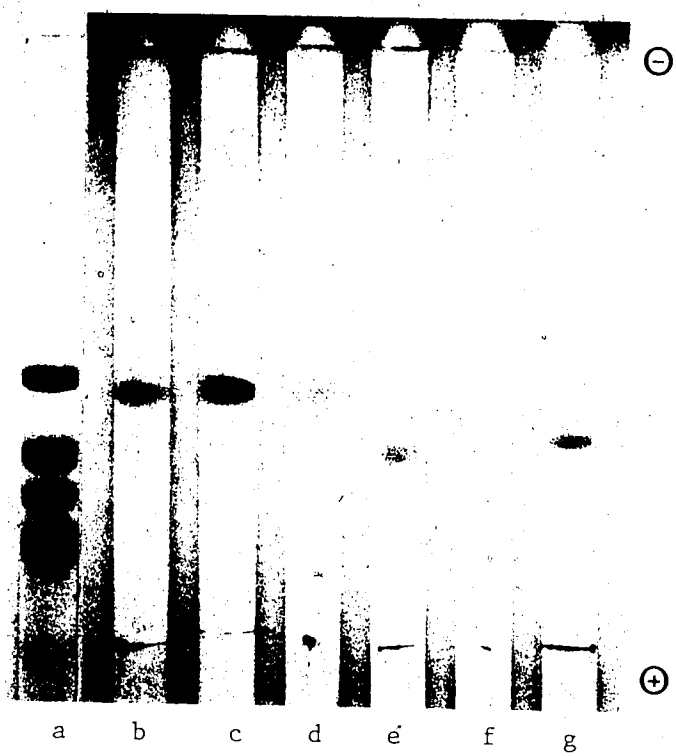


PLATE III. Apparent subunit molecular weight values of human alkaline phosphatases determined by SDS-PAGE

Samples were prepared for SDS-PAGE by incubation at 100°C for 2 min in 1% SDS and 1% 2-mercaptoethanol (10 mM phosphate buffer, pH 7.2). Electrophoresis was carried out in 4% polyacrylamide gels which were stained for protein as described in Chapter II. The subunit molecular weight markers shown in gel a were: phosphorylase a (rabbit muscle), 100000; bovine serum albumin, 68000; glutamate dehydrogenase (bovine liver), 53000; and aldolase (rabbit muscle), 40000. Gels b, c, d and g, protein bands obtained from alkaline phosphatase purified from Paget's serum, liver, intestine and placenta respectively. Gels e and f, protein bands obtained from 2 different preparations of alkaline phosphatase from kidney.

showed three components with molecular weights of 95800, 71000 and 53500. A different kidney alkaline phosphatase preparation (gel e) showed only one band which had a molecular weight of 71000. The results of the peptide mapping studies (see the section below) clearly showed that the 71000 and 53500 molecular weight components had peptides in common with the 95800 molecular weight component, and thus may represent partially degraded kidney alkaline phosphatase. This degradation may have occurred during the purification of the enzyme.

Peptide composition

The patterns of the radioiodinated tryptic peptides from human alkaline phosphatases are shown in Plate IV. The relative distribution of the peptides from liver, kidney (95800 molecular weight component), and Paget's serum were very similar (A-C). There were differences in the relative intensity of some spots, but almost all the peptides were represented in each map. The maps of the radiolabelled peptides of alkaline phosphatase from intestine or placenta (D and E) were markedly different from each other and from those for the other tissues (A-C).

More than 50 peptide maps were obtained which showed that the peptide maps were reproducible. Several maps were produced for the enzyme from each tissue source. The labelled peptides for alkaline phosphatase from four different livers were also very similar and maps from two different liver preparations are shown in Plate V (A and C). In addition, maps produced from the stained protein bands after PAGE were almost identical to those obtained after SDS-PAGE.

The most noticeable difference between maps prepared on different occasions was in the relative intensity of spots. Peptides were released from the gel slice, after tryptic hydrolysis of the protein,

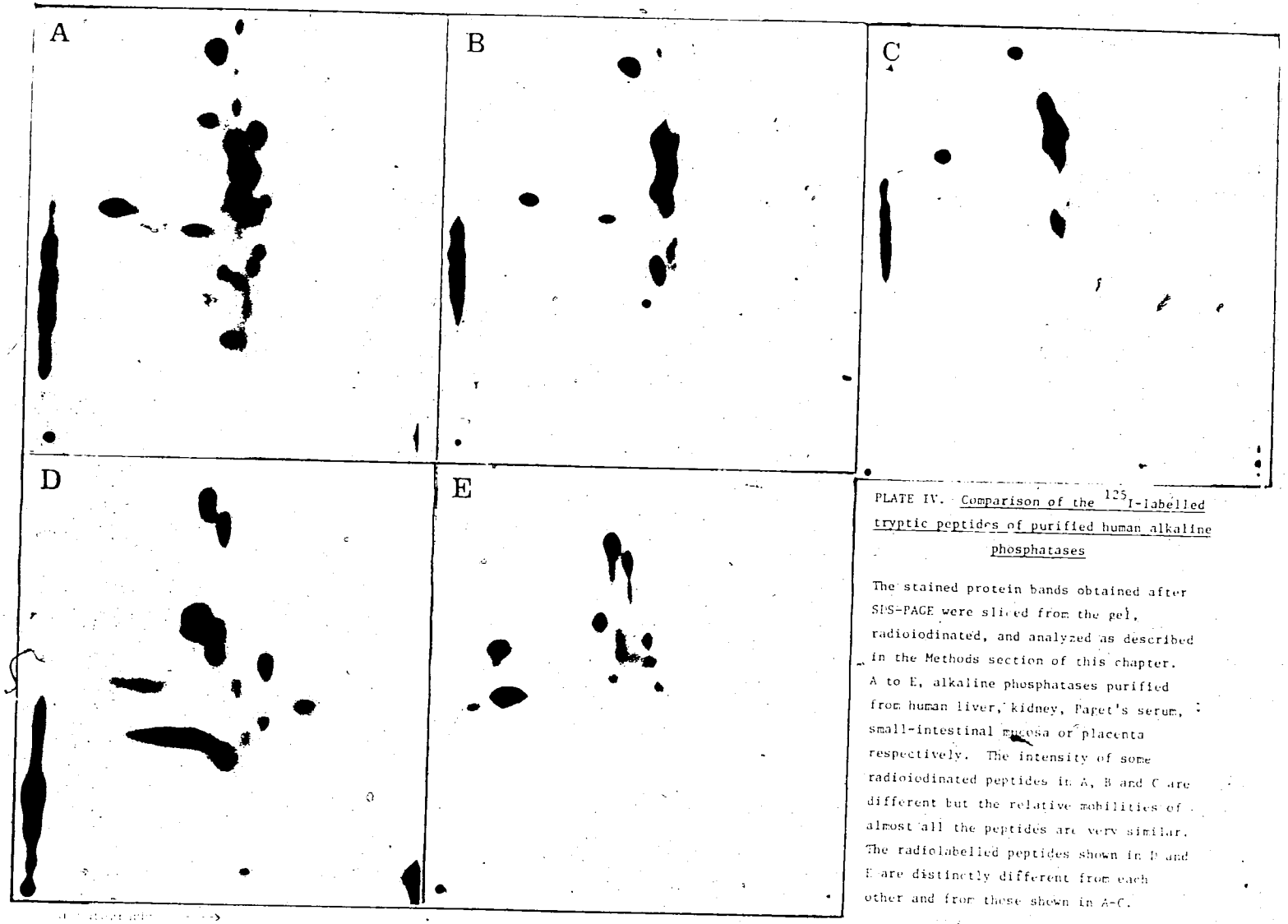


PLATE IV. Comparison of the ^{125}I-labelled tryptic peptides of purified human alkaline phosphatases

The stained protein bands obtained after SDS-PAGE were sliced from the gel, radioiodinated, and analyzed as described in the Methods section of this chapter. A to E, alkaline phosphatases purified from human liver, kidney, Paget's serum, small-intestinal mucosa or placenta respectively. The intensity of some radioiodinated peptides in A, B and C are different but the relative mobilities of almost all the peptides are very similar. The radiolabelled peptides shown in D and E are distinctly different from each other and from those shown in A-C.

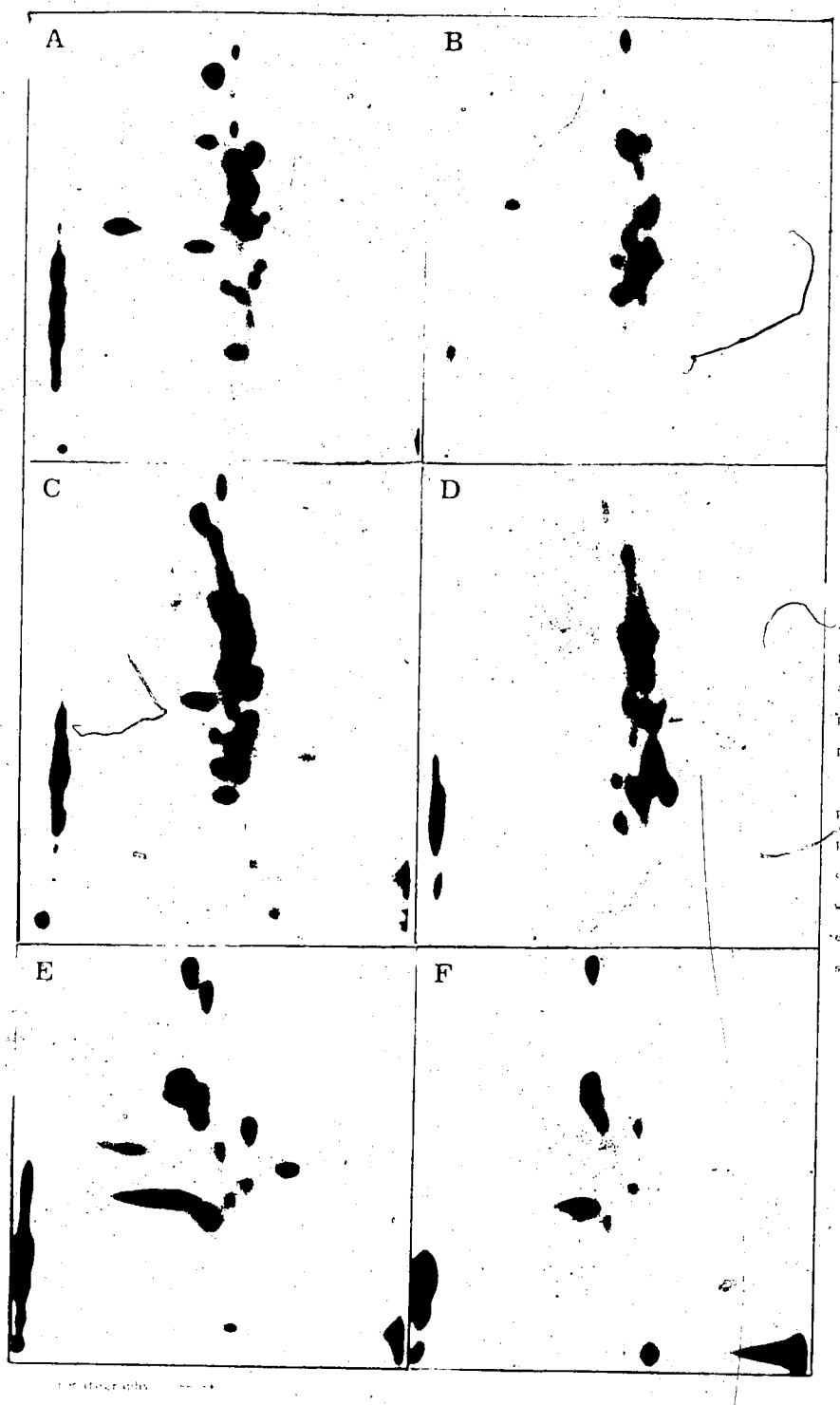


PLATE V. Influence of various treatments on the ^{125}I-labelled peptides of purified human alkaline phosphatases.

The ^{125}I-labelled peptides were prepared and analyzed as described in the Methods section of this chapter.

A and B, peptides released upon initial trypsin treatment (A) or further trypsin treatment (B) of a gel slice containing alkaline phosphatase from liver.

C and D, labelled peptides from a different liver alkaline phosphatase preparation before (C) and after (D) treatment of the peptides with neuraminidase plus endoglycosidase.

E and F, labelled peptides of alkaline phosphatase from intestine obtained from gel slices which contained active enzyme (E) or denatured subunits of the enzyme (F).

at different rates. As shown in Plate V (A and B), markedly different relative intensities were observed for the peptides released upon the initial trypsin treatment (60-80% of the recovered peptides) and the peptides released upon further trypsin treatment of a gel slice containing alkaline phosphatase. In addition, each peptide may be represented by more than one spot due to variation in the extent of radiolabelling with ^{125}I. It is likely that tyrosine residues are selectively iodinated, but diiodotyrosine derivatives as well as iodinated derivatives of phenylalanine and histidine may also be formed (Krohn et al, 1977). Thus the intensity of a particular spot may depend on the conditions for iodination.

Differences in carbohydrate content did not appear to have much influence on the peptide maps. Although alkaline phosphatase from various sources may differ in carbohydrate composition, the similarity of the peptide maps of the enzyme from liver, kidney and Paget's serum suggested that such differences, if present, were not reflected in the peptide maps. The map from a desialylated alkaline phosphatase preparation from liver was almost identical to that from the native enzyme. As shown in Plate V (C and D), treatment of the tryptic peptides with neuraminidase and endoglycosidase D had little influence on the distribution of the peptides. Elder et al (1977) have suggested that glycopeptides do not migrate in the apolar solvent system used here for thin-layer chromatography.

Three protein-stained bands were obtained after SDS-PAGE of one kidney alkaline phosphatase preparation, while only band was obtained from a different preparation (Plate III). Since the enzyme preparations had high specific activities, peptide maps were produced from each of these bands. The results clearly showed that the peptides present in

the 71000 and 53500 molecular weight components were also present in the 95800 molecular weight component. Thus the lower molecular weight components appeared to represent partially degraded forms of kidney alkaline phosphatase.

Peptide maps were also used to demonstrate that the protein bands from the SDS-PAGE gels were in fact alkaline phosphatase. As shown in Plate V (E and F), the peptide maps from intestinal alkaline phosphatase after SDS-PAGE under denaturing and non-denaturing conditions (see Chapter II) were very similar. The disappearance of the slow-moving enzymically-active band was correlated with the appearance of the faster migrating inactive band (see Plate I, Chapter III), which corresponded to the subunit of the enzyme. Both of these maps were nearly identical to the map of the enzymically-active protein obtained after PAGE. Thus there can be little doubt that the peptides were derived from alkaline phosphatase.

For comparative purposes, peptide maps were also produced for alkaline phosphatase from calf intestine and E. coli. Each of these maps was different from all the others and it was not possible to determine if any peptides were common to all the preparations.

DISCUSSION

Although the difference indicies used to compare the amino acid compositions of proteins can not absolutely demonstrate structural relationships, the low difference indices obtained when 78 pairs of alkaline phosphatase proteins were compared strongly suggest that significant structural similarities exist among the enzymes. However, the enzymes from various tissues have characteristic properties which distinguish them from each other (see Chapter I). As shown in the Results section of this chapter, human alkaline phosphatases purified from liver, kidney, intestine and placenta were found to have the heat inactivation and chemical inhibition properties that others have found to be characteristic of the enzymes from these tissues. Although the tissue source of the elevated serum alkaline phosphatase found in patients with Paget's disease of bone has not been conclusively identified, the enzyme is generally considered to be derived from increased osteoblast activity (Krane, 1977). Alkaline phosphatase purified from Paget's serum was more heat labile than the other enzymes, was strongly inhibited by L-homoarginine, and upon PAGE migrated in a diffuse band with mobility slightly less than that of the liver enzyme. These properties are characteristic of the bone enzyme (Fishman, 1974) and thus alkaline phosphatase from Paget's serum probably represents the enzyme found in bone.

Although the apparent subunit molecular weight values determined by SDS-PAGE may not be accurate since the enzymes are glycoproteins and could migrate anomalously on polyacrylamide gels (Segrest & Jackson, 1972), it is noteworthy that alkaline phosphatases from liver, kidney, intestine and Paget's serum all had apparent subunit molecular weight

values of 92000-96000. The enzyme from placenta had an apparent subunit molecular weight of 74000. These values are somewhat higher than the subunit molecular weight values reported by others (see Table I, Chapter I) for human alkaline phosphatases from liver (69000-80000), intestine (86000), and placenta (64000-65000). Corresponding values for the enzyme from human kidney or bone have not been reported. The reason for the higher values for the subunit molecular weight values reported here is unknown but could be related to the acrylamide concentrations used in the various studies.

The peptide maps showed that alkaline phosphatase purified from liver, kidney and Paget's serum had very similar tryptic peptide compositions. The maps for the enzyme from intestine and placenta were different from each other and from the maps for the enzymes from the other tissues. Allelic proteins at a single locus generally have almost identical amino acid sequences (Harris, 1975) and therefore would have very similar tryptic peptide maps. However products of different structural genes would be expected to show significant differences in amino acid sequences and therefore would have different tryptic peptide compositions. Thus the evidence from peptide maps strongly suggests that three structural genes code for human alkaline phosphatases from these sources. One of these codes for alkaline phosphatase from placenta, another for the enzyme from intestine, and a third for the enzymes from liver, kidney and bone. The results confirm the finding of Badger & Sussman (1976) that alkaline phosphatase from liver and placenta represent different structural gene products.

Since alkaline phosphatase from liver, kidney and Paget's serum appears to represent the same gene product, the differences observed among these enzymes must be due to post-translational modification of

the enzymes. Uy and Wold (1977) have presented evidence for the occurrence in proteins of at least 120 covalently modified amino acids. Thus processes such as deamidation, methylation, acetylation, proteolysis, and glycosylation may represent tissue-specific modifications of the enzymes.

It is not known whether the alkaline phosphatases from other human sources such as brain, thymus, pancreas, milk and bile represent products of additional structural genes. Hamilton et al (1979) have recently presented evidence based on antigenic, functional and structural properties that alkaline phosphatase from human milk is closely related to the enzyme from human liver and suggested that both enzymes may be coded by the same gene. The approach used here to classify alkaline phosphatase on a structural basis is suitable as a general procedure for the identification of the enzyme. Maps of the radioiodinated peptides can readily be obtained in only a few days using less than 2 µg of alkaline phosphatase protein. In addition, it is not necessary to purify the protein to homogeneity, provided that it can be separated sufficiently from other proteins by SDS-PAGE (or other techniques) to allow excision of the stained protein band from the gel. Structural classification of alkaline phosphatases will avoid the potential inaccuracies present when classifying gene products on the basis of heat stability, differential chemical inhibition, immunologic specificity, electrophoretic mobility or other functional properties.

CHAPTER EIGHT

GENERAL DISCUSSION

The three basic aims of this study, outlined in Chapter I, have been largely accomplished. A general procedure for the purification of homogeneous alkaline phosphatase from a number of human tissues has been developed, some catalytic properties of alkaline phosphatase from human liver have been studied, and a structural comparison of some of the multiple-molecular enzyme forms has been carried out.

Human alkaline phosphatases have been poorly characterized because of the small amounts of the purified enzymes that have been available. The protocol described here for the purification to apparent homogeneity of alkaline phosphatase from kidney, intestine, placenta, and the serum of a patient with Paget's disease of bone, appears to be suitable, with minor modification, for use as a general procedure for the purification of the enzyme from human tissues.

The biochemical function(s)of alkaline phosphatase remain unknown. Data has been presented here that shows that the enzyme from human liver may be responsible for the hydrolysis of biologically-important phosphomonoesters, and may be regulated by phosphate and vanadate. Magnesium and calcium ions may also be involved in regulation by formation of metal-substrate complexes which are not hydrolysed by the enzyme. Further studies are required before the physiological role(s) of alkaline phosphatase can be assigned. The transphosphorylation reactions should be carefully evaluated, as well as the possible involvement of the enzyme in the phosphorylation and dephosphorylation of proteins. Studies should be carried out, perhaps by electron

microscopy of immunolabelled enzyme, to determine the exact orientation of the enzyme in the membrane. Does the enzyme function independently or, is it a component of a specialized membrane complex? Alkaline phosphatase is primarily associated with the plasma membranes of tissues actively involved in transport processes. Does inhibition of alkaline phosphatase have any effect on these transport processes? Further comparative studies of the purified enzymes and membrane preparations of the enzymes may be useful in this regard. It is hoped that the in vitro study, reported herein, of some catalytic properties of purified alkaline phosphatase from human liver will ultimately prove useful in the elucidation of the physiological function of human alkaline phosphatases.

Alkaline phosphatases from all sources studied were remarkably stable in SDS solutions. Although removal of sialic acid residues increased the rate of inactivation by SDS of alkaline phosphatase from human liver, the stability in SDS was not dependent solely on the presence of carbohydrate. The enzyme from E. coli, which does not contain carbohydrate, was also stable in SDS solutions. This stability could be used to tentatively identify the alkaline phosphatase protein in impure preparations. In a crude placental preparation, the pattern of the protein bands was the same, except for the alkaline phosphatase band, when samples were prepared for SDS-PAGE under denaturing or non-denaturing conditions as described in Chapter II.

The removal of sialic acid residues from liver alkaline phosphatase by neuraminidase-treatment caused the isoelectric point to change from 4.0 to 6.5, but had no influence on the specific activity of the enzyme, the K_m values for six substrates, or the inhibition by L-homoarginine.

Thus the role of the carbohydrate residues remains undefined. It is likely that the carbohydrate residues are important in determining the orientation of the enzyme within the membrane and also in its interaction with other membrane components. The carbohydrate moieties may also be involved in determining the biological half-life of the enzyme.

Evidence from heat inactivation, chemical inhibition, subunit molecular weight determination and peptide maps indicated that three different structural genes code for the protein moiety of alkaline phosphatase from human liver, kidney, intestine, placenta and Paget's serum. It has not been known previously whether some of the enzyme forms from various tissues were caused by differences in amino acid sequences or tissue-specific post-translational modifications. The evidence presented here shows that alkaline phosphatase from liver, kidney and Paget's serum have identical or nearly identical amino acid sequences and therefore represent the product of a single structural gene. The amino acid sequence of alkaline phosphatase from placenta and intestine have different amino acid sequences and represent the product of two additional structural genes. Further study is required to determine if the enzyme forms from other tissues represent the products of additional structural genes. The significance of the existence of the multiple forms of alkaline phosphatase are not evident at present since the function of the enzyme remains unknown.

The purification protocol and the procedure for mapping the tryptic peptides from alkaline phosphatase radioiodinated within polyacrylamide gels appear to be generally useful for the characterization and structural identification of alkaline phosphatase. Thus it will be possible to classify, on a structural basis, all human alkaline

phosphatases including fetal forms (intestinal and placental origins) and those found in tumor tissues. A definitive classification of the tumor-associated alkaline phosphatases, that appear to represent placental and non-placental enzyme forms is relevant to the study of gene expression in cancer. Since most of the genes expressed in cancer cells are also expressed in normal cells, then cancer can be considered to be a disease due to disordered controls of gene expression (Fishman, 1974). Therefore, the structural classification of oncoalkaline phosphatases will be valuable in characterization of gene expression in neoplasia.

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APPENDIX

PUBLICATIONS ARISING FROM THIS THESIS

Trépanier, J. M., Seargeant, L. E. & Stinson, R. A. (1976) Affinity Purification and some Molecular Properties of Human Liver Alkaline Phosphatase. Biochem. J. 155, 653-660

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