ISOENZYME STUDIES ON HUMAN ALKALINE PHOSPHATASES

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SUMMARY.

About thirty years ago it was thought that the alkaline phosphatases obtained from different human tissues were closely similar or even identical. However, as knowledge of the properties of these enzymes has increased, differences between phosphatases from several human organs have been revealed in their reaction to specific antibodies, and in enzymatic properties such as their response to various inhibitors. These observations were for the most part based on studies using impure enzyme preparations, and nothing was known about the molecular basis for these distinctions. The object of the present study was to purify alkaline phosphatase from various human tissues and to investigate the properties of the purified enzymes to find out whether the differences previously reported were genuine properties of the alkaline phosphatase molecules or due to the presence of impurities. In addition, it was hoped to throw light upon the molecular origin of any differences between the isoenzymes.

Human liver, intestinal and bone alkaline phosphatases were purified about 2000, 1000 and 700-fold respectively, by means of a sequence of procedures including gel-filtration and anion-exchange chromatography. The properties of the purified enzymes were then compared in respect of their physical properties, catalytic properties and molecular structure.

The physical properties studied included the molecular weight of the purified enzymes, their reaction to neuraminidase,

their mobility on starch-gel electrophoresis, and their rates of inactivation by various denaturing agents. On the basis of their molecular weight and their reaction to neuraminidase the liver and bone enzymes were found to be similar, but were markedly different from the intestinal enzyme. The three isoenzymes differed in their mobility on starch gel electrophoresis and in their resistance to inactivation.

The substrate specificity of the three purified alkaline phosphatases was investigated. Although alkaline phosphatases were originally thought to be solely orthophosphatases, all three enzymes were found to hydrolyse inorganic pyrophosphate and several organic pyrophosphates. This activity was shown to be a property of the alkaline phosphatase molecule; it was not due to the presence of a mixture of enzymes of differing specificities. The relative rates of hydrolysis of a range of substrates varied with the tissue source of the enzymes. The effect of metal ions on the enzymatic activity and the Michaelis constants were specific for the tissue of origin. Measurement of the catalytic centre activity showed that the liver and intestinal enzymes differed in this respect.

The molecular structure was investigated by labelling the enzyme molecule with ^{32}P-phosphate followed by hydrolysis of the protein so that the patterns of labelled peptides could be compared. However, this did not yield any information about the structure of the alkaline phosphatase molecule and possible reasons for the failure of this experiment are discussed.

In summary, the alkaline phosphatases from human liver, small intestine and bone were found to differ in many of their properties. It is concluded that there is a fundamental difference between the enzymes, but the molecular basis of their differences has not yet been elucidated.

ACKNOWLEDGEMENT.

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CHAPTER 1 INTRODUCTION

The history of enzymology can be traced back to the discovery in 1833 by Payen and Persoz of a heat labile substance which converted starch into sugar and which they named "diastase". The name "enzyme" was not coined until 1878 when Kuhne proposed that the agents which caused fermentation and digestion should be denoted "enzymes". From this beginning over 100 years ago the number of known enzymes has grown to about 700 (Dixon and Webb, 1964).

The first description of an enzyme which caused the liberation of inorganic phosphate from phosphate esters was by Suzuki, Yoshima and Takahishi in 1907, who found an enzyme in rice and wheat bran which split phytin. This was quickly followed by the discovery of a similar enzyme in calf liver by McCollum and Hart (1908) and of an enzyme in cat kidney, intestine, lung, liver and spleen which hydrolysed glycerophosphate (Grosser and Husler, 1912). Since then the number of enzymes reported as hydrolysing phosphomonoesters has increased to 19 (Dixon and Webb, 1964). Some of these are specific for one substrate (e.g. nucleotide phosphohydrolase, E.C.3.1.3.5.) while others have a broad specificity (e.g. acid and alkaline phosphatases, E.C.3.1.3.2. and 3.1.3.1.). The work described in this thesis is concerned with the non-specific phosphoric monoester hydrolase which has a pH optimum on the alkaline side of neutrality and is commonly known as alkaline phosphatase (E.C.3.1.3.1.).

The first description of a phosphatase with a pH optimum greater than 7 was made by Robison (1923) who found that extracts

of rabbit and rat bone could hydrolyse β-glycerophosphate and hexose monophosphate esters. This enzyme, he later found, was concerned with bone ossification (Robison and Soames, 1924). It was also identified in kidney, liver, spleen (Robison, 1923), milk (Graham and Kay, 1933) and intestinal mucosa (Levene and Dillon, 1930).

Kay (1932) suggested that the phosphatases from bone, intestine, kidney and plasma were identical since they had many properties in common. Bodansky (1937), however, found that phosphatase extracted from the intestine of various species differed from the phosphatase from bone and kidney in that the latter were inhibited by bile acids added to the assay mixture while the former was insensitive to them. Since then differences with respect to inhibitors (e.g. L-phenylalanine; Fishman, Green and Inglis, 1962), antigenic properties (Schlamowitz and Bodansky, 1959) and sensitivity to various denaturing agents (e.g. heat; Birkett, Conyers, Neale, Posen and Brudenell-Woods, 1965) have been found between the phosphatases from different tissue sources and in different species.

Not only are there variations between catalytically analogous enzymes from different species and different tissues, but, more recently, heterogeneity with respect to a given enzyme activity within one organ has also been reported, first of all for lactate dehydrogenase (E.C.1.1.1.27.) (e.g. by Wieland and Pfleiderer, 1957; Vesell and Bearn, 1958; Sayre and Hill, 1957) and later for alkaline phosphatase (Moss and

King, 1962).

In 1959 Markert and Møller coined the name "isozyme" to define "the different molecular forms in which proteins may exist with the same enzymatic specificity"; the name "isoenzyme" is now preferred (Webb, 1964). Markert and Møller (1959) considered that, because of their broad specificity, alkaline phosphatases could not be considered as isoenzymes, as within each tissue there might be a family of closely related enzymes, rather than variants of a single enzyme. The term "isoenzyme" has subsequently been used in two senses. Firstly, to refer to different enzyme proteins with the same enzymatic specificity from the same species and the same tissue (e.g. Wieland and Pfleiderer, 1962), and secondly to refer to enzymes having the same catalytic activity prepared from different tissues of the one species (e.g. Moss, 1964). The term is used in the latter sense in this work. Despite their broad specificity the alkaline phosphatases are not thought to be a family of associated enzymes in each tissue (Landau and Schlamowitz, 1961).

1.1. SOURCES AND PURIFICATION OF ALKALINE PHOSPHATASE.

Alkaline phosphatase has been identified in most human tissues. Morton (1954) found that the alkaline phosphatases of calf intestinal mucosa and cow mammary gland were associated with the microsomal particles and were firmly bound to lipoprotein. Several methods for the isolation of alkaline phosphatase have been suggested. Morton's (1950) extraction with butanol has largely superseded the older methods of controlled autolysis

(Albers and Albers, 1935) and tryptic digestion (Schmidt and Thannhauser, 1943). The butanol method gave a greater yield of alkaline phosephatase than autolysis and, although alkaline phosphatase was thought to be resistant to trypsin, there is the possibility of modification of the enzyme protein in the latter procedure.

A few workers have used highly purified preparations to examine the properties of the enzyme (e.g. Ahmed and King, 1960b; Binkley, 1961a) while others have worked with blood serum or tissue homogenates (e.g. Robinson and Pierce, 1964). Contamination of the enzyme extracts with similar enzymes and non-specific proteins and metal ions, and the consequent vitiation of the results, makes it advisable in all enzyme studies to use only preparations of the highest possible purity.

Table 1.1 shows the highest specific activities so far obtained for alkaline phosphatases from a variety of sources. No preparations from human tissues of any great purity have so far been reported, except that of Ahmed and King (1960a).

Table 1.1. Specific Activities of some Purified Alkaline Phosphatases.

units: Roche units/mg. nitrogen (Roche and Bouchillaux, 1950).

Reference	Specific Activity	Tissue Source of Enzyme
Morton, 1954	83,500	Calf intestine
Ahmed and King, 1960a	99,750	Human placenta
Mathies, 1958	103,000*	Swine kidney
Alvarez and Lora-Tamayo, 1958	166,000	Swine kidney
Binkley, 1961a	300,000	Swine kidney
Portmann, 1957	400,000	Calf intestine
Engstrom, 1961a	400,000	Calf intestine

* from the calculation by Binkley (1961a) to compare with his preparation.

1.2. OUTLINE OF THE AIM OF THE INVESTIGATION.

The aim of this work was to investigate whether or not the alkaline phosphatases isolated from different human tissues were identical. The general characteristics of the alkaline phosphatase reaction (e.g. substrate specificity, kinetics, inhibition) are discussed first and then the properties of the isoenzymes are compared under the three headings: physical properties, enzymic properties and molecular structure.

1.3. GENERAL CHARACTERISTICS OF THE ALKALINE PHOSPHATASE REACTION.

The general reaction of alkaline phosphatase is the hydrolysis of an orthophosphoric monoester with the production of inorganic phosphate and the corresponding alcohol. By employing water labelled with ^{18}O, it has been shown that the point of attack by the water on the substrate is the oxygen - phosphorus bond (Cohn, 1949; Stein and Koshland, 1952).

$$R - O - \overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}} - OH + H_2{}^{18}O \longrightarrow R - O - H + H^{18}O - \overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}} - O^-$$

1.3.1. Substrate Specificity.

The broad substrate specificity of alkaline phosphatase has long been recognised (Kay, 1932) and some doubt about whether alkaline phosphatase represents a family of closely related enzymes or one enzyme of broad specificity has been expressed (Markert and Møller, 1959). Morton (1955) carried out an extensive study of the specificity of calf intestinal phosphatase towards the organic moiety of the substrate. All monophosphate esters and diphosphate esters (e.g. fructose 1:6 diphosphate) were hydrolysed. This has been confirmed by Folley and Kay (1936) with various human phosphatases; by Schmidt and Thannhauser (1943) with calf intestinal phosphatase; by Garen and Levinthal (1960) with alkaline phosphatase from E.coli; and by Trubowitz, Feldman, Morgenstern and Hunt (1961) with human leucocyte phosphatase.

Activity towards pyrophosphates (e.g. inorganic pyrophosphate,

ADP and ATP) has been reported by some workers while others have shown that their alkaline phosphatase preparations were free from pyrophosphatase activity. Kay (1932) found activity towards pyrophosphate in his preparations of alkaline phosphatase and a highly purified preparation of calf intestinal phosphatase of Schmidt and Thannhauser (1943) also had pyrophosphatase activity. However Morton (1955), using purified calf intestinal phosphatase, found no activity towards pyrophosphates, either organic or inorganic. This was confirmed by Portmann (1957), also using calf intestinal phosphatase. E. coli alkaline phosphatase was similarly found to be devoid of activity towards pyrophosphates (Garen and Levinthal, 1960; Torriani, 1960).

However, ability to hydrolyse pyrophosphate and nucleoside phosphates was found in alkaline phosphatase obtained from E. coli (Heppel, Harkness and Hilmoe, 1962) and from human leucocytes (Trubowitz et al, 1961). In each case the authors presented evidence that the pyrophosphatase activity was a property of the alkaline phosphatase protein, and was not due to contamination of the preparations.

Alkaline phosphatase has no action on phosphate diesters (Morton, 1955; Heppel et al, 1962).

1.3.2. Kinetics.

Enzymes can be characterised not only by their substrate specificity but also by study of the kinetics of the reaction between enzyme and substrate. Henri (1902) suggested that the

enzyme first combined with the substrate (S) to form a complex (ES) which then broke down to free enzyme (E) and products.

$$\text{i.e.} \qquad E + S \rightleftharpoons ES$$

$$ES \longrightarrow E + \text{products}$$

From these equations, Michaelis and Menten (1913) derived an expression for the equilibrium constant.

If e is the total enzyme concentration

s the free substrate (the amount combined with the enzyme is negligible so the substrate concentration is much greater than the enzyme concentration)

and p the concentration of ES

then the equilibrium constant, $K_s = \frac{(e-p)s}{p}$

$$\text{i.e.} \qquad p = \frac{es}{K_s + s}$$

If k is the velocity constant of the reaction ES $\longrightarrow$ E + products and v is the velocity of the overall reaction then

$$v = kp$$

and

$$v = \frac{ek}{K_s/s + 1}$$

At maximum velocity (V_{max}) then p = e and

$$v = \frac{V_{max}}{K_s/s + 1}$$

Michaelis and Menten (1913) assumed in this derivation that the equilibrium governed by K_s is attained very rapidly and is maintained. The effect on the equilibrium of the reaction for the breakdown of ES to enzyme and products was ignored.

Briggs and Haldane (1925) derived a similar equation based on the postulate that at any moment rates of formation and breakdown of ES were equal.

$$\text{i.e. } E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + \text{products}$$

$$pk_{+2} + pk_{-1} = s(e - p)k_{+1}$$

(The symbols have the same connotation as before)

This reduces to $v = \dfrac{V_{max}}{K_m/s + 1}$

where $K_m = \dfrac{k_{-1} + k_{+2}}{k_{+1}}$

K_m, the Michaelis constant, is the value of s which is experimentally found to give half the maximum velocity. When k_{+2} is considerably less then k_{+1} then $K_m = K_s$.

K_m and V_{max} may be found by measuring the velocity of the reaction at various substrate concentrations. Several methods of plotting the results have been suggested, the best known of which is probably that of Lineweaver and Burk (1934), when 1/v is plotted against 1/s. The intercept on the 1/s-axis is then $-1/K_m$, and the intercept on the 1/v-axis is $1/V_{max}$. Another method is to plot s/v against s when the x-axis intercept is

- K_m (Hanes, 1932), or to plot v against v/s when the gradient is $-K_m$ (Hofstee, 1959).

Folley and Kay (1935) and Ross, Ely and Archer (1951) found that the pH optimum for alkaline phosphatase activity varied with the substrate concentration. If velocity measurements were made at a fixed pH and varying substrate concentration and K_m was calculated, then K_m was found to vary with the enzyme concentration used (Motzok, 1959). By making velocity measurements at the optimum for each substrate concentration this was avoided (Motzok, 1959; Moss, Campbell, Anagnostou - Kakaras and King, 1961a).

1.3.3. Effect of Temperature and pH.

Measurement of the velocity of a reaction at different temperatures shows a temperature optimum. This is due to a combination of two effects; enzyme instability and enzyme activity. The rate of denaturation of an enzyme increases with increase in temperature. The enzymatic activity, as with all chemical reactions, also increases with increasing temperature and in consequence, the velocity of the reaction at any temperature is a balance between the rate of denaturation and the kinetic effect of temperature on reaction rates. Many determinations of enzyme activity are carried out at 37°C since this is the normal human body temperature and one at which enzymes are comparatively stable. The effect of heat on the stability of the alkaline phosphatases is discussed later.

Generally enzymes are only active over a restricted range

of pH values and usually an optimum pH can be found. The optimum may be due to an effect of pH on the stability of the enzyme, or an effect on V_{max}, or an effect on K_m, or may reflect a combination of these effects. Enzymes being proteins contain many ionisable groups and so the enzyme, the substrate, and the enzyme-substrate complex may exist in more than one ionic form depending on the pH. Probably only one of the forms of the substrate, the enzyme and the enzyme-substrate complex will have the correct ionic configuration to permit it to take part in the reaction. The effect of pH on V_{max} is produced solely by variations in the amount of ES in the correct form. The effect on K_m is an effect on the affinity of the enzyme for the substrate and the ionisable groups of the enzyme involved in the catalytic action. At certain pH values the groups will be in an ionic form less favourable for binding the substrate: the affinity is, therefore, reduced and the K_m increased. The effect of pH on stability is considered under physical properties.

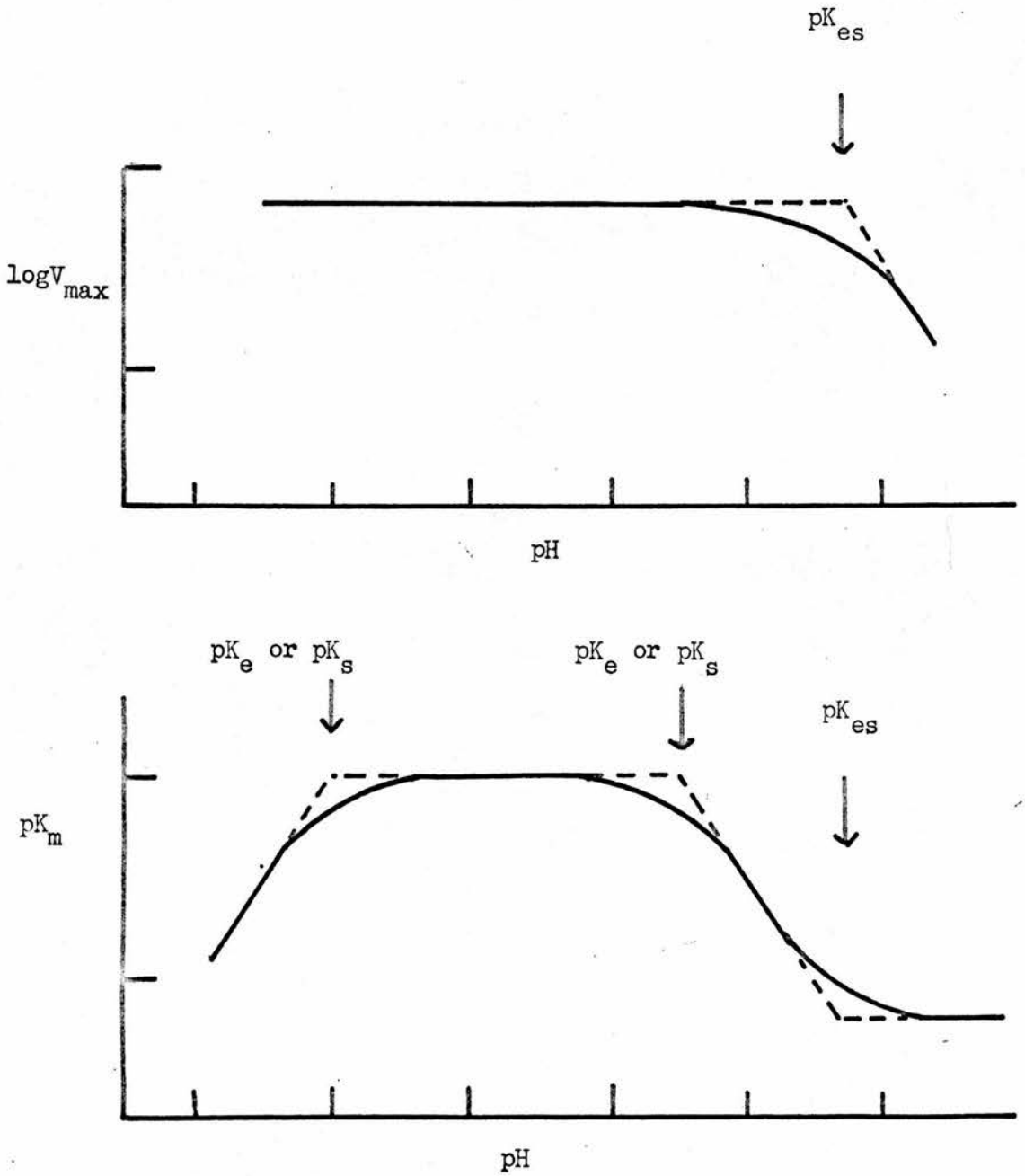
Dixon (1953a) derived a relationship between log V_{max} and pH, and between pK_m and pH, whereby the ionisation constants (K) of ES, E and S could be derived. If log V_{max} is plotted against pH, the graph which is obtained consists of linear sections of gradient 0, 1, or 2, with short curved portions joining them (Fig. 1.1.). If lines of the appropriate slope are drawn through the straight line parts then the points of intersection give the pK values for the ionisation of ES. If a gradient of 2 is obtained then two groups of ES are ionising simultaneously. Plots of pK_m against pH show a similar picture, but in this case

the intersections of the straight portions are pK values for the ionisation of E, S and ES (Fig. 1.1.). Since the ionisation constants for the substrate can be readily obtained by other means, pK for the enzyme can be defined. The pK_e values are the ionisation constants of the groups of the enzyme involved in the attachment of the substrate and, hence, in the active site of the enzyme. By measuring pK_e, it would, therefore, be possible to identify the amino acid groups involved in the active centre, were it not for the fact that a group may not have the same pK value when it is combined in a protein molecule and surrounded by other ionising groups as it does as a free amino acid. pK_e values can only, therefore, give some indication of the amino acid group involved in the reaction. Plots of pH against pK_m for chick alkaline phosphatase (Motzok, 1959) and for calf intestinal phosphatase (Morton, 1957) have been published, but no similar work has been reported for the human enzymes.

1.3.4. Inhibition of the Hydrolysis Reaction.

i) Kinetics: Enzyme inhibition may be divided into two main types, reversible and irreversible, depending on whether or not, on dialysis of the inhibitor and the enzyme, the inhibition is removed. In the case of reversible inhibition, there is set up between the enzyme and the inhibitor an equilibrium which is characterised by a constant, K_i, known as the inhibitor constant. K_i is the reciprocal of the affinity of the enzyme for the inhibitor. The degree of inhibition with a reversible inhibitor

Fig.1.1 Diagram of plots of $\log V_{max}$ and pK_m against pH.



is dependent on the concentration of the inhibitor and is, once equilibrium has been reached, independent of time.

On the other hand, with irreversible inhibition there is no equilibrium and the inhibition is progressive with time, reaching 100% inhibition if the inhibitor concentration is greater than the enzyme concentration. This inhibition is characterised by a velocity constant. An example of irreversible inhibition is the action of cyanide on xanthine oxidase, while the inhibition of cytochrome oxidase by cyanide is reversible.

Since the inhibitors of alkaline phosphatase which have been studied are all reversible inhibitors, the kinetics of irreversible inhibition will not be discussed.

Reversible inhibitors may be subdivided into three main classes - competitive, non-competitive and uncompetitive. Competitive inhibitors, by competing with the substrate for the active site of the enzyme, decrease the affinity of the enzyme for the substrate and so increase the K_m. Non-competitive inhibitors have no effect on K_m but reduce V_{max} while uncompetitive inhibitors affect both K_m and V_{max}. Mixed types of inhibition have also been noted.

a) Competitive Inhibition:

These are generally substances with a similar structure to the substrate and can combine with the active site of the enzyme, effectively blocking it, but not breaking down into free enzyme and products. In this case the enzyme combines

with the substrate or the inhibitor but not both. These are called fully competitive inhibitors. A partially competitive inhibitor is one which combines with the enzyme, not at the active site but at a point sufficiently close to it to reduce the affinity of the enzyme for the substrate although still allowing the substrate to combine with the enzyme.

In the fully competitive type there are two possible reactions of the enzyme:

$$E + S \rightleftharpoons ES \longrightarrow E + \text{products}$$
$$E + I \rightleftharpoons EI$$

where I is the inhibitor.

An expression for v can be derived for these equations, viz.

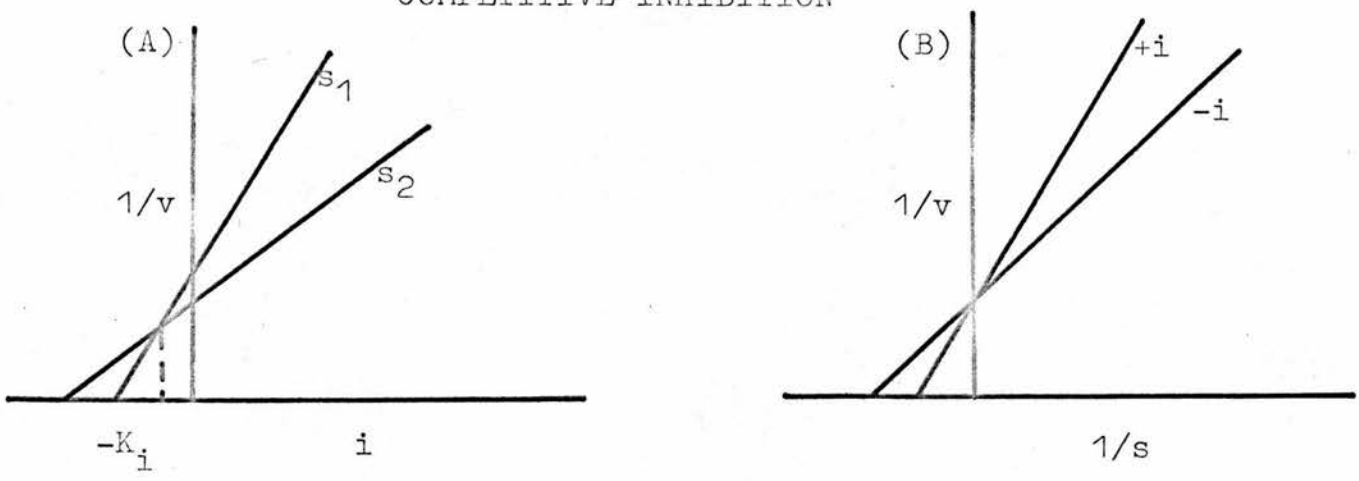
$$v = \frac{V_{max}}{1 + K_m/s\ (1 + i/K_i)}$$

Where K_i is the inhibitor constant and is a measure of the affinity of the enzyme for the inhibitor.

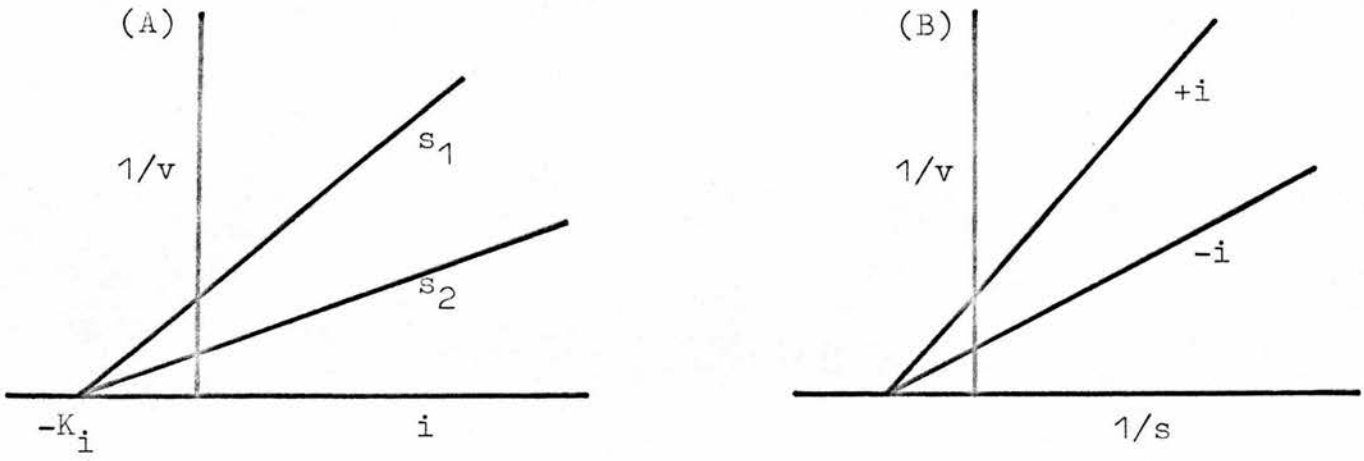
Again there are several ways of plotting the results. The velocity with a range of substrate concentrations in the absence and in the presence of an inhibitor may be plotted as 1/v against 1/s (Lineweaver and Burk, 1934). With competitive inhibitors V_{max} is unaffected by the presence of the inhibitor, so the lines intersect on the 1/v-axis and the point of intersection with the 1/s-axis is $-1/K_m(1 + i/K_i)$ in the presence of the inhibitor (Fig. 1.2.). Dixon (1953b) suggested measuring the

Fig. 1.2. Diagram showing Dixon's (1953b) method (A) and Lineweaver and Burk's (1934) method (B) of representing the effect of the three main types of inhibitors.

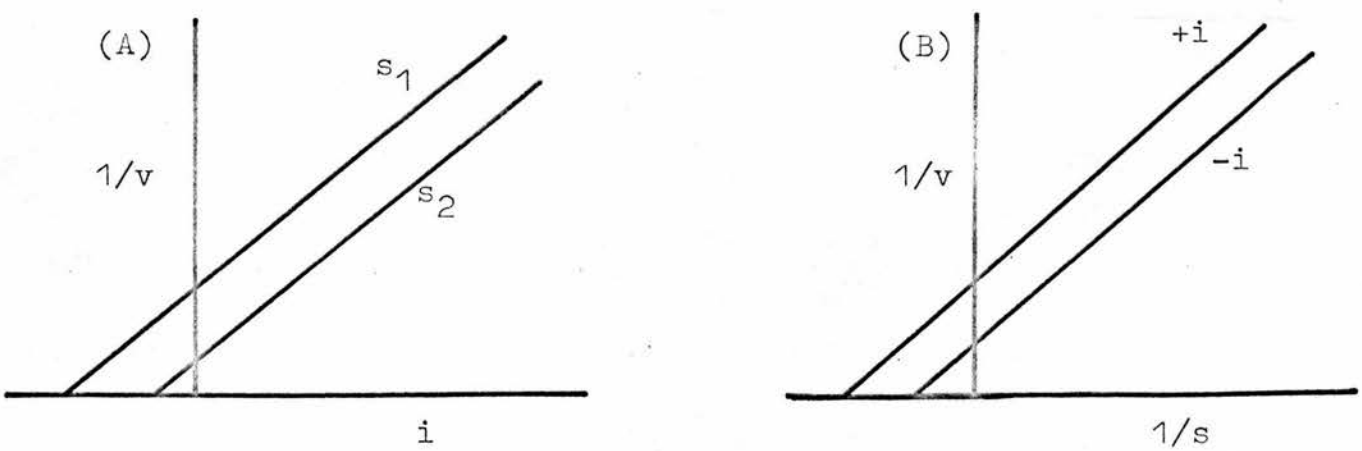
COMPETITIVE INHIBITION



NONCOMPETITIVE INHIBITION



UN-COMPETITIVE INHIBITION



velocity at a range of inhibitor concentrations and plotting 1/v against inhibitor concentration for each substrate concentration. The point of intersection, which lies between the two axes, has an abscissa of $-K_i$ (Fig. 1.2.).

In the partially competitive type of inhibition the enzyme can combine with both I and S

i.e.
$$E + S \rightleftharpoons ES \longrightarrow E + \text{products (P)}$$
$$E + I \rightleftharpoons EI$$
$$ES + I \rightleftharpoons EIS \longrightarrow E + P$$
$$EI + S \rightleftharpoons EIS \longrightarrow E + P$$

Since, by definition, the inhibitor affects only K_m and not V_{max}, the velocity of breakdown of EIS must be the same as that for ES, and the overall velocity is the sum of the two separate velocities. When the method of Dixon (1953b) is used to represent the results of this partially competitive type of inhibition, the plots are similar to the fully competitive type, but at high inhibitor concentrations the velocity may increase more rapidly than would be expected, due to the fact that the inhibitor concentration is greater than the enzyme concentration and the enzyme is saturated with inhibitor and the inhibition has reached a maximum value.

b) Non-competitive inhibition:

These inhibitors combine with the enzyme at a point some way removed from the active site; they have no effect on the affinity of the enzyme for the substrate, but affect the velocity of the

breakdown of the enzyme - substrate complex. This type of inhibition may also be subdivided depending on whether or not EIS breakdown occurs. In the former case it can be proved that

$$v = \frac{V_{max}}{(1 + i/K_i)\ (1 + K_m/s)}$$

If the results are plotted by the method of Dixon (1953b), the point of intersection lies on the x-axis and is equal to $-K_i$ (Fig. 1.2.). When EIS does break down, the velocity of the reaction is the sum of the velocities of the two reactions, $ES \longrightarrow E + P$ and $EIS \longrightarrow EI + P$. Calculation of K_i by the usual methods is not possible in this case.

It is also possible to have a "mixed" type of inhibition. In this case, the inhibition is a combination of partially competitive and one of the two types of non-competitive inhibition and if the results are plotted by the method of Lineweaver and Burk (1934) the lines do not intersect on either axes nor are they parallel.

c) Uncompetitive Inhibition:

In this case the inhibitor can combine only with the ES complex and it can be shown that

$$v = \frac{V_{max}}{K_m/s + 1 + i/K_i}$$

This type of inhibition is characterised by parallel lines in the double reciprocal plots of Lineweaver and Burk (1934) and in the Dixon (1953b) plots (Fig. 1.2.).

ii) Examples of Inhibitors: Some inhibitors of alkaline phosphatase appear to affect all the isoenzymes while others are specific for the enzyme from one tissue source. Examples of the former group are the metal ion complexing agents, some amino acids, and some monophosphate esters. EDTA and cyanide inhibit alkaline phosphatases from a variety of animal sources by a non-competitive mechanism (e.g. Schmidt and Thannhauser, 1943; Plocke, Levinthal and Vallee, 1962). Human leucocyte and placental phosphatases are also inhibited by metal-binding reagents (Ahmed and King, 1960b, Trubowitz et al, 1961). Phosphate, as the product of the hydrolysis reaction, acts as a competitive inhibitor (Lazdunski and Ouellet, 1962). Other monophosphate esters and arsenate, which has a chemical structure similar to phosphate, also inhibit competitively the enzyme from E. coli (Garen and Levinthal, 1960). Bodansky (1948) found that many amino acids inhibited alkaline phosphatase activity.

Inhibitors specific for certain isoenzymes are discussed in section 1.4.2.

1.3.5. Effect of Metal Ions.

Although some enzymes are unaffected by metal ions, many others are markedly activated or inhibited. The effect of a particular metal varies from enzyme to enzyme and from substrate to substrate. Concentration of the ion, pH of the solution, and even the purity and age of the enzyme solution alter the effects of the metal. The actions of the metal ions may, therefore, be very complex and the precise method and conditions

of treatment of the enzyme with metals and full details of the source of the enzyme and the method used for its preparation are necessary if effects observed by different workers are to be satisfactorily compared.

Plots of metal concentration against the velocity of the enzyme reaction resemble those for substrate concentration, and a constant (K_a) analogous to the Michaelis constant can be obtained. However the interpretation of K_a is not simple.

There are several ways in which an enzyme may be activated by a metal. The metal may form an essential part of the active centre of the enzyme, or it may act as a link between the enzyme and the substrate. Although not actually involved in the enzymic reaction a metal may produce a change in the equilibrium constant by a combination with, and removal of, the products of the reaction or an inhibitor present in the enzyme preparation.

Alkaline phosphatase, like most other enzymes acting on phosphorylated substrates, is activated by magnesium ions. Most methods of assay include the addition of Mg^{2+} to the assay mixture (Clark and Porteous, 1965). This activation is also produced by other divalent ions, e.g. manganese and to a lesser extent by calcium, zinc and cobalt. In each case the ions activate at low concentration but are strongly inhibitory at higher concentrations (Morton, 1955; with calf intestinal phosphatase). The only alkaline phosphatase reported as not being affected by magnesium ions is the enzyme obtained from E. coli (Binkley, 1961b). Alkaline phosphatases from different sources have been found to show different degrees of activation

by magnesium ions (e.g. by Ahmed, Abul-fadl and King, 1959). This may be due to differences in the enzymes, or to variations in the amount of the endogenous magnesium still remaining after purification.

When alkaline phosphatase from E. coli is dialysed against metal chelating agents there is progressive loss in activity, which can be restored with Zn^{2+} and to a lesser degree by other divalent ions (e.g. Plocke and Vallee, 1962). This evidence, together with metal analysis (Engstrom, 1961b) has been interpreted as an indication of the presence of zinc as an essential part of the active site of the enzyme.

The metal ion inhibitions which have been reported are generally with high concentrations of Ca^{2+}, Zn^{2+} and Co^{2+}. Beryllium, unlike these metals, inhibits at high concentrations but does not activate at a lower concentration (Grier, Hood and Hoagland, 1949).

1.4. THE IDENTITY OR NON-IDENTITY OF THE ISOENZYMES OF HUMAN ALKALINE PHOSPHATASES.

In considering the properties of alkaline phosphatase, the question arises whether or not the isoenzymes from different tissues are identical. If they are not, then to what is the difference due? This problem is not only of academic importance, but it has also clinical significance. An increase in the serum alkaline phosphatase activity may be due to a number of causes e.g. infective hepatitis, obstructive jaundice, cirrhosis of the liver, Paget's disease, hyperparathyroidism, etc. If alkaline phosphatases are not identical, a simple method of

distinguishing them in serum would facilitate the identification of the tissue source of serum alkaline phosphatase in disease and so aid the diagnosis of the cause of the elevation in enzyme level.

The possibility that all the alkaline phosphatases from different tissues are identical was suggested by Kay (1932) when he studied the alkaline phosphatases from bone, intestine, plasma and kidney and found that they all had the same optimum pH, all hydrolysed inorganic pyrophosphate, were all stimulated by the same concentration of magnesium ions, all had the same relative rates of hydrolysis with a series of substrates, and all synthesised phosphoric esters. Since then, however, various workers have found properties of alkaline phosphatase which indicate that there may be differences between the iso-enzymes. These will be discussed under three headings - namely, physical properties, enzymic properties and molecular structure.

1.4.1. Physical Properties.

a) Molecular weight: The molecular weights of proteins may be determined by various means, the two most frequently employed for alkaline phosphatase being sedimentation in an ultra-centrifuge, and filtration through a column of "Sephadex G-200" when the elution volume is compared with values obtained for proteins of known molecular weight. Table 2.2. shows values for human alkaline phosphatases. (Those from animal sources have been added for comparison).

Table 1.2. Molecular Weights of Some Alkaline Phosphatases.

Tissue Source	Molecular Weight	Method	Reference
Human kidney	120,000-130,000	ultracentrifugation	Boyer, 1963
Human placenta	120,000-130,000	ultracentrifugation	Boyer, 1963
Bovine milk	180,000	gel-filtration	Andrews, 1965
Bovine liver	150,000	gel-filtration	Engstrom, 1964a
Calf intestine	100,000	ultracentrifugation	Engstrom, 1961a
E. coli	75,000-80,000	ultracentrifugation	Garen and Levinthal, 1960

Boyer (1963) found that the behaviour of the human kidney and placental enzymes on ultracentrifugation was identical. A failure to separate the phosphatases from placenta, liver, bone and intestine on "Sephadex" confirmed the similarity of their molecular weights (Birkett et al, 1965).

b) Electrophoresis: Various supporting media have been used in zone-electrophoresis of alkaline phosphatase. Ahmed and King (1960a) used paper as supporting medium for electrophoresis in their method for purifying placental alkaline phosphatase. A difference between the mobility of bone and liver alkaline phosphatase on paper electrophoresis was found by Nath and Ghosh (1964). Agar-gel electrophoresis of alkaline phosphatase was developed by Stevenson (1961), and characteristic bands for liver and bone isoenzymes were demonstrated, the bone enzyme migrating slightly more slowly than liver phosphatase at pH 8.4 (Haije and DeJong, 1963).

Starch-gel, however, is the most frequently used medium for electrophoretic separation of enzymes, and there are numerous reports of electrophoretic separation of alkaline phosphatase in serum and in tissue extracts using starch-gel as the supporting medium. Both the molecular size and the charge on the molecule affect the rate of migration of the ion in the gel (Smithies, 1962). There is some variation in the number of bands of alkaline phosphatase activity seen after starch-gel electrophoresis of human serum at pH 8.6. Boyer (1961) reported 16 bands which could be divided into 6 main zones, though not all were observed in any one individual, while Kowlessar, Haeffner and Riley (1961) and Kreisher, Close

and Fishman (1965) found two main bands of activity in normal serum. This variation was partly due to a difference in the state of the patient from whom the serum was taken and partly to the technique of starch-gel electrophoresis.

The more cathodic of the two bands seen in normal serum after starch-gel electrophoresis has been identified as originating from the intestine, by testing for its sensitivity to L-phenylalanine, by examining its resistance to thermal denaturation (Kreisher et al, 1965), and by its reaction with anti-intestinal phosphatase antibody (Boyer, 1961). The occurrence of this component in the serum of certain individuals is associated with the ABO blood groups (Arfors, Beckman and Lundin, 1963).

The more anodic band is from the liver (Hodson, Latner and Raine, 1962). In cases of bone disease, which caused an elevated alkaline phosphatase level, a band of activity slightly cathodic to the liver band was seen (Hodson et al, 1962). Since the difference in migration between the liver and bone enzyme is small, many workers have found similar migration (e.g. Chiandussi, Greene and Sherlock, 1962) and some have stated that the main band in normal sera originated from bone (e.g. Kowlessar et al, 1961). Antibody experiments also supported this (Schlamowitz and Bodansky, 1959). Boyer (1961) found that three of the six main zones of activity only occurred in pregnancy and were presumably placental in origin. Electrophoresis of placental alkaline phosphatase has been reported to produce several different distributions of activity, which

appear to be genetically determined (Robson and Harris, 1965).

Electrophoresis of tissue extracts has shown that there are several bands for each tissue. Moss and King (1962) found that liver alkaline phosphatase had three or four bands; intestinal phosphatase, two; kidney phosphatase, three; and bone phosphatase, two. In each case one component migrated slowly, parallel with the lipoprotein fraction of the serum proteins. Urinary alkaline phosphatase migrated more rapidly than the liver or kidney enzymes (Butterworth, Moss, Pitkanen and Pringle, 1965). The zone of activity associated with kidney alkaline phosphatase is broad but the leading edge is approximately parallel with that of the liver. The variation in the appearance of the minor bands can be due to different methods of preparation (Moss, 1962) or to degradation (Moss and King, 1962; Moss, 1965). Moss and King (1962) found no difference between the different bands from one tissue source with respect to K_m values and heat stability, and they suggested that there was a single alkaline phosphatase in each tissue, the main zone on starch-gel electrophoresis being free enzyme and the other bands arising from aggregation or disaggregation of the free enzyme or its association with protein fractions.

c) Reaction to Neuraminidase: Neuraminidase (E.C.3.2.1.18.) catalyses the removal of sialic acid residues from mucoproteins (Gottschalk, 1958), and Robinson and Pierce (1964) tested its effect on serum alkaline phosphatase. Starch-gel electrophoresis of serum produced a number of bands of activity, varying from one to four with different sera, and they found

that all but one of these was altered by treatment with neuraminidase. The unaffected band of alkaline phosphatase was inhibited by L-phenylalanine, and they concluded that it was of intestinal origin.

d) Denaturation of Various Agents: Many processes and agents will cause a change of an enzyme from the "native" or active state into a "denatured", inactive form. Physical agencies such as X-rays, ultraviolet light, heat, and violent shaking will cause denaturation. Some organic solvents, extremes of pH and strong solutions of urea, salicylate and guanidine salts also inactivate. Denaturation is generally irreversible being accompanied by loss of enzyme activity; it represents a more disordered arrangement of the protein molecule, reflected by changes in the physical properties of the enzyme, e.g. changes in the isoelectric point, the solubility in water, and the reaction to specific antibodies.

Urea denaturation: High concentrations of urea have been known for some time to exert a powerful action on proteins by breaking of the hydrogen bonds which maintain the secondary, tertiary and quaternary structure of the molecule; urea is often used to break molecules into subunits. Generally, if the urea concentration is less than about 4M (at neutral pH) there is only a reversible inhibition of enzyme activity; but at higher concentrations a reversible denaturation occurs (Steinhardt and Beychok, 1964). Urea has been shown to produce two such effects on human alkaline phosphatases and their isoenzymes. The

concentration of urea at which the inhibition changes from reversible to irreversible varies with the isoenzyme, the placental enzyme being the most stable and the bone enzyme the least stable (Birkett et al, 1965). Callaghan and Martin (1962 and 1963) found a similar critical value for the effect of urea on albumin and globulin and interpreted the effect of high concentration of urea as being due to the rupture of hydrogen bonds and consequent unfolding of the protein molecule.

Heat denaturation: Enzymes lose activity slowly at room temperature, but the rate of denaturation increases rapidly with increase in temperature and is also time dependent. Considerable variation between the isoenzymes of alkaline phosphatase has been found with respect to their heat stability. The enzyme preparations of Moss and King (1962) from human tissues, in order of increasing stability at 55°C and pH 7.0, were bone, kidney, intestine and liver alkaline phosphatase, the time taken for the activity to fall to 50% of the original activity being 8 minutes for the bone enzyme and 18 minutes for the liver phosphatase. Biliary alkaline phosphatase is more stable than the bone and liver enzymes (Posen, Neale and Clubb, 1965), while the placental enzyme is much more resistant to heat - it is unaffected by heating to 56° for 30 minutes (Neale, Clubb, Hotchkis and Posen, 1965).

Acid denaturation: Reduction of the pH of an enzyme solution will generally cause loss of activity which may or may not be recovered when the pH is readjusted to neutrality, depending on the pH and the length of time of inactivation.

Comparisons of the effect of low pH on human alkaline phosphatases have not been reported. With E. coli phosphatase, however, treatment with acid below pH 3.0 causes the enzyme to dissociate into two inactive subunits (Schlesinger and Barrett, 1965).

e) Immunological Properties: A general method of distinguishing protein molecules is by the immune response. Repeated injection of a foreign protein into a suitable animal (e.g. rabbit or horse) causes the production of an antibody to that protein. These antibodies, which are γ-globulins, are specific to the injected protein and will react only with the injected protein (apart from possible cross-reactions with proteins closely resembling the antigen) causing, under appropriate conditions, co-precipitation. This provides a means of distinguishing proteins, and hence enzyme molecules, not by their catalytic activity but by the structure and conformation of the protein chain. Antibodies to alkaline phosphatase were first made by Schlamowitz (1958) for the enzyme from dog intestine, and later by Schlamowitz and Bodansky (1959) for human alkaline phosphatases. They found that the antibody to human intestinal phosphatase precipitated only human intestinal enzyme and not the bone, kidney or liver enzymes. Their antibody to bone phosphatase, however, reacted with liver, kidney and bone phosphatases and to a lesser extent with the intestinal enzyme. They concluded that, although it appeared that liver, bone and kidney enzymes were immunologically similar and all differed from the intestinal enzyme, it could be that their bone preparation contained small

quantities of the liver and kidney enzymes and that antibodies to these were also being produced. Nisselbaum, Schlamowitz and Bodansky (1961) confirmed that the anti-bone antibodies precipitated the liver and kidney enzymes. Boyer (1963) suggested that there were three antigenic classes of alkaline phosphatase; group one consisting of liver, bone, spleen and the major component of phosphatase activity in kidney; group two consisting of intestinal phosphatase and the minor kidney component; and a third group consisting of the placental enzyme, which he considered to be immunologically different from all the others.

1.4.2. Enzymic Properties.

Some variation in substrate specifically between human phosphatases was found by Landau and Schlamowitz (1961). The relative rates of hydrolysis for several monoesters by the intestinal enzyme differed from those for the phosphatases from liver, bone, spleen and kidney. They were, however, unable to find a clear-cut difference between the Michaelis constants of these enzymes with β-glycerophosphate as substrate. On the other hand, Moss et al (1961a), using β-naphthyl phosphate, found that the isoenzymes from tissue homogenates of liver, bone, kidney and intestine had different K_m values. In the serum of patients with elevated alkaline phosphatase activities due to disease of the liver, the K_m of the phosphatase resembled the value for the liver isoenzyme, whereas in patients with raised activities due to bone disease, the K_m resembled the

value for bone phosphatase. In cases where bone and liver phosphatases both contributed to the elevated serum alkaline phosphatase activity then the K_m had an intermediate value (Moss, Campbell, Anagnostou - Kakaras and King, 1961b).

Distinction between the isoenzymes has also been made on the basis of the effect of certain inhibitors on the enzyme reaction. Two inhibitors which are organ-specific are the bile acids and L-phenylalanine. In 1937 Bodansky showed that preparations of intestinal enzyme from rat, rabbit, cat and man were unaffected by bile acids, while extracts from bone and kidney were inhibited. This was the first evidence that the phosphatases from different organ sources were not identical. Fishman et al (1962) found that L-phenylalanine was a specific inhibitor of the intestinal phosphatase of rat, while the bone, blood, lung, liver and kidney phosphatases were unaffected. The D-isomer of phenylalanine had no inhibitory effect. L-phenylalanine was later shown to be a specific inhibitor of human intestinal phosphatase also (Fishman, Green and Inglis, 1963). Derivatives of phenylalanine (e.g. formyl phenylalanine) and other isomers (e.g. β-phenyl-β-alanine and α-phenyl-β-alanine) did not inhibit (Fishman et al, 1962).

1.4.3. Molecular Structure.

A study of the isoenzymes at a molecular level should indicate whether the physical differences between them are due to differences in basic structure or to differences in non-protein constituents of the molecule.

Structure of the protein molecule and the active centre: Attempts have not yet been made to elucidate the primary structure of any of the phosphatases.

Various methods have been used for the identification of the different groups in the active centre of enzymes. Firstly, by measuring the pK values of the groups in the enzyme which react with the substrate, and by deducing which amino acids are involved. The kinetics of this have already been discussed. Morton (1957) presented evidence based on kinetic experiment that the phenolic group of tyrosine might be in the active site of calf intestinal phosphatase. However, he does suggest that the ϵ - amino group of lysine might be involved since the two groups have similar pK values. Although this method is valid as a means of characterising the different isoenzymes, it is open to criticism as a means of identifying amino acid residues in the active centre, since the presence of adjacent groups can profoundly affect the pK of an ionisation as compared with the pK for the free amino acid.

A second method involves inactivation of the enzyme with specific chemical reagents. Lazdunski and Ouellet (1962) found that reagents which were specific for sulphhydryl groups inhibited the alkaline phosphatase from calf intestine. This inhibition could be reversed on addition of cysteine. They concluded that there was a sulphhydryl group in the active centre of the enzyme, and that it was involved in the enzymic reaction.

A third method is based on partial degradation of the

enzyme molecule, either by removal of parts of the peptide chain followed by testing to find out whether enzymic activity still remains or has been lost, or by first labelling the active centre and then examining the fragments of degradation to find the label. The former method has been employed by Anfinsen (1956) on ribonuclease (E.C.2.7.7.16.), and the latter method has been applied to many enzymes, using an organophosphorus compound as label. This organophosphate reacts with esterases to give permanently bound phasphoryl groups at the active centres of these enzymes (Adrian, Feldberg and Kilby, 1947).

More recently, isotopically labelled substrates have been employed to label the active centre before degradation (Milstein and Sanger, 1961; Naughton, Sanger, Hartley and Shaw, 1960). This method has been applied to the alkaline phosphatase from E. coli by Milstein (1964), who effected the incorporation of phosphate labelled with ^{32}P and found that the label was attached to a serine residue lying between aspartic acid and alanine residues. The alkaline phosphatase from Serratia marscescens has a similar sequence around the active serine residue (Zwaig and Milstein, 1964), and the same sequence has been found for calf intestinal phosphatase (Engstrom, 1964b). Since the serine residue can be isolated with the phosphate attached the linkage must be covalent.

Very little is known about the active centre of human alkaline phosphatase though it may be inferred that, since such widely varying enzymes as calf intestinal and E. coli phosphatases have the same sequence in the active centre,

human phosphatases are likely to be similar.

Non-protein content: Metal ions not only activate the enzyme reaction but are also intimately involved in the enzyme structure. Since the zinc concentration increases with purification of the enzyme, and metal chelating agents inhibit the purified enzyme, Plocke et al concluded that the E. coli phosphatase contained zinc (Plocke and Vallee, 1962; Plocke, Levinthal and Vallee, 1962). The protective effect of orthophosphate against EDTA inhibition, and the fact that orthophosphate is a competitive inhibitor of phosphatase action, indicates that the metal ion is at the active centre of the enzyme molecule (Garen and Levinthal, 1960). Evidence that zinc ions are essential for enzymic action has been reported with human leucocytes (Trubowitz et al, 1961), with calf intestinal phosphatase (Engstrom, 1961a), and with swine kidney phosphatase (Mathies, 1958).

1.5. CONCLUSIONS AND AIMS.

If all the known properties are taken into consideration, it would appear that human alkaline phosphatases are not all identical. There are three enzymic properties in which the isoenzymes have been found to differ: relative rates of hydrolysis of different substrates; Michaelis constants; and reaction to certain inhibitors. There are also differences in four physical properties; mobility on starch-gel electrophoresis; reaction to neuraminidase and denaturing agents; and antigenic properties. On the basis particularly of the effect of neuraminidase, the reaction to inhibitors, the

relative rates of hydrolysis with different substrates and the antigenic behaviour, the phosphatases can be divided into intestinal and non-intestinal groups, the latter group comprising the liver, bone, kidney and possibly the spleen enzymes. However, the Michaelis constants, the starch-gel electrophoresis patterns and the reaction to denaturing agents show further differences between the isoenzymes, including those in the non-intestinal group. The placental enzyme appears to be different from both the intestinal and non-intestinal groups of phosphatases with respect to its antigenic properties and its heat stability.

Since in the earlier investigation of these properties, serum or relatively impure enzyme preparations were used for the most part, there was a possibility that these variations in properties reflected differences in contaminants isolated concomitantly with the phosphatase, and were not due to differences in the enzyme molecule itself. To establish whether the differences between the isoenzymes were real, it would be necessary to reinvestigate the properties using purified enzyme preparations.

If the isoenzymes are not identical and the differences cannot be accounted for by contaminants present in the enzyme preparation, the basis of their non-identity then requires explanation. Differences could occur, for instance, in the non-protein part of the molecule (e.g. neuraminic acid content), or in the primary structure, or in the conformation of the protein chain or chains.

The aim of this investigation was, therefore, firstly to purify alkaline phosphatase from several different human tissues and then establish whether these alkaline phosphatases were identical, by studying the properties of the purified enzymes. If differences were found, the aim was to elucidate the molecular basis of these differences.

CHAPTER 2. ENZYME PURIFICATION AND ASSAY METHODS.

2.1. MEASUREMENT OF ENZYME ACTIVITY.

2.1.1. General method of assay.

In order to be able to follow the progress of enzyme purification at all stages a rapid and reproducible method of measuring the enzyme activity is necessary. Alkaline phosphatase hydrolyses monophosphates with release of inorganic phosphate. Either the phosphate or the organic group may be measured and in the literature both methods are described. The methods for measuring phosphate have the advantage that they can be used for any substrate, but have the disadvantage that the measurement of phosphate is not simple and generally involves the addition of several reagents consecutively.

Choice of Substrate: The first substrate to be used was β-glycerophosphate by Robison (1923) and by Demuth (1925) who measured the inorganic phosphate released. King and Armstrong (1934) suggested phenylphosphate as a substrate with the measurement of the phenol produced. Other assays which have been used are the measurement of phenolphthalein liberated from phenolphthalein diphosphate (Huggins and Talalay, 1945), p-nitrophenol from p-nitrophenyl phosphate (Bessey, Lowry and Brock, 1946), α-naphthol from α-naphthyl phosphate (Moss, 1960) and 4-umbelliferol from 4-umbelliferyl phosphate (Fernley and Walker, 1965). With each of these the organic moiety is readily measured.

p-nitrophenyl phosphate (pNPP) was chosen for the present work because the p-nitrophenol (pNP) produced is easily

measured in the presence of unaltered substrate. In alkaline solution p-nitrophenol is yellow with a maximum absorbance at 404mμ, while p-nitrophenyl phosphate is colourless and has a maximum absorbance at 312 mμ. The molar absorptivity of p-nitrophenol was found experimentally to be 17.7×10^3 at pH values greater than 9.5. This compares well with Jacobsson's (1960) value of 18×10^3. Enzymatic activity is expressed as μmoles substrate hydrolysed per min. per ml. of enzyme solution under the conditions of assay used.

Buffer system: The carbonate-bicarbonate buffer (pH 9.9) of Delory and King (1945) was used. Other buffers in general use for alkaline pH (e.g. borate, glycine) were not used since they have been reported to inhibit alkaline phosphatase activity (Delory and King, 1945). The optimum pH varies with the concentration of the substrate (Ross et al, 1951), but, in accordance with the general assay methods for alkaline phosphatase, the enzyme was measured at pH9.9 with a 2mM substrate concentration (See e.g. King and Armstrong, 1934). The buffer was prepared by mixing equal quantities of 0.1N sodium carbonate and 0.1N sodium bicarbonate. The pH of the buffer was found to decrease slowly with time and it was, therefore, checked each day before use on a Radiometer pH meter fitted with a glass electrode.

Magnesium ions have been found to stimulate alkaline phosphatase activity at low concentration. In order to eliminate the effect of varying amounts of residual magnesium

ions in the preparation, a constant amount of magnesium was added to the assay solution. A concentration of 5mM was found to be optimal. The effect of magnesium ions on alkaline phosphatase activity is examined in detail in section 4.2.1.

Amount of enzyme added: 20 to 100 µl of enzyme solution (or diluted enzyme solution) were added per assay and the time of incubation was generally five minutes. The velocity of the reaction is linear up to about 20 min.

Summary of method: 1ml. of 0.1M carbonate-bicarbonate buffer containing 10mM magnesium chloride and an appropriate amount of enzyme solution were preincubated at 37° for about five minutes. 1ml. of prewarmed 4mM pNPP was added and after a measured time interval 1 ml. 0.4N NaOH with 0.02M EDTA was added to terminate the reaction. The addition of EDTA prevents the precipitation of magnesium hydroxide. The absorbance of the solution was read at 400 mµ in a spectrophotometer. In a few cases the initial velocity was determined by continuous measurement in a direct recording spectrophotometer; Gilford 2000. This general method was used for the measurement of activity throughout the preparative stages. Modifications for kinetic studies are reported at the appropriate point.

2.1.2. Other methods of assay.

When studying the substrate specificity of the enzyme it was necessary to have a method of assay which depended upon measurement of the inorganic phosphate released. Two different

methods were used, both based on the reaction of ammonium molybdate with inorganic phosphate and the reduction of the phosphomolybdic acid formed to molybdenum blue. The method of Delsal and Manhouri (1958) uses p-methylamino-phenol sulphate (rhodol) as the reducing agent. The enzymatic reaction is terminated with 2 ml 2.3M acetate buffer (pH 4.0) containing copper ions and then 0.5 ml. 5% ammonium molybdate and 0.5 ml. rhodol solution are added. The inorganic phosphate in a standard of known phosphate content is measured in parallel. The molybdenum blue complex required about 10 min. to form after the addition of the rhodol and was only stable for a further 20 min.

In some of the later experiments the method of Baginski Foa and Zak (1967) was employed; this uses ascorbic acid as reducing agent. The reduced phosphate - molybdate complex is stable for 24 hours and any phosphate released subsequent to the addition of the colour reagent has no effect on the intensity of the final blue colour since one of the reagents, arsenite-citrate, complexes with the excess molybdate. This method proved to be more sensitive and more accurate than that of Delsal and Manhouri (1958).

2.2. ENZYME PREPARATION.

2.2.1. Extraction and Fractionation.

Specimens of human liver, small intestine and rib with no gross evidence of disease were obtained within 24 hours of death and stored at -20° until extraction. Eight to twelve

livers (wet weight 8-12 kg.) or about twelve intestines (wet weight 5 kg.) were washed with tap water, minced and then extracted by a modification of Morton's (1950) butanol method. In a pilot experiment the tissues were extracted with various proportions of water and n-butanol, the highest yield of enzyme being obtained when the ratio of water to butanol was 2:1. The minced tissues was, therefore, stirred for about 1 hour in 2 litres of water and 1 litre of n-butanol/kg. wet weight of tissue and then centrifuged. The fat layer and the butanol phase, which contained no enzyme activity, were discarded and the aqueous layer containing the enzyme was decanted for subsequent purification.

About twelve pieces of rib were broken, while still frozen, into small fragments (wet weight about 600g.). The method of Albers and Albers (1935) which involves autolysis in chloroform and water and the butanol method of Morton (1950) were compared. The latter gave a higher yield of enzyme activity. The bone fragments were, therefore, stirred in a 2:1 mixture of water and n-butanol for about 3 hours. The mixture was centrifuged and all but the aqueous layer discarded.

The enzyme solution from each tissue was then cooled and fractionated with acetone at -1°. Pilot experiments with small quantities of butanol extract were carried out to ascertain the amounts of acetone required to give an 80-90% yield of enzyme with the maximum increase in specific activity. Concentrations of acetone used in the full-scale extraction are shown in Table 2.1. The protein precipitate was separated by centrifugation, dissolved in 0.01M tris-HCl buffer, pH 7.7, and dialysed

against this buffer to remove the acetone which would otherwise have interfered with the next stage of purification. Since acetone tends to cause inactivation of the enzyme, the solution was kept at 0^{o} throughout the procedure.

The enzyme solutions were then fractionated with ammonium sulphate (AnalaR grade) at room temperature. The optimum fraction for each tissue was again found by pilot experiments. (Details of quantities are given in Table 2.1.). The protein precipitates were separated by filtration or centrifugation and dissolved and dialysed against 0.01M tris-HCl buffer.

The orthophosphatase activity was assayed in each fraction after acetone and ammonium sulphate fractionations and the protein content was measured by the extinction of the solution at 280 mμ; $E_{280}^{1cm.} = 1.0$ has been taken as equivalent to a protein concentration of 1mg./ml. (Warburg and Christian, 1941). A reversal of the order of the acetone and ammonium sulphate fractionations resulted in lower yields and smaller increases in specific activity. These two procedures increased the specific activity of the enzyme 10-20 fold (See Table 2.1.).

2.2.2. Gel filtration.

Sephadex G-200 (Pharmacia AB, Uppsala, Sweden) was swollen in distilled water for about 24 hours and the "fines" removed by decantation. The gel was packed under gravity in columns 100 x 3cm. diameter or, for larger quantities of enzyme, 90 x 8.8cm. The buffer, 0.01M tris-HCl pH 7.7 with 0.1M NaCl and 0.1% n-butanol as a preservative, was pumped upwards through the column. The effluent fractions (10-25 ml. depending on

the size of the column) were collected in a fraction collector. The enzyme solution after ammonium sulphate fractionation was concentrated by dialysis against "carbowax" solution (polyethylene glycol) and pumped on to the column. The rate of flow was between 1 and 4 ml./hr./sq. cm.

The orthophosphatase activity and the protein concentration of the effluent fractions were measured. Typical separations are shown in Figs. 2.1. to 2.3. If the $E_{254m\mu}$ transmittance of the effluent prior to collection was monitored in an absorptiometer, a trace consisting of three or four "protein" peaks was obtained, the alkaline phosphatase activity coming between the first two. On some columns a small peak of enzyme activity (about 10-20% of the total activity) with approximately the same effluent volume as the first protein peak, occurred. This fraction was mainly associated with the slow moving band on starch-gel electrophoresis (see section 3.2.).

On the small column the maximum sample volume for good resolution was about 5 ml. while for the larger column 6g. protein in 100 ml. buffer was the maximum load. Loss of activity on the gel filtration columns was of the order of 10%, but after selection of the central portion of the peak and concentration against "carbowax", the overall yield was only about 60%. There was little difference in yield whether the columns were run at 4^{o} or at room temperature. Gel-filtration increased the specific activity of the enzyme about 10-fold. When the leading and trailing edges of the main enzyme peak were concentrated separately and rerun on

Fig. 2.1. Gel filtration (Sephadex G-200) of liver extract. Fraction volume: 15 ml.

——— Alkaline phosphatase activity (μmoles pNPP hydrolysed /min./ml.)

– – – Pyrophosphatase activity (μmoles pyrophosphate hydrolysed /min./ml.)

—·— E_{280}.

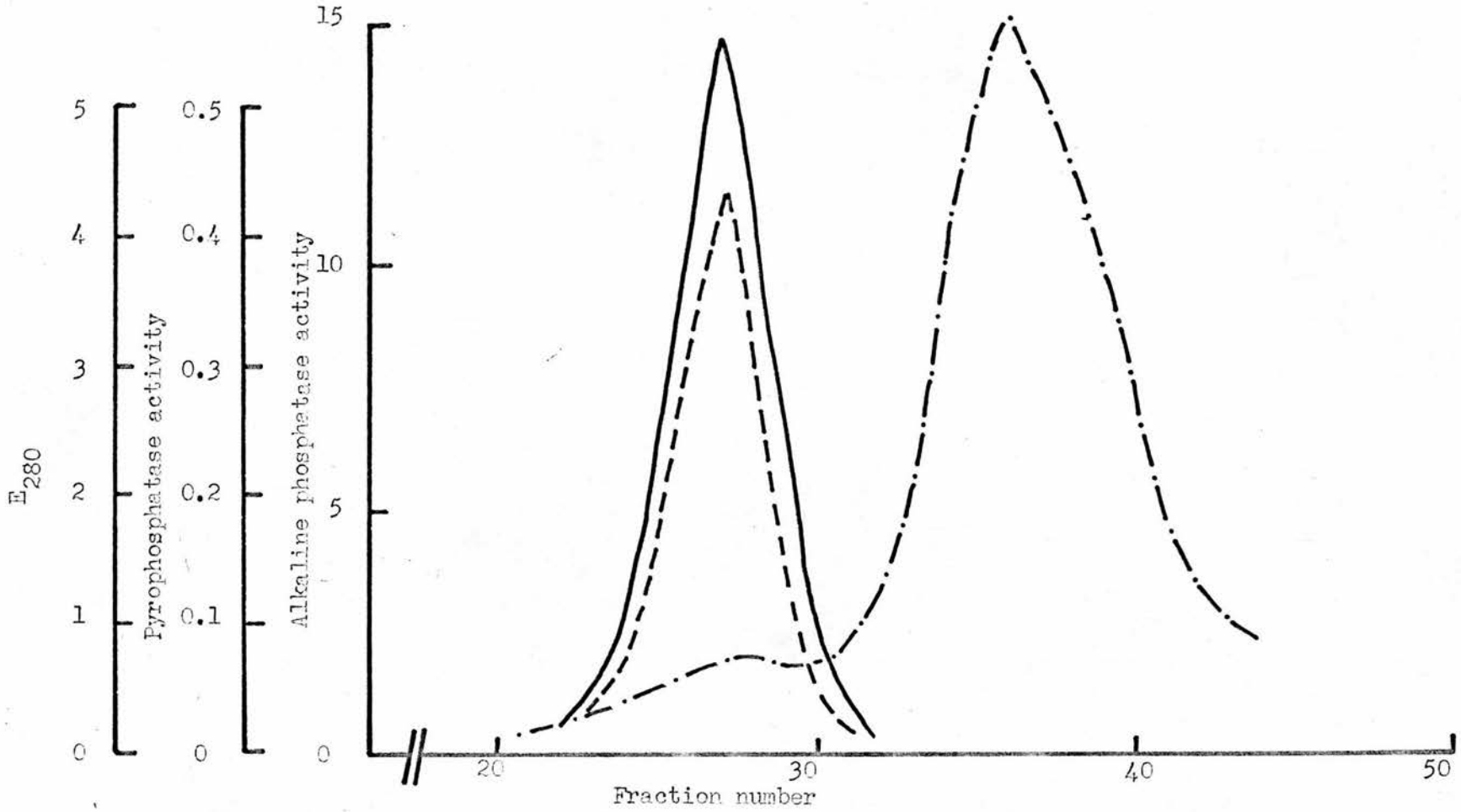


Fig.2.2. Gel filtration (Sephadex G-200) of small-intestinal extract. Fraction volume :15 ml. ——— Alkaline phosphatase activity (μmoles pNPP hydrolysed /min./ml.) ; — — Pyrophosphatase activity (μmoles pyrophosphate hydrolysed /min. /ml.); —· —E_{280}.

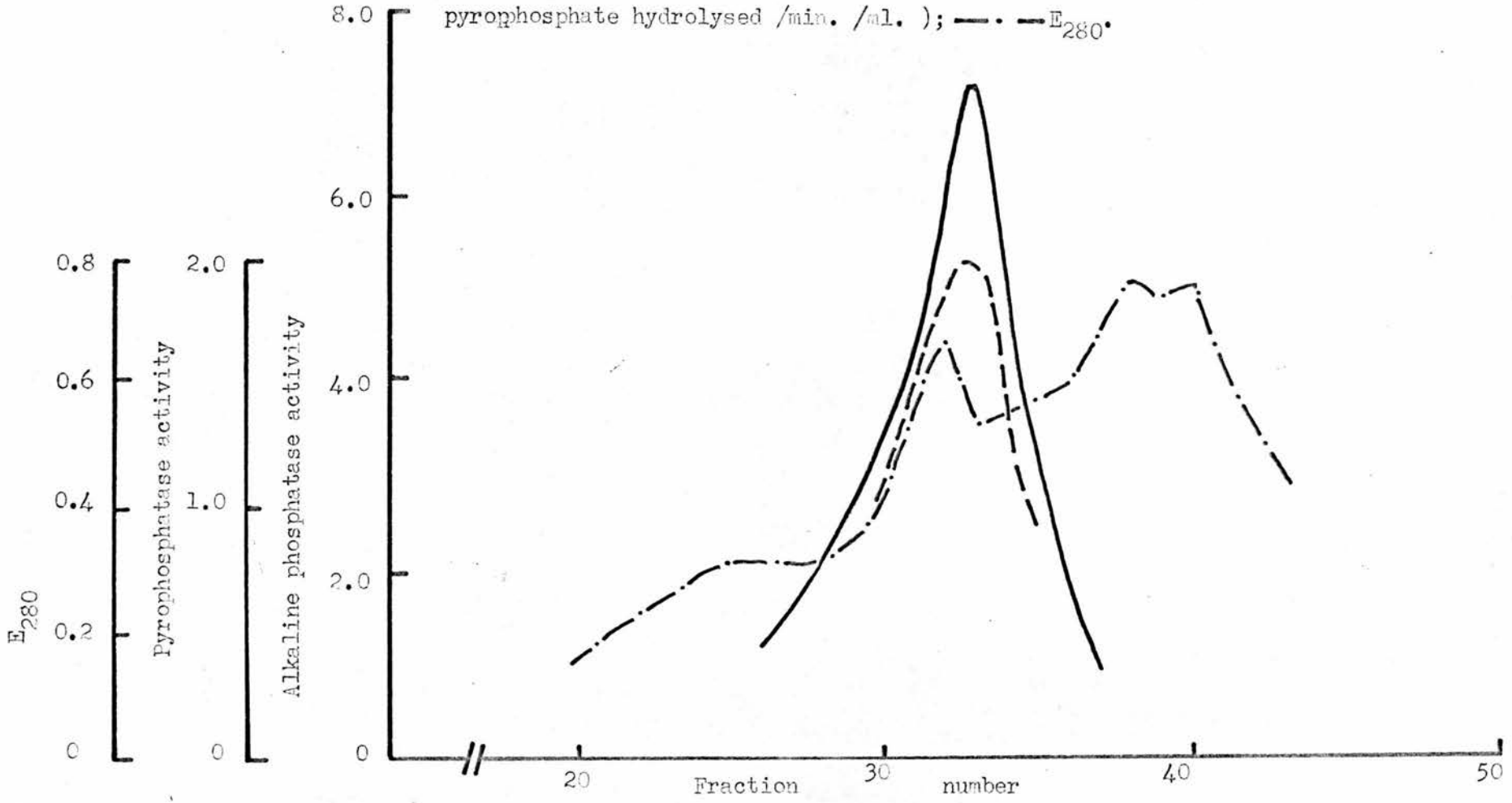
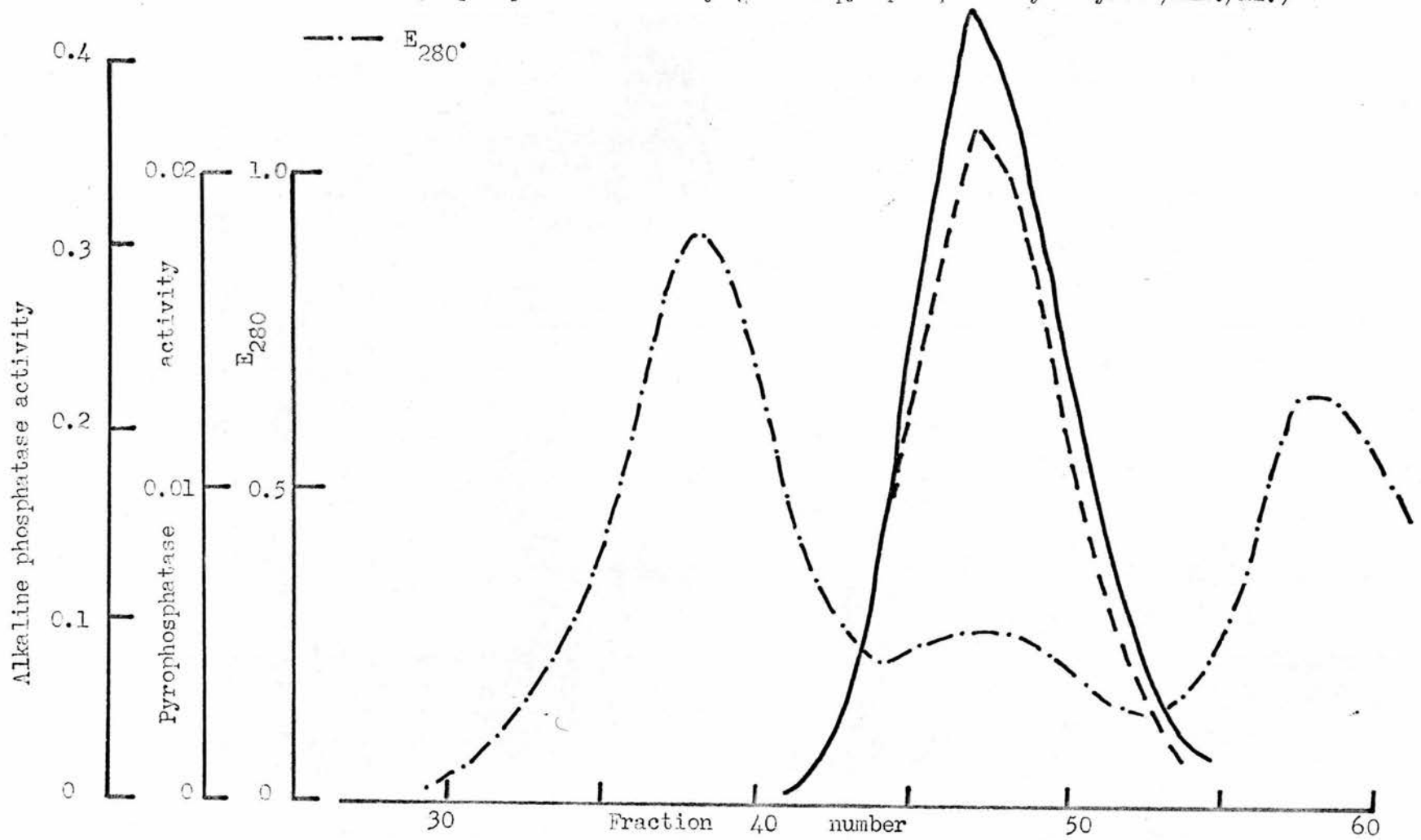


Fig. 2.3. Gel filtration (Sephadex G-200) of bone extract. Fraction volume: 8.6ml.

——— Alkaline phosphatase activity (μmoles pNPP hydrolysed /min./ml.);

– – – Pyrophosphatase activity (μmoles pyrophosphate hydrolysed /min./ml.)

—·— E_{280}.



Sephadex G-200 the elution volumes were unaltered. Each isoenzyme is, therefore, homogeneous with respect to molecular size.

2.2.3. Ion-exchange chromatography.

The enzymes were submitted to cation-exchange chromatography on cellulose phosphate pH6.0, and anion-exchange chromatography on DEAE-cellulose at pH 6.8, 7.7 and 9.0. The highest yields and greatest increase in specific activity was obtained on DEAE at pH 7.7.

DEAE-cellulose (Whatman No. 11 or No. 52) was soaked in distilled water, then treated consecutively with 0.5M HCl and 0.5M NaOH and equilibrated with 0.01M tris-HCl, pH 7.7. The column used for the liver and intestinal phosphatases measured 90 x 2.5 cm. diameter; but for the bone phosphatase a column 30 x 2.5 cm. was used since there was considerable loss of activity on the longer column. The cellulose was packed under gravity and the sample (the concentrated effluent from gel filtration), was applied to the top of the bed. 50 to 100 ml. starting buffer (dilute tris-HCl, pH 7.7) was passed through the column and the enzyme eluted with an increasing concentration of NaCl. As 2 litres of buffer passed through the 90cm. column the salt concentration was gradually increased from 0 to 0.5M NaCl while on the 30cm. column the salt gradient was 0-0.5M NaCl over 1 litre. In both cases gradients of 0-0.3M were used latterly. These gave broader peaks of enzyme activity, but an increase in specific activity. Effluents were collected as before and assayed for phosphatase

activity and protein content (Fig. 2.4-2.6). Since at low salt concentrations the enzyme is adsorbed on to the exchanger, there is theoretically no limit to the volume in which the sample may be applied to the column. For good separation, a volume of about 30 ml. (protein concentration; 0.1g./ml.) was found to be the maximum load, but less protein was generally applied.

Enzyme recoveries as low as 25% were occasionally found with the liver and intestinal phosphatases. Pre-treatment of the DEAE-cellulose with buffer containing 0.1mM zinc and inclusion of zinc in the elution buffer increased the yield to about 70%. Alternatively, addition of Zn^{2+} to the assay mixture increased the activity of the enzyme fractions from the columns. This loss of activity was attributed to removal of Zn^{2+} from the enzyme during chromatography. On the short columns the recovery of the bone phosphatase was not increased when the column was washed with buffer containing zinc ions nor was the activity of the effluent fractions increased when the metal was added to the assay system.

On anion-exchange chromatography at 4^{o} overall yields of about 50% were obtained for the liver and intestinal phosphataes when zinc ions were included in the buffer and for the bone phosphatase both in the presence and absence of zinc: at room temperature the yields were considerably reduced. The increase in specific activity was 3 to 5-fold. If anion-exchange chromatography preceeded gel filtration the overall yield of the two procedures was lower.

Fig.2.4. Anion-exchange chromatography (DEAE-cellulose, pH7.7) of liver extract.

——— Alkaline phosphatase activity(μmoles pNPP hydrolysed /min. /ml.);

– – – Pyrophosphatase activity (μmoles pyrophosphate hydrolysed /min./ml.)

—·— E_{280}.

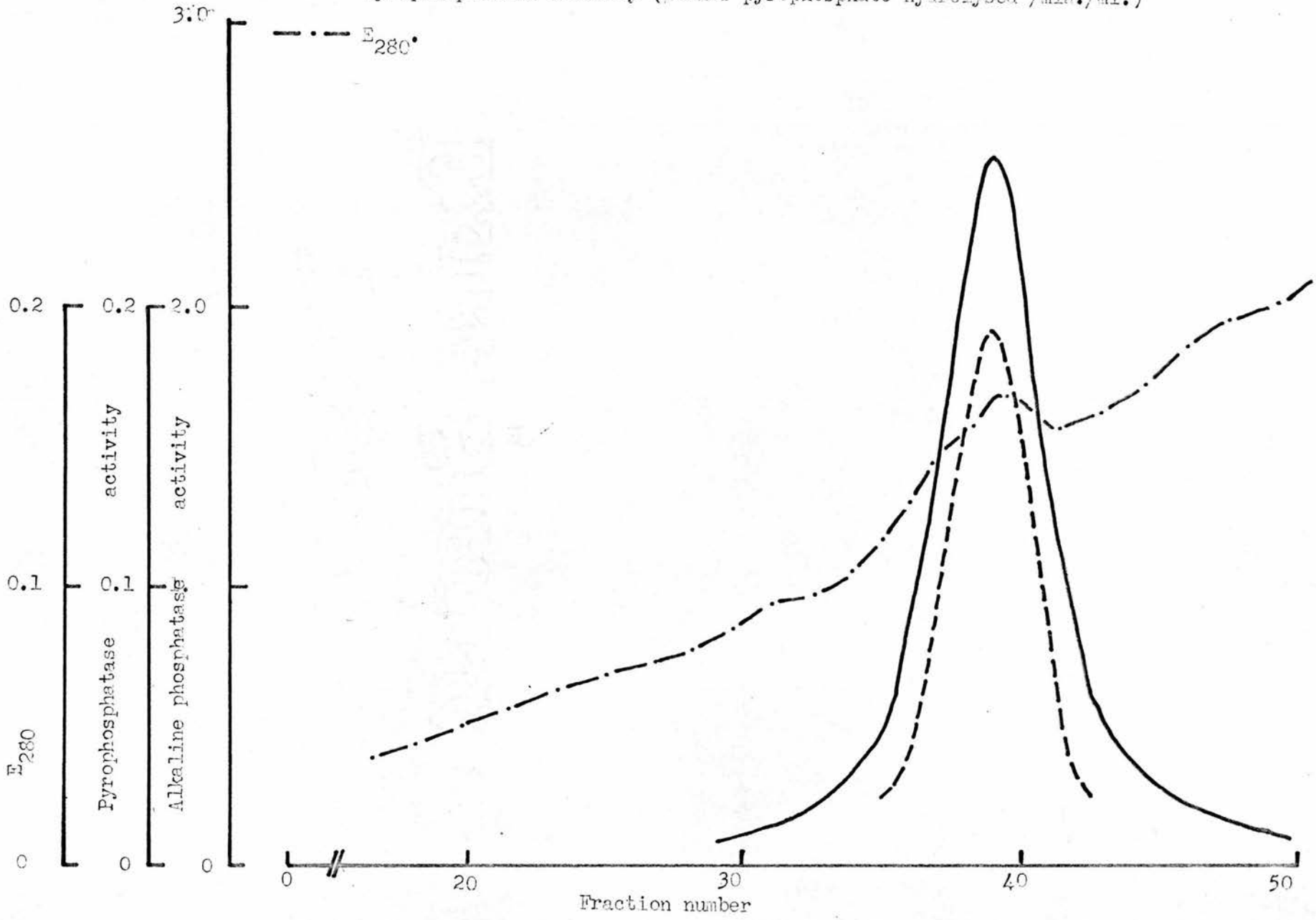


Fig.2.5. Anion-exchange chromatography (DEAE-cellulose, pH7.7) of intestinal extract. Fraction volume: 15ml. ——— Alkaline phosphatase activity (μmoles pNPP hydrolysed /min./ml.); — — — Pyrophosphatase activity (μmoles pyrophosphate hydrolysed /min./ml.); —·— E_{280}.

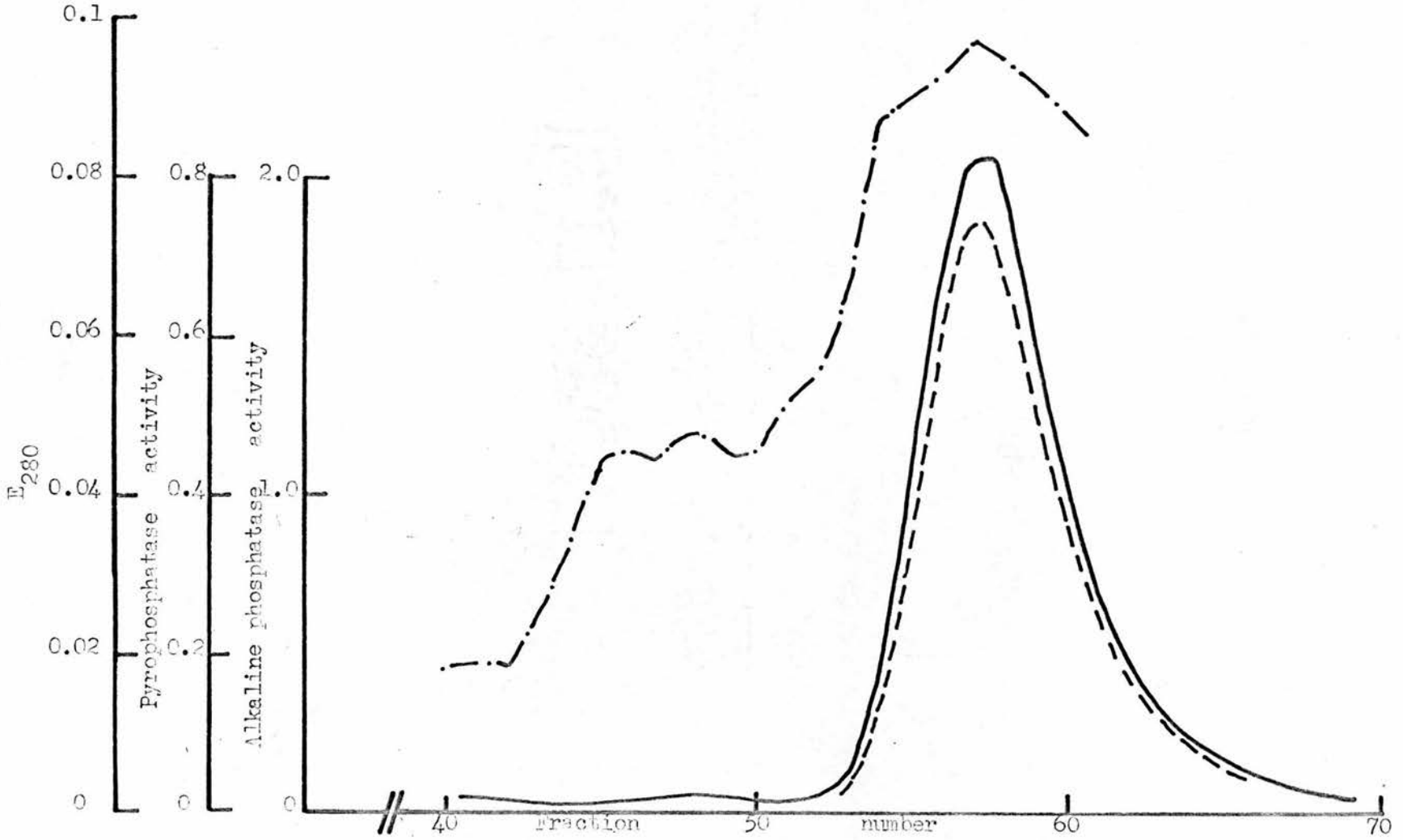
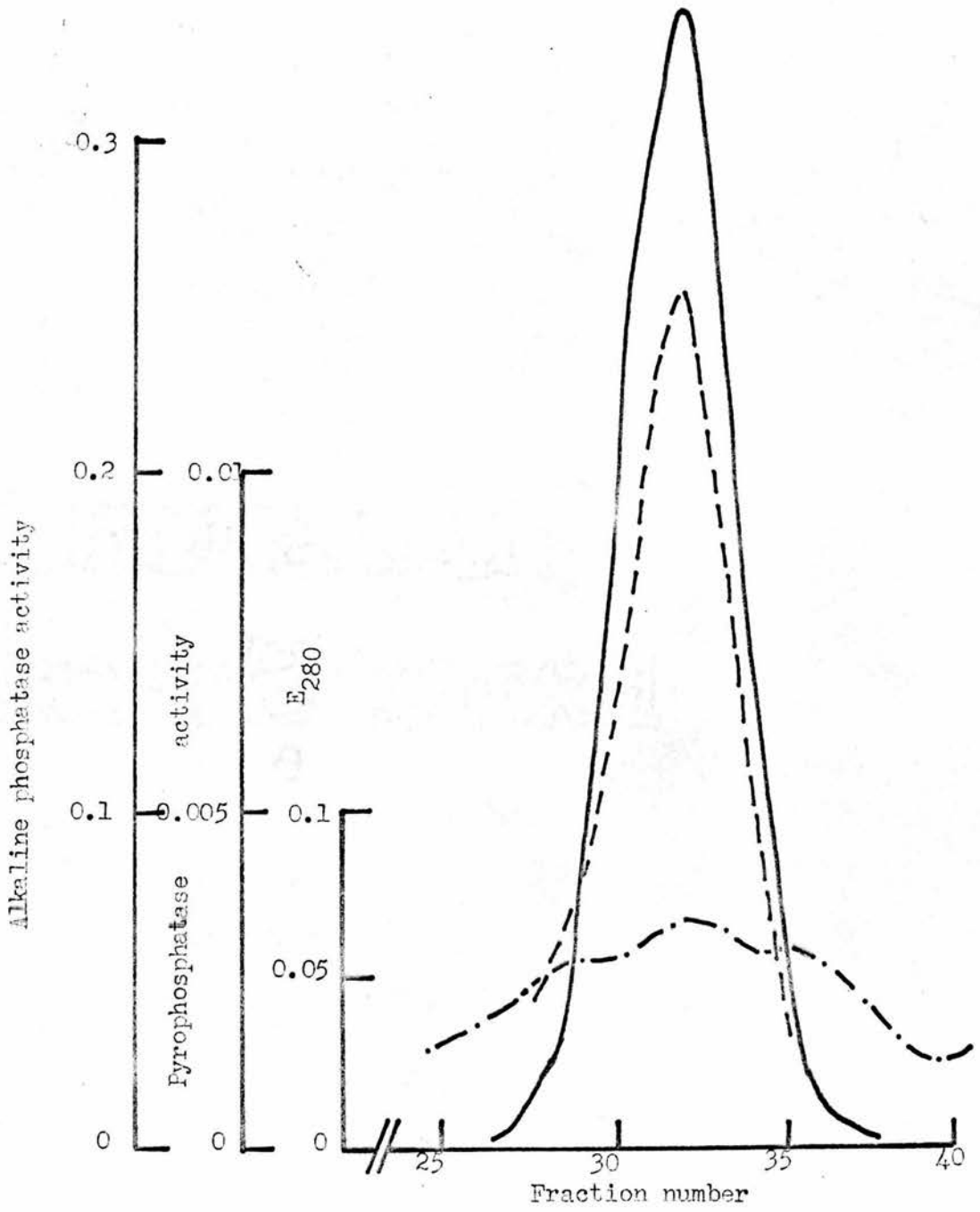


Fig.2.6. Anion-exchange chromatography (DEAE-cellulose, pH7.7) of bone extract. Fraction volume :10.5ml. ——— Alkaline phosphatase activity; (μmoles pNPP hydrolysed /min./ml.); — — — Pyrophosphatase activity (μmoles pyrophosphate hydrolysed/ min./ml.); —·— E_{280}.



2.2.4. Recycling chromatography.

Two substances which differ only slightly in molecular size may be separated either by gel filtration through a very long column or by cycling repeatedly them through a shorter column. The latter method was used after anion-exchange chromatography in an attempt to separate the phosphatases from proteins of similar size, which had eluted very close to the enzyme on the first gel filtration.

Two columns, 35 x 2.5cm. and 90 x 3.0cm. were packed with Sephadex G-200. The concentrated enzyme solution from anion-exchange chromatography was pumped through the small column; then the fraction containing the enzyme (identified by the E_{254} monitor trace on the basis of a trial passage through the column) was diverted to the base of the larger column. The enzyme was cycled twice through the large column and the fractions were collected as before (Figs. 2.7 and 2.8): the progress of the enzyme through the column being monitored on a second absorptiometer. If more than two cycles were attempted the fast fraction overtook the slow fraction.

Considerable dilution occurs during recycling chromatography and, since the total activity of the bone phosphatase was small, recycling chromatography was only applied to the liver and intestinal phosphatases. The increase in the specific activity with recycling gel filtration was three-fold.

A summary of a typical purification for each tissue is shown in Table 2.1. At each stage the enzymes were submitted to

Fig.2.7. Recycling gel filtration (Sephadex G-200) of liver extract. Fraction volume: 10ml. ——— Alkaline phosphatase activity (μmoles pNPP hydrolysed /min./ml.); —·— E_{280}.

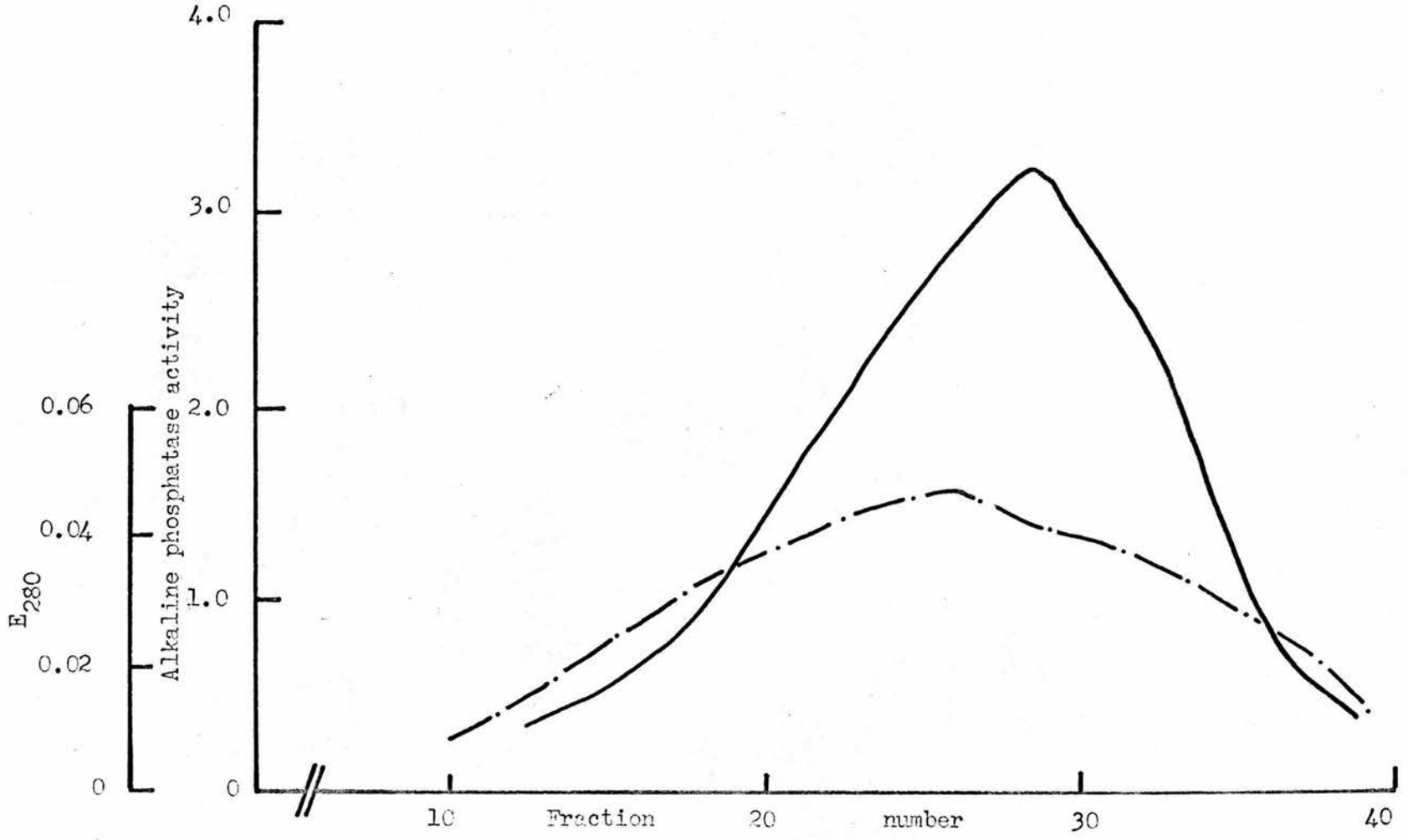
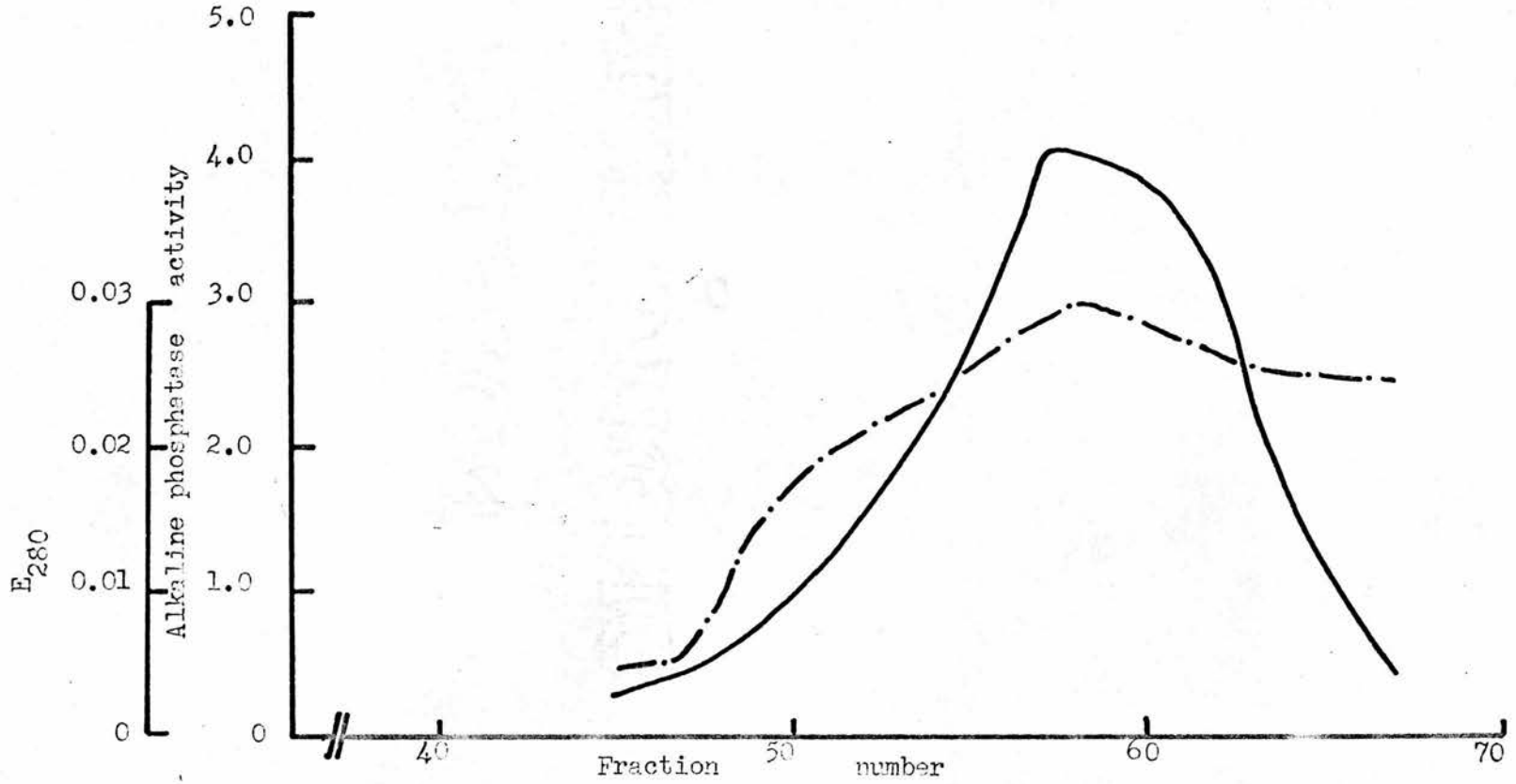


Fig.2.8. Recycling gel filtration (Sephadex G-200) of intestinal extract. Fraction volume: 10ml. ——— Alkaline phosphatase activity (μmoles pNPP hydrolysed /min./ml.); —·— E_{280}.



starch-gel electrophoresis.

Table 2.1. Summary of Purification of Human Alkaline Phosphatases.

Phosphatase activity is expressed as μmoles pNPP hydrolysed per min. Specific Activity as μmoles pNPP hydrolysed per min. per mg. protein. Results given are for individual procedures starting with 7.5 kg. liver, 8 kg. small intestine and 570 g. bone. With bone, after the ammonium sulphate fractionation 1/5 of the total activity was taken through the next two stages. Allowance is made for this in the calculation of yield.

a) Liver:

	Total Enzyme Activity	Total "Protein"mg.	Specific Activity	Overall Yield %	Purification Factor
Original Extract	19,700	291,000	0.068	-	-
Acetone 33-43% (v/v)	17,200	24,300	0.709	87	10
$(NH_4)_2SO_4$ 50-70% sat.	13,300	10,700	1.24	68	18
Gel Filtration	7,750	535	14.5	39	213
Anion Exchange Chromatography	3,640	72.5	50.2	18	738
Recycling Gel Filtration	1,460	9.9	148	7.4	2175

Table 2.1. (Contd.)

b) Intestine:

	Total Enzyme Activity	Total "Protein"mg.	Specific Activity	Overall Yield %	Purification Factor
Original Extract	18,000	212,000	0.085	-	-
Acetone 33-50% (v/v)	16,500	62,500	0.26	92	3
$(NH_4)_2SO_4$ 40-70% sat.	10,400	11,820	0.88	58	10
Gel Filtration	6,160	576	10.7	34	126
Anion Exchange Chromatography	2,726	84	32.4	15	381
Recycling Gel Filtration	1,870	21.6	86.7	10.4	1020

c) Bone:

	Total Enzyme Activity	Total "Protein"mg.	Specific Activity	Overall Yield %	Purification Factor
Original Extract	536	43,900	0.012	-	-
Acetone 33-50% (v/v)	556	6,070	0.092	100	8
$(NH_4)_2SO_4$ 25-100% sat.	400	2,670	0.15	75	12
Gel Filtration	62	43	1.44	58	120
Anion Exchange Chromatography	28	3.5	8.0	26	667

CHAPTER 3. PHYSICAL PROPERTIES OF THE ALKALINE PHOSPHATASES.

3.1. MOLECULAR WEIGHT.

This was determined by gel-filtration techniques. Andrews (1965) measured the ratio of elution volume (V_e) to void volume (V_o) for a number of proteins of known molecular weight on columns of Sephadex G-200 under different conditions and plotted the results as log. (molecular weight) against V_e/V_o. This procedure was used in the present work to determine the molecular weights of the phosphatases.

The void volume was found by measuring the elution volume of a substance which is completely excluded from the gel. "Blue Dextran" 2000 (Pharmacia, Uppsala, Sweden), which has a molecular weight of about 2×10^6, was used. Average ratios of V_e to V_o for the liver and intestinal enzymes respectively were 1.44 and 1.53. These were reproducible to within 3% when measured on different columns and with different batches of Sephadex and correspond to molecular weights of 220,000 and 195,000 respectively (Andrews, 1965). For the bone phosphatase an average ratio of 1.54 was found with the same column and batch of Sephadex. The molecular weight of the bone phosphatase is, therefore, about 200,000. Although the enzymes eluted as broad peaks this did not represent a variation in molecular size, since concentration of the extremes of the peak and a second filtration on a smaller column gave similar peaks of enzyme activity with the same elution ratios.

3.2. MOBILITY UNDER STARCH-GEL ELECTROPHORESIS.

The method of starch-gel electrophoresis was based on

that of Smithies (1955). Perspex trays (10 x 17cm.) were used, with a removable glass plate at the bottom to facilitate removal of the gel after electrophoresis. Gel thickness was about 0.9cm. 150 ml. gel was prepared by the method of Smithies (1955) using hydrolysed starch (Connaught Medical Research Lab.) in tris-citrate buffer, pH 8.6 (Poulik, 1957). Contact with the electrode buffer (concentrated boric acid - NaOH buffer, pH 8.6; Smithies, 1955), was via four thicknesses of Whatman 3MM paper. The gel was cooled to 4° and four samples were applied on pieces of 3MM paper inserted into 1cm. slots about 1-2cm. from one end of the tray. Latterly, larger trays ($16\frac{1}{2}$ x 18cm.), which required 300 ml. gel, were used to permit up to eight samples to be run in parallel. The gels were run horizontally with the samples towards the end nearer the cathode. The applied voltage was about 150 volts for the smaller trays and 250 volts for the larger for 18 hours at 4°. A current of 10-20 mA and 30-40 mA, respectively developed.

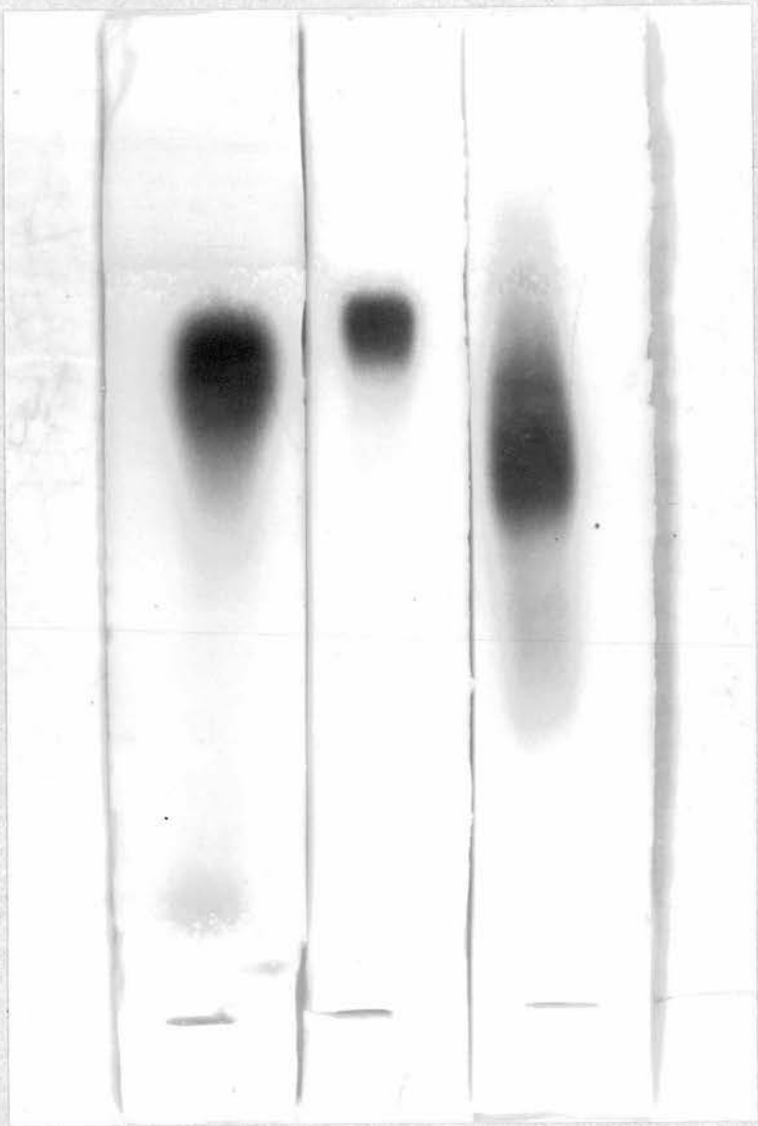
After electrophoresis the gel was sliced horizontally. Since there is distortion of the protein zones at the surfaces of the gel, the alkaline phosphatase activity was located on the cut surfaces of the gel, using the method of Estborn (1959). A solution of about 10 mg. sodium α-naphthyl phosphate in 10 ml. carbonate-bicarbonate buffer, pH 9.9 (Delory and King, 1945) containing 10 mM $MgCl_2$ was poured over the gel surface. The progress of the reaction in the bands of enzyme activity in the gel was observed by the appearance of α-naphthol, which gave a pale blue fluorescence in ultra-violet light.

After 5-60 mins., depending on the enzymic activity the gel was washed with an aqueous solution of tetrazotised o-dianisidine, which couples with the α-naphthol to give an insoluble purple complex. Finally the gel was placed in a wash solution (water: acetic acid: methanol = 5:1:4 v/v) to terminate the coupling reaction and dehydrate the gel.

A modification of the "direct" method of Allen and Hyncik (1963) was used to locate the position of enzyme activity towards inorganic pyrophosphate and orthophosphates whose organic moieties neither fluoresce nor couple with diazonium salts. The gel was incubated for up to 60 mins. at 37° in 0.1M tris-HCl buffer containing 5mM substrate and 3mM lead nitrate; the pH of the buffer was the optimum for the substrate used. The inorganic phosphate released from the substrate formed lead phosphate. After washing thoroughly with tap water for about 1 hour, the lead phosphate was converted to black lead sulphide by placing the gel in 5% ammonium sulphide solution for 1-2 mins. The excess sulphide was removed by washing with water and the gel placed in a wash solution (water: acetic acid: methanol = 5:1:4 v/v). This method was considerably less sensitive than the method of Estborn (1959), and the black zones representing enzymic activity faded in a few days even when the gels were stored in the dark.

The results of a typical electrophoresis of the phosphatases is shown in Fig. 3.1. Liver extracts at the ammonium sulphate stage of purification showed two bands; in 18 hours the leading edge of the major band migrated about ¾ of the distance from

Fig. 3.1. Alkaline phosphatase zones after starch-gel electrophoresis at pH 8.6.(B) Bone preparation at the ammonium sulphate stage of purification; (L) liver and (I) intestine preparations after gel-filtration. Anodes are at the top and the sample insertion slots near the bottom.



B L I

the point of insertion of the sample to the front, visible as a yellow line in Poulik's (1957) discontinuous buffer system. The minor band only migrated a short distance towards the anode. This less mobile fraction was readily separable from the main enzyme fraction by gel filtration and electrophoresis of liver extract beyond this stage only showed the main band.

Bone alkaline phosphatase at the ammonium sulphate stage of purification also had more than one fraction on starch-gel electrophoresis. The major fraction was generally a broader zone of activity than that of the liver enzyme and the leading edge of this zone migrated slightly more slowly than the front of the main liver phosphatase zone (Fig. 3.1.). Again a minor fraction near the origin was seen when the sample had not previously been submitted to gel filtration. A third zone of activity which was less mobile than the major fraction sometimes occurred instead of the major zone, generally when the enzyme solution had been stored for some time. This was considered to be the result of a loss of part of the bone phosphatase molecule or a change in it which did not affect the enzymic activity.

The major zone of activity on electrophoresis of the intestinal phosphatase had a mobility of about 80% of the mobility of the liver enzyme (Fig. 3.1.). The minor band was less intense and had a lower mobility than the corresponding liver zone.

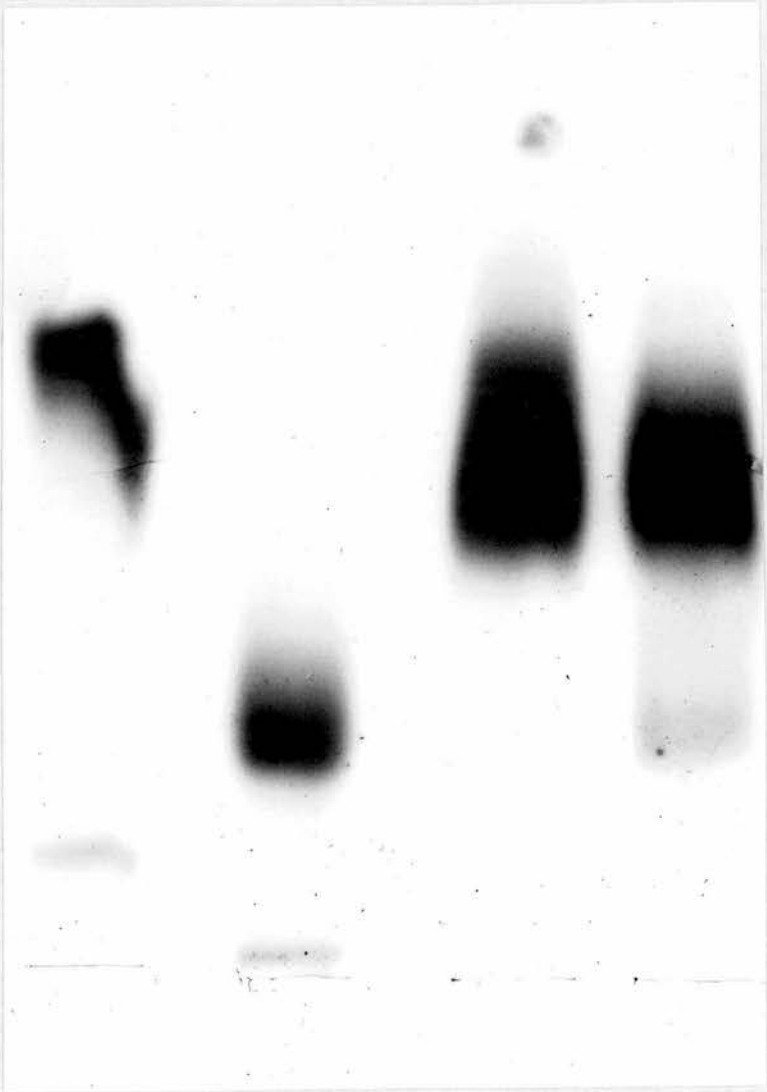
3.3. REACTION TO NEURAMINIDASE.

0.1 ml. of each phosphatase-containing tissue extract was

incubated separately with 0.4 ml. neuraminidase solution (from Clostridium perfringens: Sigma Chemical Co. Ltd., type V: 1mg./ml. in tris-HCl buffer, pH 7.7) for 24 hours at 37°. Control tubes containing 0.4 ml. buffer and 0.1 ml. phosphatase extract were also incubated. Measurement of alkaline phosphatase activity in the control and in the test tubes after 24 hours showed no loss of activity due to the action of the neuraminidase. The treated and untreated enzyme solutions were submitted to starch-gel electrophoresis and the zones of activity were located as described above.

Both the major zone of the liver enzyme and the minor fraction near the origin were retarded after neuraminidase treatment (Fig. 3.2.). The mobility of the bone enzyme was also reduced. The position of the bone phosphatase after neuraminidase treatment was slightly cathodic to the liver phosphatase after neuraminidase treatment. On the other hand, the mobility of almost all of the intestinal enzyme was unaffected; only a small fraction of the main band appearing to be sensitive to neuraminidase (Fig. 3.2.b). Partial purification of the tissue extracts by gel filtration did not affect their sensitivity or insensitivity to neuraminidase. To the neuraminidase solution, which had been incubated with intestinal enzyme for 24 hours, some liver extract was added and this was then incubated for a further 24 hours. Starch-gel electrophoresis showed two zones of enzyme activity, one in the position of the intestinal enzyme and the other in the position of the neuraminidase treated

Fig. 3.2. Comparison of alkaline phosphatase zone of liver and small intestine on starch-gel electrophoresis at pH 8.6, showing the effect of incubation with neuraminidase. (a) liver extract; (b) small-intestinal extract. In each case the sample incubated with neuraminidase is on the right and the control incubated without neuraminidase on the left. The sample slots are near the bottom and the anode at the top.



(a) (b)

liver enzyme. The lack of effect of neuraminidase on the intestinal enzyme cannot, therefore, be due to inactivation of the neuraminidase or to inhibitory factors in the intestinal extract.

Incubation with muramidase (E.C. 3.2.1.17.) and hyaluronidase, enzymes which also hydrolyse mucoproteins, produced no effect on the electrophoretic mobility of the phosphatases.

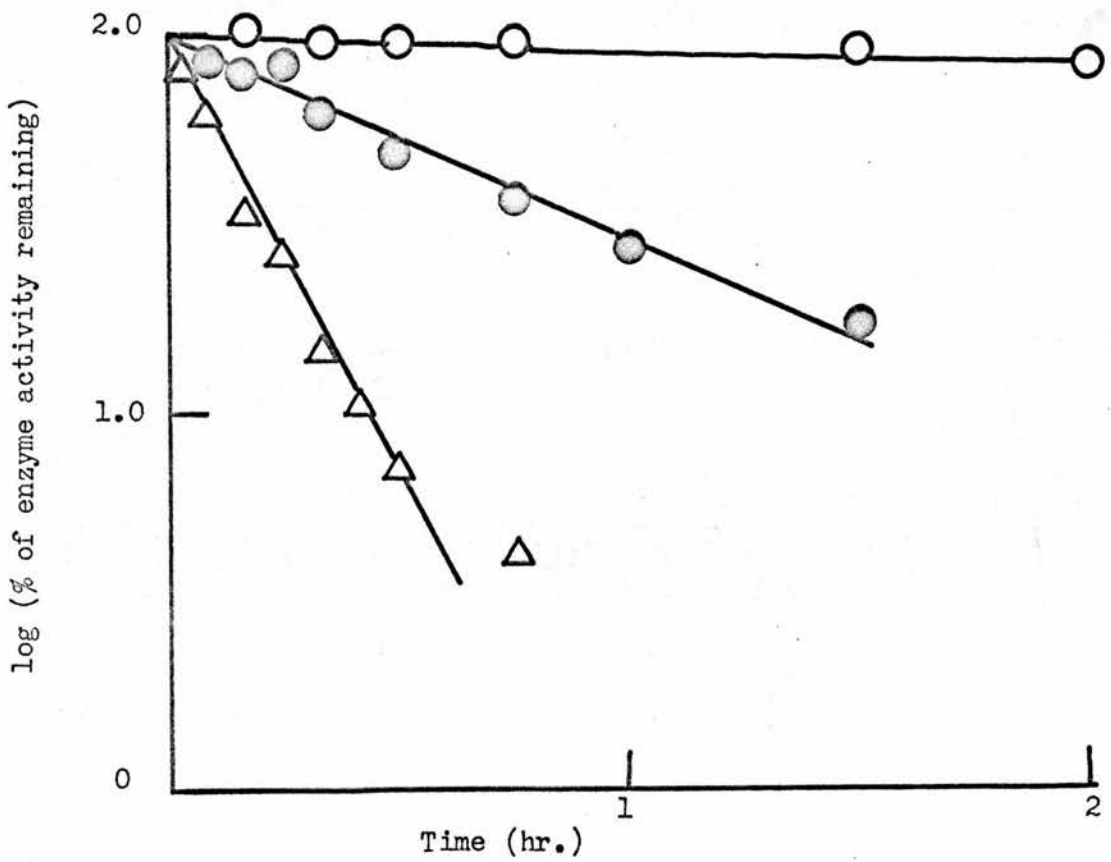
3.4. INACTIVATION BY VARIOUS REAGENTS.

3.4.1. Inactivation by heat.

A small quantity of tissue extract in dilute tris-HCl buffer, pH 7.7. was heated at 54°-55°. Aliquots were removed before heating and at intervals throughout the inactivation procedure and assayed at pH 9.9. The rates of inactivation may be expressed as the half-life of the enzyme, i.e., the time taken for the activity of the enzyme to fall to 50% of the original value. Half-lives of 5, 20 and greater than 90 mins. were recorded for the bone, liver and intestinal phosphatases respectively (Fig. 3.3.). The rate of inactivation is strongly dependent upon temperature. The half-life for the bone enzyme varied between 5 and 9 mins. and for the liver enzyme from 20 to 40 mins. in different experiments. An attempt was made to identify other factors which affected the rates of inactivation. The addition of 2% albumin, 0.1mM Zn^{2+} or 5mM Mg^{2+} had no effect on the stability of the phosphatases.

When mixtures of two of the isoenzymes were inactivated, a definite break occurred in the plots of log (% of enzyme

Fig.3.3. Inactivation of intestinal, liver and bone phosphatases during incubation at 55° and pH 7.7. Substrate: p-nitrophenyl phosphate. ○ intestinal phosphatase; ◎ liver phosphatase; △ bone phosphatase.



activity remaining) against time (Fig. 3.4. and 3.5.). The first part of the curve represents the inactivation of the thermo-labile bone phosphatase and a small portion of the more thermo-stable liver or intestinal enzymes. The inflection occurs when all the bone enzyme has been inactivated. These experiments indicate that the observed differences in stability are properties of the enzymes themselves, and not effects due to differences in the composition of their solutions.

3.4.2. Inactivation by urea.

Inactivation of the phosphatases by urea was carried out in freshly prepared urea solutions in 0.01M tris-HCl buffer, pH 7.7. The residual activity was determined at pH 9.9 as before. Urea in the assay mixture causes inhibition of the enzyme activity. However, the amount of enzyme removed for assay (generally 20 µl) was sufficiently diluted in the assay system (2 ml.) for the effect to be negligible even when the original urea concentration was high. The rate of inactivation of the phosphatases is dependent on the urea concentration (Fig. 3.6.) and on the temperature of inactivation (Fig. 3.7.). As with heat inactivation, the enzyme from bone tissue is the most sensitive to urea and that from the intestine the least (Fig. 3.8. and Table 3.1.).

Fig.3.4. Inactivation of mixtures of intestinal and bone phosphatases during incubation at 54° and pH 7.7. Substrate: p-nitrophenyl phosphate. ○ 100% intestinal phosphatase; △ 75% intestinal and 25% bone phosphatases; ◎ 50% intestinal and 50% bone phosphatases; □ 25% intestinal and 75% bone phosphatases; △ 100% bone phosphatase.

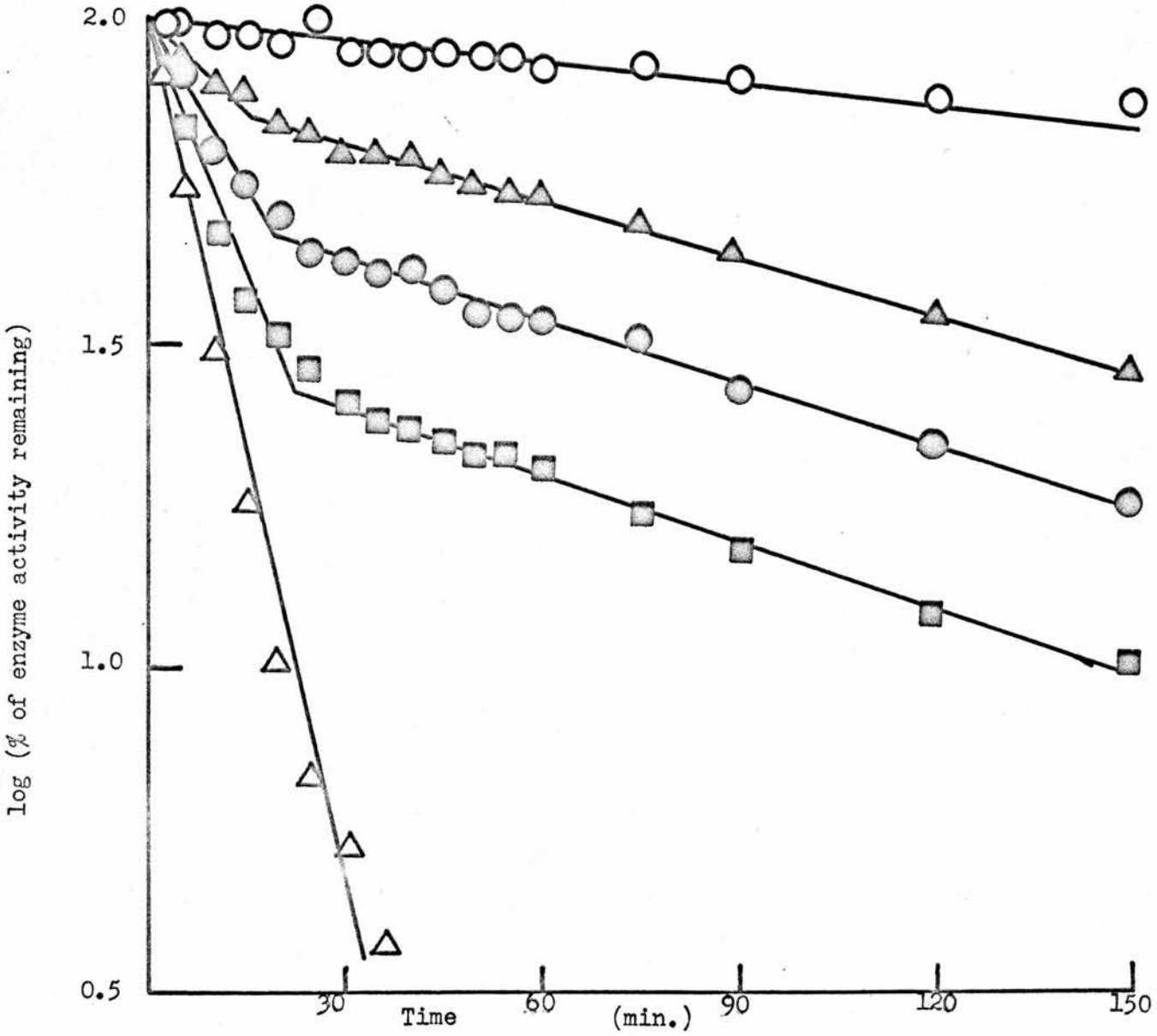


Fig.3.5. Inactivation of mixtures of liver and bone phosphatases during incubation at 55° and pH 7.7. Substrate: p-nitrophenyl phosphate.

○ 100% liver phosphatase; ▲ 75% liver and 25% bone phosphatases;

◍ 25% liver and 75% bone phosphatases; △ 100% bone phosphatase.

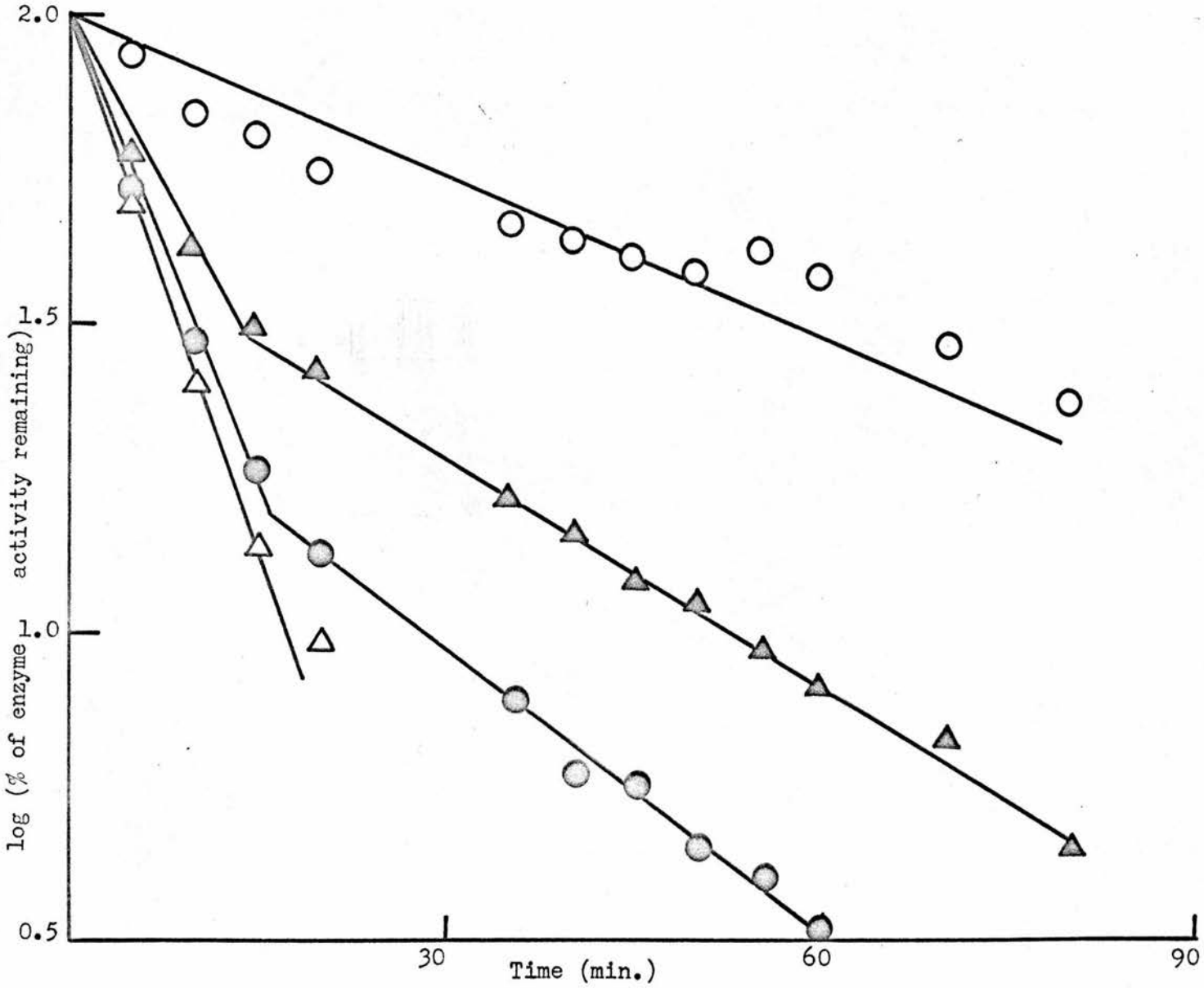


Fig.3.6. Inactivation of bone (○) and liver (◎) alkaline phosphatase in freshly prepared urea solutions at 20°C and pH 7.7. Urea concentration : (a) 3M ; (b) 5M ; (c) 6M. Residual activity was determined at pH 9.9.

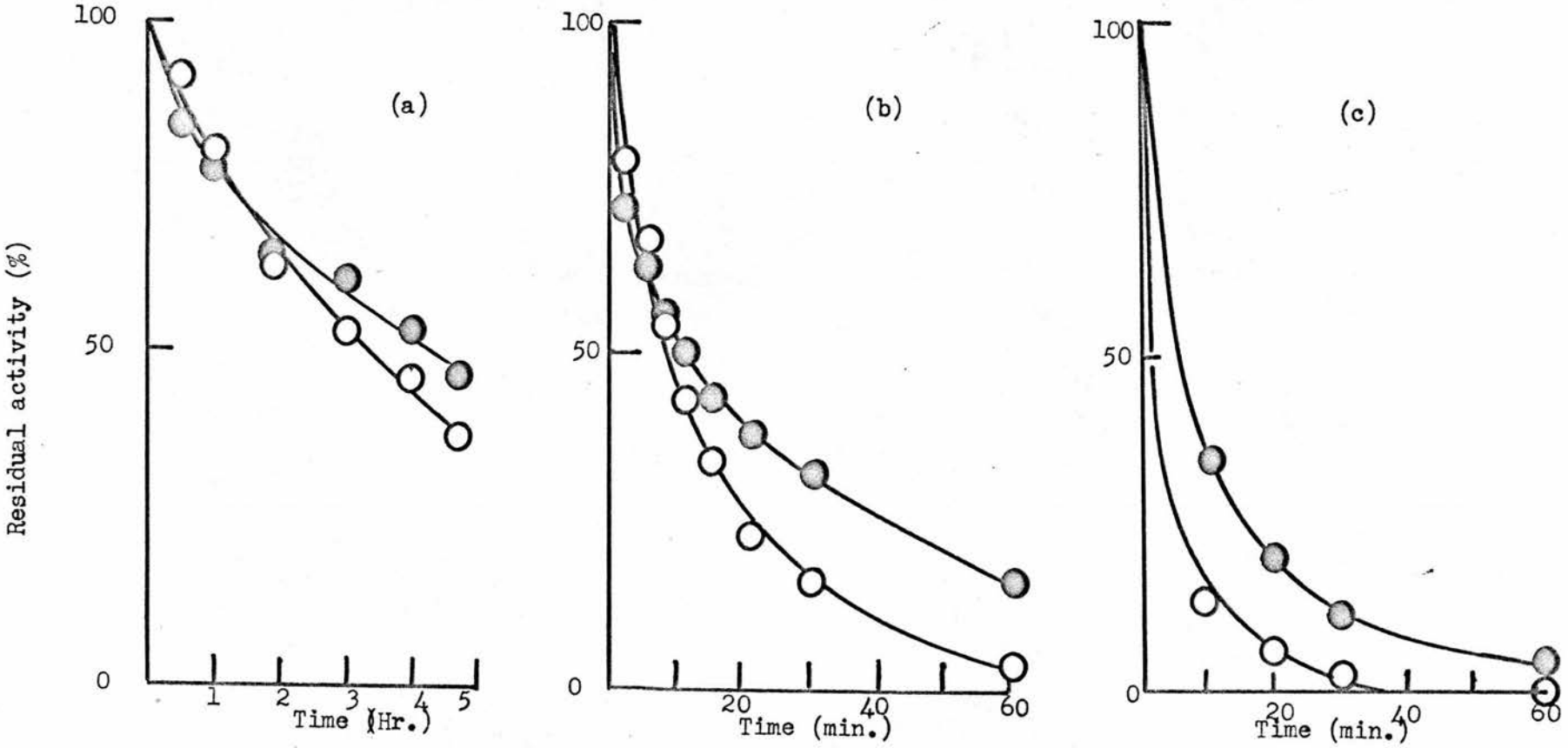


Fig.3.7. Inactivation of intestinal alkaline phosphatase in freshly prepared urea solutions at pH 7.7 and various temperatures. At ○ 0°C; ◐ 20°C; △ 37°C. Residual activity was determined at pH 9.9.

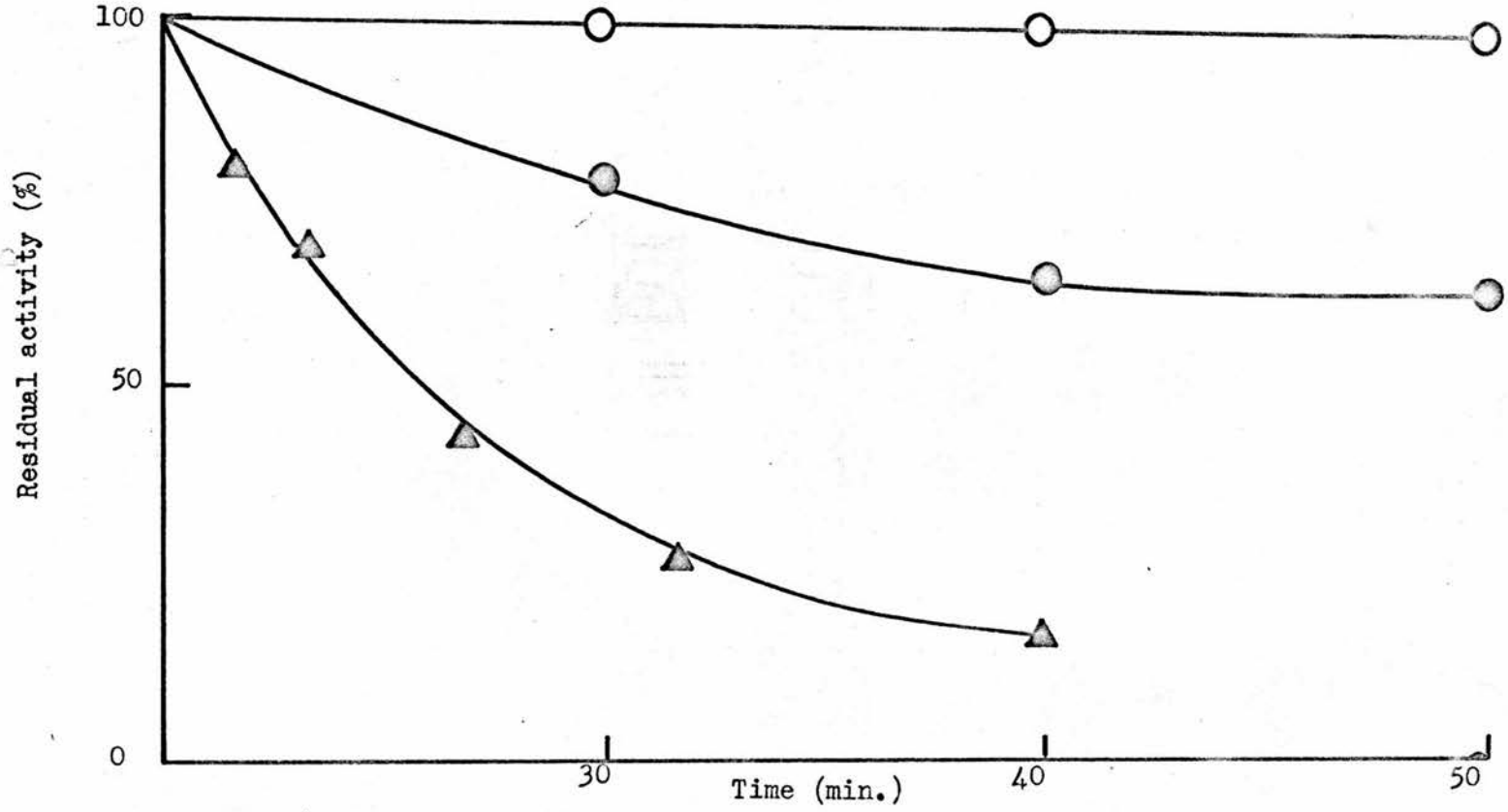


Fig.3.8. Inactivation of liver, bone and intestinal alkaline phosphatases in freshly prepared urea solutions (6M) at 20°C and pH 7.7. ○ Intestinal phosphatase; △ liver phosphatase; □ bone phosphatase. Residual activity was determined at pH 9.9.

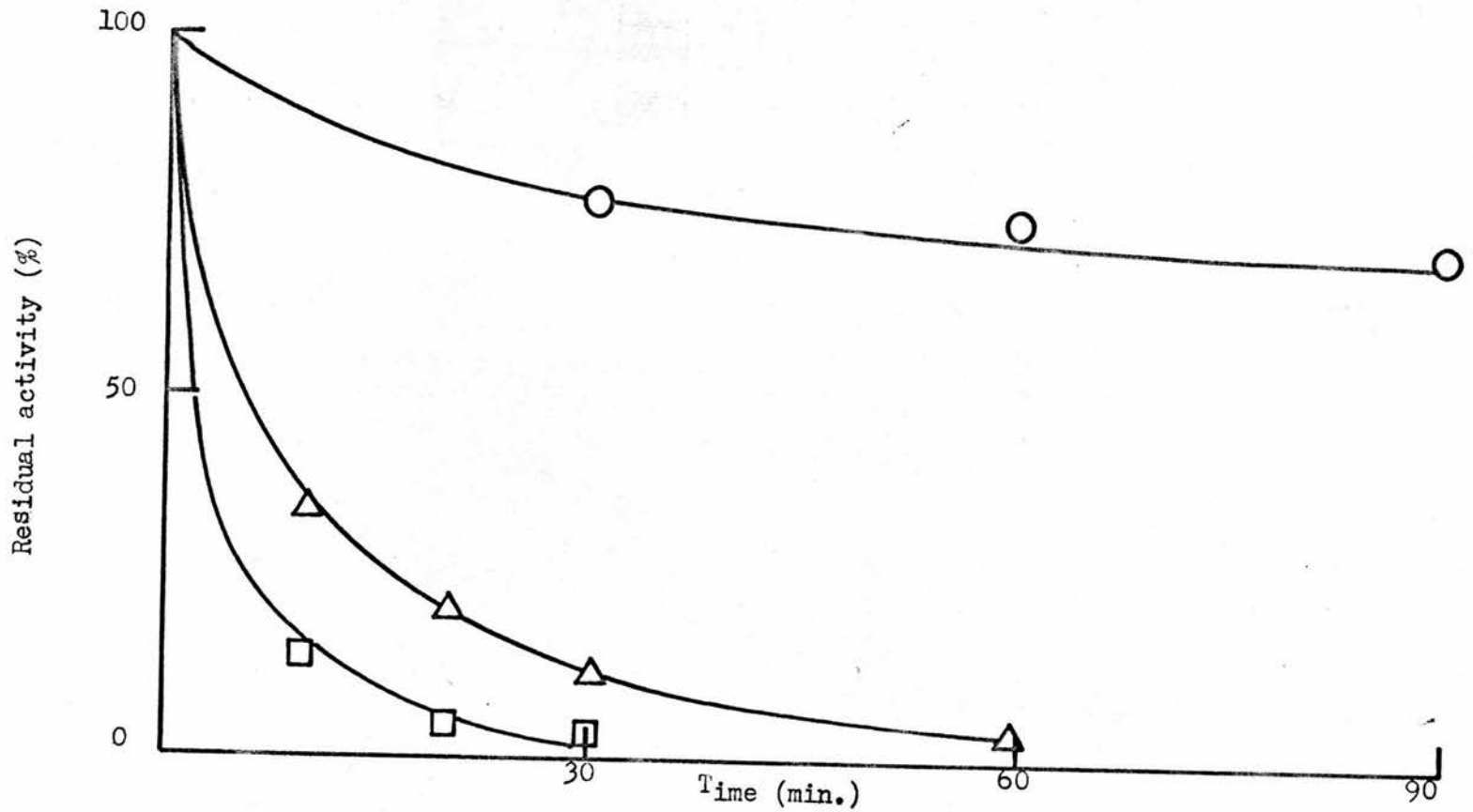


Table 3.1. Half-lives of the Phosphatases in Urea Solutions.

Urea Concentration (M)	Temperature of inactivation	Half-lives		
		Liver Enzyme	Bone Enzyme	Intestinal Enzyme
6	20°	6 mins.	4 mins.	90 mins.
6	37°	about ½ min.	-	19 mins.
5	20°	11 mins.	9 mins.	-
4	25°	48 mins.	-	> 19 hours.
3	20°	about 4½ hours	about 3¼ hours	-

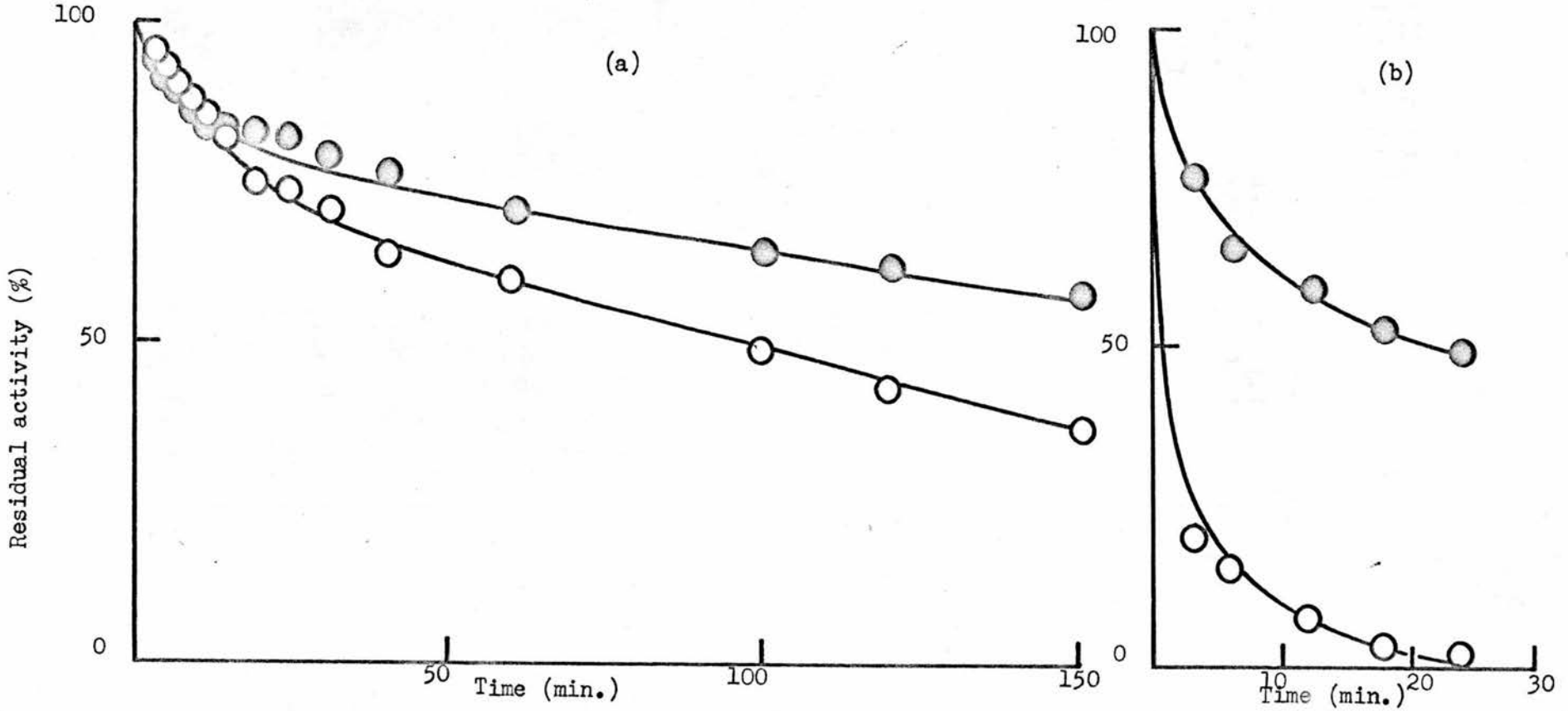
3.4.3. Inactivation by acid.

The liver and bone phosphatases were inactivated at 0° in succinate - NaOH buffer, pH 4.4 and in maleate - tris buffer, pH 3.5. The residual activity was measured at pH 9.9. The bone enzyme was more sensitive to low pH than the liver enzyme (Fig. 3.9.). Scutt and Moss (1968) found the liver phosphatase to be more readily inactivated by acid than the intestinal enzyme.

3.5. SUMMARY OF FINDINGS.

The phosphatases from liver, intestine and bone differed in their molecular weights and in their mobility under starch-gel electrophoresis. The alkaline phosphatases from bone and liver are sensitive to neuraminidase while the intestinal

Fig.3.9. Inactivation of bone (○) and liver (●) alkaline phosphatase at (a) pH 4.4, 0°C (0.2M succinate- NaOH buffer) and (b) pH 3.5, 0°C (0.05M maleate-tris buffer). Residual activity was determined at pH 9.9.



enzyme is unaffected by treatment with neuraminadase. The order of increasing rate of inactivation by urea and by heat is intestinal phosphatase, liver phosphatase and bone phosphatase.

CHAPTER 4. ENZYMIC PROPERTIES OF ALKALINE PHOSPHATASE.

4.1. SUBSTRATE SPECIFICITY.

Inorganic pyrophosphatase and organic pyrophosphatase activities were found in preparations of alkaline phosphatase from liver, bone and small intestine at all stages of purification. Since it has frequently been denied that alkaline phosphatases have pyrophosphatase activity (e.g. Morton, 1955) and has instead been contended that any such activity was to contamination, it was necessary to prove that the two activities were properties of the same enzyme protein.

4.1.1. Inorganic Pyrophosphate as a Substrate of Alkaline Phosphatase.

Pyrophosphatase activity was measured as follows. A small quantity of enzyme solution (20-100µl.) was added to 1ml. buffer-substrate mixture at 37°, containing 3.3mM sodium pyrophosphate and 1mM magnesium chloride in 0.1M tris-HCl buffer, pH 8.5 (Cox and Griffin, 1965). After incubation at 37° the reaction was terminated by the addition of acetate buffer, pH 4.0, and the liberated inorganic phosphate measured by the method of Delsal and Manhouri (1958). The activity is expressed as µmoles substrate hydrolysed/min./ml. enzyme solution. Orthophosphatase activity was estimated as described in Chapter two.

Ratio of Activities: The ratio of orthophosphatase activity to pyrophosphatase activity for the liver enzyme was 15:1 in the crude extract, and about 12:1 with the most highly purified preparations. This variation could be accounted for as the

effect of metal ions or other non-specific inhibitors present in the crude extract but removed as the purification proceeded. The ratio for the intestinal and bone phosphatases remained constant throughout the purification at 3:1 and about 20:1 respectively.

When the pyrophosphatase activity was measured in the effluent from the gel filtration and anion-exchange chromatography stages of purification, the orthophosphatase and pyrophosphatase activities had coincident peaks; furthermore, the ratio of the two activities remained constant across the peaks for the two enzymatic activities from each of the three tissues (Fig. 2.1. - 2.6.). Examination of fractions of the effluents from anion-exchange chromatography of the liver enzyme at pH 9.0 (0.01M tris-HCl) also showed coincident peaks and constant ratios for the two activities (Fig. 4.1. and 4.2.). Similarly, cation-exchange chromatography of the liver phosphatase at pH 6.0 (0.01M sodium acetate-NaOH) on CM-cellulose phosphate showed that the two activities could not be separated (Fig. 4.3.).

Evidence that Orthophosphatase and Pyrophosphatase Activities are Properties of the same Enzyme Protein: Mixed substrate experiments using purified enzyme from each tissues were carred out. Aliquots of enzyme were incubated separately with 2mM sodium pyrophosphate and with 2mM pNPP, and, in a third experiment with both 2mM pyrophosphate and 2mM pNPP simultaneously. The concentration of magnesium ions was 1mM and the buffer was 0.1M tris-HCl, pH 8.5, in each incubation. The inorganic phosphate liberated was estimated by the method of

Fig.4.1. Anion-exchange chromatography (DEAE-cellulose, pH 9.0) of liver extract. Fraction volume: 15 ml. ——— Alkaline phosphatase activity (μmoles pNPP hydrolysed /min. /ml.); — — — Pyrophosphatase activity (μmoles pyrophosphate hydrolysed /min. /ml.); —·—· E_{280}.

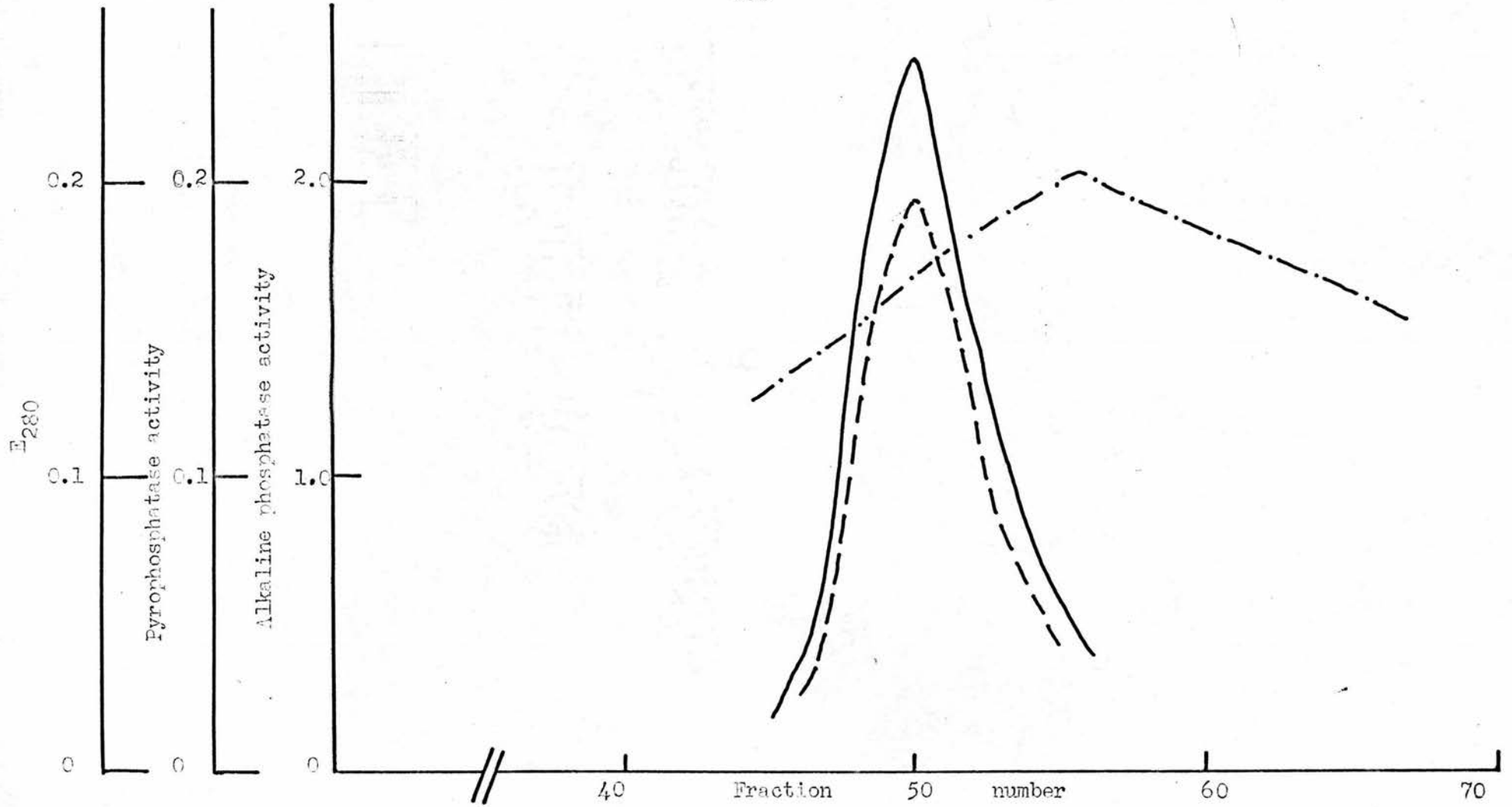


Fig.4.2. Anion-exchange chromatography (DEAE-cellulose, pH5.6) of liver extract. Fraction volume: 15ml. ——— Alkaline phosphatase activity (μmoles pNPP hydrolysed /min. /ml.); — — — Pyrophosphatase activity (μmoles pyrophosphate hydrolysed /min. /ml.); —·—· E_{280}.

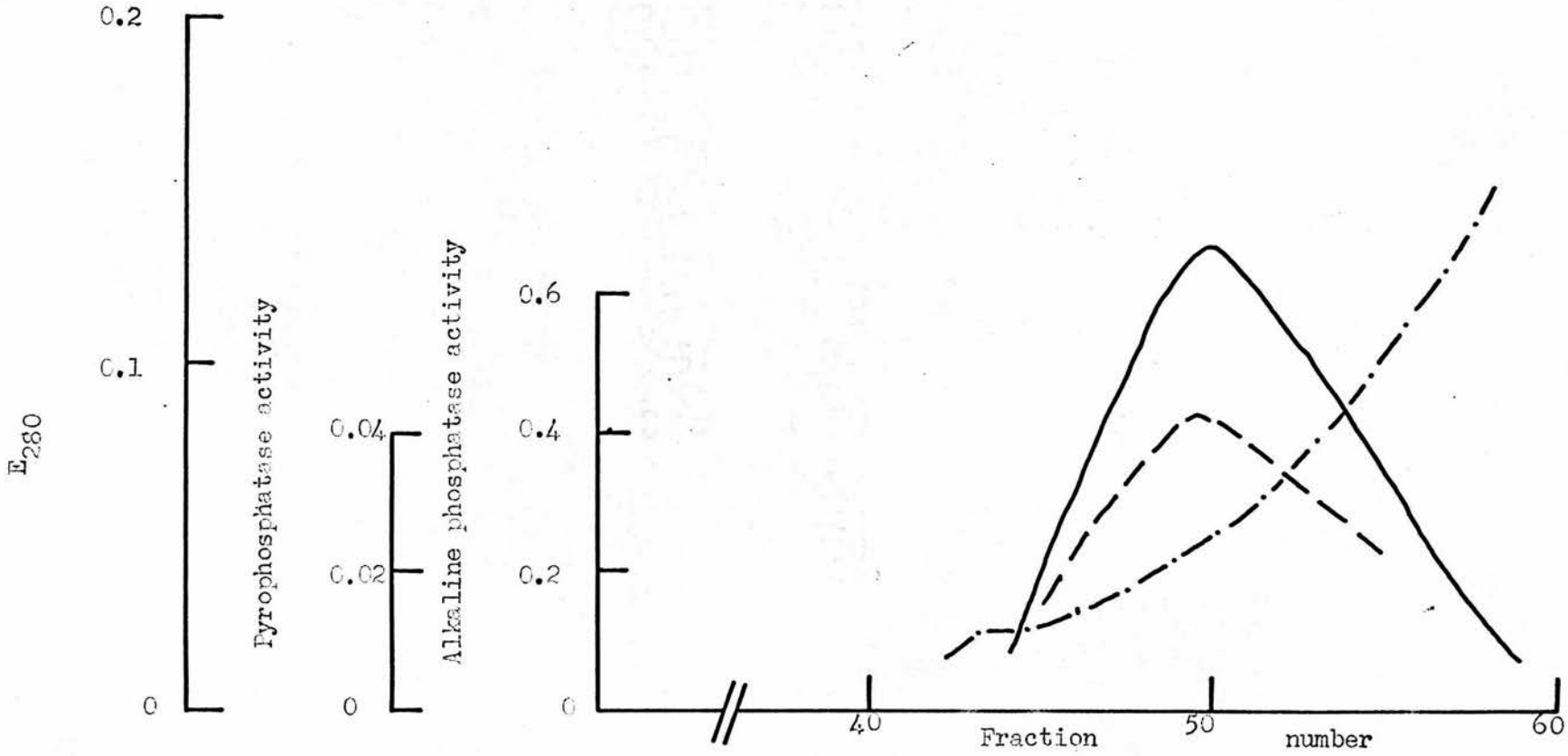
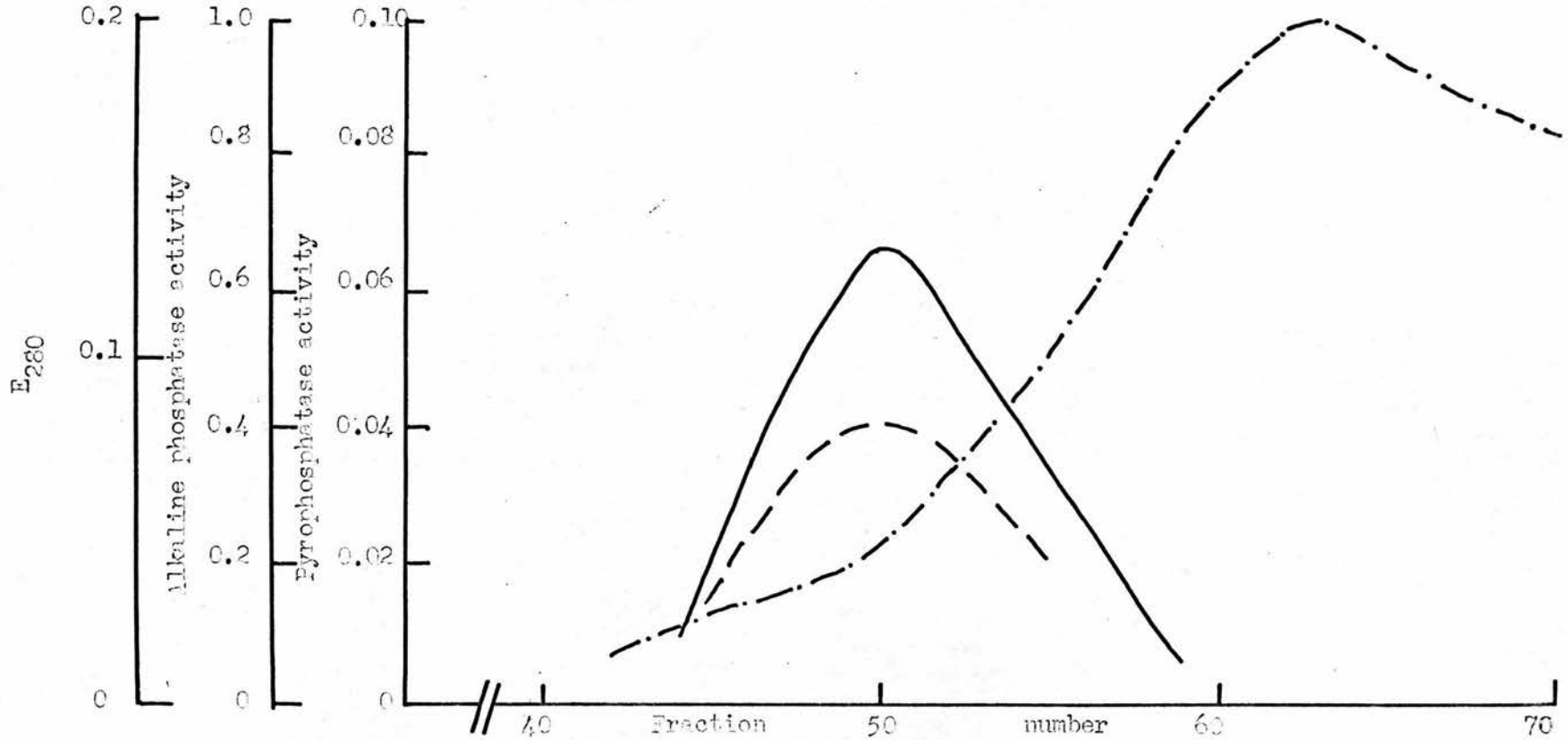


Fig.4.3. Cation-exchange chromatography (CM cellulose, pH 6.0) of liver extract. Fraction volume : 15ml. ——— Alkaline phosphatase activity (μmoles pNPP hydrolysed/min./ml.) ; — — — Pyrophosphatase activity (μmoles pyrophosphate hydrolysed/min./ml.) ; —·—· E_{280}.



Delsal and Manhouri (1958) in all incubation mixtures and, where pNPP was included, p-nitrophenol was also measured. For each enzyme the velocity of the hydrolytic reaction in the presence of both substrates together was less than the sum of the two individual velocities, showing that pyrophosphate and pNPP were not being independently hydrolysed (Table 4.1.). The pyrophosphatase activity was inhibited more than the orthophosphatase activity for the phosphatases from all three tissues.

Liver, bone and intestinal phosphatase were submitted to electrophoresis on starch-gel at pH 8.6. After electrophoresis the gel was cut horizontally; on one of the cut surfaces the orthophosphatase activity was developed with α -naphthyl phosphate and on the other surface pyrophosphatase activity was visualised using the direct method of Allen and Hyncik (1963), using inorganic pyrophosphate as the substrate in pH 8.5 buffer. The positions of the zones of the two activities were identical on the two halves of the same gel, both for the main zone of activity and for the minor zone near the origin.

In a second experiment samples of liver and intestinal phosphatase were treated with neuraminidase prior to electrophoresis. When starch-gel electrophoresis was carried out following neuraminidase treatment the orthophosphatase and pyrophosphatase activities of the liver enzyme were found to have been reduced equally in mobility, whereas the mobilities of the intestinal enzyme bands were unaffected (Fig. 4.4.).

Fig. 4.4. Sketch of a photograph showing the positions of the alkaline phosphatase zones in a liver preparation after starch-gel electrophoresis at pH 8.6. (b) and (c) have been treated with neuraminidase prior to electrophoresis. Substrates: (a) and (b) inorganic pyrophosphate; (c) and (d) ∝ -naphthyl phosphate. Anodes are at the top and the sample insertion slots at the bottom.

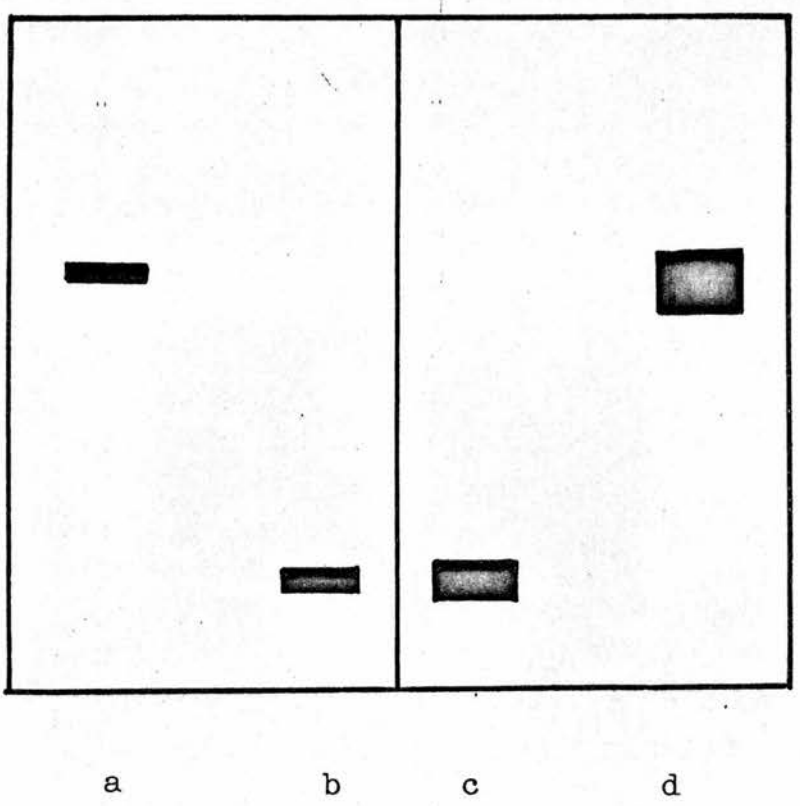


Table 4.1. Mixed-substrate Experiments with pNPP and Sodium Pyrophosphate.

Hydrolysis by purified phosphatase preparation of pNPP, sodium pyrophosphate and a mixture of the two at 37° and pH 8.5. Magnesium concentration 1mM throughout. Enzyme activities are expressed as µmoles inorganic phosphate (P_i) or pNP liberated/min.

	Liver enzyme	Intestinal enzyme	Bone enzyme
Pyrophosphate (2mM) µmoles P_i/min:	0.299	0.388	0.656
pNPP (2mM) umoles P_i/min:	0.148	0.085	0.528
µmoles pNP/min:	0.166	0.080	0.547
Pyrophosphate (2mM) +pNPP (2mM) µmoles P_i/min:	0.101	0.138	0.316
µmoles pNP/min:	0.082	0.060	0.258
Activity if hydrolysis is independent µmoles P_i/min:	0.456	0.471	1.193
Inhibition (%) of each type of activity in mixed-substrate.			
Pyrophosphatase:	94	80	91
Orthophosphatase:	48	27	52

Heat inactivation of the liver and intestinal enzymes was carried out at 55° and pH 7.7 (0.01M tris-HCl). The orthophosphatase and pyrophosphatase activities were determined at the start and at 5 or 10 minute intervals up to 45 mins. The liver enzyme was inactivated more rapidly than the intestinal phosphatase, but the ratio of orthophosphatase to pyrophosphatase activity remained constant following varying periods of heat inactivation for both enzymes (Fig. 4.5.).

In certain types of liver and biliary disease (e.g. infective hepatitis, obstructive jaundice, cirrhosis of the liver) and bone disease (e.g. Paget's disease, hyperparathyroidism) the serum alkaline phosphatase level frequently rises. Seventy-two human sera with alkaline phosphatase levels ranging from normal (3 - 13 King-Armstrong units: Varley, 1962) to about ten times the upper normal value were assayed for orthophosphatase and pyrophosphatase activity. The orthophosphatase activity was measured using pNPP as the substrate at pH 10.0 with suitable enzyme blanks to compensate for jaundiced sera, and the pyrophosphatase activity was determined at pH 8.5 by a modification of the method previously described in which the enzymic reaction was terminated by the addition of trichloroacetic acid and the liberated phosphate estimated in the supernatant. The alkaline phosphatase in each serum was classified as originating from liver or from bone tissue on the basis of other biochemical evidence and clinical data.

The orthophosphatase activity was plotted against the pyrophosphatase activity for each serum and the regression lines were calculated (Fig. 4.6.). The equation for the

Fig.4.5. Effect of incubation at 55° and pH 7.7 (tris-HCl buffer) on alkaline phosphatase and inorganic pyrophosphatase activities of purified liver and intestinal enzymes. ▲ Liver alkaline phosphatase; ▲ liver pyrophosphatase; O intestinal alkaline phosphatase; ● intestinal pyrophosphatase.

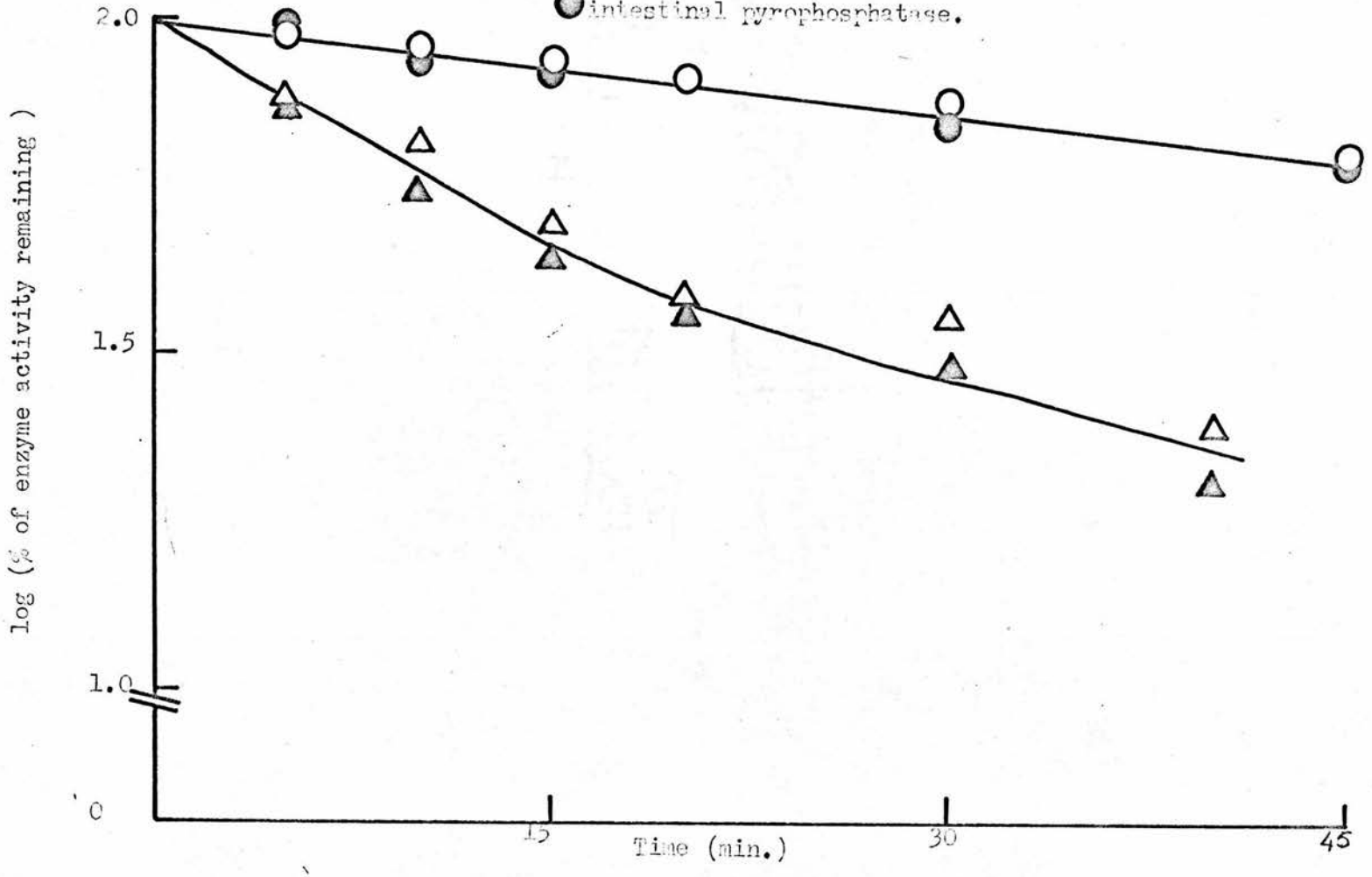
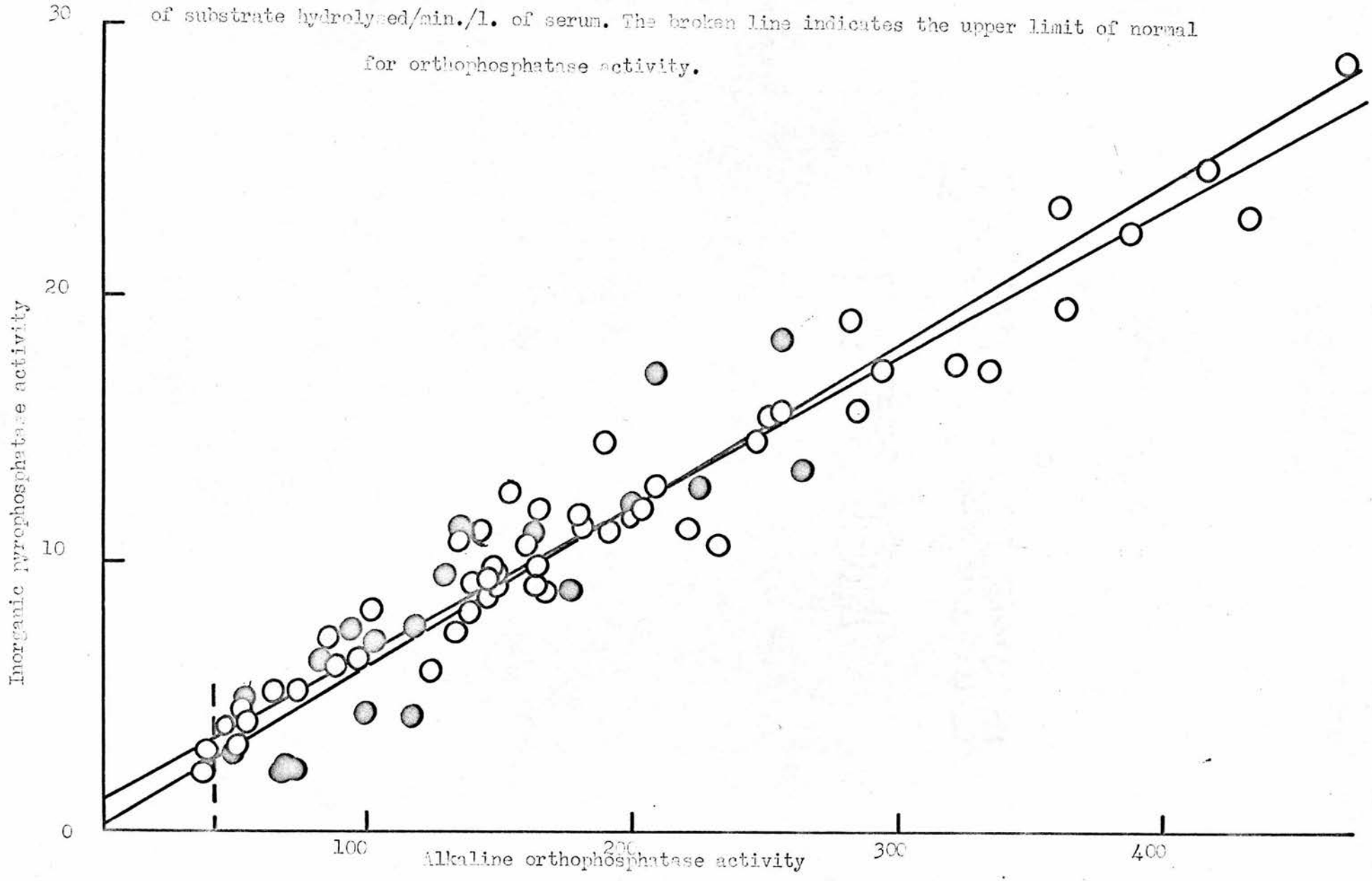


Fig.4.6. Alkaline orthophosphatase and inorganic pyrophosphatase activities in blood sera from patients with hepatobiliary (○) and bone (●) diseases. Activities are expressed as μmoles of substrate hydrolysed/min./l. of serum. The broken line indicates the upper limit of normal for orthophosphatase activity.



regression lines of inorganic pyrophosphatase on orthophosphatase (a) is y = 1.174 + 0.055x and of orthophosphatase on pyrophosphatase (b) is y = 0.354 + 0.060x. There is strong positive correlation between the levels of the two activities with a correlation coefficient of 0.96. The average ratio of orthophosphatase to pyrophosphatase activity is 15.7 to 1 and is, therefore, similar to that found for purified liver and bone phosphatases. No significant difference between the sera containing alkaline phosphatase which presumably originated from the liver, and those in which it appeared to arise from bone, could be seen.

4.1.2. Organic pyrophosphates as substrates of alkaline phosphatase.

Nucleoside phosphates were used to test the specificity of the phosphatases towards organic pyrophosphates. The derivatives of one pyrimidine base, uracil, and one purine base, adenine, were tried. Thiamine pyrophosphate (TPP), although not a nucleotide, is a compound of great physiological importance and it was also tested. In each case the monophosphates (AMP and UMP) as well as the di- and tri-phosphate esters were included for comparison, although it is well known that alkaline phosphatase will hydrolyse AMP (Morton, 1955).

Relative Rates of Hydrolysis: The relative rates of release of inorganic pyrophosphate from each substrate by the three phosphatases were measured at the pH optimum for that substrate, using 2mM concentrations of substrate both in the presence (10mM) and absence of magnesium ions. Since the rate of

spontaneous hydrolysis of the di- and tri-phosphates is relatively high, each was examined by paper chromatography in an ethanol-ammonium acetate system (Smith, 1960a) and all were found to be chromatographically homogeneous. Each enzyme preparation had been previously dialysed against dilute tris-HCl buffer, pH 7.7, to remove magnesium ions.

The relative rates of release of inorganic phosphate are given as a percentage of the activity of the enzyme towards pNPP, the reference substrate, in the presence of magnesium (Table 4.2.). The liver and bone phosphatases, unlike the intestinal enzyme, hydrolyse the monophosphates (AMP and UMP) more rapidly than the corresponding pyrophosphates (ADP etc.) in the absence of added magnesium ions. Under similar conditions the order of decreasing rates of hydrolysis for the intestinal enzyme is the diphosphate, the triphosphate and then the monophosphate for each nucleoside. If magnesium ions are added to a concentration of 10mM in the assay system, then the monophosphate is the ester most readily hydrolysed with each enzyme. The different effect of magnesium ions on the hydrolysis of the various phosphate esters will be considered further when the effects of other metal ions are being discussed.

Evidence that Orthophosphatase and Pyrophosphatase Activities are Properties of the same Enzyme Protein: Mixed-substrate experiments involving organic pyrophosphatases and orthophosphates were carried out in carbonate-bicarbonate buffer as described previously (page 61). The pH of measurement was the pH that had been found to be optimal for each substrate

Table 4.2. Relative Rates of Release of Inorganic Pyrophosphate from Different Substrates by the Purified Enzyme with and without the Addition of 10mM Magnesium ions.

All rates are given as a percentage of the rate of hydrolysis of pNPP at pH 9.9 and in the presence of 10mM magnesium. Substrate concentration: 2mM. The values are the mean of several determinations.

	Substrate	Relative rates. Without Mg^{2+}	With Mg^{2+}
Liver phosphatase	pNPP	45	100
	AMP	35	55
	ADP	22	19
	ATP	10	5
	UMP	45	60
	UDP	30	20
	UTP	15	11
	TPP	50	40
Intestinal phosphatase	pNPP	77	100
	AMP	95	125
	ADP	150	50
	ATP	115	15
	UMP	45	50
	UDP	130	40
	UTP	75	10
	TPP	110	50
Bone phosphatase	pNPP	22	100
	AMP	18	69
	ADP	15	33

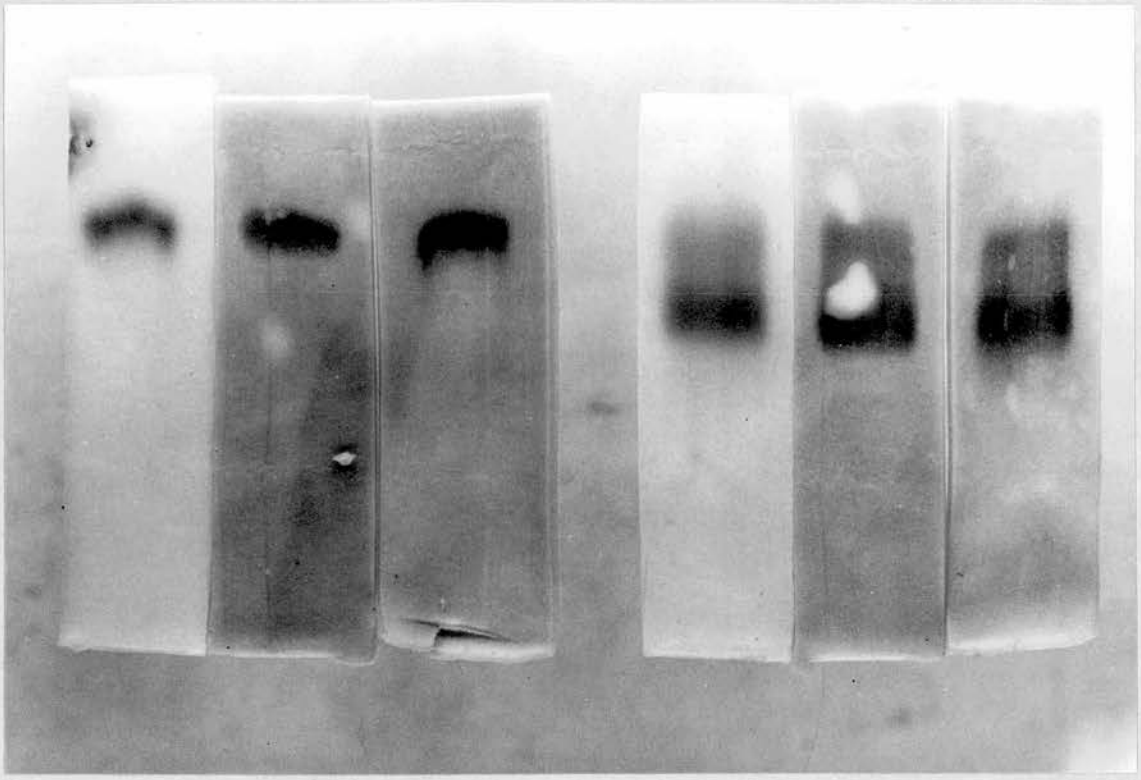
Table 4.2. (Contd.)

	Substrate	Relative rates. Without Mg^{2+}	With Mg^{2+}
Bone phosphatase (Contd.)	ATP	5	7
	UMP	18	54
	UDP	11	34
	UTP	2	13
	TPP	8	17

when using 2mM substrate concentrations, and for the adenine and uracil derivatives and TPP these were in the range pH 9.0 to 9.5, but in these mixed-substrate experiments the concentrations of each substrate was 1mM throughout. Magnesium ions were omitted from the assay system since the effect of magnesium and the optimum magnesium ion concentration is strongly dependent on the nature of the substrate. The inorganic phosphate liberated was measured by the method of Baginski et al (1967). Table 4.3. shows the results of mixed substrate experiments with each of the adenine and uracil derivatives and pNPP or AMP, for both the liver and intestinal phosphatases. In each case the velocity of the hydrolysis of the two substrates together was less than the sum of the two separate reactions.

Liver and intestinal phosphatases were submitted to starch-gel electrophoresis at pH 8.6, and the zones of activity demonstrated using a number of different nucleotides as substrates. The pattern of the zones of activity on the gel was identical whichever nucleotides were used as substrate in the development of the phosphatase bands (Fig. 4.7.).

Fig. 4.7. Alkaline phosphatase zones in liver (L) and small intestinal (I) preparations after starch-gel electrophoresis at pH 8.6. Substrates: (a) ∝ -naphthyl orthophosphate; (b) ADP; (c) UTP. Anodes are at the top, sample insertion slots at the bottom.



(a) (b) (c) (a) (b) (c)

L I

Table 4.3. Mixed-substrate Experiments with Organic Pyrophosphates and Orthophosphates.

Hydrolysis by purified phosphatase preparations of mixtures of pairs of substrates at 37° and pH 9.3.

The concentration of each substrate was 1mM and results are expressed as µmoles P_i liberated/min./ml. of enzyme solution.

	Liver		Intestine	
Substrate	observed velocity	Expected velocity for independent hydrolysis	observed velocity	Expected velocity for independent hydrolysis
AMP	2.79		1.72	
ADP	2.06		2.28	
AMP + ADP	3.54	4.85	1.89	4.00
AMP	1.80		0.413	
ATP	0.53		0.462	
AMP + ATP	1.75	2.33	0.472	0.875
pNPP	5.29		1.04	
UMP	3.34		0.66	
pNPP + UMP	5.56	8.63	1.00	1.70
pNPP	5.89		1.10	
UDP	2.03		2.12	
pNPP + UDP	5.26	7.92	1.49	3.22
pNPP	5.13		1.21	
UTP	1.17		1.33	
pNPP + UTP	3.61	6.30	1.28	2.54

The liver and intestinal phosphatases were inactivated by incubating at 54° and pH 7.7 for various times as described in Chapter three, and the residual activity assayed at pH 9.9. The activity towards each of the substrates declined in parallel. The marked difference between the rate of inactivation of the liver and intestinal enzymes was seen with all the nucleotides (Fig. 4.8.). During inactivation of the liver enzyme (in 3M urea) and of the intestinal enzyme (in 6M urea) in pH 7.7 buffer at room temperature, similar parallel losses of activity with all the substrates were seen with both liver and intestinal phosphatase (Fig. 4.9.). The decline in activity towards UMP with the intestinal enzyme may, however, differ from that for the other substrates. The difference in stability towards urea between the two isoenzymes is again independent of substrate.

Mode of Hydrolysis: Alkaline phosphatase might hydrolyse the di- and tri-phosphate nucleosides either by removal of each phosphate group consecutively as orthophosphate, or by removal of pyrophosphate and its subsequent cleavage to inorganic phosphate, and an attempt was made to identify the intermediates in the hydrolysis reaction. Liver and intestinal phosphatase were incubated for 2-3 hours at 37° with ADP, ATP, UDP and UTP separately in buffer at the optimum pH for each substrate. Since there is considerable spontaneous hydrolysis of the triphosphates, controls without enzyme were also incubated. The incubation mixtures were run on chromatograms overnight in an ethanol-ammonium acetate system (0.3M ammonium

Fig.4.8. Inactivation of (a) liver and (b) intestinal phosphatases during incubation at 54°. Substrates: △ AMP; □ ADP; ▽ ATP; ▲ UMP; ■ UDP; ▼ UTP; ○ p-nitrophenyl phosphate.

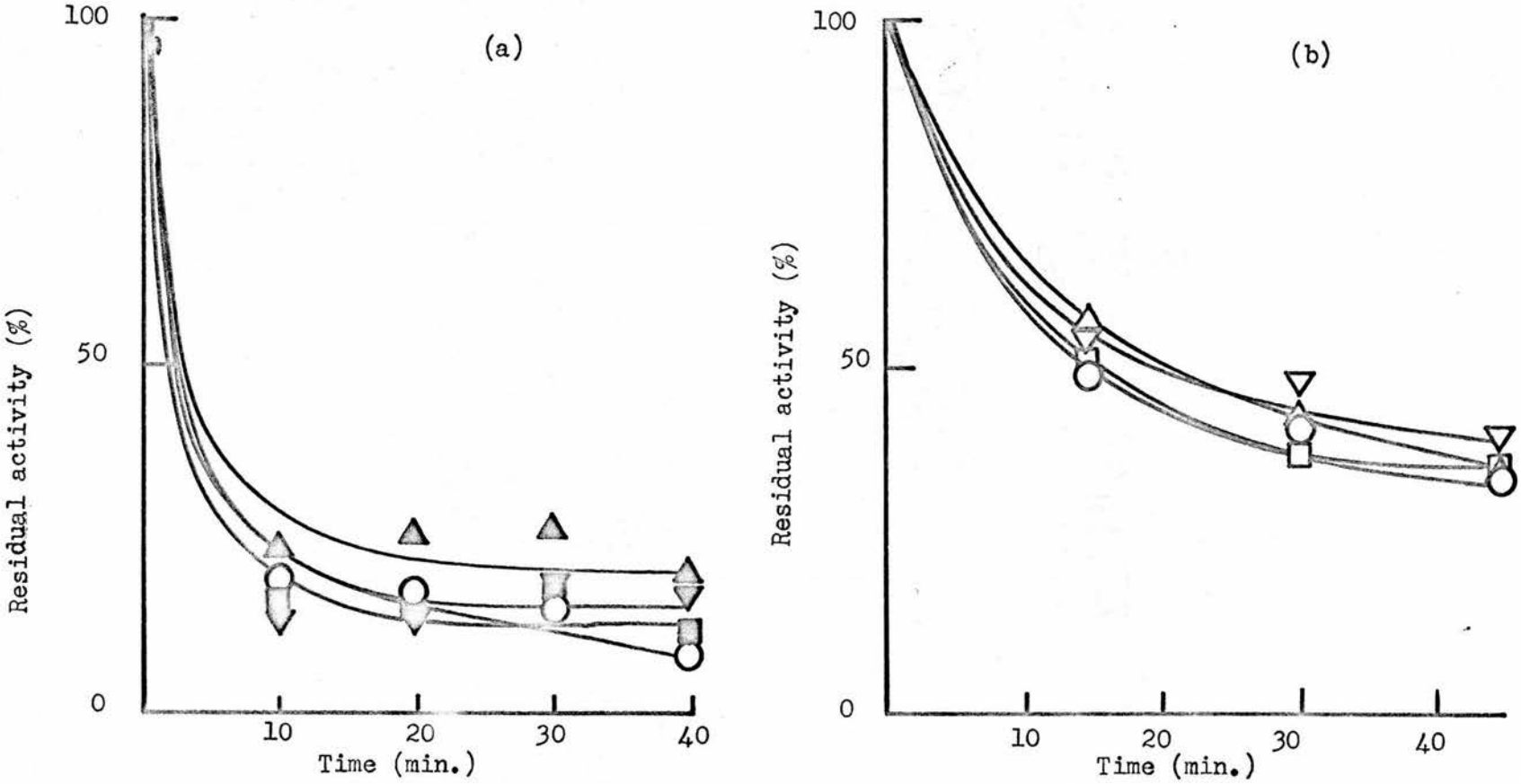


Fig.4.9.a. Inactivation of liver phosphatase with 3M-urea during incubation at 20°c and pH 7.7. Substrates: △ AMP; □ ADP; ▽ ATP; ○ pNPP.

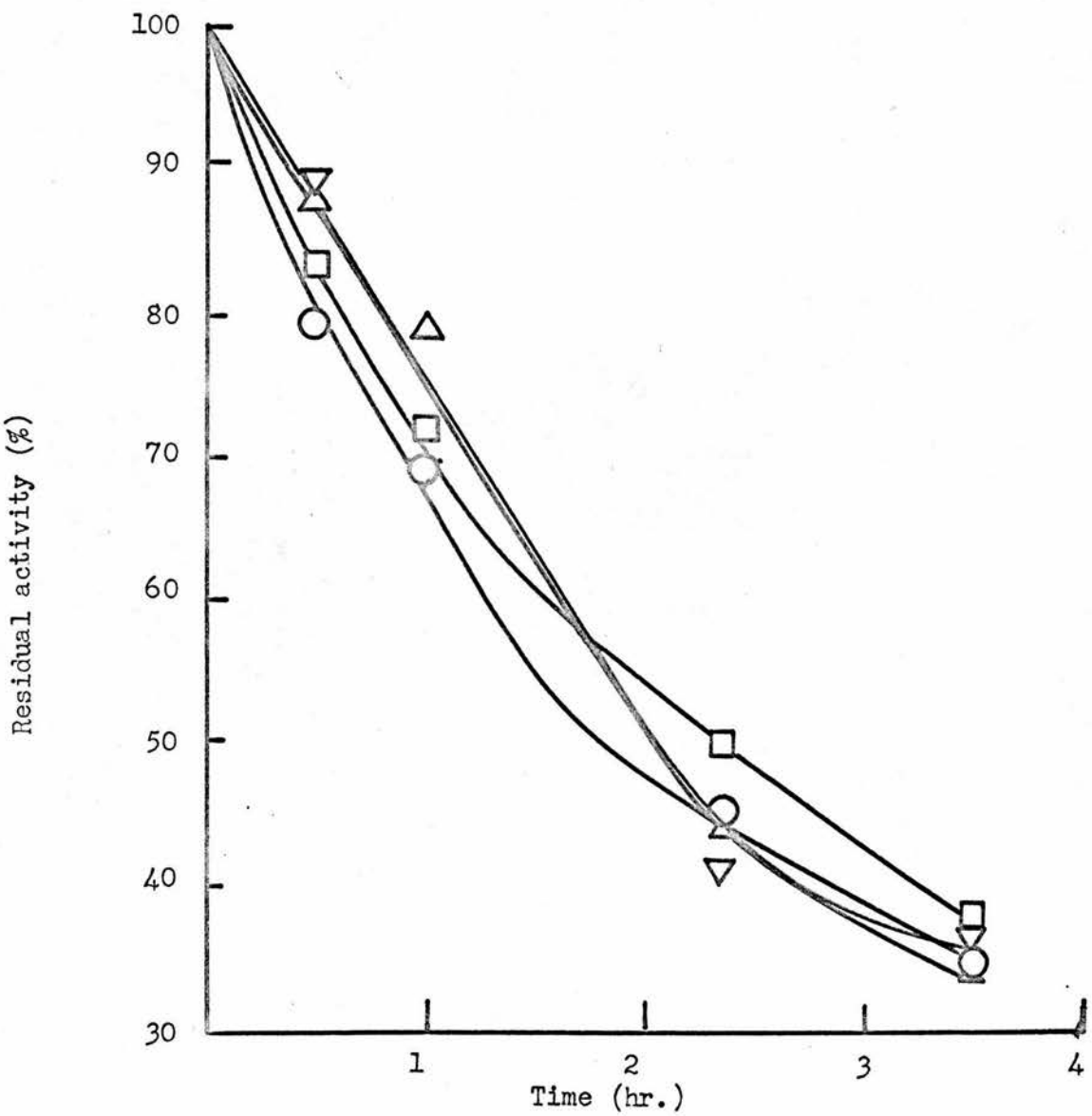
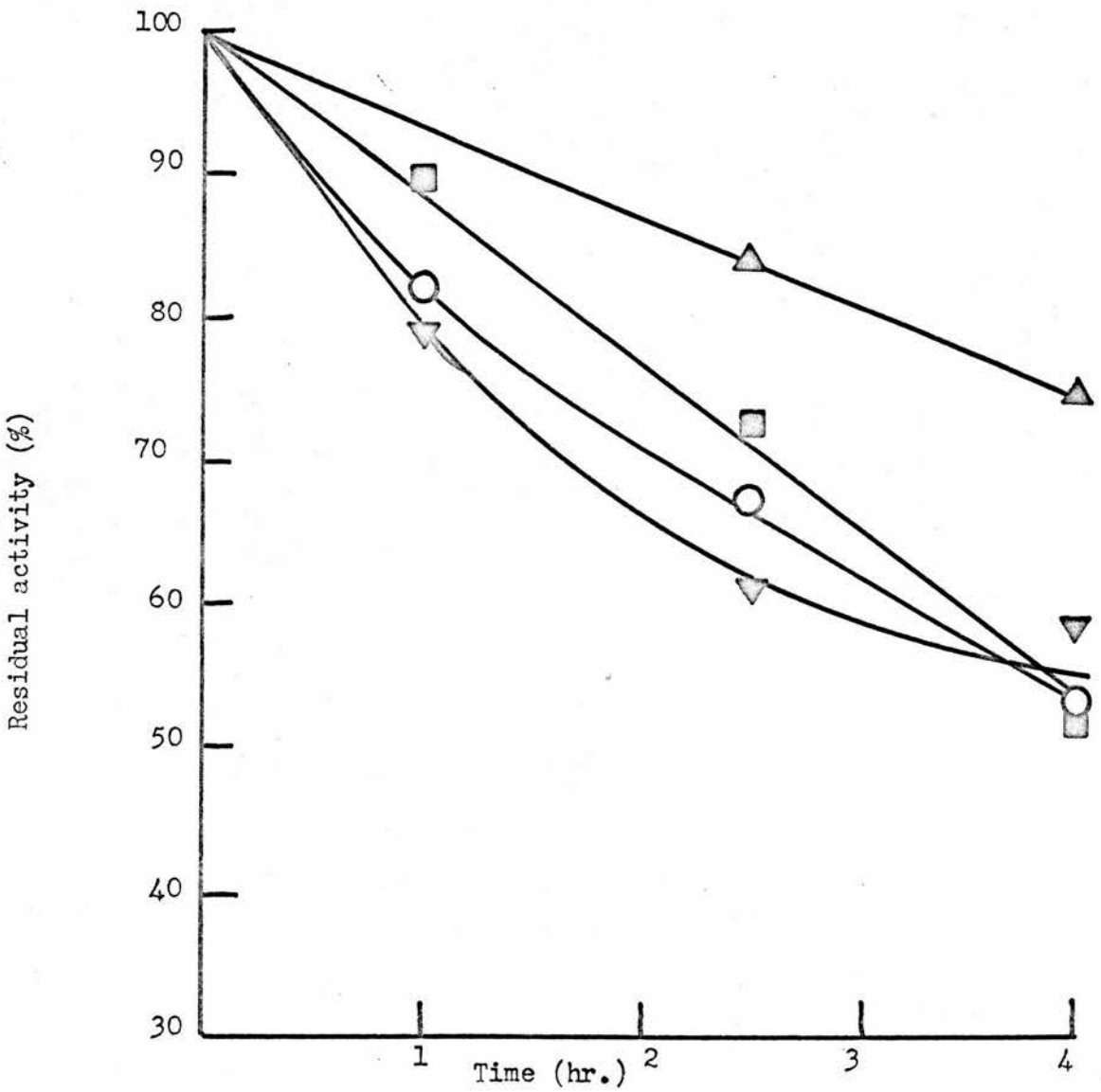


Fig.4.9.b. Inactivation of intestinal phosphatase with 6M-urea during incubation at 20° and pH 7.7. Substrates: △ UMP; □ UDP; ▽ UTP; ○ pNPP.



acetate, 3mM EDTA in 63% ethanol; Smith, 1960a). After drying the chromatograms the nucleotides were detected as dark spots under ultra-violet light. The separation between the di- and tri-phosphates in this system was small and ATP tended to break down into ADP spontaneously. It was not, therefore, possible to ascertain whether or not the diphosphates were intermediates in the hydrolysis of the triphosphates. However the monophosphates, AMP and UMP, were produced during the hydrolysis of ADP and ATP and of UDP and UTP, respectively, with both the liver and the intestinal enzymes. Similar experiments with the bone enzyme were not carried out.

4.1.3. Other substrates.

None of the phosphatases hydrolysed bis (p-nitrophenyl) phosphate, diphenyl phosphate or NAD.

4.2. THE EFFECT OF METAL IONS.

4.2.1. The Effect of Magnesium.

The effect of magnesium ions on the action of the phosphatases varies with the type of substrate, the substrate concentration, and with the tissue source of the phosphatase. The effect on the hydrolysis of orthophosphates (e.g. pNPP) at pH 9.9 is to produce a pronounced increase in the rate of the reaction with even a small increase in the magnesium concentration. For instance, with a final concentration of 0.5mM Mg^{2+} in the assay mixture, and a substrate concentration of 1mM, the activity of the bone phosphatase is increased about three-fold;

increasing the concentration further produces a gradual increase in activity which levels off at about 4-10 mM Mg^{2+}. This effect is seen with all three phosphatases, but the overall increase in reaction rate appears to be greater with the liver and bone enzymes (Fig. 4.10.); this difference may be due to differences in the residual metal-ion content of the three preparations. Table 4.4. shows that changes in substrate concentration in the range 0.2 to 5mM have little effect on the stimulation of activity by magnesium.

Table 4.4. The Effect of Magnesium on the Hydrolysis of pNPP.

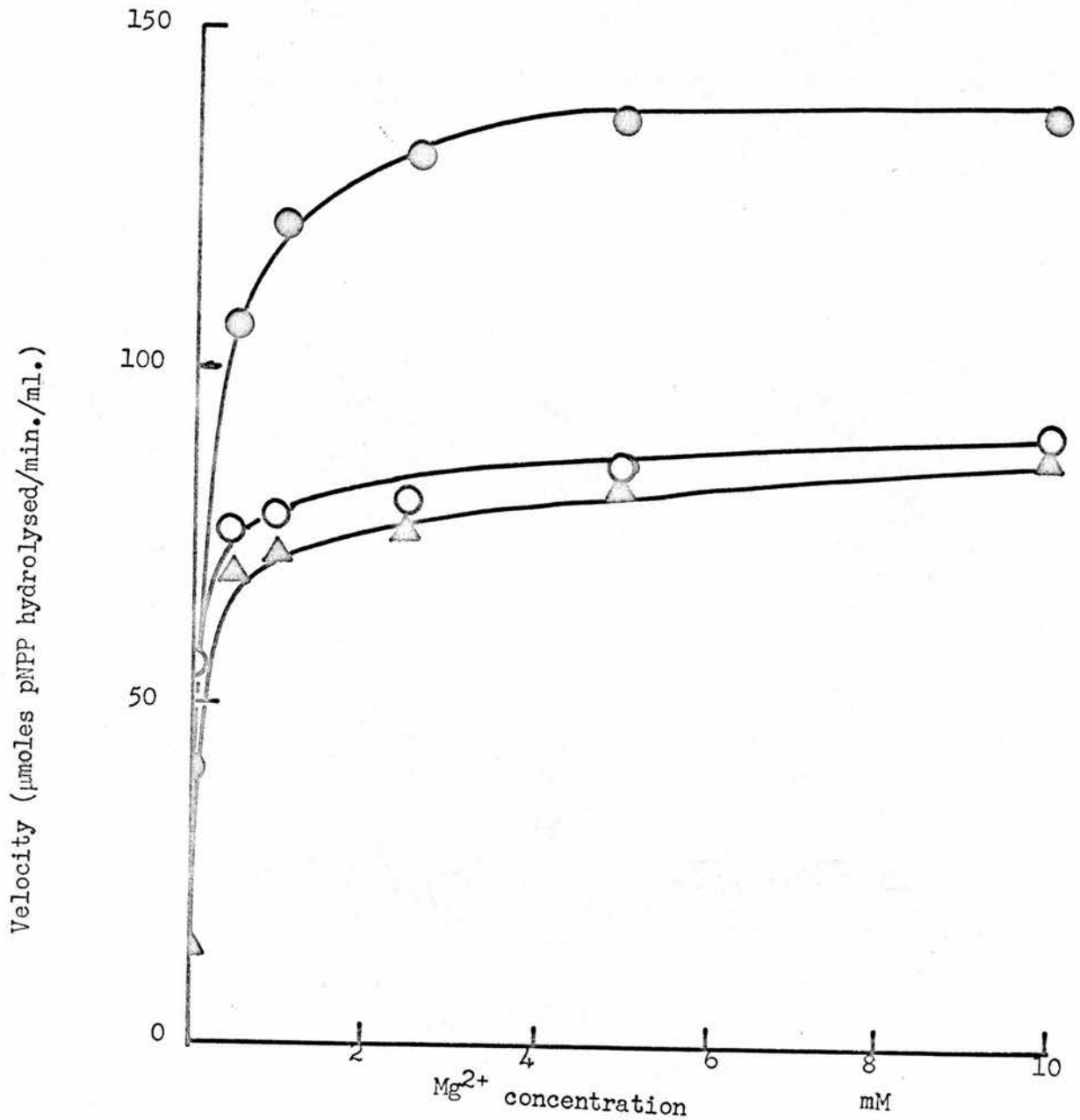
Relative rates of hydrolysis of pNPP at pH 9.9. at different substrate concentrations with and without the addition of 10mM Mg^{2+}.

Rates are expressed as a percentage of the rate of hydrolysis in the presence of 10mM Mg^{2+}.

Enzyme	0.2mM pNPP		5mM pNPP	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
Bone phosphatase	15	100	21	100
Liver phosphatase	21	100	17	100
Intestinal phosphatase	47	100	62	100

A different effect is seen with the hydrolysis of pyrophosphates, e.g. inorganic pyrophosphate at pH 8.5, since the effect of magnesium is in this case strongly dependent on the substrate

Fig. 4.10. Relationship between magnesium ion concentration and rate of hydrolysis of p-nitrophenyl phosphate by bone (●), liver (△) and intestinal (○) phosphatases at pH 9.9. Velocity: X50 for bone phosphatase and X5 for liver phosphatase.



concentration. At low substrate concentrations (1-2mM) the rate of hydrolysis increases with increasing magnesium concentration up to about 0.5 mM Mg^{2+}, but decreases rapidly if the magnesium ion concentration exceeds 1mM. At substrate concentrations of 2-4mM the optimum magnesium concentration is 1-3mM. Fig. 4.11 shows the relationship between magnesium concentration and the rate of hydrolysis of inorganic pyrophosphate by the intestinal enzyme. Bone (Fig. 4.12.) and liver phosphatases show similar relationships.

When the effect of magnesium on the hydrolysis of the organic orthophosphates (e.g. AMP) and pyrophosphates (ADP, UTP, etc.) by purified liver and intestinal phosphatases was investigated, it was found that the hydrolysis of the monophosphates (at 2mM substrate concentration and the optimum pH for each substrate) was stimulated in a similar manner to the hydrolysis of pNPP. The hydrolysis of the pyrophosphates, at 2mM substrate concentration, was activated by concentrations of magnesium ions less than 1mM but inhibited at higher concentrations, particularly with the intestinal enzyme (Fig. 4.13 and 4.14). The effect of Mg^{2+} on the hydrolysis of ATP by the bone enzyme was similar to the findings with the liver enzyme. The difference between the phosphatases may be due to differences in the amount of residual magnesium ions in the preparations rather than to differences between the actual enzymes.

The difference in effect of magnesium ions on the hydrolysis of orthophosphates and pyrophosphates accounts for the difference in specificity of the enzymes towards these two

Fig.4.11. Relationship between Mg^{2+} concentration and rate of hydrolysis of inorganic pyrophosphate by intestinal phosphatase at pH 8.5. Substrate concentrations (mM): ○, 0.5; △, 1.0; □, 1.5; ○, 2.0; △, 3.0; □, 4.0.

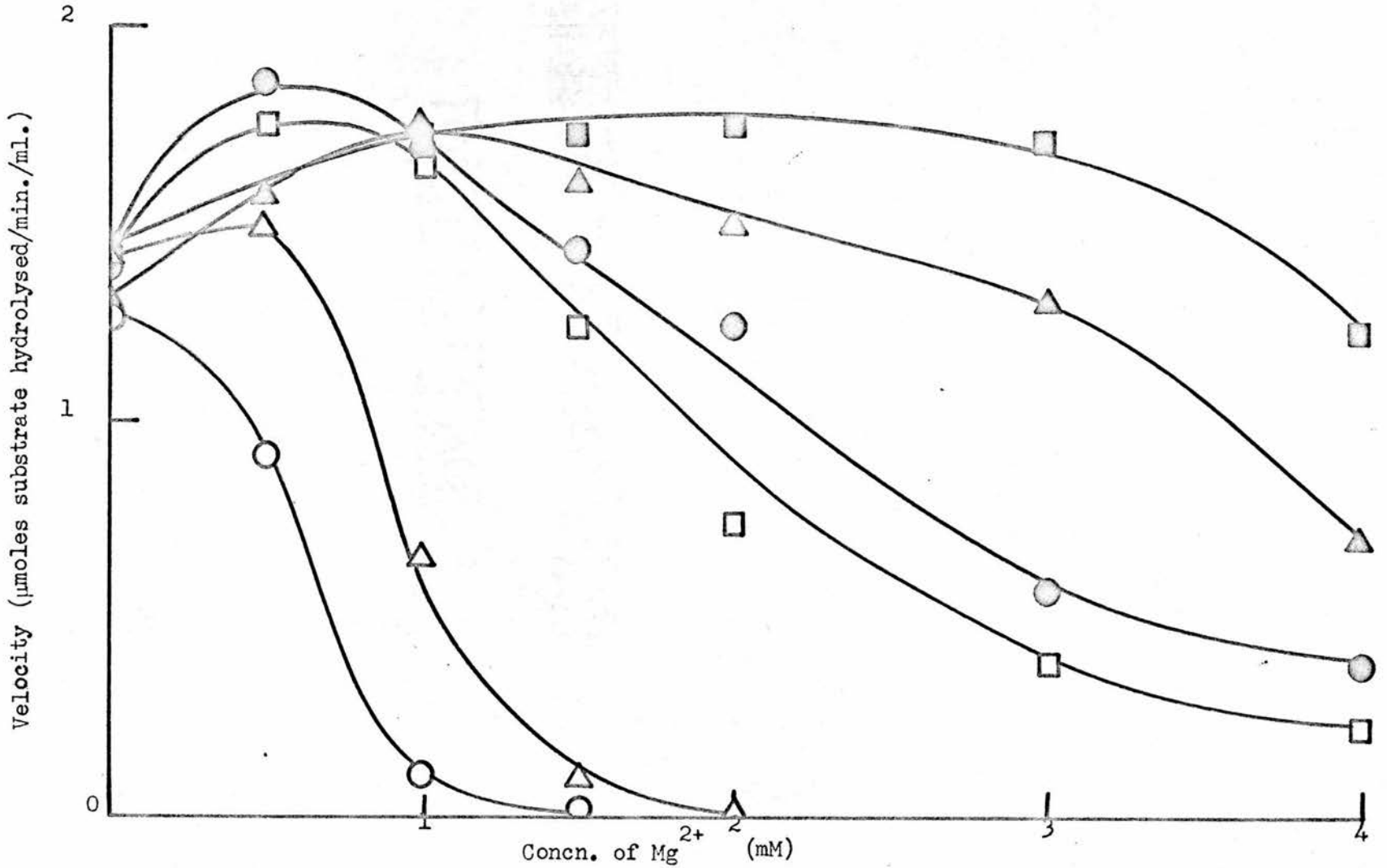


Fig.4.12. Relationship between Mg^{2+} concentration and rate of hydrolysis of inorganic pyrophosphate by bone phosphatase at pH 8.5. Substrate concentrations (mM): ○ , 0.5; △ , 1.0; ◎ , 2.0; ▲ , 3.0; □ , 4.0.

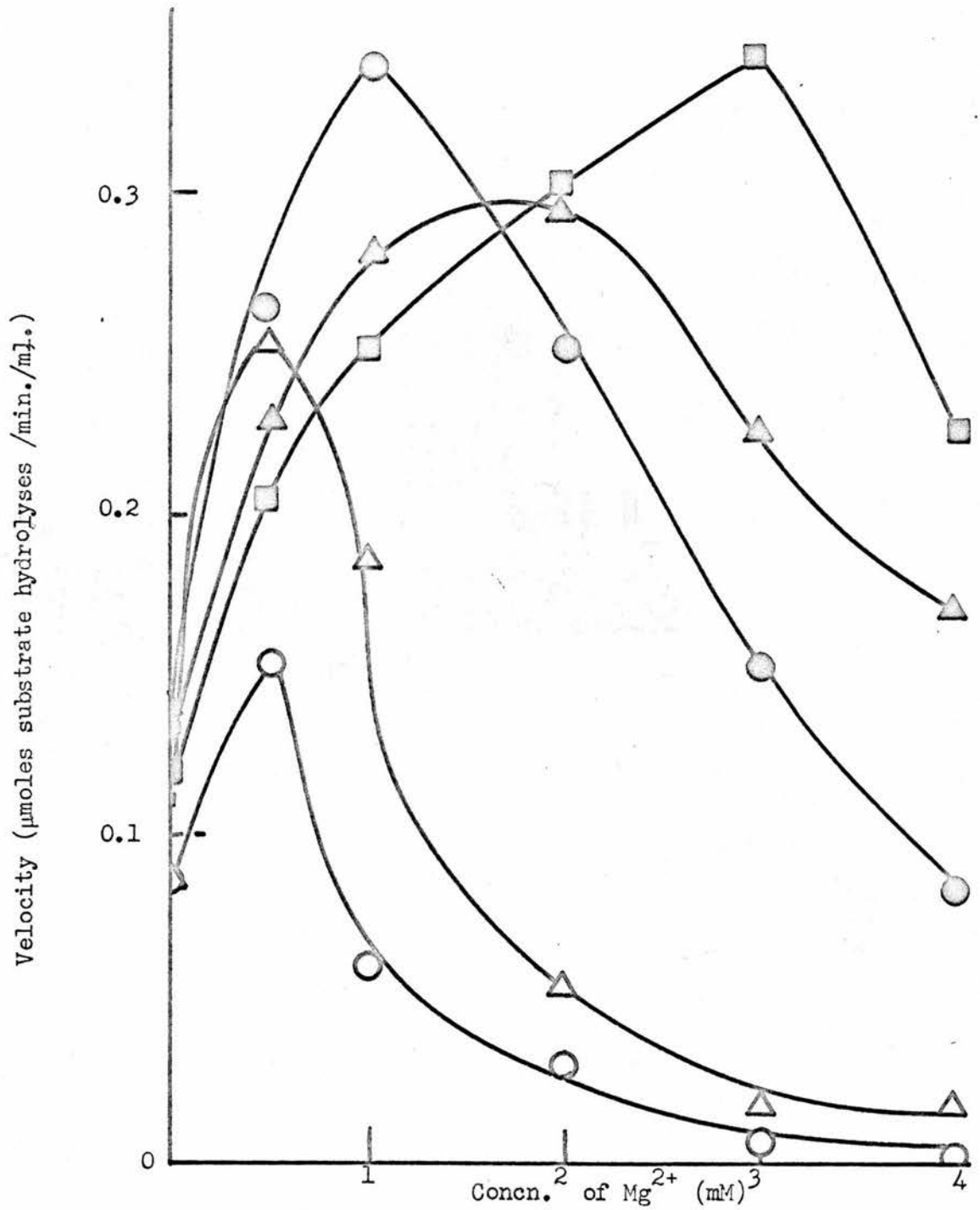


Fig.4.13. Effect of Mg^{2+} ions on rate of release of P_i from (a) adenine nucleotides and (b) uridine nucleotides (concn. of each 2mM) by intestinal phosphatase. Substrates: ▽ , AMP; □ , ADP; △ , ATP; ▽ , UMP; □ , UDP; △ , UTP; ○ , p-nitrophenyl phosphate (hydrolysis measured by release of p-nitrophenol).

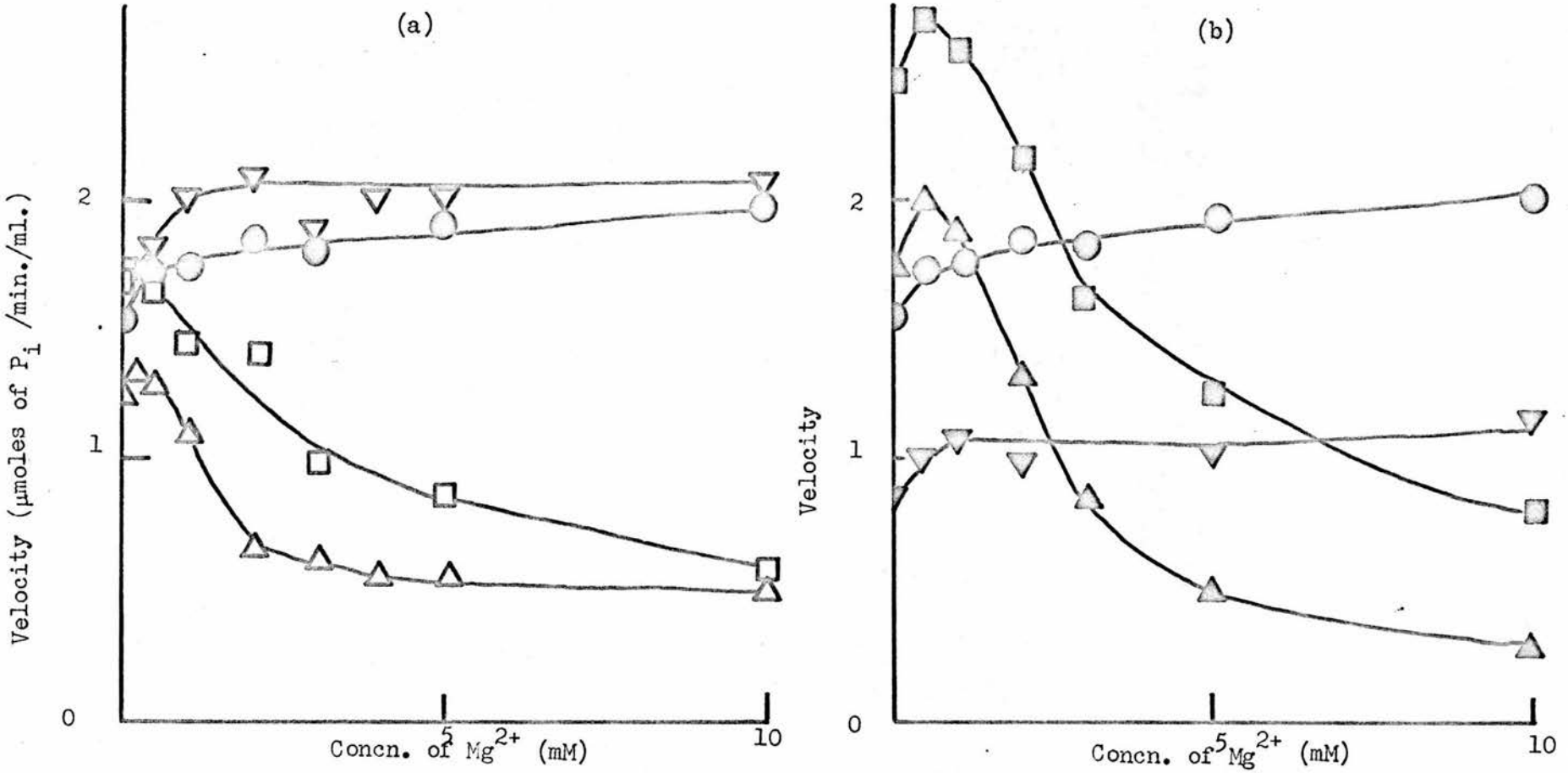
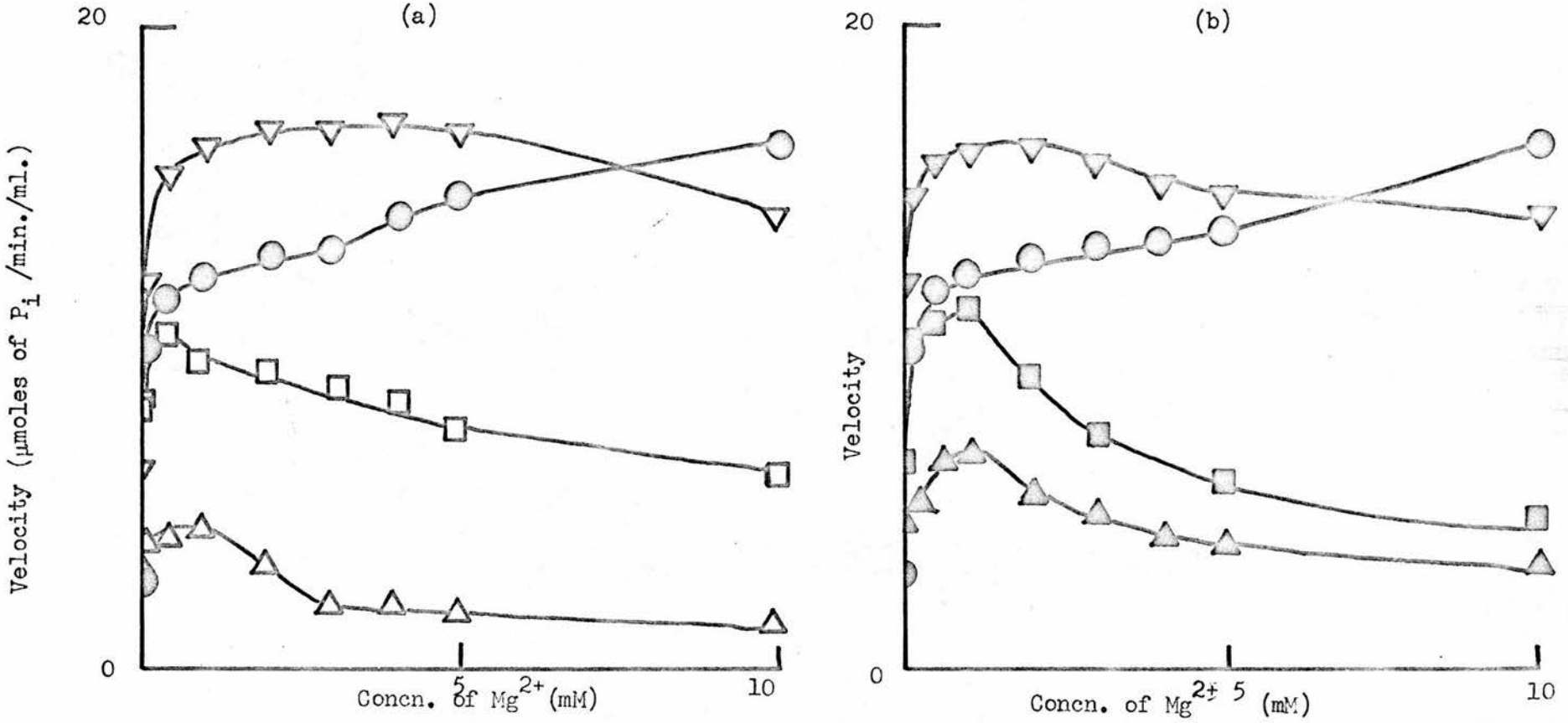


Fig.4.14. Effect of Mg^{2+} ions on the release of P_i from (a) adenine nucleotides and (b) uridine nucleotides (concn. of each 2mM) by liver phosphatase. Substrates: ▽, AMP; □, ADP; △, ATP; ▽, UMP; □, UDP; △, UTP; ○, p-nitrophenyl phosphate (hydrolysis measured by release of p-nitrophenol). Rates for AMP, ADP, ATP, UMP, UDP, UTP x2.



groups of substrates seen in the presence and absence of 10mM Mg^{2+} (Table 4.2.).

4.2.2. The Effect of Zinc.

With all three phosphatases, the presence of 1.0μM Zn^{2+} in the assay system for the hydrolysis of pNPP (2mM pNPP, 5mM Mg^{2+} in carbonate-bicarbonate buffer, pH 9.9) caused a slight activation. However, a further increase in zinc concentration produced a marked inhibition. The activity in the presence of 100 μM Zn^{2+} was about 20% of that in the absence of added zinc ions. The pyrophosphatase activity (in the presence of 1mM Mg^{2+}) was also slightly activated by 1.0 μM Zn^{2+}, but 100 μM Zn^{2+} did not cause further inhibition. In the case of the liver phosphatase, when the same enzyme preparation was used for the orthophosphatase and for the pyrophosphatase experiments, a zinc concentration of 10mM was required to reduce the pyrophosphatase activity to 20% of its original level.

4.3. KINETICS.

4.3.1. The effect of varying Substrate Concentration.

The effect of substrate concentration on the velocity of the hydrolysis reaction is dependent on pH and the type of substrate, and is generally similar for phosphatases from all tissues. When the effect of varying the substrate concentration was measured in the absence of added magnesium ions, and at the pH optimum for hydrolysis with a substrate concentration of 2mM, the orthophosphates (AMP and UMP) showed an optimum substrate

concentration at about 4mM with only slight inhibition at 8mM. With the pyrophosphates (ADP, UTP, etc.) the optima, however, were at 2-4 mM with considerable inhibition at higher substrate concentrations, particularly with the adenine derivatives and with thiamine pyrophosphate (Fig. 4.15 and 4.16).

Ross et al (1951) found that the pH optimum varied with the substrate concentration, when studying the action of rat intestinal phosphatase on β-glycerophosphate. This finding was observed in the present work for the purified enzymes of human origin, both with orthophosphatase and pyrophosphatase activities. The plots for the bone enzyme are shown in Fig. 4.17. There is a much greater variation in the optimum pH with substrate concentration for orthophosphatase than for pyrophosphatase activity. This has been further demonstrated by plotting log.(substrate concentration) against optimum pH in the manner adopted by Ross et al (1951) and by Motzok and Branion (1959) (Fig. 4.18).

4.3.2. K_m Values.

Michaelis constants were calculated from graphs of 1/v against 1/s, using the adenine and uracil derivatives as substrates at pH values corresponding to the optimum pH for 2mM substrate concentrations. No magnesium ions were added (Table 4.5.). Substrate concentrations were in the range 2mM to 0.2mM.

Fig.4.15. Relationship between substrate concentration and rate of release of P_i from various substrates for liver phosphatase. There was no added Mg^{2+} and measurements were made at the pH optimum for each substrate at 2mM concentrations. Substrates: ▽ , AMP; □ , ADP; △ , ATP; ▽ , UMP; □ , UDP, △ ,UTP.

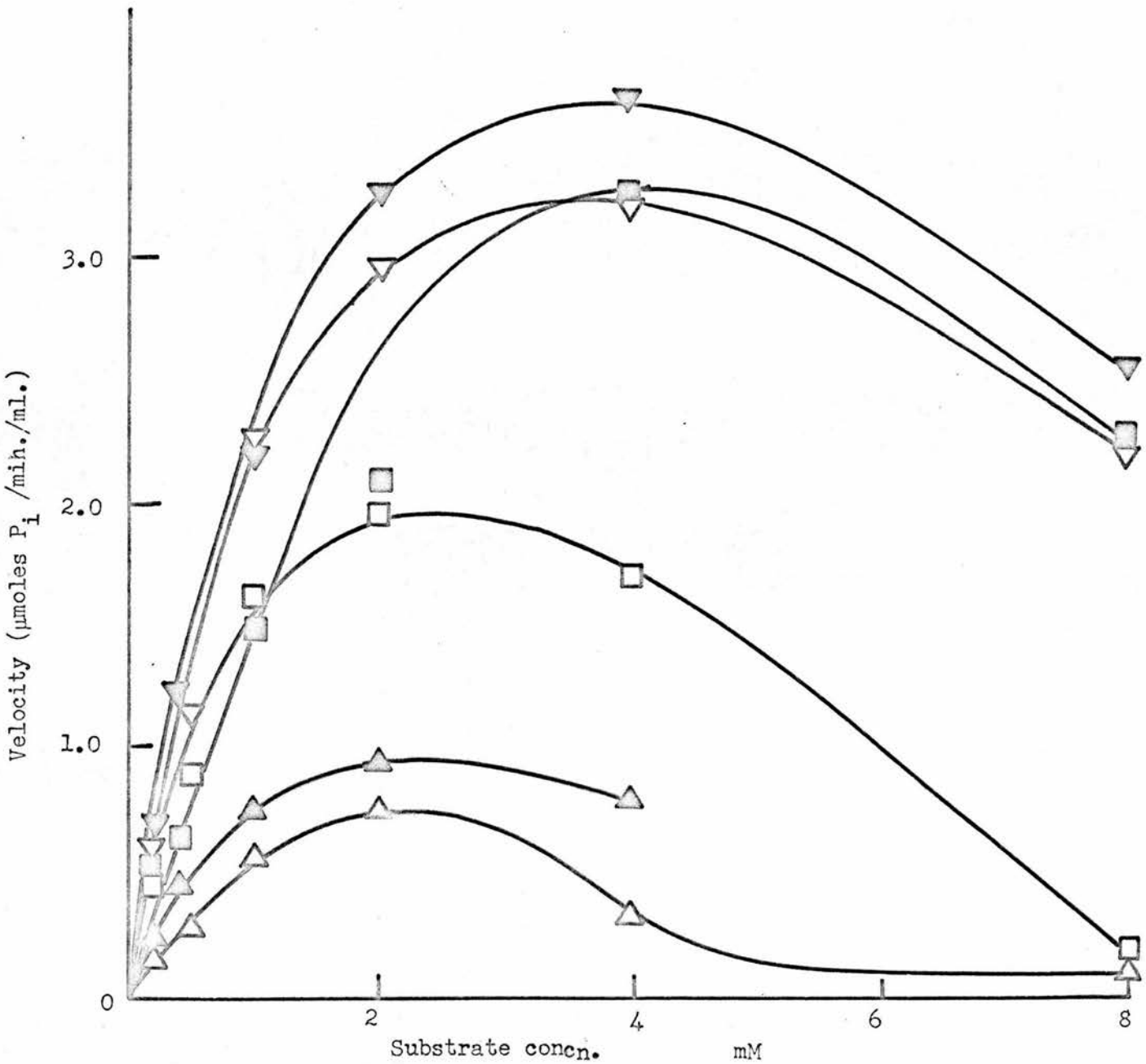


Fig.4.16. Relationship between substrate concentration and rate of release of P_i from various substates for intestinal phosphatase. There was no added Mg^{2+} and measurements were made at the pH optimum for each substrate at 2mM concentration. Substrates: ▽ , AMP; □ , ADP; △ , ATP; ▽ , UMP; □ , UDP; △ , UTP.

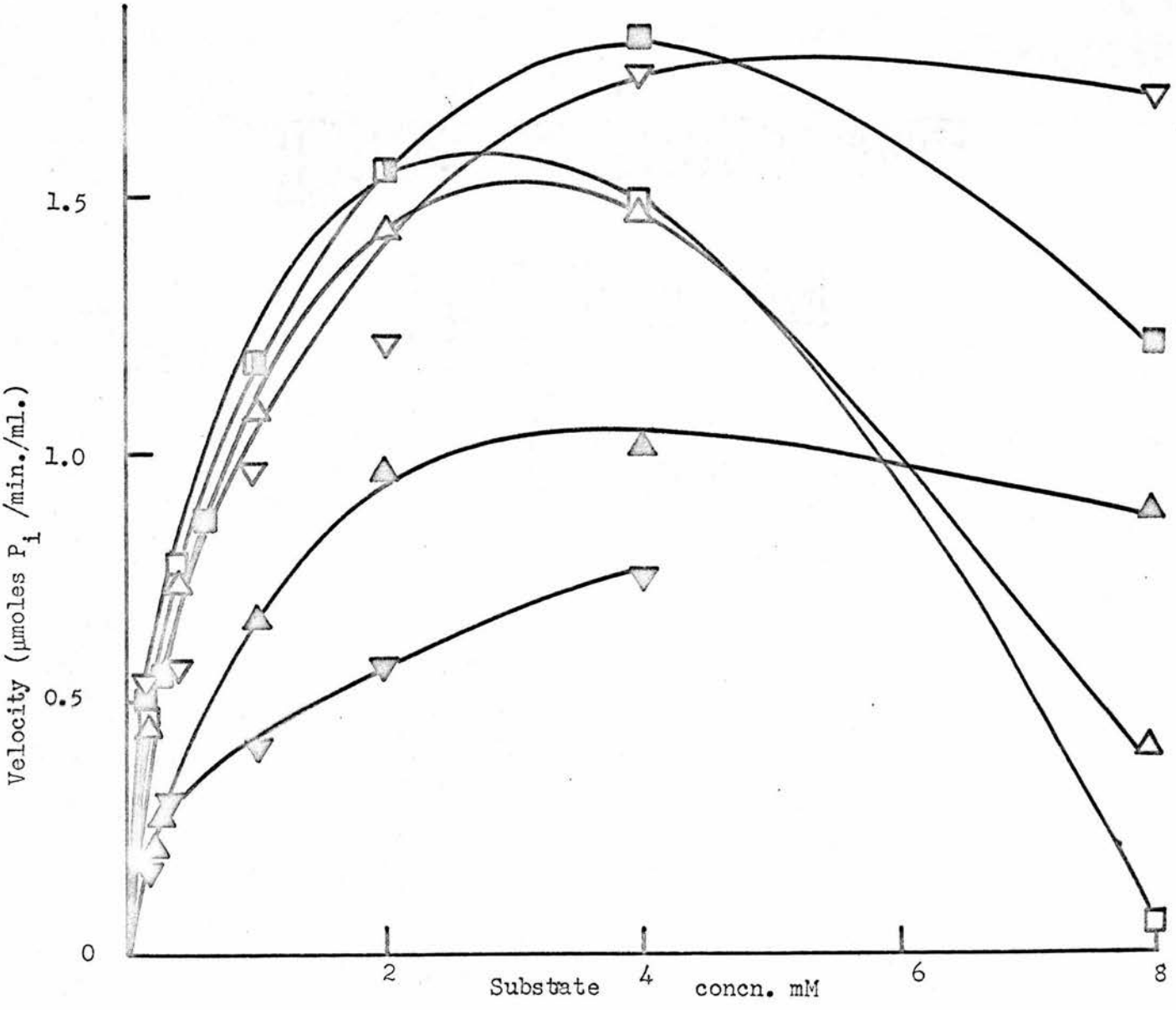


Fig.4.17. Dependence of optimum pH for hydrolysis by bone phosphatase of substrate concentration. (a) with p-nitrophenyl phosphate as substrate; (b) with inorganic pyrophosphate as substrate. Substrate concentrations (mM); ○ ,0.5; ◯ ,0.1; △ ,0.05; □ ,0.025; △ ,0.01.

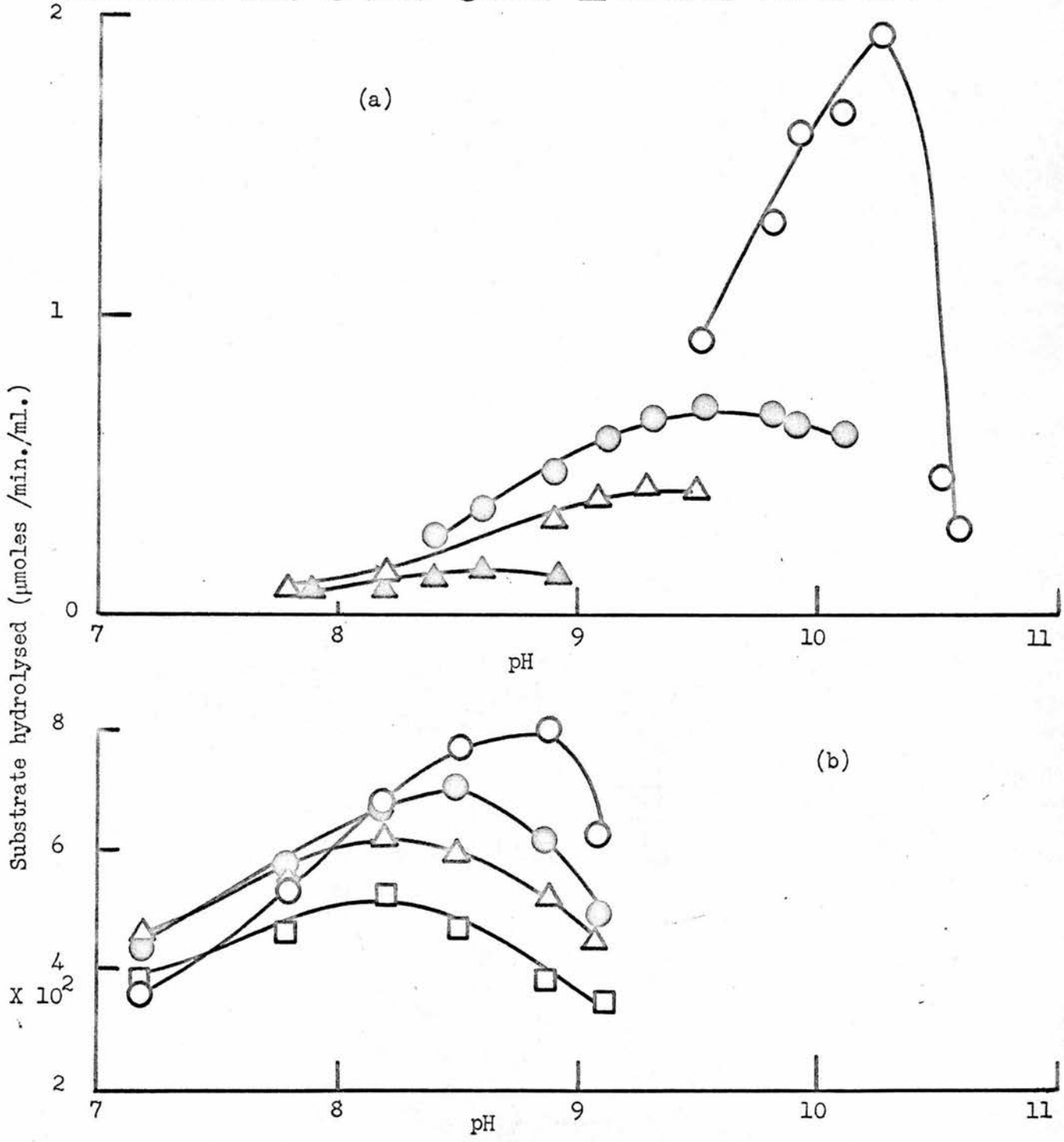


Fig.4.18. Relationship between log of substrate concentration and optimum pH for hydrolysis of inorganic pyrophosphate (○) and p-nitrophenol phosphate (◯) by bone alkaline phosphatase.

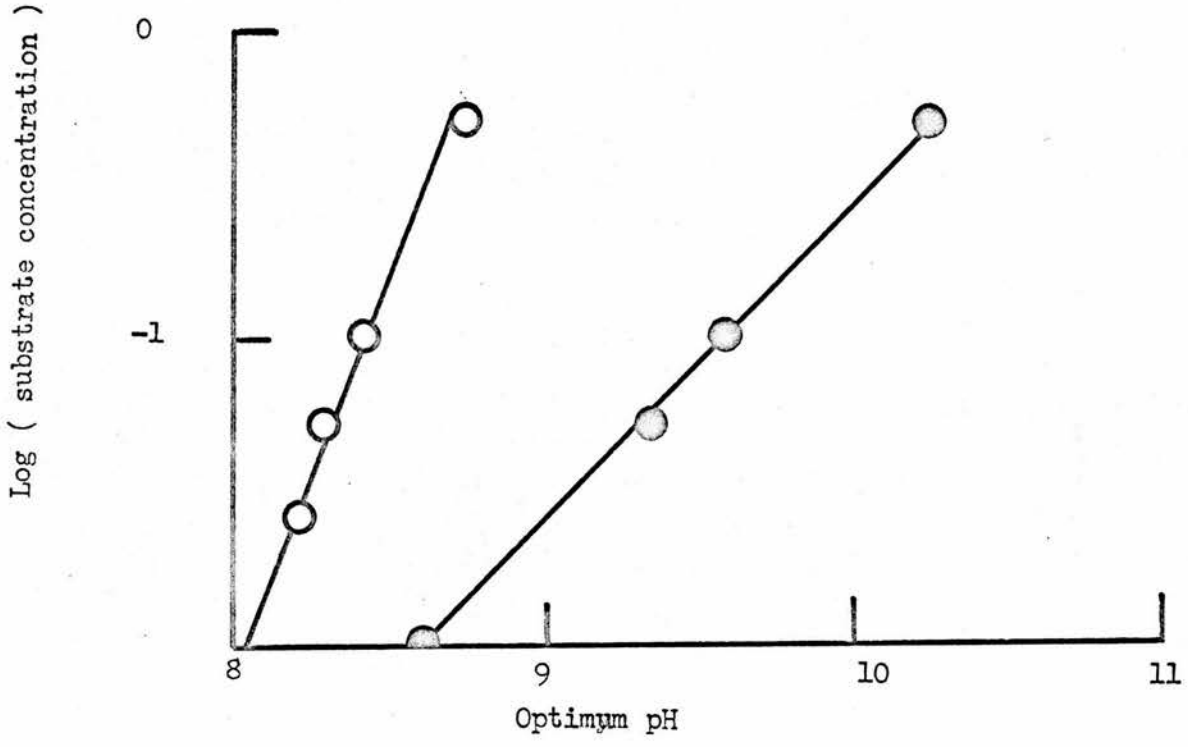


Table 4.5. K_m Values (mM) for Liver and Intestinal phosphatase.

Substrate	Liver phosphatase	Intestinal phosphatase
AMP	2.0	1.7
ADP	2.1	0.8
ATP	2.0	0.7
UMP	1.1	0.7
UDP	1.5	0.5
UTP	0.8	1.0
TPP	6.3	1.2

The K_m values for the bone phosphatase, under similar conditions, could not be determined precisely, but were estimated to be of the same order as those for the liver and intestinal enzymes.

The K_m values at different pH values were determined for each phosphatase acting on the substrates pNPP and inorganic pyrophosphate. Since the effect of magnesium ions is complex magnesium was not added to the assay mixtures. The buffers used were tris-HCl (0.1M) over the range pH 7 to 9.5, ammediol (2-amino-2methyl-1,3-propandiol)-HCl (0.1M) over the range pH 8.5 to 10.2, and in a few cases the carbonate-bicarbonate buffers of Delory and King (1945) were used at pH values greater than 10.0. The orthophosphatase activity at 37° was measured as the increase in absorption at 400 mμ in a recording spectrophotometer. When inorganic pyrophosphate was the

substrate, the incubation was for a fixed time (usually 5 mins.), and the liberated phosphate was being measured by the method of Baginski et al (1967).

K_m values were calculated from graphs of s/v against s; the velocity was expressed as µmoles substrate hydrolysed/min./ml. The use of different buffers was not found to have any effect on the K_m values. When pK_m was plotted against pH (Dixon, 1953a) a series of curves with straight portions of gradient 0,-1 or -2 fitted the experimental points quite well. An extension of the lines gave pK values for the various ionisations affecting the enzyme-substrate interaction. Fig. 4.19 shows the "Dixon plots" for the orthophosphatase activity for the three phosphatases. The results for the three enzymes are similar for their orthophosphatase activity, each having an ionisation between pH 8.5 and pH 8.8. This type of inflexion can represent either a pK_e or a pK_s but, since pNPP does not have a pK in this region, it is presumably the pK of a substrate-binding group in the enzyme molecule. The graph for liver phosphatase may have a second inflexion about pH 10.2, representing a pK of the enzyme-substrate complex (pK_{es}). The bone and intestinal enzymes do not indicate a pK_{es} around pH 10.5, but the K_m values were not determined above this pH since the enzyme activity is very low at high pH values. The overall change in K_m for the orthophosphatase activity varied by 40 to 70 times over the range from pH 8.5 to pH 10.5.

Fig. 4.20 shows the "Dixon plots" for the inorganic pyro-

Fig.4.19. Plots of pK_m (-log K_m(mM)) against pH for the action of (a) liver, (b) bone and (c) intestinal phosphatases of p-nitrophenyl phosphate. Buffer solutions: ○ , tris-HCl; △ , ammediol-HCl; □ , carbonate-bicarbonate.

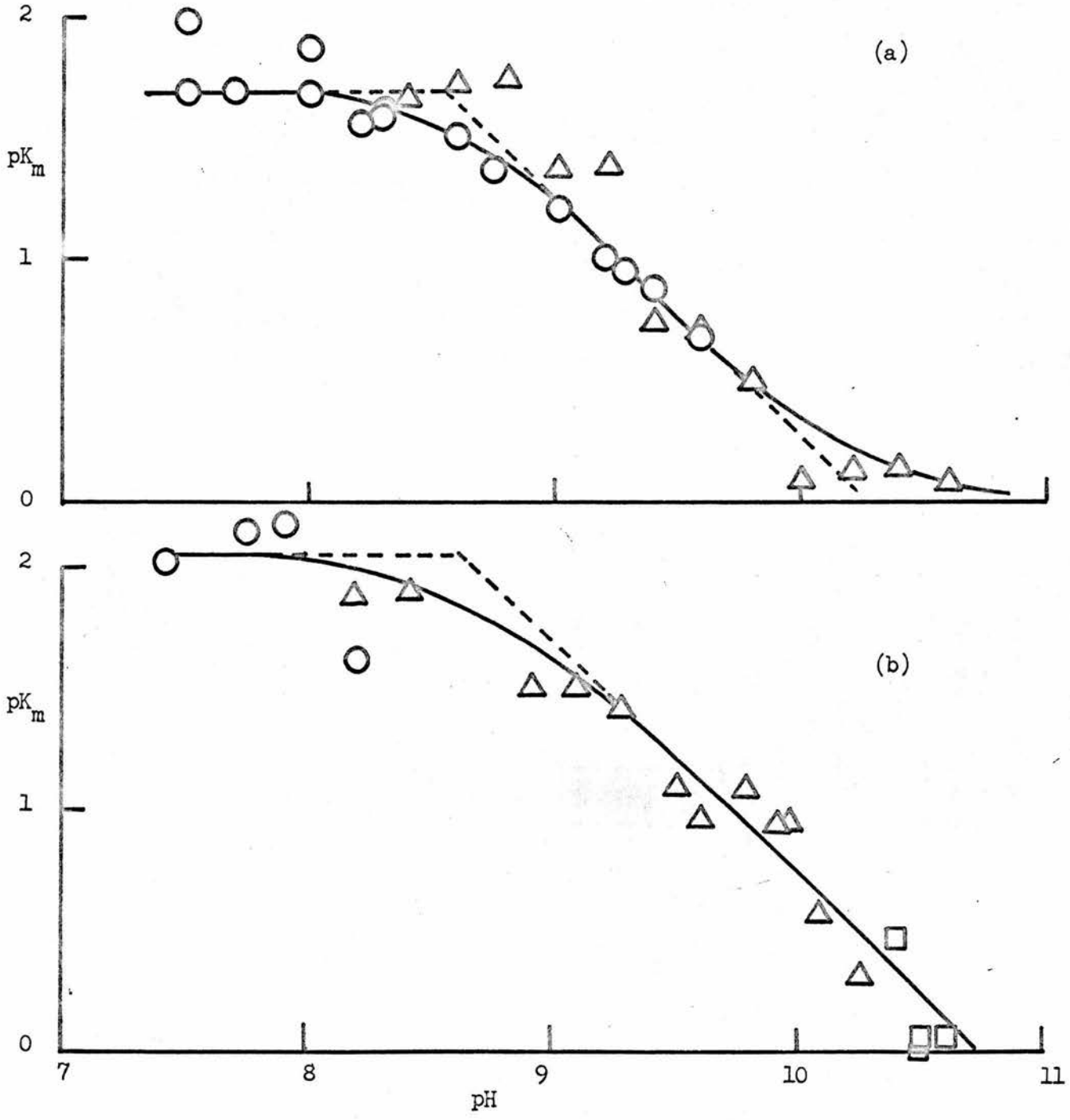


Fig.4.19. (c)

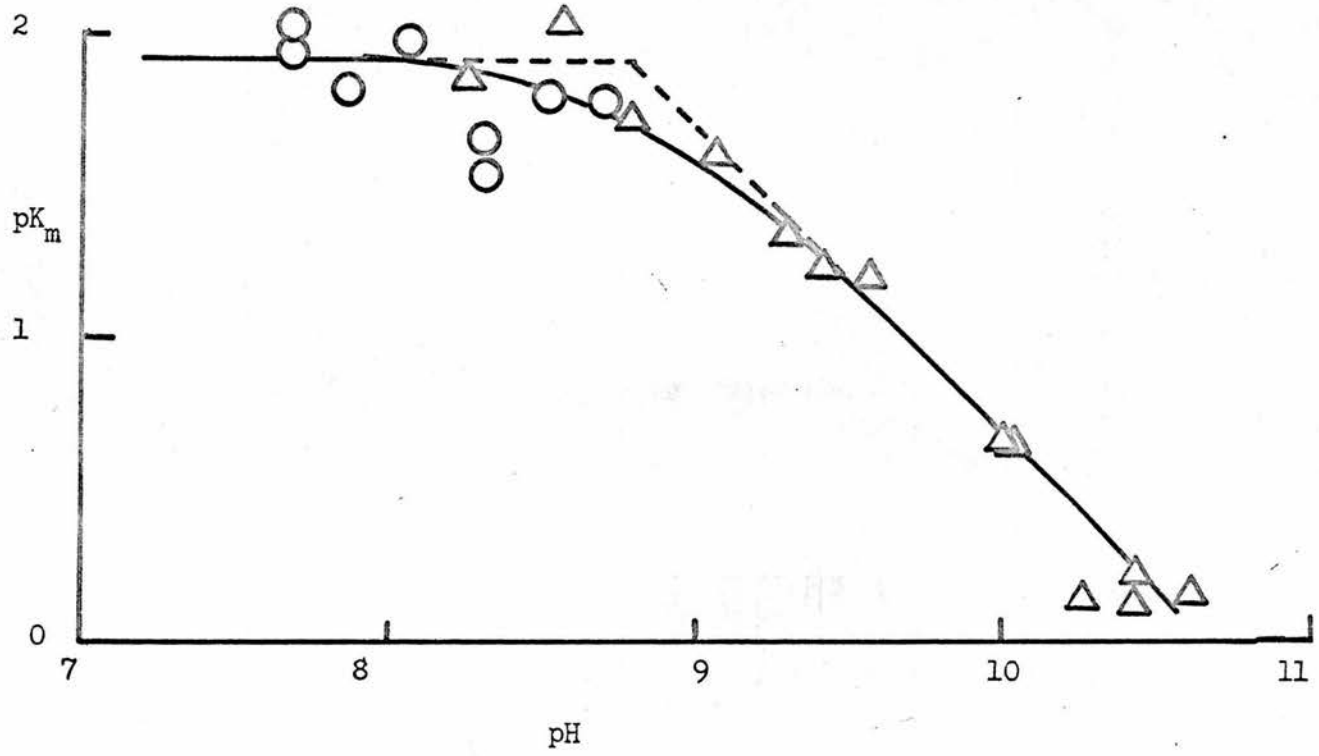
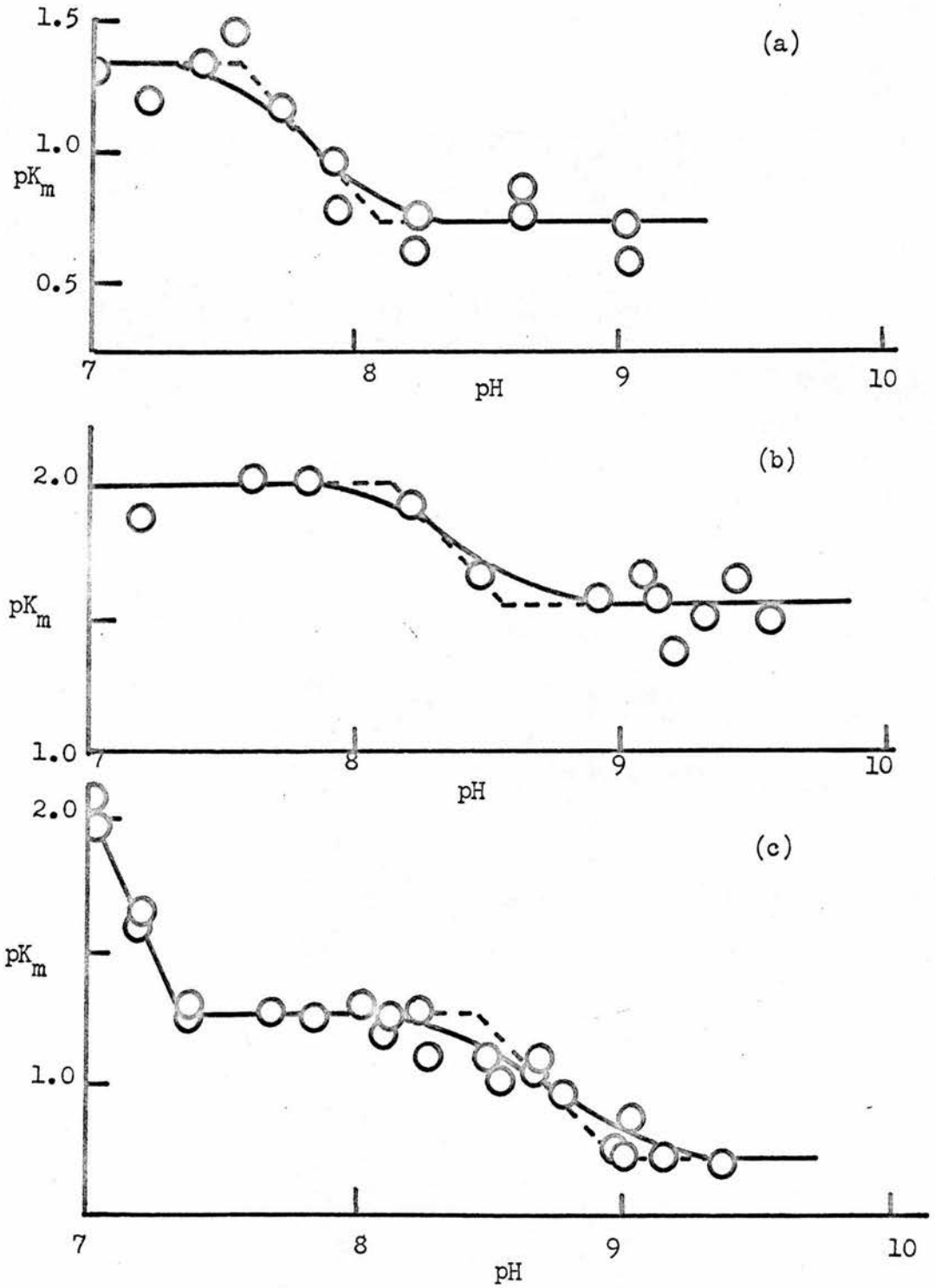


Fig.4.20. Plots of pK_m (-log K_m (mM)) against pH for the hydrolysis of inorganic pyrophosphate by (a) liver, (b) bone and (c) intestinal phosphatases. Buffer solutions: tris-HCl.



phosphatase activity. Since the pH optimum of the activity, with a substrate concentration of 1mM, is in the region of pH 8-9, the K_m values were measured over the range pH 7-9.5. The variation in K_m with pH between pH 7.5 - 9.5 with this substrate is about five fold over a range of two units of pH. Two or three inflexions were seen in each case with inorganic pyrophosphate. Table 4.6 indicates the pH values at which these occurred.

Table 4.6. pK Values for the Three Phosphatases with Inorganic Pyrophosphate as Substrate.

Change of Slope	Liver phosphatase	Bone phosphatase	Intestinal phosphatase
from -2 to 0	-	-	7.4
from 0 to -1	7.5	8.1	8.4
from -1 to 0	8.1	8.55	8.95

There is greater variation in the pK values where the gradient changes from 0 to 1, with this substrate. The pK values for the ionisation of three of the hydroxyl groups of inorganic pyrophosphate lie below 6.0; however, the fourth hydroxyl group has been reported to have a pK for its ionisation between 8.2 and 9.4 in the literature, and a pK of 9.1 for this ionisation was found by titration, at ionic strength 0.01, in the present work. Thus, the pK values at 7.5 8.1 and 8.4 must represent pK_e values, and not values for pK_s, for the three phosphatases. The dissimilarity between the three phosphat-

ases is also seen in the pK_{es} values, which vary between 8.1 and 8.95. The second concave bend found in the curve for the intestinal enzyme, at pH 7.5, may represent a second pK_{es}; since this slope has a gradient of -2, it would represent the simultaneous ionisation of two groups.

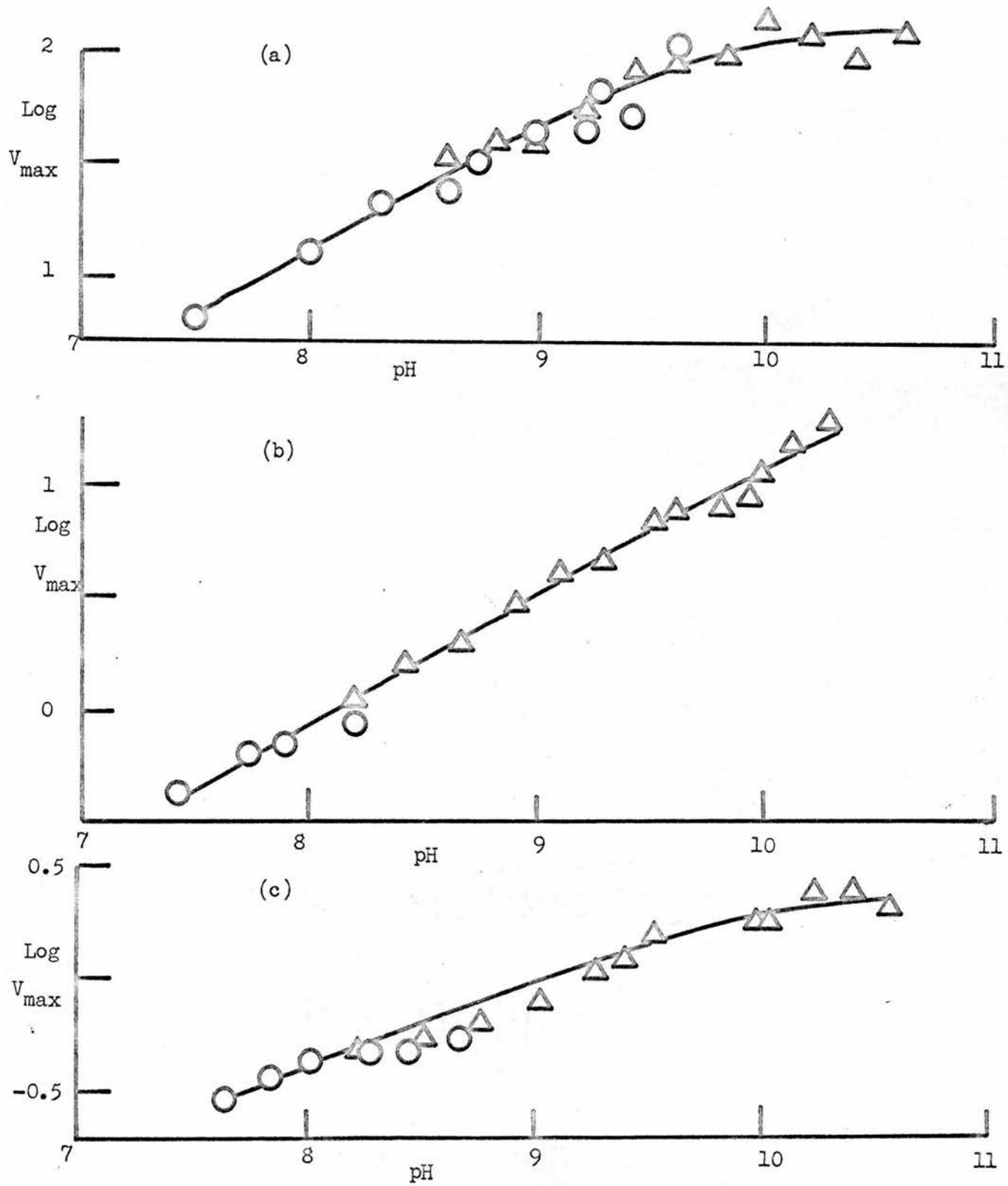
At pH 7.0 the affinities of the enzymes for the two classes of substrate are relatively unaffected by the type of substrate and by the tissue source of the enzyme (Table 4.7.). On the other hand, at pH 9.0 the liver and intestinal enzymes show a greater affinity for orthophosphate than for pyrophosphate, the difference in affinity being 5-10 fold. Increasing the pH to 10.5 produced a marked increase in the K_m for orthophosphate with all three phosphatases.

Table 4.7. A Comparison of the K_m Values (mM) for the Hydrolysis of pNPP and of Inorganic Pyrophosphate (PP_i) at Different pH Values.

Enzyme Source	pH 7.0		pH 9.0		pH 10.5
	pNPP	PP_i	pNPP	PP_i	pNPP
Liver	0.02	0.04	0.06	0.2	0.8
Bone	0.01	0.01	0.02	0.03	0.6
Intestine	0.01	0.01	0.025	0.2	0.7

V_{max} at each pH was calculated from graphs of s/v against s for orthophosphatase activity, using pNPP as substrate and log V_{max} was then plotted against pH (Fig. 4.21). These latter graphs should, in theory, consist of a series of lines

Fig.4.21. Variation of log V_{max} with pH for the hydrolysis of p-nitrophenyl phosphate by liver(a), bone(b) and intestinal(c) phosphatases. Buffer solutions: ○, tris-HCl; △, ammediol-HCl.



of zero and of unit slope joined by curved portions, the intersections of the straight portions giving pK values for the enzyme-substrate complex. However, such lines do not fit the experimental points well. A discontinuity about pH 10.0 could be postulated for the liver enzyme, which would correspond to the pK_{es} found in the pK_m plots.

Lines of unit and zero slope could be more readily fitted in the graphs of log V_{max} against pH for the inorganic pyrophosphatase activity (Fig. 4.22), and pK_{es} values of 7.9 (liver enzyme), 9.1 (bone enzyme) and 7.9 (intestinal enzyme) were found. It is probable that there is a second pK_{es} value for the liver enzyme above pH 9.0, and for the bone enzyme below pH 8.0; in each case the start of the curve can be seen. Similarly a second pK_{es} above pH 10.0 might be shown by the intestinal enzyme if it were possible to extend observations sufficiently above pH 9.0.

At pH 7.0 the orthophosphatase and the pyrophosphatase activities of the bone enzyme have similar V_{max} values, whereas at pH 9.0 the pNPP activity is three to four times greater (Table 4.8.). The ratio of V_{max} for pNPP at pH 10.5 to V_{max} for pyrophosphate at pH 8.5 is about 13 to 1, which resembles the ratio of the two activities when each is measured under optimum conditions of pH and magnesium concentration. The increase in V_{max} with increasing pH, using pNPP as substrate reflects the high pH optimum of the enzyme with that substrate.

Fig.4.22. Variation in log V_{max} with pH for the hydrolysis of inorganic pyrophosphate by liver(a), bone(b) and intestinal(c) phosphatases. Buffer solutions: ○ , tris-HCl; △ , ammediol-HCl.

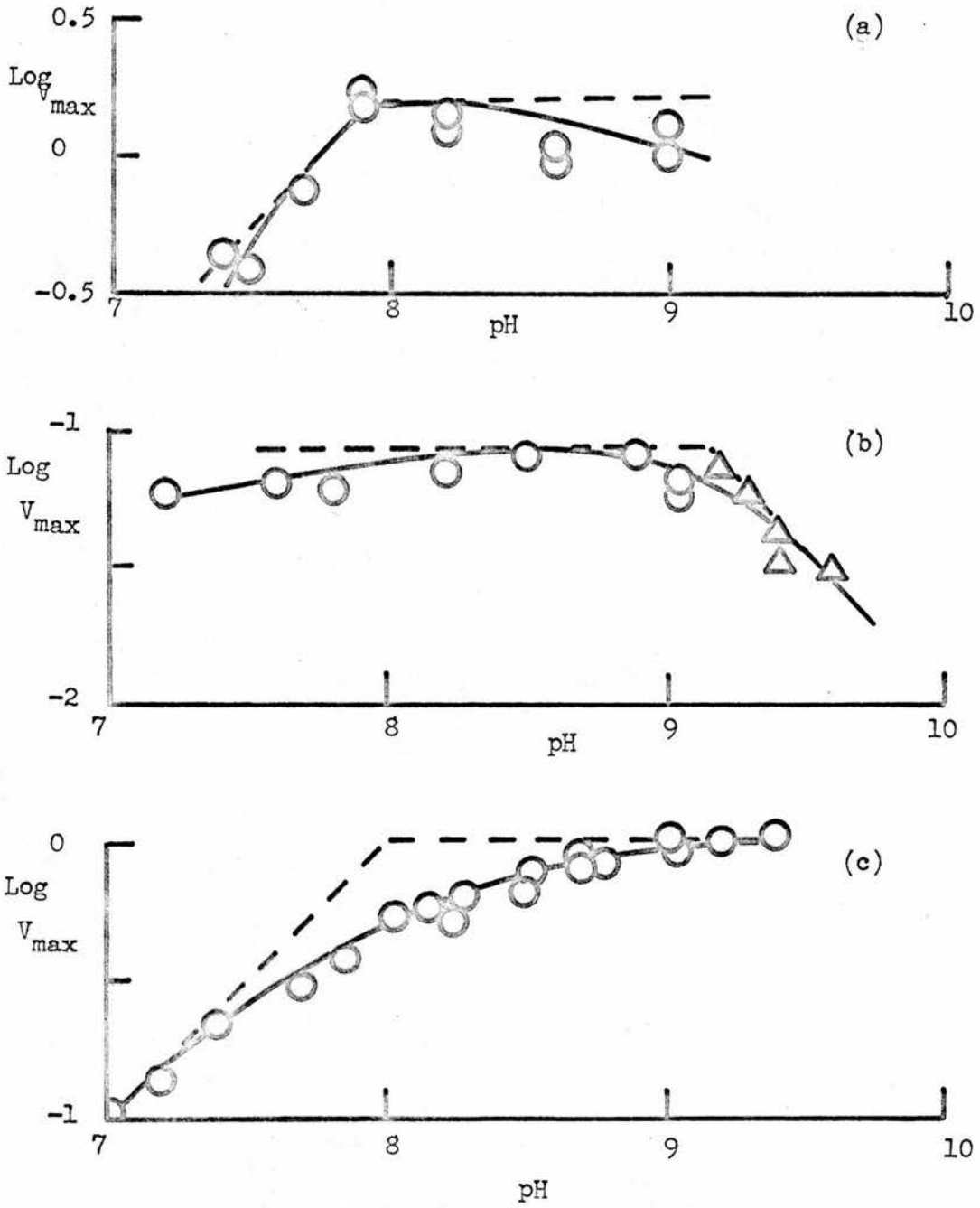


Table 4.8 A Comparison of the V_{max} Values for Orthophosphatase (pNPP) and Pyrophosphatase (PP_i) Activity for the Bone Enzyme at Several pH Values.

V_{max} is expressed as μmoles substrate hydrolysed/min./ml.

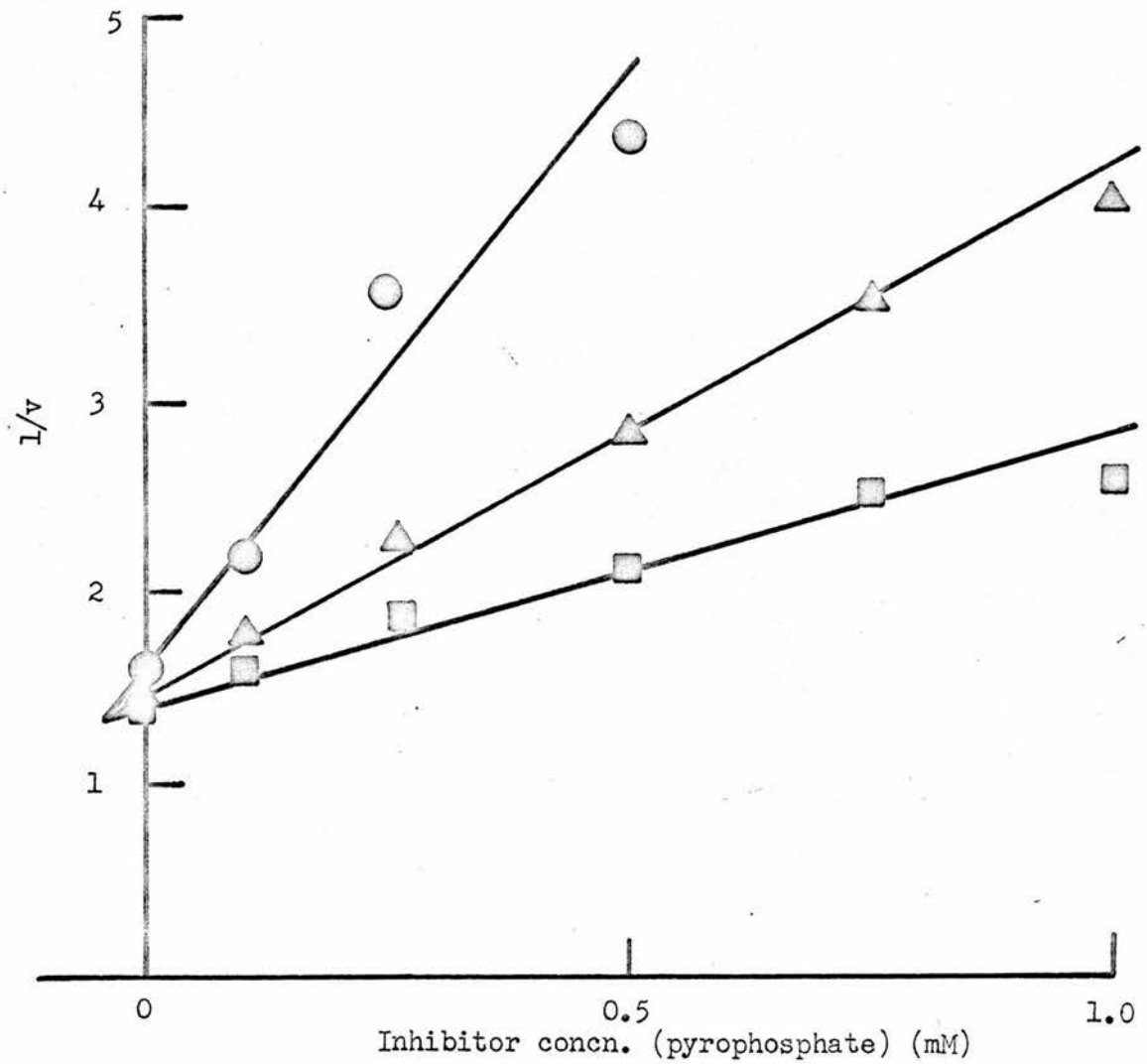
pH	pNPP	PP_i
7.0	0.02	0.05
8.0	0.065	0.08
9.0	0.25	0.07
10.5	1.05	-

Note: Corresponding tables for the V_{max} values with liver and intestinal phosphatases could not be produced from the results of the velocity determinations, since different enzyme preparations were used for the orthophosphatase and pyrophosphatase assays.

4.4. INHIBITION.

The inhibition of orthophosphatase activity by inorganic pyrophosphate was measured at pH 8.9 (0.1M tris-HCl) with pNPP as substrate. Magnesium ions were omitted. The results were plotted as 1/v against the concentration of inhibitor (Dixon, 1953b), and it was found that inhibition was competitive (Fig. 4.23.). At pH 8.9 the rate of hydrolysis of pyrophosphate is fairly low, while the orthophosphatase activity is still readily measured. In a similar experiment the inhibition of inorganic pyrophosphatase activity by pNPP was measured at

Fig.4.23. Plot of 1/v against i for inhibition of orthophosphatase activity of intestinal phosphatase by inorganic pyrophosphate at pH 8.9. Substrate (p-nitrophenyl phosphate) concentrations (mM): ○ ,0.1; △ ,0.25; □ ,0.5. Initial velocity,v, is expressed as μmoles/min./ml.



pH 8.5. The rate of breakdown of pNPP at pH 8.5 in the absence of magnesium ions is low and the incubation time was kept short. The inhibition was again found to be competitive (Fig. 4.24.). The inhibition by inorganic pyrophosphate of pNPP activity of the bone and liver enzymes at pH 8.9 was also competitive. The corresponding inhibition by pNPP was not studied.

The relationship between pyrophosphate inhibition of orthophosphatase activity and magnesium concentration was studied using α-naphthyl phosphate as substrate. The velocity of hydrolysis was observed in a recording spectrophotometer by measuring the increase in absorption at 335mμ due to the production of α-naphthol (Moss, 1966), in the presence of the carbonate-bicarbonate buffer, pH 9.9. The results for the inhibition of the orthophosphatase activity of intestinal phosphatase by inorganic pyrophosphate at different concentrations of inhibitor and varying concentrations of magnesium ions are plotted in Fig. 4.25. In the absence of inhibitor the optimum concentration of Mg^{2+} was 1mM but, as the concentration of pyrophosphate was increased, there was an upward shift in the optimum concentration of magnesium (Fig. 4.25b). This could be due to the formation of a complex between magnesium ions and pyrophosphate ions, with a consequent reduction in the effective magnesium concentration.

Fig. 4.25a shows that, in the absence of added magnesium, orthophosphatase activity was slightly inhibited by pyrophosphate, but that pyrophosphate acts as an activator with 6.7mM Mg^{2+}. This is thought to be due to the removal by inorganic pyrophos-

Fig.4.24. Plot of 1/v against i for inhibition of pyrophosphatase activity of intestinal phosphatase by p-nitrophenyl phosphate at pH 8.5. Substrate (inorganic phosphate) concns. (mM): ○ ,1.0; △ ,2.0; □ ,3.0. Initial velocity, v, is expressed as μmoles/min./ml.

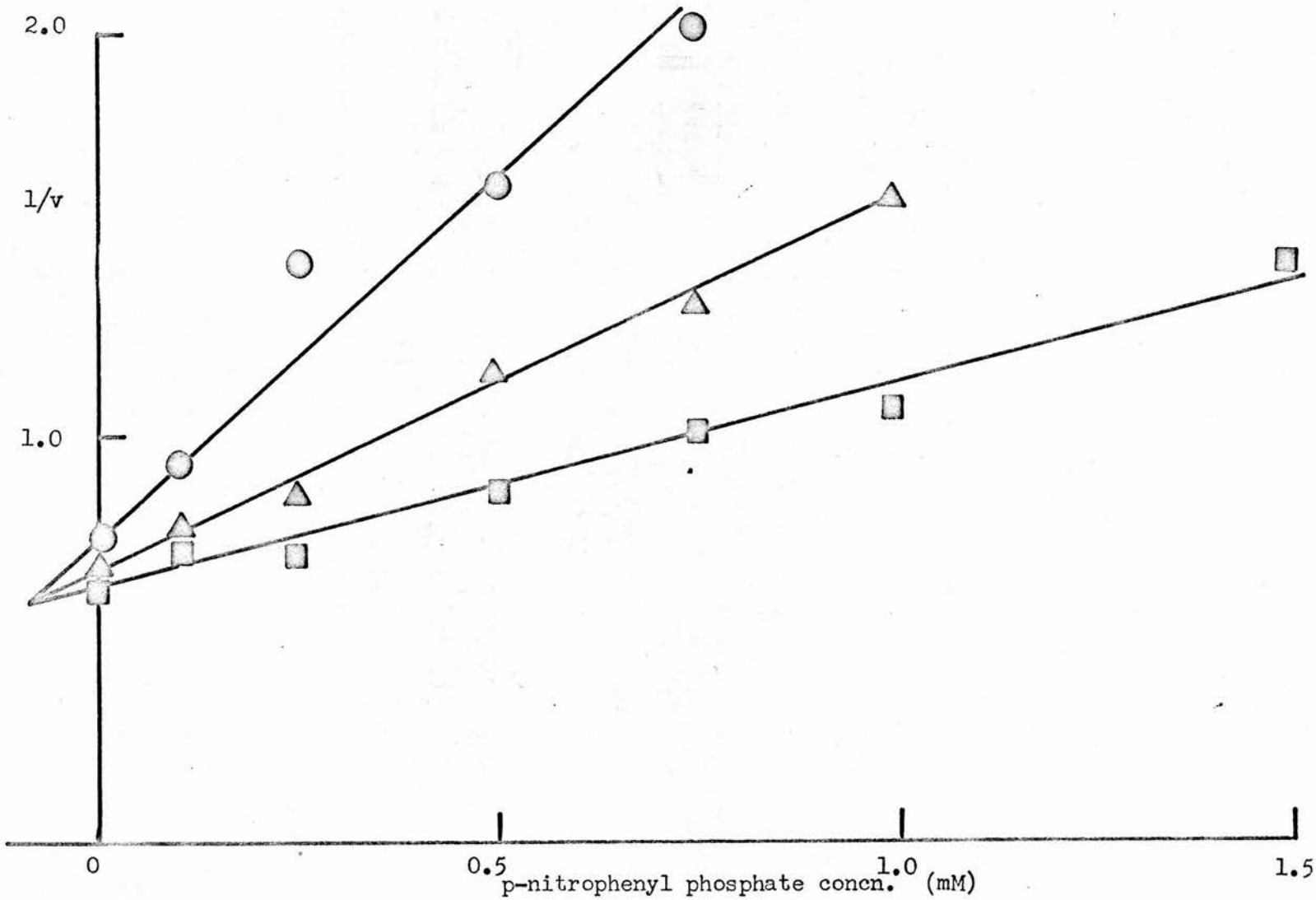
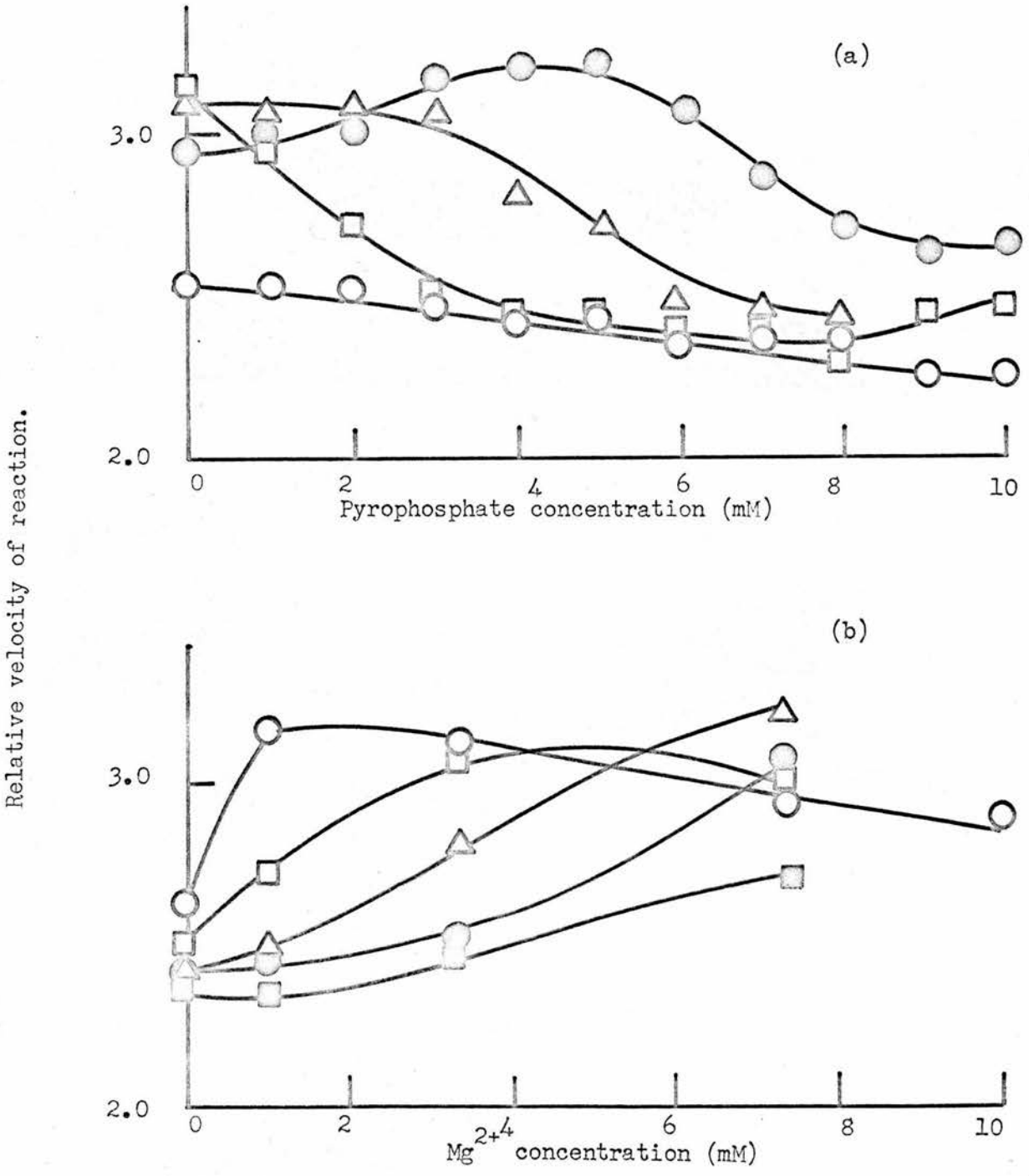


Fig.4.25.(a) Relationship between orthophosphatase activity (substrate, 3.3mM α-naphthyl phosphate) and inorganic pyrophosphate concn. at pH 9.9. Mg^{2+} concns.(mM): ○ ,0; □ ,1.0; △ ,3.3; ◯ ,6.7. Intestinal enzyme was used. (b) Replotted as v against Mg^{2+} concn. Pyrophosphate concn.(mM): ○ ,0; □ ,2; △ ,4; ◯ ,6; □ ,8.



phate of the slight inhibition produced by excess magnesium ions.

At pH 9.9, with 3.3 mM ∝-naphthyl phosphate as substrate and without the addition of Mg^{2+}, 10mM inorganic pyrophosphate inhibited the orthophosphatase activity by about 10% (Fig. 4.25). On the other hand, at pH 8.9 and in the presence of 1.0mM inorganic pyrophosphate, the inhibition of the orthophosphatase activity, measured by the hydrolysis of 0.5mM pNPP, was 44%; these findings are calculated from the results presented in Fig. 4.23. It would, therefore, appear that pyrophosphate does not bind so strongly to the phosphatase at pH 9.9 as at pH 8.9. This is in agreement with the observation that K_m increases in value with rise in pH, noted for the intestinal enzyme when using inorganic pyrophosphate as substrate (Table 4.7.).

L-phenylalanine inhibits the alkaline phosphatase from intestine but is without effect on alkaline phosphatase from other tissues (Fishman et al, 1962), and these findings have been confirmed for the enzyme studied in the present investigation. The activities of purified liver and bone phosphatases were unaffected by the addition of L-phenylalanine to the assay system. The inhibition by L-phenylalanine of the orthophosphatase activity of intestinal phosphatase was measured at pH 9.9 (carbonate-bicarbonate buffer), without the addition of magnesium ions, using pNPP as substrate. The inhibition of pyrophosphatase activity at pH 8.5 (0.1M tris-HCl) was also measured. Plots of 1/v against the concentration of inhibitor showed that the inhibition was non-competitive in type for both activities

(Fig. 4.26), and the inhibitor constants (K_i) were calculated (Table 4.9). When the inhibition of the two activities was measured at the same pH (0.1M tris-HCl, pH 8.9) the inhibitor constants were the same for both substrates (Fig. 4.27). At pH 8.9, low concentrations of pNPP must be used because a shift in the optimum pH to low values occurs with decreasing substrate concentration leads to inhibition by excess substrate as noted by Motzok (1950) and Ross et al (1951).

Sodium arsenate acted as a competitive inhibitor of the orthophosphatase and pyrophosphatase activities of all three alkaline phosphatases studied. K_i values were similar for both activities when they were measured at the same pH (Fig. 4.28-4.31), and the inhibitor constants for each enzyme are recorded in Table 4.9.

4.5. CATALYTIC-CENTRE ACTIVITY.

Barman and Gutfreund (1966) estimated the catalytic-centre activity of bovine milk alkaline phosphatase by labelling the active centre of the enzyme with inorganic phosphate labelled with ^{32}P. In the present studies, purified human liver and intestinal alkaline phosphatases were phosphorylated with ^{32}P by a modification of the method of Barman and Gutfreund (1966).

About 1-3 mg. protein, having a total enzyme activity of approximately 50 µmoles pNPP hydrolysed/min. at 37° , was incubated with varying amounts of sodium orthophosphate (0 - 2mM) and a known amount of carrier-free [^{32}P] - ortho-phosphate dissolved in sodium acetate - acetic acid buffer, pH 5.0 (0.05M and 0.035 M with respect to magnesium acetate).

Fig.4.26. Plots of 1/v against i for inhibition of intestinal orthophosphatase and pyrophosphatase by L-phenylalanine at pH 9.9 and pH 8.5 respectively. Substrate concns. (mM): p-nitrophenyl phosphate: ○ ,0.25; △ ,2.0; pyrophosphate: ◯ ,0.5; △ ,5.0. Initial velocity, v, is expressed as μmoles/min./ml.

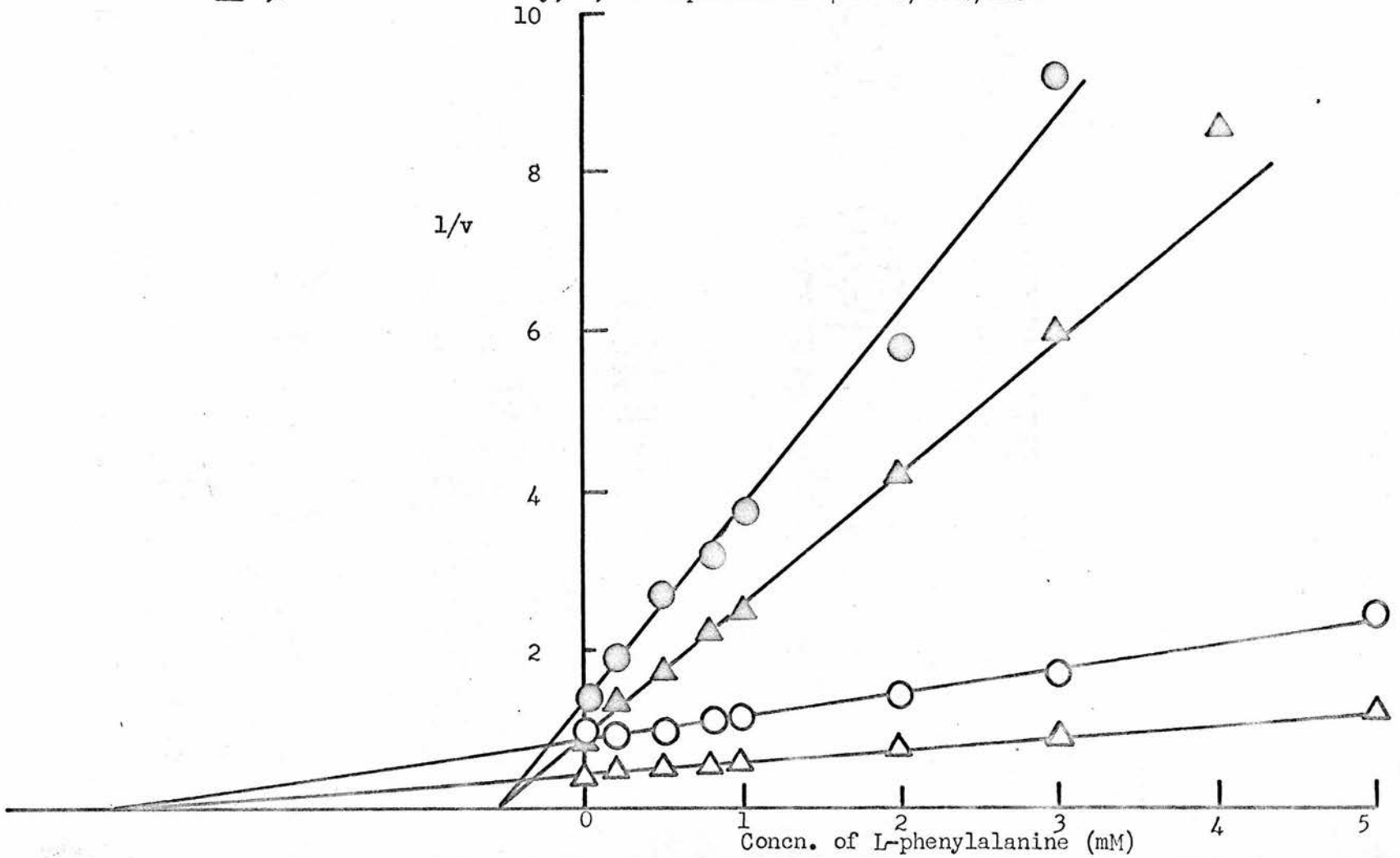


Fig.4.27. Plots of 1/v against i for inhibition of intestinal orthophosphatase and pyrophosphatase activities by L-phenylalanine at pH 8.9. Substrate concns.(mM): p-nitrophenyl phosphate: ○ ,0.02; △ ,0.1; □ ,0.5; inorganic pyrophosphate: ● ,0.5; ▲ ,3.0. Initial velocity, v, is expressed as μmoles /min./ml.

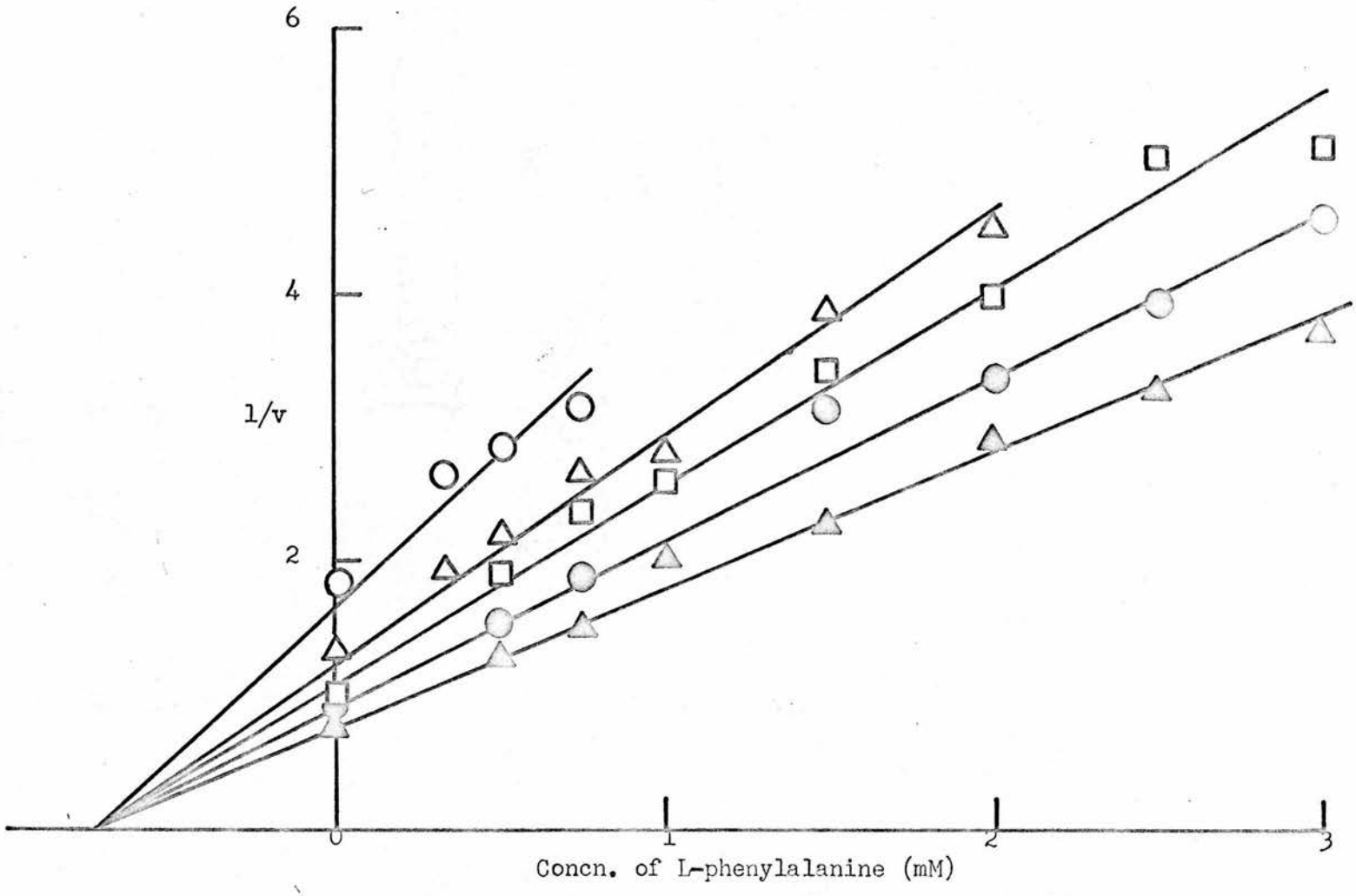


Fig.4.28. Plots of 1/v against i for inhibition of intestinal orthophosphatase and pyrophosphatase activities by arsenate at pH9.9 and pH 8.5 respectively. ○ , △ , □ , orthophosphatase; ○ , △ , □ , pyrophosphatase: Substrate concns. (mM): p-nitrophenyl phosphate: ○ ,0.5; △ ,1.0; □ ,2.0; pyrophosphate: ○ ,0.5; △ ,1.0; □ ,3.0. Initial velocity, v, is expressed as μmoles/min./ml.

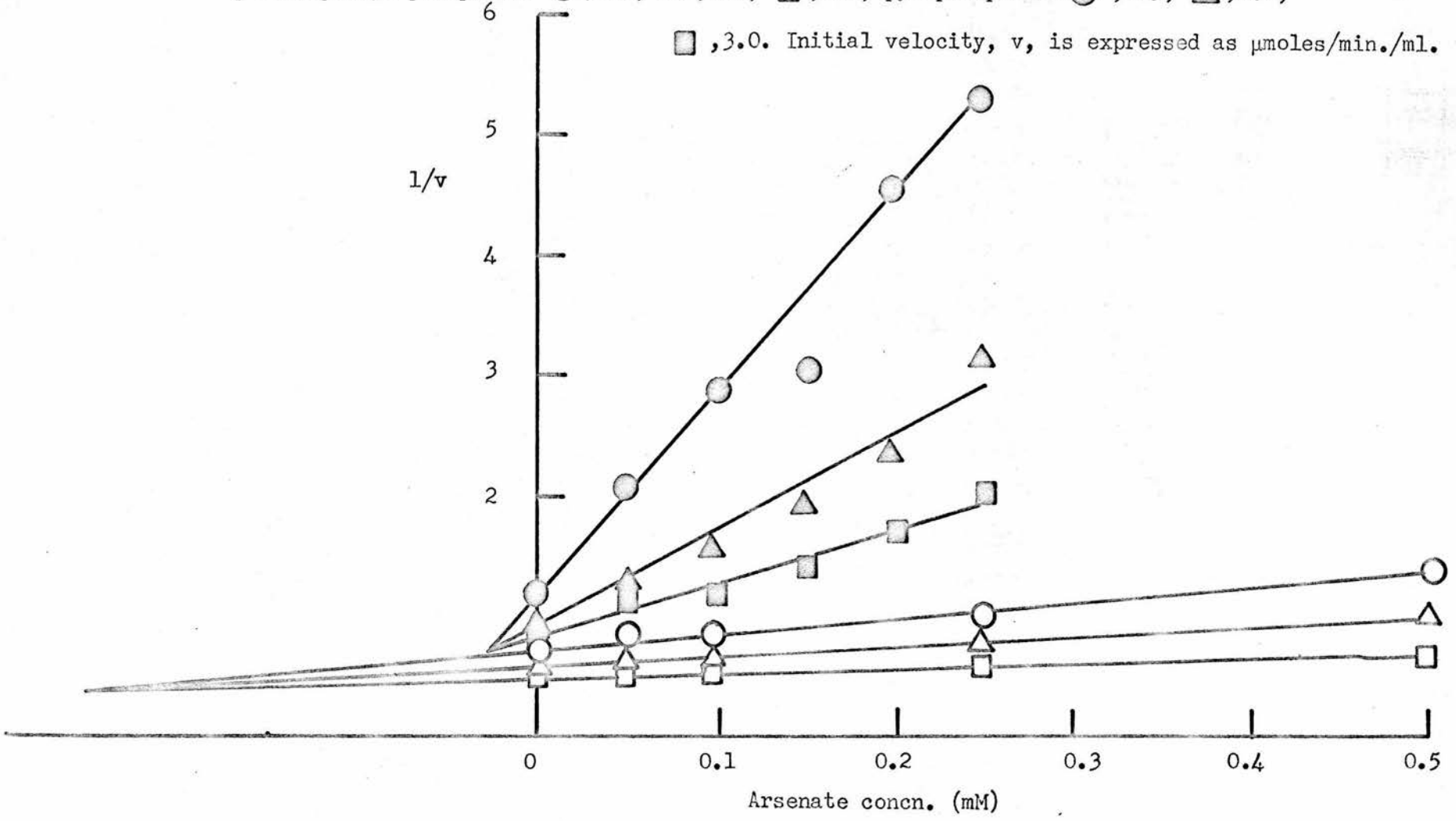


Fig.4.29. Plots of 1/v against i for inhibition of intestinal orthophosphatase and pyrophosphatase activities by arsenate at pH 8.9. ○ , △ , □, orthophosphatase; ○, △ , □ , pyrophosphatase. Substrate concns. (mM):p-nitrophenyl phosphate: ○ ,0.1; △ ,0.25; □ ,0.5; pyrophosphate: ○ ,0.5; △ ,1.0; □ ,3.0. Initial velocity, v, is expressed as μmoles/min./ml.

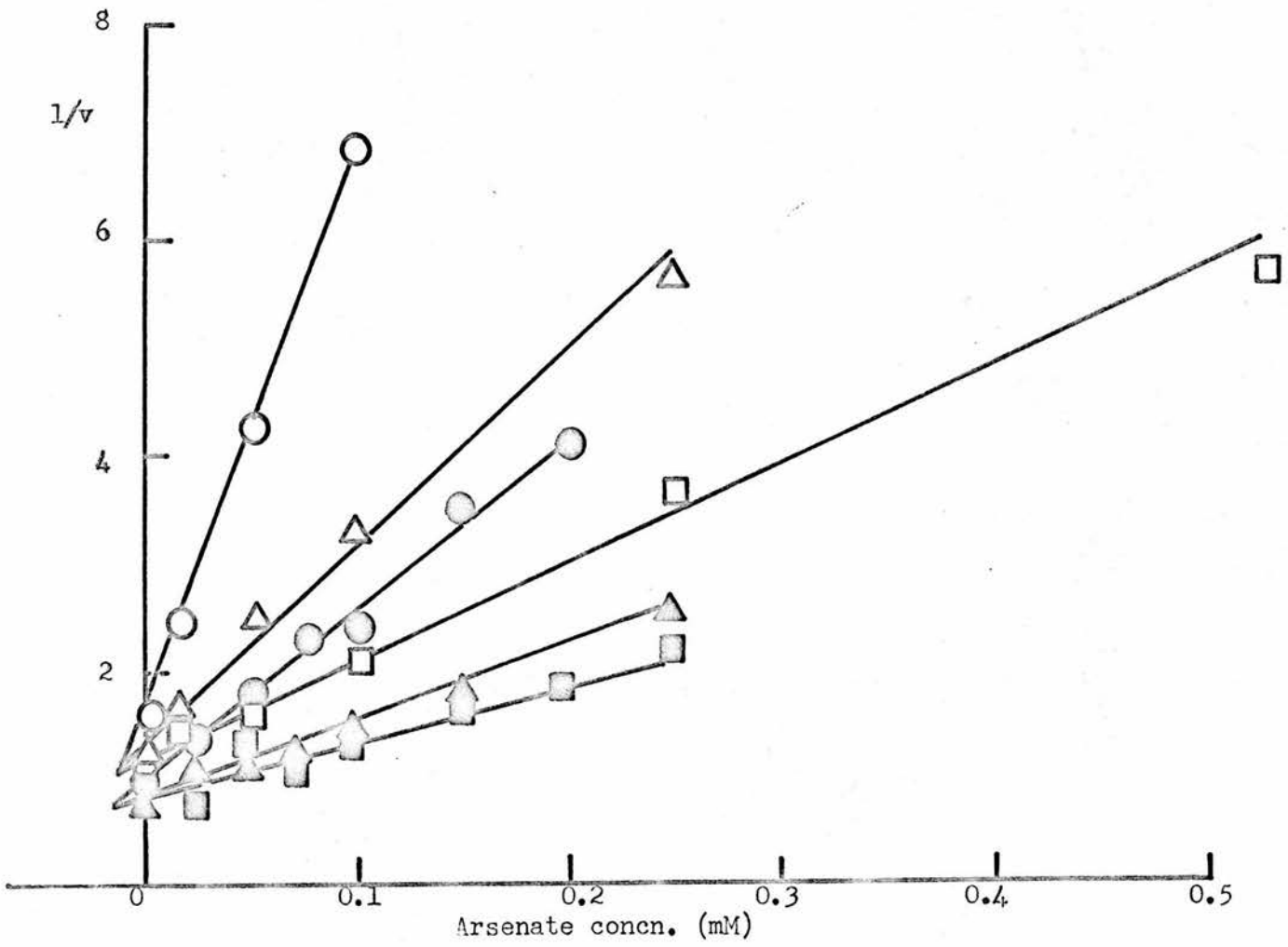


Fig.4.30. Plots of 1/v against i for inhibition of liver orthophosphatase and pyrophosphatase activities by arsenate at pH 8.9. Substrate concns.(mM): p-nitrophenyl phosphate: ○ , 0.1; △ , 0.25; □ , 0.5; pyrophosphate: ○ , 0.5; △ ,1.0; □ , 3.0. Initial velocity, v, is expressed as μmoles/min./ml.

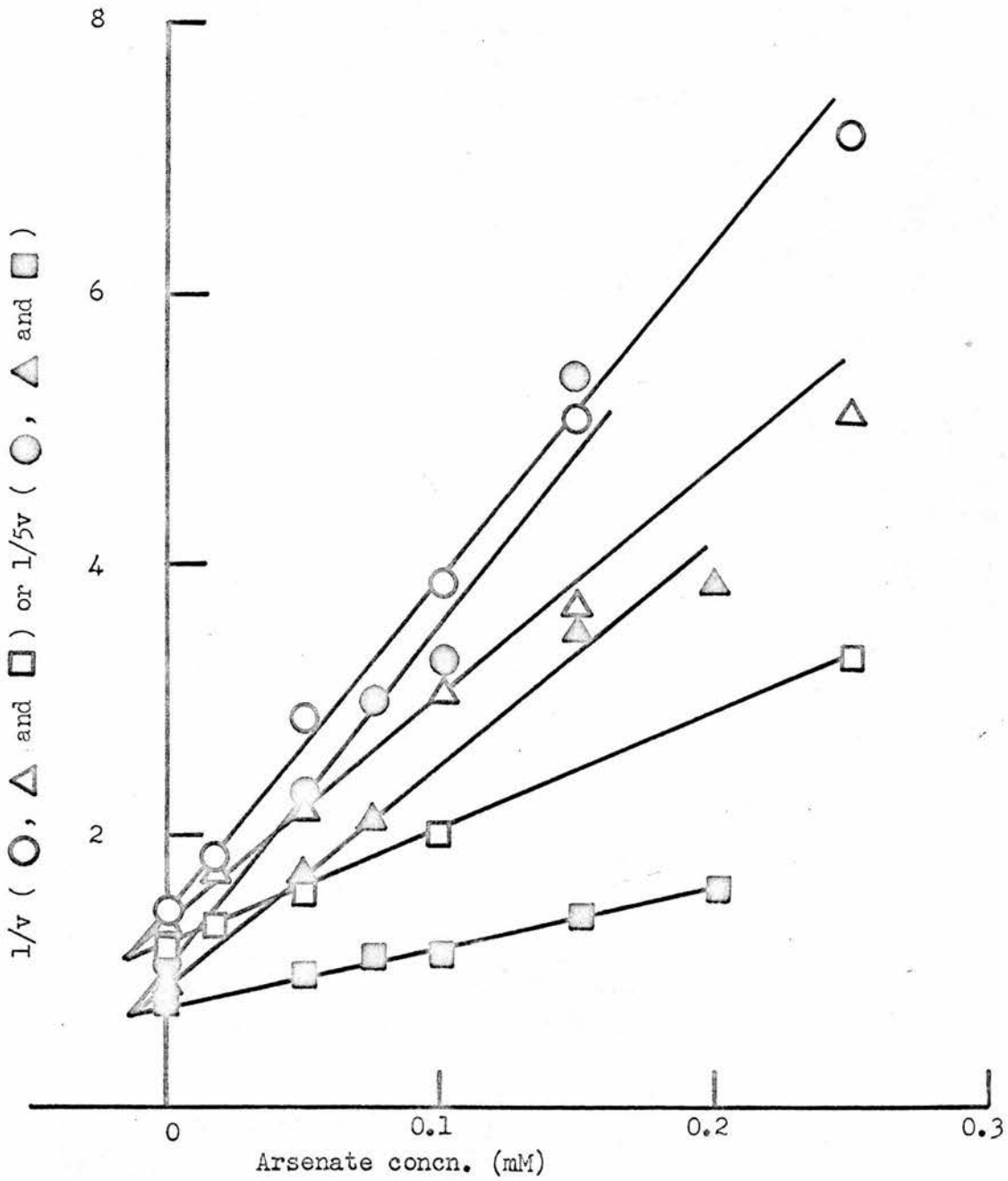


Fig.4.31. Plots of 1/v against i for inhibition of bone orthophosphatase and pyrophosphatase activities by arsenate at pH 8.9. Substrate concns. (mM): p-nitrophenyl phosphate: ○ , 0.125; △ , 0.25; □ , 0.5: pyrophosphate: ◯ , 0.3; △ , 1.5. Initial velocity, v, is expressed as μmoles/min./ml.

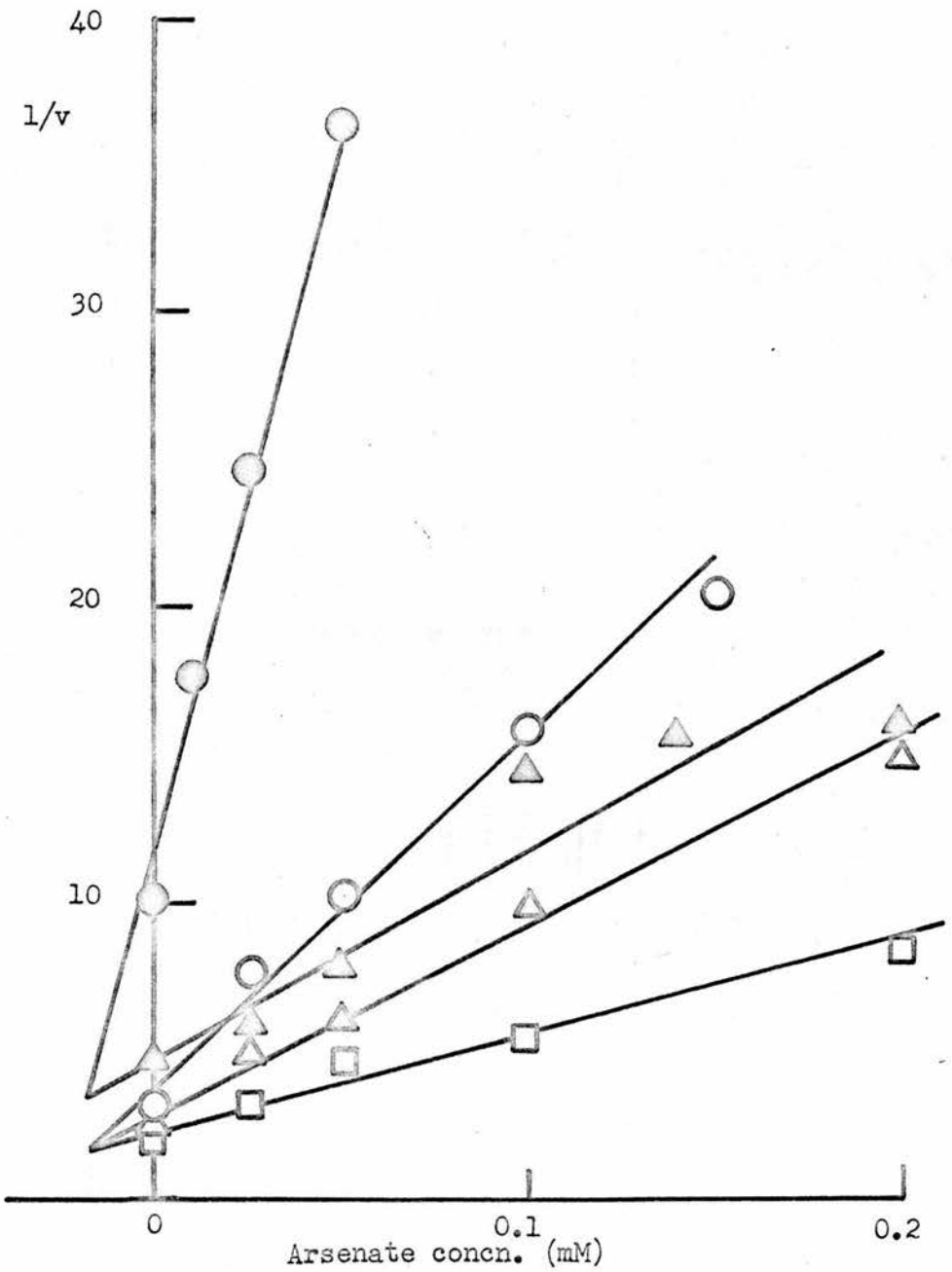


Table 4.9. Values of K_i (mM) for the Inhibition of Orthophosphatase and Pyrophosphatase Activities of Purified Intestinal, Liver and Bone Preparations.

Enzyme	Substrate	Inhibitor	pH	K_i	Type of inhibition
Intestinal phosphatase	Pyrophosphate	pNPP	8.5	0.09	competitive
	pNPP	Pyrophosphate	8.9	0.05	competitive
	Pyrophosphate	L-phenylalanine	8.5	0.5	non-competitive
	Pyrophosphate	L-phenylalanine	8.9	0.8	non-competitive
	pNPP	L-phenylalanine	8.9	0.8	non-competitive
	pNPP	L-phenylalanine	9.9	3.5	non-competitive
	Pyrophosphate	Arsenate	8.5	0.025	competitive
	Pyrophosphate	Arsenate	8.9	0.02	competitive
	pNPP	Arsenate	8.9	0.02	competitive
	pNPP	Arsenate	9.9	0.25	competitive
Liver phosphatase	Pyrophosphate	Arsenate	8.5	0.02	competitive
	Pyrophosphate	Arsenate	8.9	0.02	competitive
	pNPP	Arsenate	8.9	0.02	competitive
	pNPP	Arsenate	9.9	0.4	competitive
Bone phosphatase	Pyrophosphate	Arsenate	8.9	0.02	competitive
	pNPP	Arsenate	8.9	0.02	competitive
	pNPP	Arsenate	9.9	0.38	competitive

This solution (total volume: 1.0ml.) was kept at 0° for 3 mins. and then γ-globulin (1mg. in 0.1ml. water) followed immediately by 0.2ml. of 20% (v/v) perchloric acid were added. The γ-globulin was added to assist in the precipitation of the enzyme. Control tubes for each phosphate concentration were incubated similarly except that an amount of γ-globulin equivalent to the enzyme protein was added instead of the enzyme. The precipitates were washed five times with a total volume of 30ml. 5% (v/v) perchloric acid and were then dissolved in 0.5ml. 90% formic acid. The total radioactivity in the solution was measured by liquid scintillation counting (Packard, Tricarb). The liquid scintillator contained 6 gm. diphenyloxazole, 0.3 gm. 1,4 bis-5(-phenyl oxazol-2-yl) -benzene, and 100 gm. naphthalene in 1 litre dioxan. Considerable quenching was observed during counting but a correction was applied by the addition of internal standards of ^{32}P.

For a simple dissociation of phosphoryl enzyme into free enzyme and orthophosphate, the equation $EP \rightleftharpoons E + P$ can be written.

Then, $K = \frac{[E]\ [P]}{[EP]}$ where K is the dissociation constant.

If EP = the concentration of phosphoryl enzyme

P_o = the total orthophosphate concentration

E_o = the total enzyme concentration

then $[E] = E_o - EP$ and $[P] = P_o - EP$.

Since EP is very small compared with P_o, P is approximately

equal to P_o, the expression becomes

$$K = \frac{(E_o - EP)\ P_o}{EP}$$

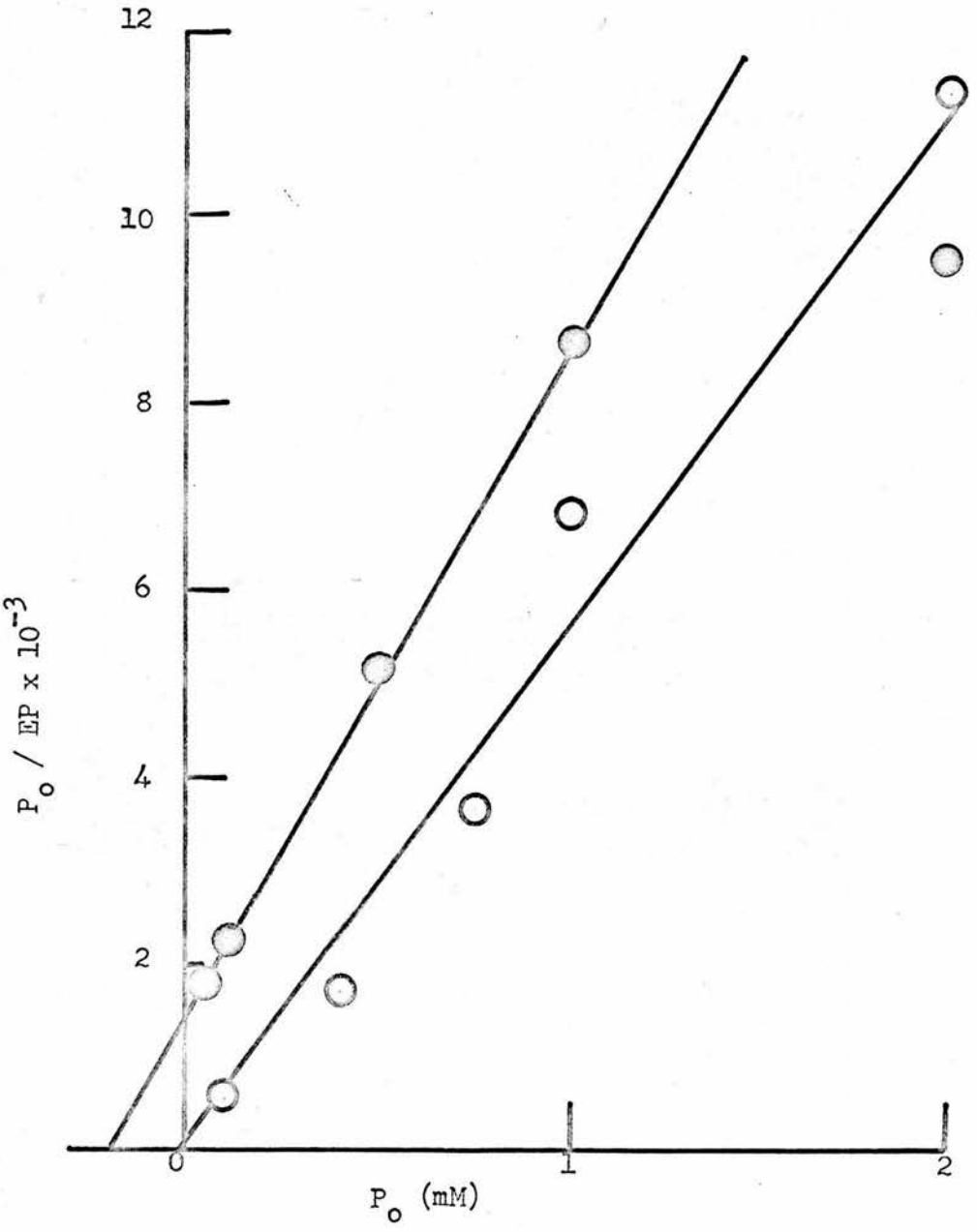
and $$\frac{P_o}{EP} = K.\frac{1}{E_o} + P_o \frac{1}{E_o}$$

If P_o/EP is plotted against P_o then a straight line of gradient $1/E_o$ and with an intercept on the x-axis equal to -K is obtained E_o represents the concentration of sites on the enzyme which bind phosphate at pH 5.0. If it is assumed that this is the concentration of catalytic-centres in the molecule, and that they are all equally labelled, then the catalytic-centre activity of the enzyme can be calculated.

The results for the liver and intestinal phosphatases were plotted in this manner, and the results are shown in Table 4.10 and Fig. 4.32. From the graphs the concentrations of active sites were found to be 0.18 μM and 0.14 μM for the liver and intestinal phosphatases respectively, and the catalytic-centre activities were calculated to be 5030 $sec.^{-1}$ and 6550 $sec.^{-1}$ for pNPP at 37° and pH 10.0. The dissociation constant for the phosphorylated enzyme from intestine is 2 x 10^{-4}M, but the value for the liver enzyme is too low to be measured with accuracy. To make sure that the uptake of ^{32}P was due to incorporation into active enzyme and not the result of non-specific absorption, labelling with ^{32}P was carred out with a set of controls containing heat-inactivated enzyme, and with another set in the presence of a high

Fig.4.32. Plot of P_o/EP against P_o where P_o is the total orthophosphate concentration and EP the concentration of catalytic centres combined with phosphate.

○ liver phosphatase; ◎ intestinal phosphatase.



concentration of a substrate (20mM pNPP); the uptake of radioactivity was reduced in both instances.

Estimation of the purity of the enzyme preparations: If the molecular weights of the liver and intestinal phosphatases are taken as 220,000 and 195,000 respectively (Chapter three), the purity of the best preparations can be calculated. For the liver enzyme the average specific activity obtained at the final stage of purification was 148 µmoles pNPP hydrolysed/min./mg. protein (Chapter two). Assuming one catalytic-centre/enzyme molecule, the specific activity of the pure enzyme would be 1370 µmoles pNPP hydrolysed/min./mg. protein; the purity of this preparation is thus about 11%. Similarly, the highest specific activity found for the intestinal enzyme was 136 µmoles pNPP hydrolysed/min./mg., which corresponds to a purity of 7%.

If the molecular weights of the human alkaline phosphatases are compared with the value for the E.coli phosphatase, which has a molecular weight of about 80,000 (Garen and Levinthal, 1960), and account is taken of the fact that E.coli phosphatase consists of two subunits (Schlesinger and Barrett, 1965) this would suggest that alkaline phosphatase in human tissues is composed of four subunits. If this is the case and each subunit has a catalytic centre, then the purities of the best preparations studied in the present experiments would be only about 3% for the liver and 2% for the intestinal phosphatases.

Table 4.10 Incorporation of ^{32}P by Liver and Intestinal Phosphatases at pH 5.0.

Expt. A: Liver phosphatase, enzyme activity 54.4 μmoles pNPP hydrolysed/min., protein content 2mg. Initial radioactivity 2.994×10^7 cts./min.*

Expt. B: Intestinal phosphatase, enzyme activity 55.1 μmoles pNPP hydrolysed/min., protein content 2.7 mg. Initial radioactivity 2.318×10^7 cts./min.

* Cts./min. are quoted after correction has been made for quenching.

	Orthophosphate concentration (mM)	Radioactivity incorporated in enzyme precipitate (cts./min.)	Concentration of catalytic centre combined with phosphate (uM)
Expt. A	0.10	57,229	0.191
	0.40	18,152	0.242
	0.75	8,238	0.206
	1.00	4,388	0.147
	2.00	2,639	0.176
Expt. B	0.05	13,562	0.029
	0.10	10,239	0.044
	0.50	4,481	0.097
	1.00	2,713	0.117
	2.00	2,472	0.213

The bone phosphatase was not obtained in sufficient quantity and purity to permit the catalytic-centre activity to be estimated.

4.6. SUMMARY OF FINDINGS.

The alkaline phosphatases from liver, intestine and bone all hydrolyse inorganic pyrophosphate and several organic pyrophosphates (e.g. ADP, UTP, TPP, etc.) but do not hydrolyse phosphate diesters. In each case the pyrophosphatase and orthophosphatases activities are properties of the same enzyme molecule. The ratio of the two activities varies with the isoenzyme; the intestinal preparation having relatively the greatest pyrophosphatase activity and liver and bone phosphatases approximately equal relative activities.

The isoenzymes varied with respect to the effect of metal ions on the rates of hydrolysis both with orthophosphatase and pyrophosphatase activities. Kinetics of the hydrolysis reaction also varied with the tissue source of the alkaline phosphatase. Arsenate inhibited both the inorganic pyrophosphatase activity and the orthophosphatase activity of all three isoenzymes competitively. On the other hand phenylalanine inhibited the intestinal enzyme noncompetitively but had no effect on the enzymes from bone and liver. A study of the catalytic centre activities showed liver and intestinal phosphatases to differ in this respect.

CHAPTER 5. AN EXAMINATION OF THE MOLECULAR STRUCTURE.

5.1. INTRODUCTION.

An examination of the amino acid sequence at the active centre of alkaline phosphatase molecules has been made by Engstrom (1964b) on calf intestinal phosphatase, and by Milstein (1964) on E. coli alkaline phosphatase. Engstrom (1961b) found that, if calf intestinal phosphatase was incubated with ^{32}P-orthophosphate and subsequently hydrolysed with hydrochloric acid at 100°, serine phosphate with a radioactive label could be isolated. The incorporation was maximal at pH 5.0 and complete in 20 secs; there was no incorporation with enzyme that had previously been inactivated by heat. By partial hydrolysis of the enzyme labelled in this way, the amino acid sequence around the serine residue in the phosphatase molecule was found to be aspartic acid-serine-alanine for both the calf intestinal enzyme (Engstrom, 1964b) and the phosphatase from E. coli (Milstein, 1964).

It therefore appeared probable that there was a serine residue at the active centre of human alkaline phosphatase and attempts were made to verify this. It was also hoped to show whether liver and intestinal alkaline phosphatases differ in molecular structure in the region of their active centres, by investigating whether differences existed in the patterns of labelled peptides prepared from the two enzymes.

5.2. LABELLING OF LIVER ALKALINE PHOSPHATASE WITH ^{32}P-PHOSPHATE: PRELIMINARY EXPERIMENTS.

Liver phosphatase (enzyme activity: 18 µmoles pNPP hydrolysed/min. Total protein: 20mg.) was incubated with ^{32}P-

phosphate (about 1mCurie) and 10mμmoles orthophosphate in acetate buffer pH 5.0 (total volume: 1.8ml.). The radioactivity of the incubation mixture was measured in a Geiger-Müller counter. After 5 mins. at 0°, 0.3ml. 3N HCl was added and the precipitate removed by centrifugation. The radioactivity in the supernatant was measured in a Geiger-Müller counter and the supernatant discarded. The precipitate was washed twice with acetone - HCl (8 vol. acetone and 2 vol. 0.5N HCl) and, after drying overnight in vacuo over KOH, was dissolved in 1ml. 0.01N HCl and its radioactivity measured. The results of a typical labelling experiment are given in Table 5.1.

The solution containing the protein labelled with ^{32}P, was divided into two fractions. One of these was hydrolysed with 8 mg. pepsin at 37° and pH 2.0 for 24 hours and the other by 11N HCl for 3½ days at 37°.

Table 5.1. Labelling of Liver Phosphatase with ^{32}P-Phosphate.

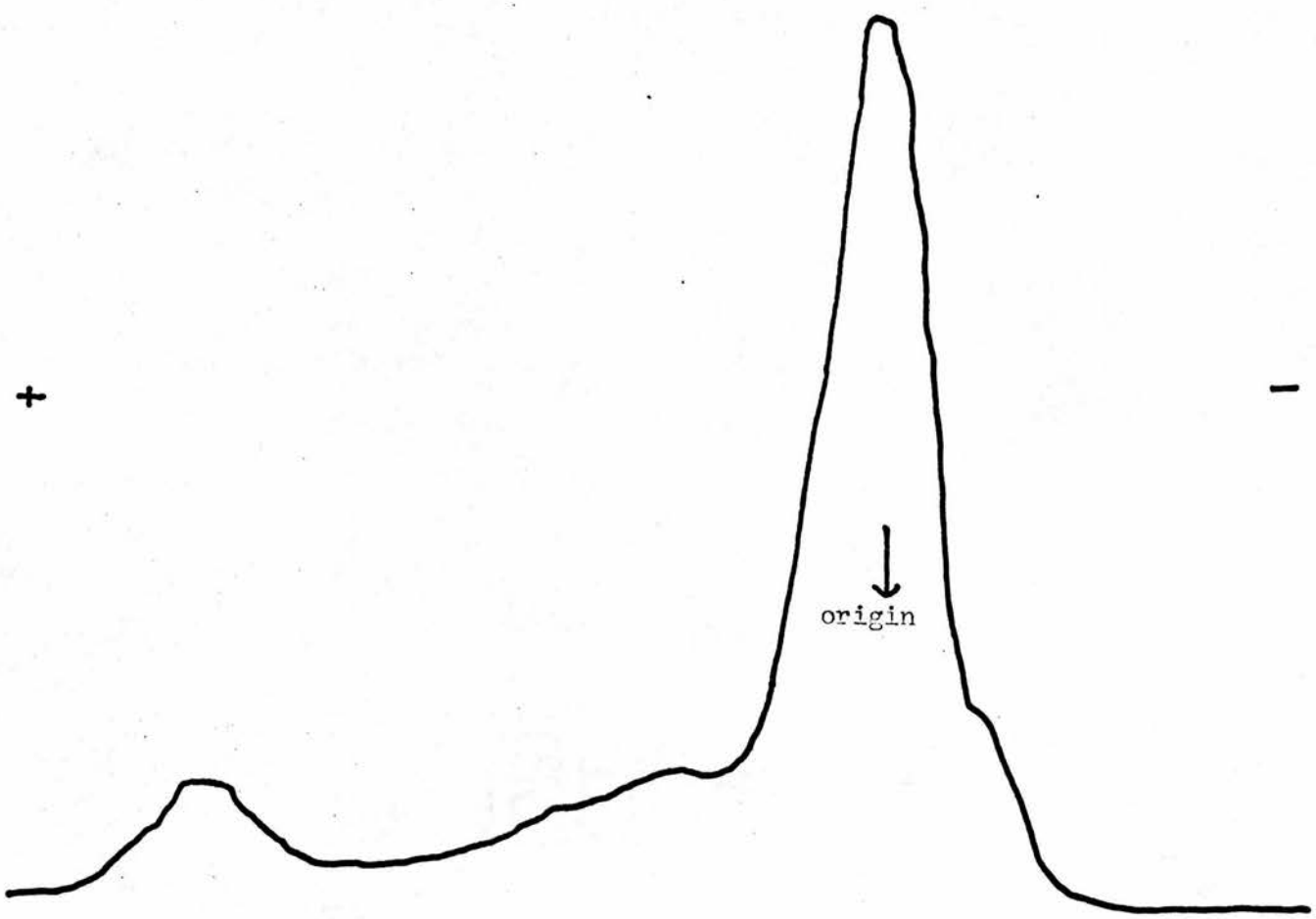
Sample counted	Total cts./sec.	% original
Original incubation mixture	4×10^6	100
Supernatant after precipitation of labelled enzyme	3×10^6	75
First washing	7.6×10^5	19
Second washing	1.2×10^4	0.3

High voltage electrophoresis of the mixture of peptides was carried out as follows. A piece of Whatman 3MM filter paper, approximately 41 by 38cm., was soaked in buffer and

excess buffer removed by pressing between sheets of filter paper. The sample was then applied as a one inch streak about 10 cm. from one end, and the paper placed on the pre-cooled plate of a "Mini Pherograph" (L. Hormuth, Heidelberg). The electrical contact was via platinum block-electrodes pressing on to filter paper strips previously soaked in buffer, the anode being at the end further from the sample. A voltage of 1500 volts was applied for 1 to 1½ hours. After electrophoresis the paper was removed, dried and the strip containing the sample cut out and scanned for radioactivity on an automatic scanner (Nuclear-Chicago) or used for auto-radiography. Unlabelled inorganic phosphate was run in parallel with the sample, and was located by dipping the strip containing the phosphate into a freshly prepared molybdate solution consisting of 8 vol. 12½% (w/v) ammonium molybdate, 3 vol. 11N HCl, 3 vol. 12N perchloric acid and 86 vol. acetone (Smith, 1960b). The paper was dried and exposed to ultra-violet light for at least half an hour, by which time the phosphate marker appeared as a blue spot.

After pepsin treatment the hydrolysate was submitted to electrophoresis at pH 6.5 in pyridine-acetic acid buffer (pyridine: glacial acetic acid; water = 10:1:100, v/v). The radioactivity scan showed two peaks, one at the origin and one that had moved towards the anode (Fig. 5.1a). With the other fraction, after acid hydrolysis, electrophoresis at pH 3.5 (pyridine; glacial acetic acid; water = 1:10:100 v/v) showed one peak at the origin. Chromatography of the acid-hydrolysed material in butanol-acetic acid (n-butanol:

Fig.5.1.a. Radioactivity scan of electrophoresis at pH 6.5 of the peptic hydrolysate of liver phosphatase labelled with ^{32}P-phosphate.



acetic acid: water = 120:30:50, v/v) for 18 hours resulted in a separation of the radioactivity into two peaks, one of which ran with the same R_f as inorganic phosphate (Fig. 5.1b).

To determine whether hydrolysis of the enzyme was occurring, a portion of phosphorylated enzyme was incubated with concentrated hydrochloric acid (37^{o} for 3 days) and was then dialysed overnight. Fig. 5.2. shows the result of electrophoresis at pH 3.5 of both the fraction within the dialysis sack and that outside.

Since only inorganic phosphate and small peptides can pass through the sack, some hydrolysis of the protein must have occurred. Amounts of radioactivity were too small to allow elution and re-chromatography of the peaks.

5.3. LABELLING OF E.COLI ALKALINE PHOSPHATASE WITH ^{32}P-PHOSPHATE.

To ensure that the method of labelling and hydrolysis of the human enzyme was effective, attempts were made to reproduce the results of Milstein (1964) with E.coli alkaline phosphatase obtained from Sigma Chemical Co., London. 5 mg. of the enzyme suspension (enzyme activity: 140µmoles pNPP hydrolysed/min./ml.) were labelled with radioactive phosphate by the method of Milstein (1964). Table 5.2. shows the results of the labelling.

Table 5.2. Labelling of E.coli Phosphatase.

Sample counted	Total cts./sec.	% original
Original incubation mixture	3.8×10^{7}	100
Supernatant after precipitation of labelled enzyme	2.9×10^{6}	8
First washing	1.4×10^{5}	1
Second washing	5.6×10^{4}	0.1

Fig.5.1.b. Radioactivity scan of chromatography (butanol:acetic acid:water) of the acid hydrolysate of liver phosphatase labelled with ^{32}P-phosphate.

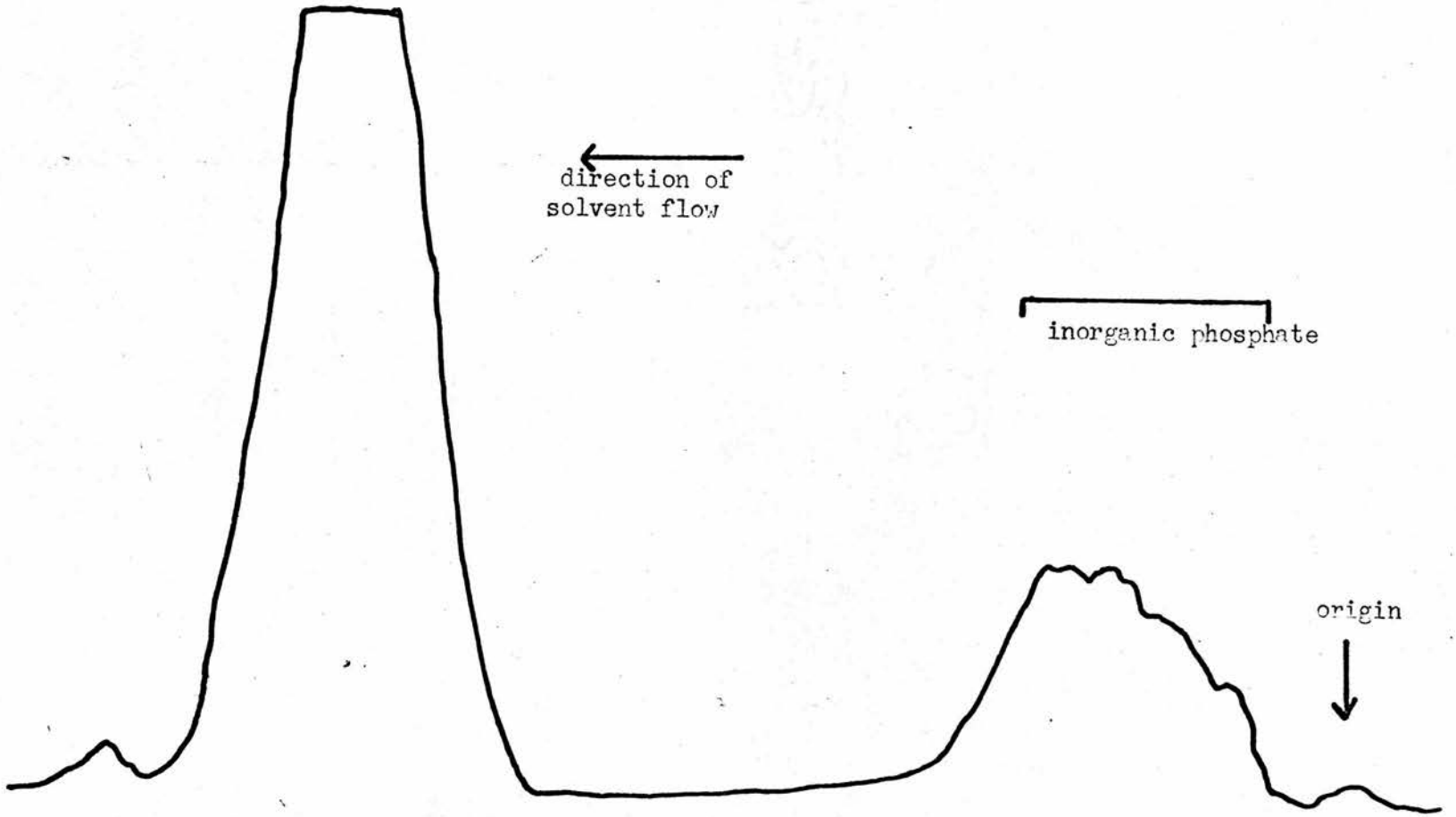
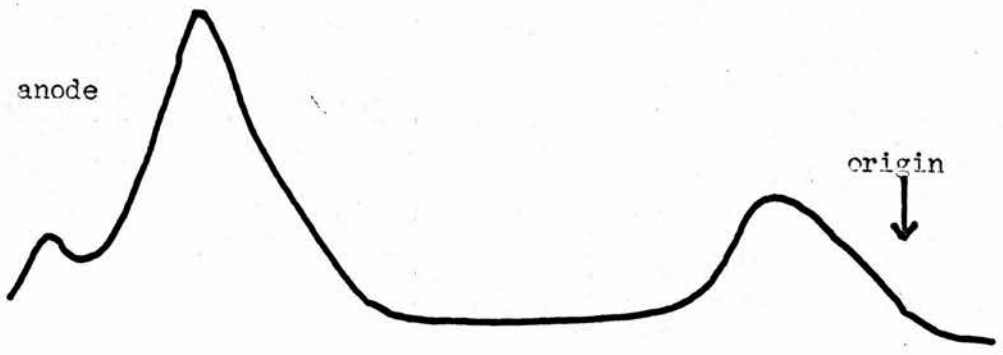
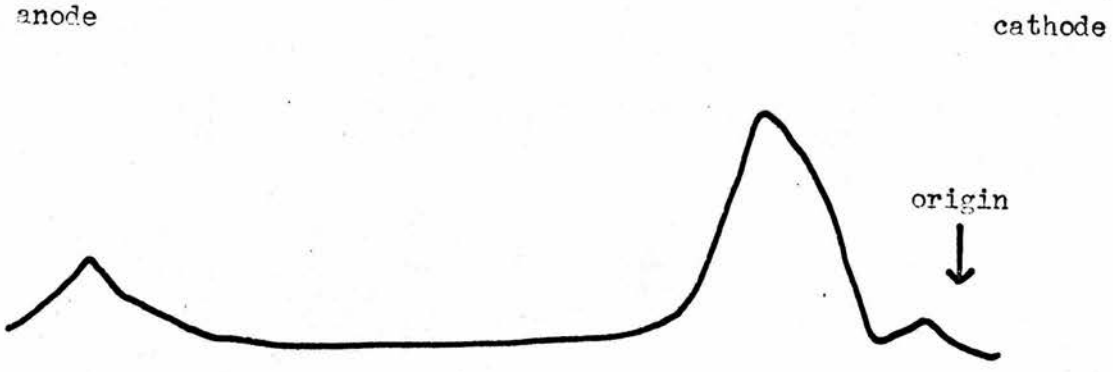


Fig.5.2. Radioactivity scans of the electrophoresis at pH3.5 of the acid hydrolysate of the liver phosphatase labelled with ^{32}P-phosphate. The hydrolysate was dialysed for 18hr. against buffer.

a) fraction outside the dialysis sack

b) fraction inside the dialysis sack.





The labelled enzyme was subjected to digestion with pepsin after oxidation with perchloric acid. Electrophoresis of the digest at pH 6.5 gave four peaks of radioactivity (Fig. 5.3.b). This tracing closely resembled the results of Milstein (1964) under similar conditions, except that Milstein was able to resolve peak three (Fig. 5.3.b) into three separate peaks. Peak three was eluted in water, evaporated to a small volume, hydrolysed with HCl and submitted to electrophoresis at pH 3.5. Ten bands of radioactivity were visible (Fig. 5.4.). An autoradiograph showed more clearly the separation between bands 4, 5 and 6, and between 9 and 10. There was also an indication of a band cathodic to the origin. In this electropherogram the inorganic phosphate was allowed to run off the paper. Milstein (1964), with an electropherogram of about twice the length, had 16 distinct bands, but, the bands of greatest intensity observed in the present work were similar to those of Milstein (1964).

Hydrolysis by hydrochloric acid for varying lengths of time gave a trace consisting of four peaks (Fig. 5.3.a). There was no significant difference between traces for different lengths of time of hydrolysis. Milstein (1964), in a separation of about twice the length, observed 16 bands of radioactivity of varying intensity. It would, therefore, appear from the results of the peptic hydrolysates that the E.coli phosphatase has been labelled and hydrolysed.

Fig.5.3.a. Trace of radioactivity scans of the electrophoresis at pH 3.5 of the acid hydrolysate of ^{32}P-phosphate labelled E.coli phosphatase.

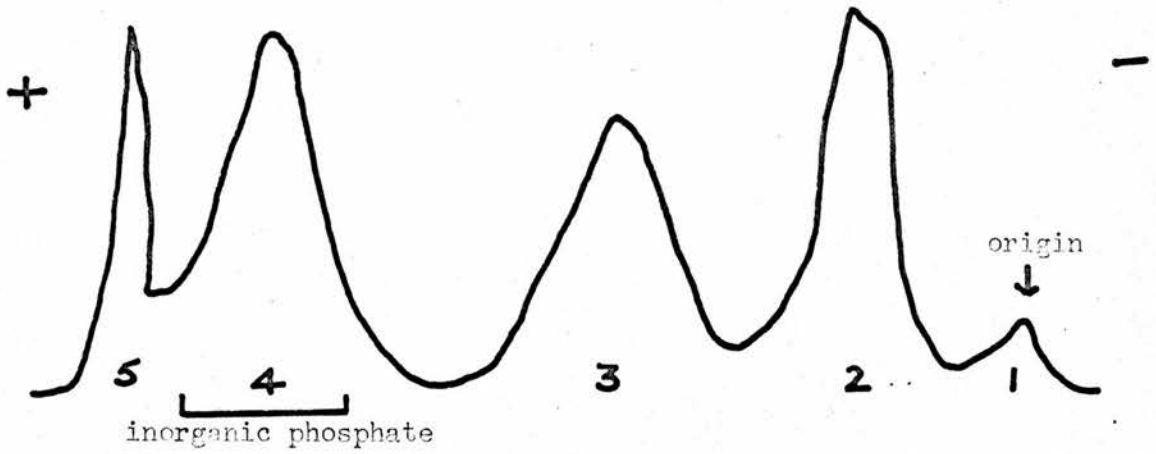


Fig.5.3.b. Trace of radioactivity scan of the electrophoresis at pH 6.5 of the peptic hydrolysate of ^{32}P-phosphate labelled E.coli phosphatase.

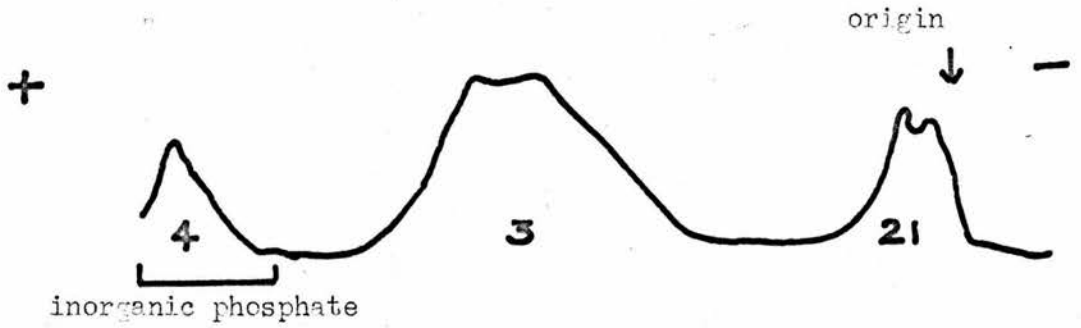
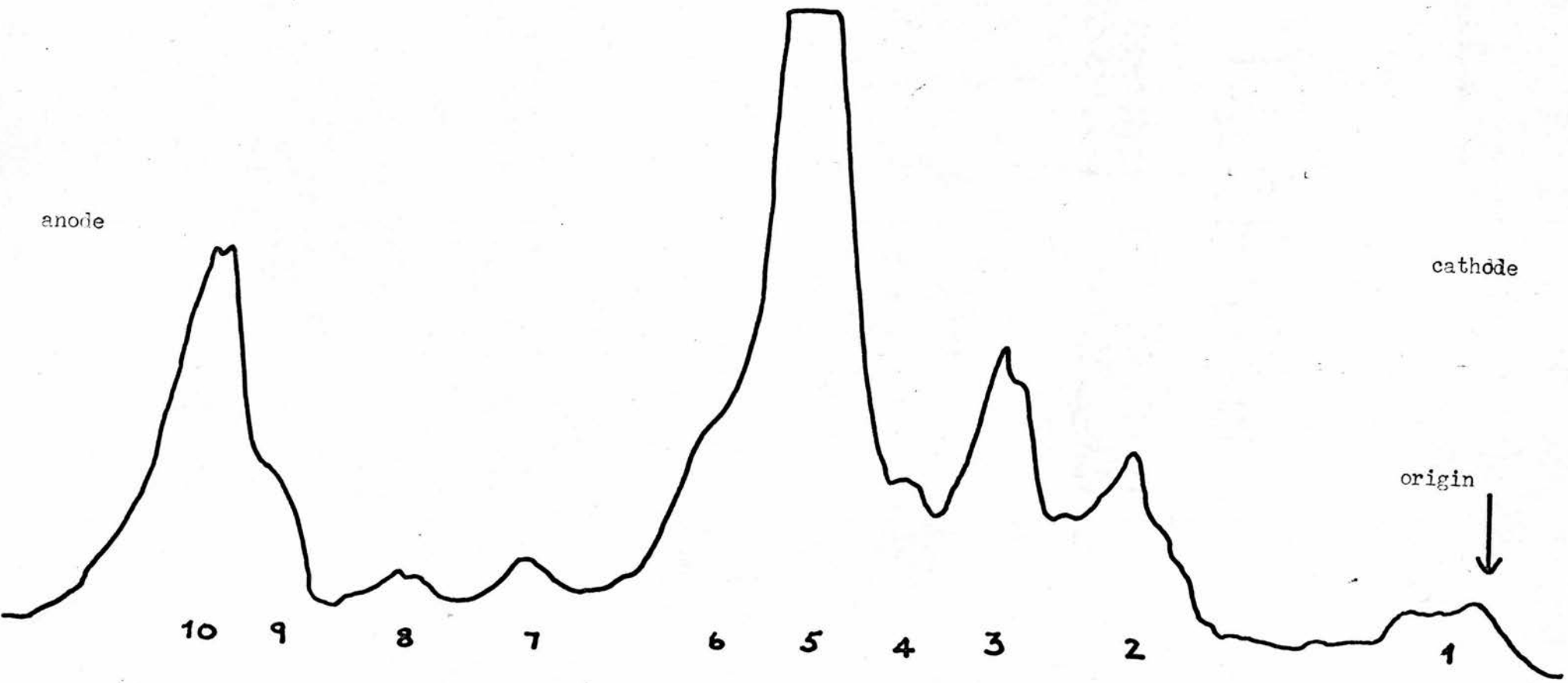


Fig.5.4. Trace of radioactivity scan of the electrophoresis at pH6.5 of peak 3 (fig.5.3.a) of the peptic hydrolysate after rehydrolysis with HCl. E.coli phosphatase.



5.4. A SECOND ATTEMPT TO LABEL LIVER PHOSPHATASE.

Since a repetition of Milstein's (1964) experiment with E.coli phosphatase had been achieved, a second attempt to label the liver enzyme was made. Phosphatase (enzyme activity: 119 µmoles pNPP hydrolysed/min.; protein: 3mg.) was incubated with ^{32}P-phosphate as described previously. Less than 1% of the radioactivity in the incubation mixture was incorporated into the enzyme protein.

A portion of the solution containing the phosphorylated enzyme was oxidised with performic acid and incubated with pepsin at pH 2.0. Electrophoresis at pH 6.5 resulted in tracings consisting of three peaks (Fig. 5.5.a). This tracing differed from that found when the liver phosphatase was first labelled with ^{32}P-phosphate and hydrolysed with pepsin (compare Fig. 5.1.a with 5.5.a). The difference may be due either to the fact that in the second experiment the protein was oxidised with performic acid prior to incubation with pepsin or to the fact that during the experiments with E.coli phosphatase the method of electrophoresis was modified. Originally the contact between the electrode and the electrophoresis paper was via a strip of filter paper soaked in buffer, but a better separation between the bands was obtained when the electrodes were placed in buffer reservoirs containing 1 litre of buffer and 3MM filter paper was used as a bridge.

Peak two (Fig. 5.5.a) was eluted, hydrolysed with acid and subjected to electrophoresis at pH 3.5. The radioactivity trace showed a double peak, one migrating at the same rate as

Fig.5.5.a. Radioactivity scan of electrophoresis at pH6.5 of peptic hydrolysate of liver phosphatase labelled with ^{32}P-phosphate.

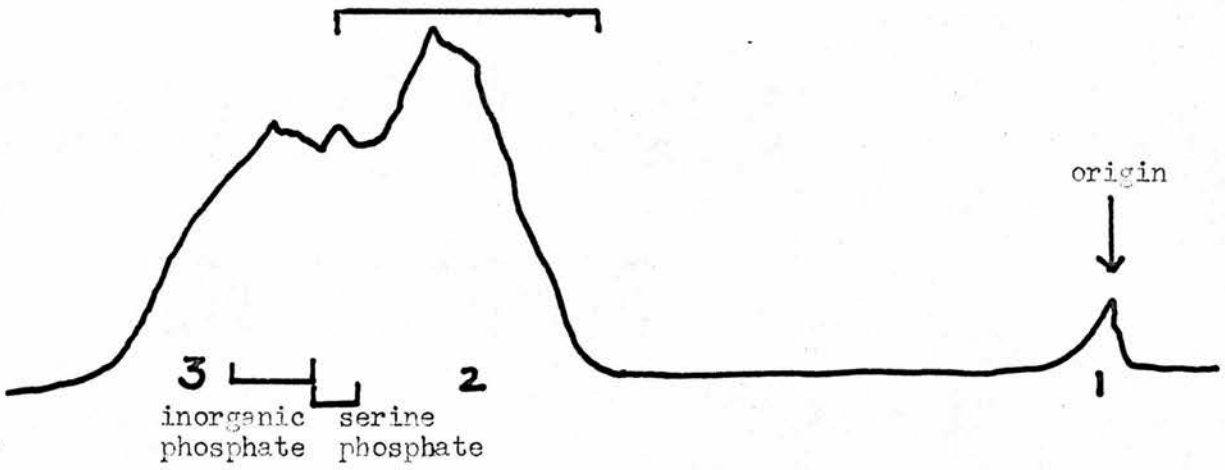
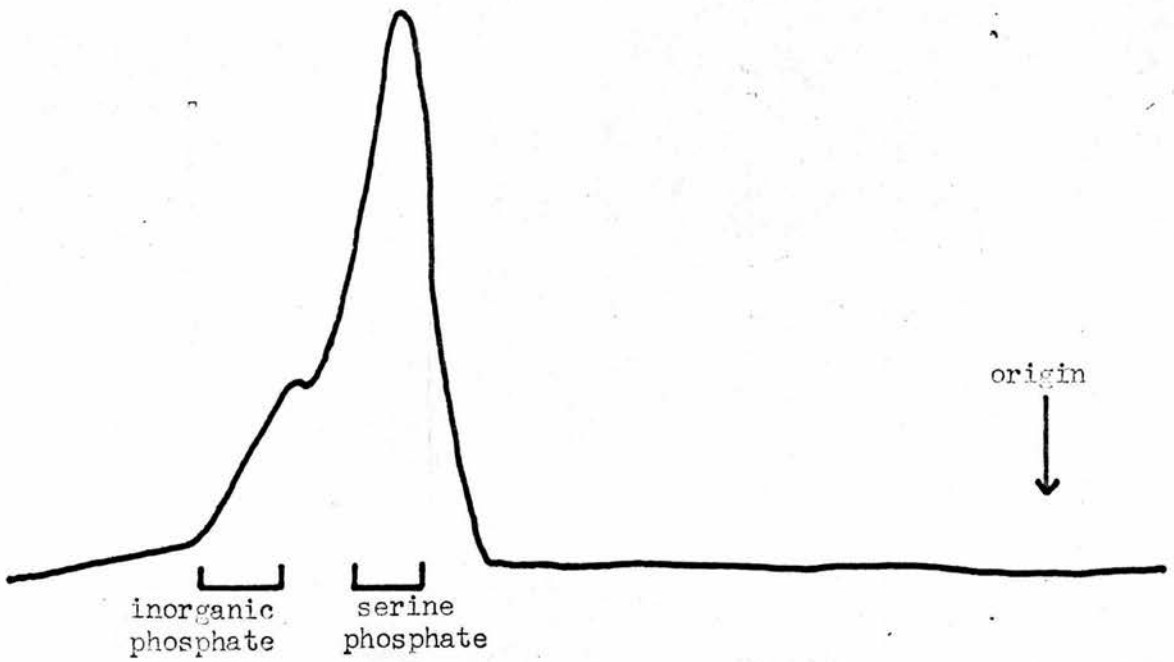


Fig. 5.5.b. Radioactivity scan of electrophoresis at pH 3.5 of acid hydrolysis of peak 2 of fig.5.5.a.



inorganic phosphate and the other parallel to a serine phosphate marker (Fig. 5.5.b).

Aliquots of the phosphorylated enzyme were hydrolysed with hydrochloric acid at 100° for periods from 10 to 90 min., and then subjected to electrophoresis at pH 3.5. In each case the radioactivity trace consisted of one peak between the origin and the phosphate marker. There was no difference in the relative intensity of the bands with different periods of incubation with acid. Electrophoresis at other pH values of the phosphorylated enzyme after acid hydrolysis did not achieve any improvement in the separations.

Electrophoresis of the unhydrolysed material at pH 3.5 and pH 6.5 gave a band of radioactive material with the same rate of migration as inorganic phosphate and a second peak at the origin.

Since there remained some doubt as to whether or not the liver phosphatase had been labelled, an experiment was devised to test this. Barman and Gutfreund (1966) found that although the enzyme - phosphate band was stable at pH 5.0, at pH 10.0 inorganic phosphate was liberated. The alkaline phosphatase from liver was incubated with ^{32}P-phosphate and washed with acetone-HCl as described previously. Half the precipitate was dissolved in pH 5.0 buffer and the other half in pH 10.0 buffer and after about 18 hours the protein was precipitated with acetone and the radioactive phosphate in the supernatant measured.

Table 5.3 shows the amounts of radioactivity at each stage. Since more radioactive material was released in the alkaline

than the acid buffer, it would appear that phosphate had been incorporated into the enzyme. However, the difference between the results for the two experiments and the total number of counts incorporated, is small.

Table 5.3. Incubation of Phosphorylated Enzyme at two pH Values.

Expt. A: Phosphorylated enzyme dissolved in pH 5.0 buffer.

Expt. B: Phosphorylated enzyme dissolved in pH 10.0 buffer.

Sample counted	Expt. A Total cts./ sec.	Expt. B Total cts./ sec.
Original incubation mixture	2.1×10^5	2.3×10^5
Supernatant after precipitation of labelled enzyme	2.0×10^5	2.0×10^5
First washing	6.8×10^3	6.7×10^3
Second washing	5.3×10^2	6.1×10^2
Supernatant after dissolving in buffer (18 hours)	1.7	10

The method of phosphate incorporation was further investigated by carrying out the labelling experiment at pH 4.0, 5.0 and 6.0. In each case about 90% of the radioactivity was found in the first supernatant. Electrophoresis at pH 3.5 of the acid hydrolysed material gave two bands for the enzymes labelled at pH 6.0. Allowing the incorporation to proceed for longer periods of time or increasing the phosphate concentration did not result in greater yields of phosphory-

lated enzyme.

No attempt was made to label the intestinal or bone enzyme.

CHAPTER 6. DISCUSSION.

6.1. INTRODUCTION.

With the exception of placental phosphatase (Ahmed and King, 1960b) the properties of human alkaline phosphatases have generally been studied with unpurified tissue extracts or sera. When such enzyme sources are used, other enzymes may interfere with the study of the properties, either by attacking the substrate or the product of the reaction under examination, or by altering the enzyme itself. Purified enzymes are of particular importance in an examination of the substrate specificity of an enzyme. In order, then, to eliminate these unknown factors in the study of the properties of the alkaline phosphatase, steps were taken to obtain purified enzyme from the tissue extracts.

6.2. ENZYME PREPARATION.

The yields of enzyme per kg. tissue are 195, 234 and 49 units (μmoles pNPP hydrolysed/min.) from liver, intestine and bone respectively, for the preparations outlined in Table 2.1. Bone is, therefore, a much poorer source of alkaline phosphatase than liver or intestine, and this is confirmed by the low initial specific activity. The bones used were adult ribs: a better source, though not so readily available, is bone from young children.

The highest specific activity for the liver and intestinal enzyme, 165 and 135 units/mg. protein respectively, can be compared with those of other workers when their results are converted into these units by experimental comparison of the

methods. Ahmed and King (1960a), with human placental phosphatase, obtained a specific activity of 59 units/mg. protein, the highest so far for a human phosphatase. However, Engstrom (1961a) and Portmann (1957) purified calf intestinal phosphatase to a specific activity of about 360 units/mg. protein. The most highly purified alkaline phosphatase is that of Binkley (1961a), with a specific activity of 1440 units/mg. protein using swine kidney as the source. Without recycling gel filtration, the specific activity of the bone enzyme prepared in the present work remains very much lower than any of these other values.

As the experiments to estimate the purity of the enzymes by means of ^{32}P labelling show, the liver and intestinal enzymes are still contaminated with other proteins. For the liver enzyme, the main contaminant is probably a haptoglobin-methaemoglobin complex, as evidenced by its mobility on starch-gel electrophoresis, peroxidase activity, absorption spectrum and molecular weight. Barman and Gutfreund (1966) estimated that their bovine milk alkaline phosphatase preparation had a purity of 3.6%, and they calculated that the preparation of Lyster and Aschaffenburg (1962) was 62% pure. The possible limitations of the labelling method are discussed below.

Human placental alkaline phosphatase has recently been purified 4000-fold and crystallised (Ghosh and Fishman, 1968). In the present work it was not found possible to improve the purity of the liver and intestinal enzymes further, and the question arose as to whether a purity of about 10% was

sufficient for kinetic studies to be of value. This represents, for the liver and intestinal enzymes, a purification of about 2000 and 1000-fold respectively. Ideally, the present work should have been carried out with enzymes of 100% purity, but attempts to separate them from other proteins of the same charge and molecular size were unsuccessful. It was not possible to obtain the bone enzyme in anything like such a high degree of purity, as has already been noted, since the enzyme was present in the tissue of origin in much smaller amounts. The final enzyme preparations, though far from pure, were the best that could be obtained.

Slight variations in the conditions of isolation of the three phosphatases, e.g. the ammonium sulphate concentration effective in precipitation, do not necessarily reflect differences between the isoenzymes, but may instead indicate differences in the nature or concentration of the contaminants (e.g. lipid content).

6.3. ARE THE ALKALINE PHOSPHATASES FROM DIFFERENT TISSUES IDENTICAL?

This question can most readily be answered by comparing the physical and enzymic properties, and the molecular structure of the isoenzymes.

6.3.1. Physical Properties.

Molecular weight: The similarity of the molecular weights of the three phosphatases (220,000, 195,000 and about 200,000 for the liver, intestinal and bone enzymes respectively) found in

the present work is confirmed by the results of Birkett et al (1965) who found that the isoenzymes all had the same elution volume on "Sephadex G-200". Boyer (1963) found that the kidney and placental enzymes had molecular weights of about 130,000, as measured by ultracentrifugation. Recently, the molecular weight of one of the variants of the placental enzyme has been shown to be 70,000 by ultracentrifugation; another variant is thought to be an aggregate of this and have a molecular weight of over 200,000 (Ghosh and Fishman, 1967). There thus appears to be some variation between the molecular weight as measured by gel-filtration and by ultracentrifugation. However, Andrews (1964) found that molecular weights measured by the method of gel-filtration never varied by more than 10% from the ultracentrifugation results.

The effluent volume on gel-filtration is in fact not so much dependent on molecular weight as on the shape and size of the molecule, for the rate at which molecules pass through a gel is inversely proportional to the fluid volume accessible to them within the column. However, within a group of macromolecules, size and molecular weight are closely related (Andrews, 1962). In a later paper Andrews (1964) suggested that, if a protein contains a large quantity of carbohydrate, it may have a more expanded form than proteins which do not contain carbohydrate; this would give a higher result for molecular weights measured by gel-filtration. The results of experiments with neuraminidase suggest that the intestinal enzyme contains less carbohydrate, or at least less carbohydrate susceptible to the action of neuraminidase, than the

bone and liver enzymes. If there were sufficient carbohydrate to affect the molecular weight measurement then the intestinal enzyme would have a smaller molecular weight. Andrews (1964) quoted as the upper limit to include variation due to carbohydrate content and this would give a molecular weight for the liver enzyme of 198,000. However, the amount of carbohydrate is probably not sufficient to affect the elution volume, and Saraswathi and Bachhawat (1968) found that removal of sialic acid from sheep brain alkaline phosphatase had no effect on the molecular weight, as measured by gel-filtration. If the figures obtained by gel-filtration are true estimates of molecular weight, there would appear to be a slight difference between the liver and intestinal enzymes. The bone phosphatase probably resembles the liver enzyme more closely than the intestinal enzyme.

Mobility on starch-gel electrophoresis: The main bands of the tissue extracts from liver, bone and intestine have mobilities similar to those found by Moss and King (1962) and by Hodson et al (1962). The slow band which Moss and King (1962) found in each of their preparations and which was presumed to be an enzyme-lipoprotein complex (Moss, 1962) was also seen in the present work. The band with intermediate mobility observed by Moss and King (1962) in liver phosphatase was not found. Warnock (1966) reported similar patterns following starch-gel electrophoresis, and she observed a band of intermediate mobility with bone phosphatase also. This appears to correspond in relative position to the zone of activity which was found in the present work with bone extracts which had

been stored for long periods. Warnock (1966) also confirmed that the slow "lipoprotein" bands in each tissue were of a similar nature as the main band by L-phenylalanine inhibition and heat inactivation.

Differences in mobility in starch-gel electrophoresis are partly governed by the size of the molecule and partly by its charge. Smithies (1962) devised a method for distinguishing which of these factors was the main cause for differences in mobility of two or more proteins by submitting them to electrophoresis in starch-gel having different concentrations of gel, but this type of experiment was not performed in the present work.

Recently, Smith, Lightstone and Perry (1968) were able to separate a mixture of bone and liver phosphatase on acrylamide-gel electrophoresis.

Reaction to neuraminidase: Robinson and Pierce (1964) suggested that intestinal phosphatase was insensitive to the action of neuraminidase whereas the other alkaline phosphatases found in normal serum were sensitive. It was confirmed, in the present work, that the mobilities on starch-gel electrophoresis of both the main band and the minor band of liver and bone phosphatases were altered after incubation with neuraminidase both in the early and in the final stages of preparation. The intestinal phosphatase was insensitive. Alkaline phosphatases from various other human tissues have recently been shown to be sensitive to neuraminidase, including enzymes prepared from placenta (Robinson, Pierce and Blumberg, 1966), kidney

(Butterworth and Moss, 1966) and lung (Smith et al, 1968). Recently Saraswathi and Bachhawat (1968), working with sheep brain alkaline phosphatase, found two distinct phosphatases in the same tissue. These differed in their sialic acid content and although treatment with neuraminidase reduced the mobility of the faster fraction to the mobility of the slower, other properties, e.g. substrate specificity, were still dissimilar.

The product of neuraminidase action on placental phosphatase has been shown to be sialic acid by the absorption spectrum of the complex formed from the reaction of sialic acid with thiobarbiturate (Ghosh, Goldman and Fishman, 1967). Robson and Harris (1966) have produced up to eight bands of alkaline phosphatase activity with human placental phosphatase acted on by graded neuraminidase concentrations.

The results show that the intestinal phosphatase on the one hand and the liver and bone phosphatases on the other differ, either in sialic acid content or in the availability of the sialic acid to the action of neuraminidase. However, the differences in mobility on starch-gel electrophoresis cannot be solely due to differences in sialic acid content, for the electrophoretic migrations of the liver and bone enzymes do not become identical following treatment with neuraminidase, and both enzymes then migrate more slowly than the intestinal enzyme.

Since the amount of sialic acid is probably small, as judged by the finding of 1-2% sialic acid in placental phosphatase (Fishman and Ghosh, 1967b, p.312), removal of sialic

acid would not be expected to effect much change in the size of the molecule. Consequently, it is likely that differences in mobility on starch-gel electrophoresis following neuraminidase treatment, are mainly due to alterations in charge and not to reduction in the size of the molecule. It is probable, therefore, that the differences in mobilities of the enzymes prior to neuraminidase treatment are due to differences in the total charge of the molecules. This would mean that there are differences either in amino acid residues with charged side-chains (e.g. aspartic acid) or in charged non-protein parts of the molecule.

Since there is no change in the enzymic activity after treatment with neuraminidase, the sialic acid residues do not play a vital role in the hydrolytic action of the phosphatases.

Inactivation under various conditions: The rate of inactivation during heating of the phosphatases was found, in this work, to be greatest with bone phosphatase, and least with the intestinal enzyme. Although there were considerable variation in the rate for each phosphatase with minor changes in temperature, the general pattern was consistent. This has been confirmed by Warnock (1966). The much greater stability of the placental phosphatase to heat has been noted by Neale et al (1965) and by Fishman, Inglis, Barke and Ghosh (1966). Moss and King (1962) found the intestinal enzyme to be slightly less stable than the liver enzyme. This is not in agreement with the results found here, nor with the findings of Warnock (1966). Slight variation in the temperature of inactivation,

if the inactivations are not carried out in parallel, could account for this discrepancy.

The order of increasing stability towards urea was the same as for thermal inactivation, namely, bone, liver and intestinal phosphatase. Rapid increase in the rate of inactivation by urea with temperature was also seen. Other workers have found that kidney phosphatase is slightly more rapidly inactivated by urea than the liver enzyme (Butterworth and Moss, 1967) and that placental enzyme is more stable than the intestinal enzyme (Birkett et al, 1965). The critical concentration of urea at which denaturation changes from reversible to irreversible observed by Callaghan and Martin (1962 and 1963) using albumin has also been noted by Birkett, Conyers, Neale, Posen and Brudenell-Woods (1967) with human alkaline phosphatases. At urea concentrations below about 1.5M for the bone phosphatase and 4M and 8M for the intestinal and placental enzymes respectively, urea inhibits by a mechanism which has been interpreted as due to unfolding or conformational changes in the molecule, caused by binding of the urea at sites other than at the catalytic centre. At urea concentrations above the critical value, denaturation of the enzyme is probably taking place with rupture of hydrogen bonding (Birkett et al, 1967).

At acid pH the order of increasing stability is bone, liver and intestinal phosphatase. Scutt and Moss (1968) found, by bringing the pH back to pH 7.2 after acid inactivation, that the liver and intestinal phosphatases could be reactivated.

However, the degree of reactivation depended on the pH of inactivation and on the length of time that the enzymes had been exposed to low pH. The kinetic properties, e.g. Michaelis constant, of the reactivated enzyme differed from the original value with intestinal phosphatase, but showed no change with the liver enzyme. If the intestinal phosphatase was partially inactivated, this intermediate form had the same kinetic characteristics as the enzyme which had been completely inactivated and then allowed to reactivate. They concluded that there were at least two stages in the inactivation of the intestinal isoenzyme, the first of these being rapid and the second slower. On the other hand, swine kidney phosphatase can be completely inactivated at acid pH and reactivated at neutral pH without showing any change in electrophoretic and gel-filtration properties (Butterworth, 1968).

Reynolds and Schlesinger (1967) found that, when E.coli phosphatase was inactivated at pH 2.0 and 0°, two inactive subunits with extended coil shape were produced. If the pH was then raised to pH 4.0, the subunits began to take up a more globular shape, but were still inactive. Raising the pH to 6 to 8 for one minute at 20° caused refolding of the subunits to give a monomer with the same content of α-helix as the native enzyme but with no enzymic activity; more prolonged incubation at pH 6-8 and 20° and in the presence of zinc ions, resulted in the formation of a dimer which was indistinguishable from the original enzyme molecule. Although the human phosphatases are not known to consist of subunits,

it would seem possible that they may be made up of four such units, since the molecular weight of the human enzymes is approximately twice the value for the E.coli phosphatase. The differences in stability towards acid conditions could be due to differences in the binding of the subunits, and in the structure of the subunits themselves.

The marked differences between the phosphatases in stability towards these three denaturing agents indicates that there are fundamental differences between them. Although the order of stability is the same in each case, the mechanism of inactivation and the cause of the dissimilarity between the phosphatases is not necessarily the same. Some form of intramolecular bonding is presumably being disrupted by these conditions and it would appear that the placental enzyme has the strongest bonding and that from bone the weakest. The nature and strength of the bonding will, in turn, be related to the amino acid sequences of the isoenzymes.

Immunological Properties: A recent development in the immunological study of the alkaline phosphatases has been the work of Sussman, Small and Cotlove (1968). When an antibody to purified human liver phosphatase was made, it cross-reacted only with the liver enzyme and not with bone, placental, kidney, intestinal, nucleophilic or E.coli phosphatases. Similarly the anti-placental phosphatase antibody only precipitated the placental phosphatase. They concluded that there were at least three antigenic types of phosphatase, i.e. liver, placental and one or more from the other enzymes, and suggested

that the antigens used in previous studies had been mixtures which had resulted in the simultaneous induction of several antibodies and the conclusion that there was apparently common antigenicity. Smith et al (1968) suggested that the cross-reaction previously found between anti-liver phosphatase antibody and bone and kidney phosphatases (e.g. by Boyer, 1963) might be due to the fact that most tissue preparations contain a certain amount of blood which cannot be removed by washing and, since the main isoenzyme in serum is from the liver, all preparations were contaminated with small quantities of liver enzyme. As intestine is relatively free from blood the phosphatase would have a smaller interaction with the antibody to the liver enzyme. It would appear probable, therefore, that each isoenzyme will be shown to be immunologically distinct.

6.3.2. Enzymic Properties.

Substrate Specificity: Although pyrophosphatase and alkaline phosphatase activities have been thought to be closely associated, they have not always been considered to be functions of the same enzyme protein (Roche, 1950; Morton, 1955). The findings of Heppel et al (1962) and of Trubowitz et al (1961), however, suggested that the two activities might be associated. Since the start of this work more evidence has accumulated which attributes pyrophosphatase activity to alkaline phosphatase.

In 1965 Russell found an association between low plasma and tissue levels of alkaline phosphatase and an elevated excretion of inorganic pyrophosphate. Calf intestinal alkaline phosphatase has been found to hydrolyse inorganic pyrophosphate

(Fernley and Walker, 1967; Fernley and Bisaz, 1968). Human leucocytes (Cox and Griffin, 1965), a culture of human cells (Cox, Gilbert and Griffin, 1967) and human placenta (Sussman and Laga, 1968) have each been found to contain an alkaline phosphatase which also hydrolysed inorganic pyrophosphate. The only alkaline phosphatase which has recently been shown to have no pyrophosphatase action is that from human neutrophils (Huser and Olderding, 1967). However, the enzyme preparation of these workers was also inactive towards AMP which has been recognised as a substrate for alkaline phosphatase for some time. The evidence presented here, e.g. mixed-substrate experiments and the position of the zones of activity with different substrates after starch-gel electrophoresis, as well as the observations of other workers, that human liver, intestinal, placental and bone alkaline phosphatases are also pyrophosphatases, would appear to be fairly conclusive.

The ratio of inorganic pyrophosphatase to orthophosphatase activity differs by a factor of five or six between the intestinal enzyme on the one hand, and the liver and bone phosphatases on the other. The slight variation in the ratio between the bone and liver enzymes may or may not be significant, but the intestinal enzyme must be considered as different from the other two enzymes in this respect. As with the effect of neuraminidase, the isoenzymes may thus be divided into two groups on the basis of their relative activities towards inorganic pyrophosphate.

When the relative rates of hydrolysis of various nucleotides are compared, it can be seen that again the intestinal phosphatase differs more from the liver and bone enzymes than the latter do from each other (Table 4.2). The low ratio of orthophosphatase to inorganic pyrophosphatase activity for intestinal phosphatase is reflected in the high relative rates of hydrolysis towards ADP and UDP. Apart from the hydrolysis of AMP by the intestinal enzyme, the nucleoside monophosphate esters are hydrolysed less rapidly than pNPP. But it would appear that the active centre of the molecule is relatively non-specific for the non-phosphate part of the substrate molecule, once this has reached a certain size, for there is a marked similarity between the relative rates of hydrolysis with the corresponding adenine and uracil derivatives, particularly with the bone enzyme. The relatively rapid rate of hydrolysis of AMP by the intestinal enzyme has already been noted (Landau and Schlamowitz, 1961) and, though difficult to explain, it does re-emphasise the fact that the intestinal phosphatase appears to differ from the liver and bone enzymes.

A possible explanation of why Morton (1955) was unable to detect pyrophosphatase activity in his enzyme preparations may be that he used a 10mM magnesium concentration which, from the results found here, would completely inhibit the pyrophosphatase activity. Differences in stability between pyrophosphatase and orthophosphatase activities in extracts of pig liver were suggested as evidence for the non-identity of the two activities (Roche, 1950), but such differences were not found with the

human phosphatases. Anderson and Nordlie (1967), working with E.coli phosphatase, attributed differences in heat stability with different substrates to the possibility that the non-phosphate part of different substrates might bind to different sites in the protein molecule and a difference in stability did not, therefore, preclude the two activities being properties of the same enzyme molecule.

Effect of Metal Ions: In common with most enzymes acting on phosphorylated substrates, the hydrolysis of orthophosphates by alkaline phosphatase is activated by the addition of magnesium ions to the assay system. Since Mn^{2+}, or to a lesser extent Ca^{2+}, may substitute for Mg^{2+} in the activation (Morton, 1957; Ahmed, Abul-fadl and King, 1959), and since the concentration of metal ions is not dependent on the substrate concentration, the activation by Mg^{2+} is probably non-specific. However, when inorganic pyrophosphate is the substrate a relative concentration of substrate to magnesium of about 2:1 is optimal.

With the inorganic pyrophosphatase from rat erythrocytes (E.C.3.6.1.1.) the rate of hydrolysis of inorganic pyrophosphate increased with increasing magnesium ion concentration to at least 7mM and, in this case, the true substrate was thought to be a magnesium-substrate complex, $(Mg\ P_2O_7)^{2-}$. An increase in Mg^{2+} thus produced an increase in the amount of true substrate present, and hence an increase in velocity (Bloch-Frankenthal, 1954). However, the relationship between magnesium concentration and the rate of hydrolysis with the pyro-

phosphatase activity of human alkaline phosphatase does not follow this pattern and, unlike the rat erythrocyte enzyme, there is some phosphatase activity in the absence of added magnesium. The mechanism of action of Mg^{2+} is, therefore, unlikely to be by the formation of $(Mg\ P_2O_7)^{2-}$.

The effect of magnesium on the hydrolysis of orthophosphates and of inorganic pyrophosphate does not vary with the source of the enzyme, with the possible exception that higher rates of hydrolysis were observed with pNPP and intestinal phosphatase (Table 4.4). This may be due to residual Mg^{2+} in the enzyme solution, but it may on the other hand represent a genuine difference between the isoenzymes since the enzyme solutions were dialysed before use. With the organic pyrophosphates, there is a greater difference between the isoenzymes, but this does not follow a definite pattern. (Fig. 4.13 and 14 and Table 4.2.).

When zinc ions are added, the low optimum concentration indicates that the metal is not forming a metal-substrate complex. This would be consistent with the suggestion of many workers that a metal ion, probably zinc, is a constituent of the E.coli alkaline phosphatase molecule (Garen and Levinthal, 1960; Plocke et al, 1962; Plocke and Vallee, 1962; Reynolds and Schlesinger, 1967). Recently, Fishman and Ghosh (1967a) have produced evidence to suggest that a metal, probably zinc, plays a functional role in the catalysis of the hydrolysis of phenyl phosphate by rat intestinal phosphatase.

Conyers, Birkett, Neale, Posen and Brudenell-Woods (1967)

studied the effect of EDTA on the hydrolysis of phenyl phosphate by human bone, intestinal and placental alkaline phosphatases, and found three effects. At low concentrations of EDTA (10^{-5} - 10^{-3}M) in the assay system, there was an instantaneous inhibition of the activity of each phosphatase, possibly due to the removal of some activator which was contaminating the enzyme preparation. As the concentration of the EDTA in the assay system was increased from 10^{-3}M to 10^{-1}M, however, a progressive inhibition was seen with the bone and intestinal enzymes only; the placental phosphatase was activated under these conditions, and they attributed the inhibitory effect observed to chelation of a divalent cation necessary for activity of the bone and intestinal enzymes. The third effect observed by Conyers et al (1967) was a time-dependent inactivation, not reversed by dilution, which occurred when the enzyme was preincubated with EDTA; all three phosphatases exhibited this inactivation, but the rates of inactivation differed, and they attributed this inhibition to the removal or binding of a functionally essential metal, probably zinc.

The approximately 100-fold difference between the concentration of zinc which inhibits the orthophosphatase and pyrophosphatase activities is difficult to explain and a full investigation using enzyme preparations which have been previously inactivated by dialysis against a metal-chelating agent might help to clarify this problem.

The total evidence, however, suggests that the human alkaline phosphatases are metallo-enzymes.

Kinetics of the Hydrolysis Reaction: Although the effects of high substrate concentration are different with orthophosphate and pyrophosphate substrates, there appears to be little difference between the effects with liver and intestinal phosphatases. (Fig. 4.15 and 4.16). Similarly, only a small difference between the K_m values can be seen although there is a tendency to higher values with liver phosphatase (Table 4.5). Moss et al (1961a) found a reproducible difference in K_m values for the human enzymes when the velocities were measured at the optimum pH for each substrate concentration. When the plots of pK_m against pH for orthophosphatase activity (Fig. 4.19) are studied, it can be seen that at any one pH value the K_m for the phosphatases from different tissues are dissimilar and again the liver enzyme has higher values than the bone or intestinal phosphatase.

The "Dixon plots" of pK_m against pH for orthophosphatase activity show that the pK_e values for the three phosphatases with pNPP (8.5 to 8.8) are only slightly different. It is probable, therefore, that human bone, liver and intestinal phosphatases all have the same amino acid residues involved in the binding of pNPP to each enzyme, and that the ionic environment of these residues is the same. pK values for human placental phosphatase with phenyl phosphate as substrate have been found to be 9.3 and 9.9 for the free enzyme, and 9.7 and 10.3 for the enzyme-substrate complex (Fishman and Ghosh, 1967b, p.275). If a line of unit slope is fitted to the data of Fishman and Ghosh, a pK_e of about 9.2 and a pK_{es} of 10.4 are obtained. When deriving the pK_e for calf

intestinal phosphatase and cow's milk phosphatase, using phenyl phosphate as substrate, Morton (1957) fitted lines of unit slope and found that the pK_e with both enzymes was about 9.2. Ghosh and Fishman (1966), on the other hand, found a pK_e of 8.6 with rat intestinal phosphatase and phenyl phosphate; this value becomes 8.3 if lines of unit slope are fitted. It would appear, then, that the pK_e values generally vary both with the substrate used and with the tissue used as source of the enzyme.

The variations in pK_e with substrate and with source are again seen when the graphs of pK_m against pH, with inorganic pyrophosphate as substrate, are studied (Fig. 4.20 and Table 4.6). The differences in the pK_e values for the three phosphatases could mean that different amino and residues in each phosphatase were combining with the substrate. However, it would seem more likely that the amino acids involved are the same, and that the pK values are different due to differences in the ionic environment. The pK_{es} values for pyrophosphatase activity also differ for the three phosphatases, and increase in the order liver phosphatase, bone phosphatase and intestinal phosphatase. These findings also probably indicate differences in neighbouring amino acid groups rather than differences in the active centre itself. The bone and liver enzymes may have a second pK_{es} value analogous to the value found for the intestinal enzyme. However, this second value would lie below pH 7.0, which is outside the pH range in which measurements can be made. Unlike some of the other differences between the isoenzymes, such as neuraminidase sensitivity and substrate

specificity, the isoenzymes cannot be divided into intestinal and non-intestinal groups of enzymes on the basis of pK_e and pK_{es} values, since they each show different characteristics.

A comparison of the K_m values or affinities for the two substrates (Table 4.7) shows that, at pH 7.0, the affinity of each enzyme is the same for both substrates. It is probable that the phosphate part of the substrate is binding to the enzyme molecule at this pH, while at a higher pH the attachment of the organic part assumes greater importance. The pK_e at 8.3-8.5, observed when pNPP is used as substrate, is not involved in the binding of pyrophosphate since no discontinuity occurs at this pH in the plots for pyrophosphatase activity (Fig. 4.20).

Plots of log V_{max} against pH indicate pK values for the enzyme-substrate complex. When inorganic pyrophosphate is the substrate the pK_{es} values obtained from the graphs of pK_m and log V_{max} against pH (Fig. 4.20 and 4.22) do not match very closely. The comparison of V_{max} values at different pH values (Table 4.8) shows that, below pH 8.0, alkaline phosphatase is equally effective a pyrophosphatase as an orthophosphatase.

Inhibition of the Hydrolysis Reaction: On the basis of their sensitivity to L-phenylalanine, the isoenzymes can be divided into two classes, intestinal and non-intestinal. The fact that both the pyrophosphatase and the orthophosphatase activities of the intestinal enzyme are inhibited, whereas the liver and bone phosphatases are not affected supports the theory that both activities are properties of the same enzyme molecule.

Since the K_i values are identical with both substrates, it is likely that L-phenylalanine is combining at the same site in the enzyme molecule when acting as an inhibitor of either the pyrophosphatase or the orthophosphatase activities. L-phenylalanine has recently been shown to inhibit human placental phosphatase, the inhibition being uncompetitive in type (Fishman et al, 1966), and on the basis of its sensitivity to phenylalanine the placental isoenzyme falls into the same group as intestinal phosphatase.

Fishman and Ghosh (1967b, p.285) found the inhibition of human intestinal and placental phosphatases by L-phenylalanine to be uncompetitive, but the present work on intestinal phosphatase indicates that the inhibition is non-competitive in type. Differences in the experimental conditions included the use of different substrates, and the addition, by Fishman and Ghosh, of D-phenylalanine to the control tubes.

The difference between non-competitive and uncompetitive inhibition lies in the mechanism of formation of the inhibitor - enzyme - substrate complex (EIS). With non-competitive inhibition, either an enzyme - inhibitor complex (EI) or an enzyme-substrate complex (ES) may be formed first, but in uncompetitive inhibition EIS is only formed via ES. The nature of the substrate should, therefore, have no effect on type of inhibition in these experiments with intestinal phosphatase, since the point at issue is whether or not the inhibitor can combine with the enzyme. The D-isomer of phenylalanine causes slight activation of alkaline phosphatase (Ghosh and Fishman, 1966) and this might affect the results,

leading to a different interpretation of type of inhibition, although this seems unlikely. A precise comparison of the methods with the two intestinal preparations would be necessary to clarify this problem.

Ghosh and Fishman (1966) found that the activation energy in the presence of L-phenylalanine was about three times the value observed in the presence of the D-isomer. They interpreted this finding as meaning that the inhibitor had combined with the ES complex to give an inactive, thermodyamically stable EIS complex with a consequent reduction in the amount of ES present. The results are, however, also compatible with non-competitive inhibition, when the inhibitor can combine with the enzyme.

The affinity of arsenate for the enzyme is the same irrespective of the tissue source of the enzyme and the substrate used (Table 4.9). The isoenzymes cannot, therefore, be distinguished on the basis of their inhibitor constants with arsenate.

Since the inhibitor constants at pH 8.9 for the inhibition by arsenate and L-phenylalanine are the same for both the orthophosphatase and pyrophosphatase activities, it would seem likely that both activities are located at the same site in the enzyme molecule.

Catalytic - centre Activity: The investigation of the catalytic-centre activity of the liver and intestinal phosphatases revealed differences between them. The very low dissociation constant found with the liver enzyme indicates that the equili-

brium is very strongly in favour of the enzyme-phosphate complex. Barman and Gutfreund (1966), working with a preparation of bovine milk phosphatase, reported that the catalytic-centre activity with pNPP was 2700 $sec.^{-1}$. In the present work, the activities were 5030 $sec.^{-1}$ and 6550 $sec.^{-1}$ for the liver and intestinal phosphatase respectively; this is of the same order as the value reported for the bovine enzyme, if allowance is made for the fact that the activity of the bovine enzyme was measured at 25° whereas the human phosphatases were investigated at 37°.

This method of estimating the catalytic-centre activity and investigating the purity of the enzyme preparation assumes that phosphate is binding at the catalytic-centre and that all the sites are equally and completely labelled. The former assumption is justified because phosphate is a competitive inhibitor of the action of alkaline phosphatase, and because the incorporation of ^{32}P is much reduced in the presence of substrate or heat-inactivated enzyme. Verification of the latter assumption would require detailed chemical studies of amino acid sequences.

6.3.3. Molecular Structure.

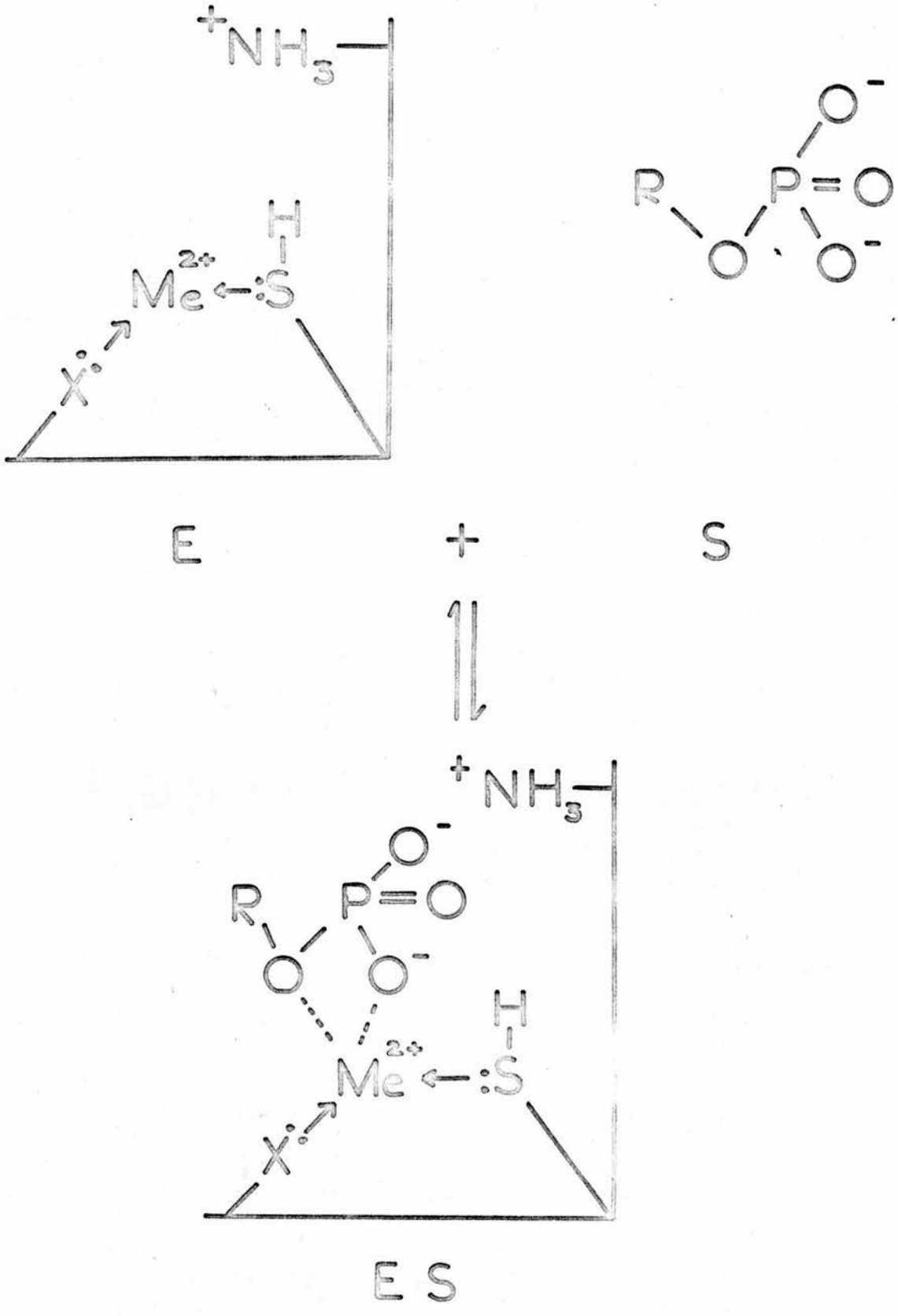
One method of studying the active centre of the phosphatases is to measure the activity of the enzymes in the presence of substances which are known to combine with particular groups in protein molecules. Recently, Fishman and Ghosh (1967a) applied this method to the study of rat intestinal phosphatase, and concluded that thiol groups and the

ϵ-amino group of lysine participated in the catalysis. Whether this is also true of the human enzymes remains to be investigated.

Fishman and Ghosh (1967b, p.282) proposed a mechanism for the hydrolysis of monoesters involving the metal ion, the sulphhydryl group and the ϵ-amino group of lysine. They suggested that the substrate becomes attached to the enzyme by co-ordination between the lone pair of electrons on the bridge oxygen and the metal and between one of the electron-rich ionic oxygen atoms and the metal. The other ionic oxygen is attracted to the ϵ-amino group while the double bonded oxygen becomes hydrogen bonded with the hydrogen-donating site of the sulphhydryl group (Fig. 6.1.). Nucleophilic attack by the hydroxyl ion of the medium on the phosphorus atom causes release of the alcohol and formation of the phosphoryl enzyme, which subsequently breaks down to inorganic phosphate and free enzyme. This suggested mechanism only takes account of the groupings thought to be directly involved in the catalytic reaction, and does not take into consideration any conformational changes in the enzyme molecule which may occur (Fernley and Walker, 1965; Barman and Gutfreund, 1966).

The attempts to investigate the amino acid sequence around the active sites of the human phosphatases were not successful, although it was possible to label and subsequently hydrolyse the E.coli phosphatase. There may be several reasons for this failure. It is possible that the enzyme was labelled but that the hydrolytic reaction was not effective and the

Fig. 6.1. Diagram of the mechanism postulated by Fishman and Ghosh (1967b) for the formation of the enzyme-substrate complex.



small number of fractions seen as peaks after scanning the electrophoresis strips would suggest this (Fig. 5.1 and 5.5). However, when the material, which had been incubated with acid, was dialysed and the fraction on the outside of the dialysis bag subjected to electrophoresis both the peaks observed (Fig. 5.2a) cannot represent free ^{32}P-phosphate and one would conclude that the other peak is a peptide or amino acid labelled with ^{32}P. Recently Goldsmith and Robinson (1968) found that human serum alkaline phosphatase was very resistant to protease action.

On the other hand, it is possible that the enzymes have not been labelled, and that the peaks seen after electrophoresis represented free radioactive phosphate together with phosphate absorbed on to contaminating proteins. The concentration of the enzyme was estimated to be not more than 1% of the total protein present and it may have been considerably less. This small amount of alkaline phosphatase may have meant that there was not sufficient present for it to become labelled with ^{32}P. The conditions necessary for labelling may also be different for the human enzymes. However, it proved possible to incorporate ^{32}P into the phosphatases when the catalytic-centre activities of the enzymes were being measured. Several groups of workers have recently labelled E.coli phosphatase (Pigretti and Milstein, 1965; Fernley and Walker, 1966), bovine milk phosphatase (Barman and Gutfreund, 1966) and calf intestinal phosphatase (Fernley and Bisaz, 1968). The method is, therefore, practicable for several

different phosphatases and it would appear necessary to repeat the experiments with the human enzymes with slight variations both in the conditions of labelling and of hydrolysis and with a greater concentration of enzyme protein. So far it has not been possible to learn anything from these experiments about the amino acid sequence at the active centre of the human alkaline phosphatases.

6.3.4. Conclusions on the Identity of the Alkaline Phosphatases.

The main differences between the alkaline phosphatases are summarised in Table 6.1. On the basis of physical properties there are four ways in which the isoenzymes differ: mobility on starch-gel electrophoresis, sensitivity to neuraminidase, rates of inactivation, and antigenicity. There are also differences in the main enzymic properties in terms of substrate specificity and the effects of inhibitors.

It would appear from the investigation of substrate specificity and from the kinetic experiments that the active centres of the isoenzymes are different. It is, however, unlikely that the amino acid sequences immediately around the active serine differ, since both E.coli phosphatase and bovine liver phosphatase have the same sequence at this point. The differences in the active centres are, therefore, probably due to variation in the amino acid residues at points in the enzyme molecule which, although remote from the active serine in the amino acid sequence, nevertheless are closely related to it in space by virtue of the tertiary structure of the molecule. This variation in the amino acids is supported by

Table 6.1. Summary of Difference Between the Human Alkaline Phosphatases.

Physical Properties:	Intestinal Phosphatase	Placental Phosphatase	Liver Phosphatase	Bone Phosphatase
1. Mobility on starch-gel electrophoresis	Cathodic to liver and bone phosphatases.	Parallel with liver phosphatase	most anodic	Just cathodic to liver phosphatase
2. Sensitivity to neuraminidase	not retarded	retarded	retarded	retarded
3. Half-lives of inactivation by				
a) 55°	90 min.	stable	20 min.	5 min.
b) urea (6M)	90 min.	more stable than intestinal phosphatase	6 min.	4 min.
c) acid	moderately stable	-	intermediate stability	rapidly inactivated
4. Antigenicity	distinct	distinct	distinct	antibody cross-reacts with liver phosphatase
Enzymic Properties:				
1. Substrate specificity	hydrolyses AMP and PP_i with respect to pNPP faster than liver and bone phosphatase	-	Similar relative rates of hydrolysis	

Table 6.1. (Contd.)

Enzymic Properties:	Intestinal Phosphatase	Placental Phosphatase	Liver Phosphatase	Bone Phosphatase
2. Inhibition by				
a) L-phenylalanine	inhibited	inhibited	not inhibited	not inhibited
b) bile acids	not inhibited	-	inhibited	inhibited
3. Catalytic-centre activity with pNPP	6550 $sec.^{-1}$	-	5030 $sec.^{-1}$	-

the fact that the differences in the electric charge carried by the isoenzymes cannot wholly be accounted for on the basis of their sialic acid content, and probably results from differences in amino acid residues with charged side-chains.

It has already been suggested that the insensitivity of intestinal phosphatase to neuraminidase results from inaccessibility of its sialic acid to the action of neuraminidase. If this is the case, then the intestinal enzyme must have a different tertiary structure from the liver and bone enzymes, which would in its turn probably indicate a difference in the primary structure of the phosphatases. Differences in the folding of the intestinal phosphatase molecule could also account for the fact that, in contrast to the liver and bone enzymes, the intestinal enzyme is inhibited by L-phenylalanine.

Fishman and Ghosh (1967b, p.284) suggested that alkaline phosphatase could exist in a certain physical state in the membranes of the striated border and that it was this native form of alkaline phosphatase which was inhibited by L-phenylalanine. Thus a tissue with microvilli active in absorption (i.e. intestine and placenta) would have alkaline phosphatase in the physical configuration necessary for L-phenylalanine inhibition. Since the intestinal and placental phosphatases vary in other properties such as heat sensitivity and sensitivity to neuraminidase this cannot be the sole difference between the isoenzymes. The great variation in stability towards heat, urea, and low pH could be accounted for on the basis of differences in tertiary, and hence primary, structure

of the enzyme molecules.

It thus appears that the human isoenzymes of alkaline phosphatase are different, and that the differences result from dissimilarities in the protein molecules at several levels of protein organisation. Further investigations are needed, particularly amino acid sequence determinations, if the differences are to be more precisely defined.

The question whether the production of the isoenzymes of alkaline phosphatase is controlled by different genes, or whether just one basic structure is formed for the alkaline phosphatases and this subsequently modified in different tissues requires consideration. Robson and Harris (1965) found six distinct phenotypes, from a study of the electrophoretic patterns of extracts of placenta, and from twin studies they concluded that the polymorphism of placental alkaline phosphatase was due to the existence of three autosomal allelic genes. There is evidence to suggest that the production of intestinal phosphatase is also genetically controlled. The appearance of an intestinal band in the starch-gel electrophoresis pattern of serum from certain individuals has been linked with the ABO blood groups and with gastric secretor status (Cepellini, 1960).

Within the non-placental, non-intestinal group of alkaline phosphatases (i.e. those from liver, bone, kidney etc.) differences between isoenzymes are less clear cut, and it would seem possible that the variations might be the result of environmental factors. In cases of hypophosphat-

asia, a genetic disease which results in deficient bone mineralisation, it has been suggested that the alkaline phosphatase level in all tissues is depressed (e.g. Schlesinger, Ludar and Bodan, 1955). However, a case with hypophosphatasia in which both the liver and duodenal juice alkaline phosphatases were normal has been reported, although in this instance cells from a tissue culture of bone showed an abundance of alkaline phosphatase activity (Scaglione and Lucey, 1956). In a recently reported series of cases of hypophosphatasia, the intestinal phosphatase was found to have either a normal or raised level (Danovitch, Baer and Laster, 1968). This would indicate the existence of separate genetic controls for bone, liver and intestinal phosphatases as well as for the placental enzyme, if the defect in hypophosphatasia is genuinely a genetically-controlled failure to produce alkaline phosphatase.

6.4. THE PHYSIOLOGICAL ROLE OF ALKALINE PHOSPHATASE.

The discovery by Robison and Soames (1924) that the immersion of bone tissue in a solution of calcium hexose monophosphate produced a deposit of calcium phosphate led to the suggestion that alkaline phosphatase played a role in bone mineralisation. Robison (1932) suggested that the enzyme induced calcification of bone by producing a local increase in phosphate concentration and, by the law of mass action, precipitation of calcium phosphate. This "booster" theory failed when it was found that extraneous tissues were normally supersaturated with respect to bone mineral (Neuman and

Neuman, 1958) and that collagen could trigger the formation of calcium phosphate crystals (Fleisch and Neuman, 1961). Fleisch and Neuman (1961) have suggested that an inhibitor might act to prevent calcification of some collagen tissue. They found that plasma contained an inhibitor which, in vitro, prevented precipitation of calcium salts and which was destroyed by alkaline phosphatase. The inhibitor was later identified as inorganic pyrophosphate (Fleisch and Bisaz, 1962). Inorganic pyrophosphate also prevented the mineralisation of chick embryo femurs grown in tissue culture (Fleisch, et al, 1964). From his finding that there was an increased urinary excretion of inorganic pyrophosphate in hypophosphatasia, Russell (1965) suggested that the defective bone mineralisation characteristic of this disease resulted from the low alkaline phosphatase level, and the consequent excessive inhibition of calcification by pyrophosphate. The discovery that human bone alkaline phosphatase acts as a pyrophosphatase supports this theory.

Recently, Jibril (1967) proposed that alkaline phosphatase might in fact have several functions in bone tissues, such as the production of a local accumulation of phosphate for mineralisation, the production of inorganic phosphate for re-utilisation in energy-producing reactions, and a control action involving limitation of the accumulation of inhibitors of calcification in the zone of active mineralisation.

With the discovery of the pyrophosphatase action of alkaline phosphatase, new light has been shed on the function

of bone phosphatase, but very little is known about the role of the other phosphatases. It has been suggested that the alkaline phosphatase which occurs in nuclei may play a role in nucleic acid synthesis by hydrolysing pyrophosphate which is a product of the reaction and which inhibits nucleic acid synthesis. The enzyme may also be involved in the phosphorylation and dephosphorylation of nuclear proteins (Cox, Gilbert and Griffin, 1967). Cox and Griffin (1967) suggest that the main function of intestinal and kidney alkaline phosphatases may be as phosphotransferases rather than as phosphatases. Physiological roles for alkaline phosphatases from tissues other than bone can, therefore, only be postulated and further investigation is obviously necessary.

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