

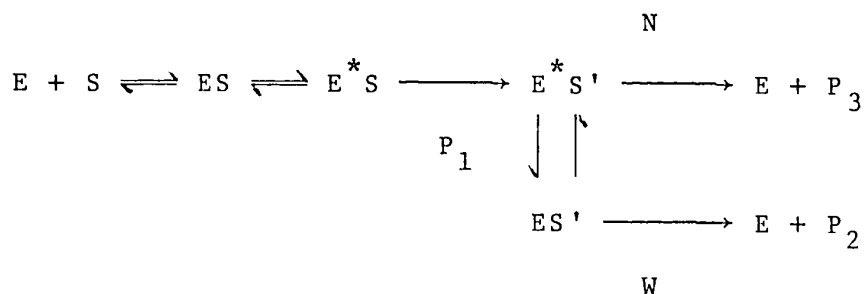
ABSTRACT

Many enzyme-catalyzed reactions, such as hydrolyses, give rise to two products  $P_1$  and  $P_2$  which are formed in different reaction steps. The second product  $P_2$  is frequently formed by hydrolysis of an intermediate such as an acyl-enzyme or a phosphoryl-enzyme. An alternative nucleophile  $N$  introduced into the system forms an additional product  $P_3$ . The kinetics of formation of  $P_1$ ,  $P_2$  and  $P_3$  in the presence of added nucleophiles has been analyzed mathematically. A number of alternative mechanisms were considered, and equations were derived for the rates of formation of the three products, and the Michaelis constant, as functions of nucleophile concentration. Graphs were presented showing the variation of these parameters with the concentration of  $N$ , for a variety of special cases. Special attention was given to the possibility of specific binding sites for the water and the nucleophile molecules.

The data for a number of enzyme systems were discussed with reference to these treatments. For reactions catalyzed by alkaline phosphatase only one mechanism (Mechanism VI, see below) was found to be consistent with the results.

An experimental study was made of the hydrolyses of phenyl phosphate and p-nitrophenyl phosphate catalyzed by chicken intestinal phosphatase. The work was done at pH 8.0 and 10.0, 25.0°C and an ionic strength of 1.0 M, and particular

attention was paid to the kinetics of formation of the products in the presence of Tris and ethanolamine. It was found that the rates of formation of phenol or *p*-nitrophenyl ( $P_1$ ) and of the phosphorylated nucleophile ( $P_3$ ) varied linearly with the concentration of added nucleophile; on the other hand the rate of formation of phosphate ( $P_2$ ) and the Michaelis constant were independent of nucleophile concentration. The ratio of products  $P_3/P_2$ , which were time-independent, were independent of the nature of the leaving group of the substrate. These results cannot be reconciled with any of the mechanisms considered with the exception of Mechanism VI, which is an elaboration of the one proposed by Trentham and Gutfreund; Mechanism VI is



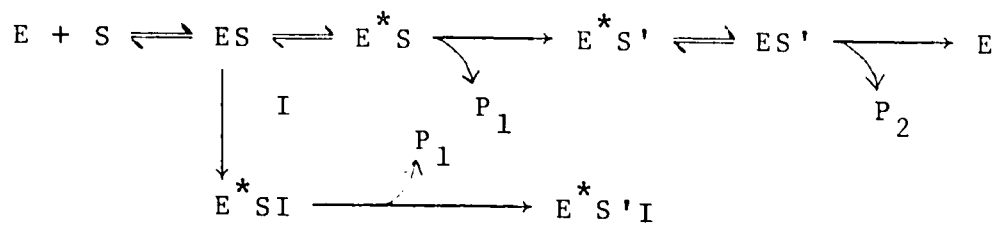
where W is water and N the alternative nucleophile. ES and  $E^*S$  are two conformers of the enzyme-substrate complex, and  $E^*S'$  and  $ES'$  two forms of the phosphorylated enzyme; only the latter can react with water and only the former with nucleophile.

A steady-state kinetic study was made of the hydrolysis of *p*-nitrophenyl phosphate by chicken intestinal

phosphatase. The work was done in barbital buffer (carbonate above pH 9.6), and covered the pH range from 7.0 to 10.0. A sufficiently wide range of substrate concentration was used to allow reliable values of  $\tilde{V}$ ,  $\tilde{K}_m$  and  $\tilde{V}/\tilde{K}_m$  to be determined. The results lead to pK values of 8.1 and 8.6 for the free enzyme, and it is concluded that the Michaelis complex and the phosphoryl intermediate only ionize on the acid side, the former also having a pK of 8.1. It is suggested that the group of pK 8.1 is probably an  $\alpha$ -amino group, and that the group of 8.6 probably corresponds to the ionization of a Zn(II)-coordinated water molecule.

An experimental study was made of the enzyme-catalyzed hydrolysis of p-nitrophenyl phosphate in the presence of different concentrations of L-phenylalanine. The enzyme used was calf intestinal alkaline phosphatase and the reactions were carried out in barbital buffer pH 9.0,  $I = 0.10$  M and at a temperature of  $25.0^\circ\text{C}$ .

The series of parallel straight lines obtained from the reciprocal plots of velocity against substrate concentration at different L-phenylalanine concentrations and the linear dependencies of both  $1/V'$  and  $1/K'$  on the concentration of L-phenylalanine indicated that the inhibition was uncompetitive. ( $V'$  and  $K'$  are the kinetic parameters of the modified Michaelis-Menten equation to which the data could be fitted.) The inhibitor constant was found to be  $(1.95 \pm .04)$  mM. The only mechanism of inhibition consistent with the known data is



## ACKNOWLEDGEMENTS

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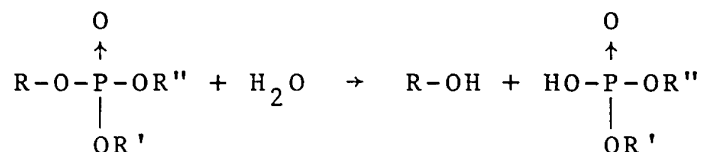
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## CHAPTER I

### GENERAL INTRODUCTION

Derivatives of orthophosphoric acid are of central importance in metabolism (1-3). Early attempts to study these phosphorylated metabolites were often frustrated by the apparently ubiquitous occurrence of enzymes that catalyzed their hydrolysis. These enzymes, collectively referred to as phosphatases (4,5), catalyze hydrolyses of the type



in which either an ester or an anhydride phosphorus-oxygen bond is broken. Studies with  $^{18}\text{O}$ -labelled  $\text{H}_2\text{O}$  (6) have established that cleavage occurs exclusively at the phosphorus-oxygen bond in both the enzyme-catalyzed reaction and in the non-enzymic alkaline hydrolysis; the acid catalyzed hydrolysis, on the other hand, often proceeds with both P-O and C-O cleavage (6-12).

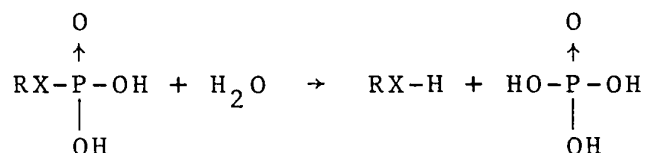
#### Classification of the Phosphatases

The phosphatase activity of most plant and animal tissues is actually due to a variety of enzymes that differ markedly in their specificity towards phosphorylated metabolites (4,5,13). These differences in specificity serve as

the basis for the classification scheme of the Report of the Commission of Enzymes (12), a simplified version of which is shown schematically in Figure 1. In this scheme the phosphomonoesterases are subdivided into two main groups, (1) the acid phosphatases with pH optima between 4.5 and 6.5 and (2) the alkaline phosphatases which have pH optima between 8 and 10. There are two types of alkaline phosphatases: (1) 'substrate specific' phosphatases and (2) 'non-specific' phosphatases.

#### Specificity of the Non-Specific Phosphatases

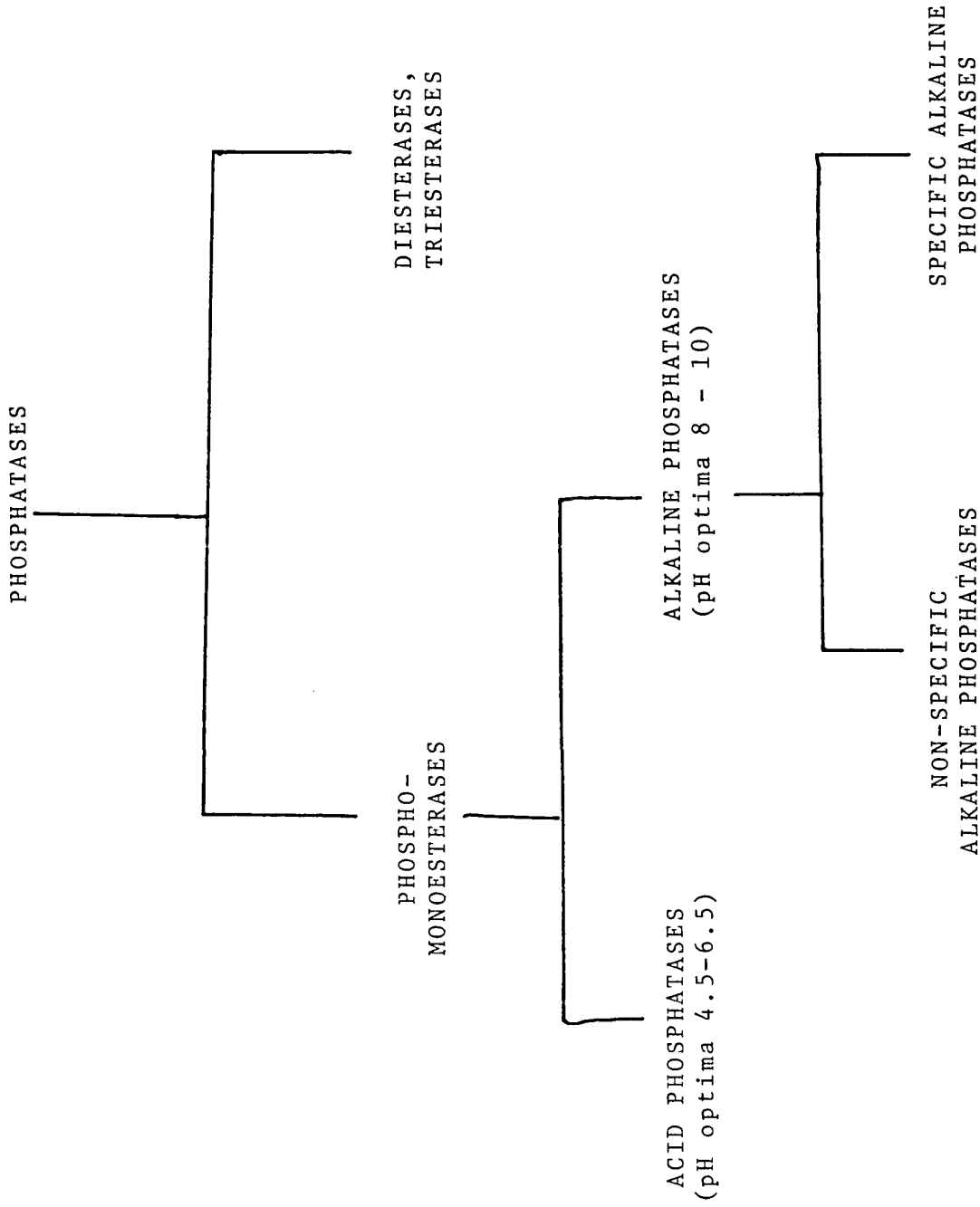
The non-specific alkaline phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) catalyze the hydrolysis of a wide variety of monoesters of phosphoric acid; the reactions catalyzed are of the form



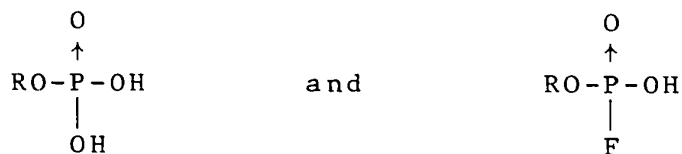
where X is an oxygen, sulfur, nitrogen, or fluorine atom and R is a hydrogen atom, an alkyl, aryl, or acyl group, etc. Substrates include phosphate monoesters of primary, secondary, and cyclic alcohols and phenols (14-23), phosphoromononucleotides (18-20), phosphoromonothioesters (24,25), monoamidophosphates (26) and various polyphosphates (19,20,27-30). In sharp contrast to the very low specificity towards the RX

Figure 1

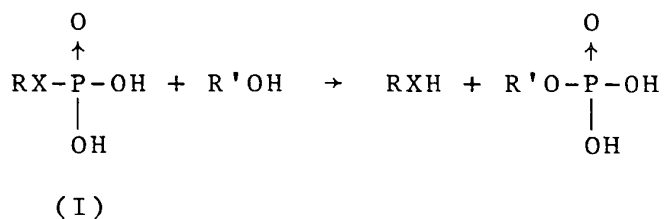
Classification of the Phosphatases



portion of the substrate is the absolute specificity for the phosphoryl group: phosphodiester and triester are not hydrolyzed; neither are mixed esters such as



In the presence of certain alcohols the non-specific alkaline phosphatases also catalyze transphosphorylation reactions of the type



in which an alcohol, R'OH, rather than H<sub>2</sub>O is the phosphoryl group acceptor (22,23,31). Since the enzymes must also catalyze the reverse reaction, all compounds of the type RXH will also be phosphoryl group acceptors. The general reaction catalyzed is therefore the transfer of a phosphoryl group from a donor of type (I) to an acceptor of the type R'X'H with cleavage of the P-X bond.

#### Distribution and Localization

Alkaline phosphatases are found in many different kinds of vertebrate cells and extracellular fluids (12,21,23, 32-34); they also appear to be very widely distributed in

various invertebrates (35-37), higher plants (21,38), fungi (39,40), and microorganisms (42-49). The localization of alkaline phosphatase activity within a particular organism, tissue, or cell has been the object of many careful investigations (50-66). These studies have shown that in vertebrates high concentrations of the enzyme are normally found in the intestinal mucosa, placental, kidney, bone, liver, lung and spleen tissues, usually in that order of abundance (23). The localization of the enzyme within these tissues is shown in Table 1. Also listed in this table is the additional detail that the enzyme from the intestinal mucosa, kidney and placental tissues is firmly associated with the lipoprotein membrane of the epithelial cells. In addition, the enzyme is also present on the chromosomes of cell nuclei of both plants and animals during mitosis. In microorganisms such as E.coli the enzyme is localized in the periplasm, the region between the protoplasmic membrane and the cell wall (67-69). Subunits of the enzyme, it appears (68-69), are synthesized on polyribosomes and transported to the periplasmic space where dimerization occurs.

#### Regulatory Mechanisms

A variety of regulatory mechanisms are now known to govern the synthesis of alkaline phosphatase. In E.coli, synthesis of the enzyme is dramatically repressed by inorganic phosphate in the growth medium (42-44). In the presence of

TABLE 1  
Localization of Alkaline Phosphatase in  
Vertebrate Tissues

Tissue	Location	Comments	References
Intestinal Mucosa	Microvilli of Epithelial Cells	Firmly Associated with the Lipoprotein Membrane of the Epithelial Cells	53-56
Kidney	Brush Border of Proximal Tubule Cells		60,61
Placenta	Trophoblastic Syncytium		62
Liver	Adjacent to Bile Canaliculi		63,64
Bone	Hypertrophic Cartilage Cells, Osteoblasts and Osteocytes		65,66

high phosphate concentrations the amount of enzyme found in this microorganism is negligible. However, when the concentration of phosphate is low enough to limit cell growth alkaline phosphatase accounts for about 6% of the total protein synthesized. Inorganic phosphate also appears to specifically repress the synthesis of alkaline phosphatase in rat kidney, since a decrease in its concentration in this tissue produced by a low phosphate diet is accompanied by a marked increase in the concentration of the enzyme (70-72). In some mammalian cells such as skin fibroblasts, L-cysteine also acts as an efficient repressor (73,74,76-78). However, induction rather than repression appears to be the more widespread mechanism regulating the level of alkaline phosphatase in higher organisms. Adrenal glucocorticoid hormones such as hydrocortisone and prednisolone and substrates such as phenyl phosphate induce the synthesis of the enzyme in human leucocytes, fibroblasts and other cell cultures such as HeLa cells (73,75,78-80). Induction of alkaline phosphatase synthesis by these substances has also been observed in various tissues of developing amphibian, chicken and rat embryos (50-52).

One important consequence of these regulatory mechanisms is that high alkaline phosphatase activity sometimes appears in tissues that usually do not show it, for example (1) in various tissues in developing embryos during the early stages of development (50-52), in the final stages

of development, however, the enzyme is found only in organs which contain it in post-embryonic life; (2) in fibroblasts during the early stages of wound healing (21,81); (3) in tumors from tissues which normally do not contain the enzyme (21) and (4) in the blood plasma of individuals with certain pathological conditions (82-88) including obstructive jaundice (82), rickets (83), and various bone diseases (84, 85). The latter two phenomena have, understandably, led to considerable clinical interest in the enzyme and the development of sensitive methods for estimating its activity as a diagnostic tool (86-88).

#### Physiological Role

Despite their widespread occurrence throughout the plant and animal kingdoms, the physiological role of the non-specific alkaline phosphatases is still unknown. Since the enzyme is repressed by orthophosphate in many strains of E.coli (42-44), it has been suggested (22) that its function is to supply the microorganism with the phosphate necessary for metabolism by the specific hydrolysis of organic phosphate esters when no other source of phosphate is available. Not all alkaline phosphatases, however, function only under conditions of phosphate deprivation: the enzyme from *Salmonella Typhimurium*, for example, is non-repressible by inorganic phosphate (45). Since the enzyme binds inorganic phosphate very tightly at low pH (21,89,90), another possibility is

that it serves to transport and concentrate phosphate from a more acidic medium to the interior of the cell.

On the basis of their substrate specificity, cellular localization and change in activity during growth, several roles have been suggested for the alkaline phosphatases of the higher organisms (12,23). For the cytoplasmic enzyme these include (1) dephosphorylation of substrate for subsequent metabolism, or as a control of some cellular processes, (2) localized production of inorganic phosphate in high concentrations (91), and (3) hydrolysis of pyrophosphate (92). In addition, since the enzyme is often localized at the absorptive surface, it may play a direct role in the active transport of nutrients across the epithelial membrane (23). The nuclear enzyme may play a role in nucleic acid synthesis - the hydrolysis of inorganic pyrophosphate, an inhibitory product of the reaction. Alternatively, the function of this enzyme may be the phosphorylation of nuclear protein, a process implicated in the control of gene activation (93).

#### Comparison of the Non-Specific Alkaline Phosphatases

The various non-specific alkaline phosphatases differ markedly in (1) molecular weight ( $8.6 - 19 \times 10^4$ ), (2) electrophoretic mobility, (3) immunological properties, (4) heat stability, (5) L-phenylalanine inhibition, and (6) specific activity (32,33,94-104). These differences, which are observed even between phosphatases from two tissues of the same organism, have been used to establish the origin

of the enzyme in extracellular fluids (97-104).

All phosphatases\* which have so far been examined in sufficient detail have been shown to possess identical substrate and acceptor specificities. In addition their amino acid sequences around what there is reason to believe is the active site of the enzyme are identical. Furthermore, all the alkaline phosphatases are now known to be zinc metalloenzymes (23). The E.coli enzyme consists of two identical subunits and active sites. However, only one of the zinc atoms in each subunit is involved in substrate binding and catalysis; the other zinc atom, which may not be located at the active site, serves to stabilize the quaternary structure of the molecule (22). The role of zinc in the other alkaline phosphatases has not yet been established, but there is some evidence which suggests that it may be similar to that in the E.coli enzyme.

#### Mechanism of Catalysis

Despite the concerted effort made in the past decade to unravel the mechanism of alkaline phosphatase catalysis (21-23), several fundamental mechanistic questions still remain unanswered. For example, what are the elementary steps of the reaction? What is the nature of the rate determining step, especially at alkaline pH? What is the nature of the groups involved in the catalytic mechanism? What role do

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\* Non-specific alkaline phosphatases.

they play in catalysis? It is the objective of this thesis to answer these questions by analyzing the results of detailed kinetic studies carried out with chicken intestinal phosphatase. The answering of these questions should lead to a much better understanding of the catalytic mechanism of this enzyme, and, in view of the similarities between the intestinal enzyme and other non-specific phosphatases, possibly also of other phosphatases.

CHAPTER II

THE KINETICS OF ALKALINE PHOSPHATASE CATALYZED  
REACTIONS IN THE PRESENCE OF ADDED NUCLEOPHILES

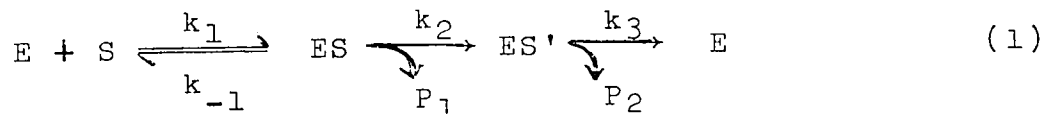
INTRODUCTION

Evidence for the Existence of a Phosphoryl-Enzyme  
Intermediate

It is now generally accepted that reactions catalyzed by alkaline phosphatase proceed via a covalent phosphoryl-enzyme intermediate. The existence of this intermediate was first postulated by Morton (105,106) to explain the phosphoryl transferase activity of the enzyme in the presence of high concentrations of alcohols (105-110) and the earlier observation that the enzyme catalyzed hydrolysis takes place exclusively at the P-O bond (111,112). Morton's hypothesis was later confirmed by Engstrom (113) and Argen (114) who showed that a phosphoprotein containing a phosphorylated serine residue is formed when the crude calf intestinal (113) or bone (114) enzyme is incubated with low concentrations of  $^{32}\text{P}$ -labeled inorganic phosphate or  $^{32}\text{P}$ -glucose-6-phosphate at  $\text{pH} < 6$ . Further studies with highly purified alkaline phosphatases from various sources (115-125) have corroborated these important initial observations, and have revealed that of the more than thirty serine residues

in the enzyme molecule only one particularly reactive serine OH group is phosphorylated by labeled substrate or inorganic phosphate. Substrates and competitive inhibitors such as sodium arsenate . . . prevented incorporation of  $^{32}\text{P}_i$  from the radioactive label (117-121), as did chemical inactivation or heat denaturation of the enzyme (121).

These results suggest that the serine OH residue that is phosphorylated in these labeling experiments is located within the active site of the enzyme - an hypothesis supported to some extent by the close similarity of the amino acid sequence about this reactive serine (116,120,123-125) to the sequence found at the active sites of many other esterases (126). It would therefore seem that alkaline phosphatase belongs to the large group of enzymes which contain a uniquely reactive serine OH residue at their active sites. Included in this group are phosphotransferases such as phosphoglucomutase and phosphofructomutase, many of the proteolytic enzymes such as trypsin, chymotrypsin, thrombin, elastase, subtilisin, and many other esterases such as acetylcholine esterase, the liver and serum esterases. The hydrolytic, phosphoryl- or acyl-transfer reactions catalyzed by these enzymes apparently all proceed via phosphoryl- or analogous acyl-enzyme intermediates according to the general scheme (105,106,121,127,128)



In this scheme E is the free enzyme; ES, the Michaelis complex in which the substrate, S, is physically attached to the enzyme; ES' is the phosphoryl- or analogous acyl-enzyme intermediate; P<sub>1</sub> is the first product of the reaction, the alcohol moiety of the substrate, ROH; and P<sub>2</sub>, the second product, such as inorganic phosphate.

In addition to the <sup>32</sup>P-labeling experiments, several indirect criteria have also been used as evidence supporting the existence of a phosphoryl-enzyme intermediate. For example, rapid flow quenching studies with both E. coli (129,130) and bovine milk (131) alkaline phosphatase are consistent with the transient formation of a phosphoryl-enzyme intermediate during the enzyme catalyzed reaction. In these experiments the enzyme was first phosphorylated at pH 5.5 with <sup>32</sup>P-inorganic phosphate. By using a specially constructed rapid flow quenching apparatus it was then possible to rapidly mix the resulting <sup>32</sup>P-labeled phosphoprotein with buffer at high pH, and quench the reaction within 5 msec after mixing. By varying the time of quenching it was possible to determine the level of labeled phosphoprotein during the course of the reaction, and, hence, the rate of dephosphorylation. The <sup>32</sup>P-labeled phosphoprotein, as expected, hydrolyzed very rapidly at pH > 7.

Kinetic studies of the transient phase of the reaction using the stopped-flow technique (132-144) have provided further support for the phosphoryl-enzyme inter-

mediate hypothesis. In these studies, carried out almost exclusively with the E. coli enzyme, it was found that when chromogenic or fluorogenic substrates such as 2,4-dinitrophenyl phosphate (138) or 4-methylumbelliferyl phosphate (132, 140) are rapidly mixed with relatively high concentrations of enzyme the liberation of the corresponding phenol appeared to pass through two phases during the course of the reaction. Initially a concentration of phenol corresponding to 1 mole of product per mole enzyme is rapidly released. This 'initial burst' is then followed by a slower, zero-order release of product. These observations can of course be readily explained with reference to the postulated phosphoryl-enzyme intermediate: the initial rapid phase corresponds to the liberation of 1 mole of phenol per mole of enzyme during the phosphorylation of the enzyme; the slower, linear phase corresponds to the steady-state liberation of phenol which is controlled by the rate-determining step.

It has been observed in the case of the E. coli enzyme that the relative rates of hydrolysis of many phosphate (145-9, 151-2) and S-phosphorothioate (150) esters with widely differing leaving groups are nearly the same. This observation seems to indicate that a phosphoryl-enzyme intermediate is involved in the catalysis. The nearly equal rates of hydrolysis can then readily be explained if it is further assumed that the rate of formation of this phosphoryl-

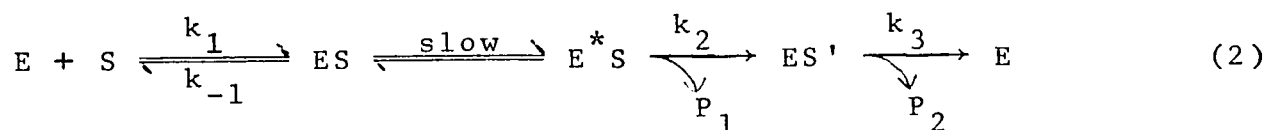
enzyme intermediate is much greater than its rate of hydrolysis. Under these conditions, the overall rate would be equal to the rate of hydrolysis of this common intermediate, and, hence, independent of leaving group.

#### Shortcomings of the Phosphoryl-Enzyme Hypothesis

A more detailed examination of the evidence presented in favor of a phosphoryl-enzyme intermediate, however, indicates that it is far from conclusive, since alternative interpretations of the data are possible. For example, Wilson and Dayan (152-154) have shown that the phosphoprotein formed by  $^{32}\text{P}$ -phosphate labeling is about  $10^6$  times more stable thermodynamically than ordinary phosphate esters such as O-phosphoryl serine and O-phosphoryl ethanolamine. Its formation, Barrett et al. (154) have pointed out, therefore is to be expected on the basis of equilibrium arguments regardless of the actual kinetic mechanism, and, for this reason, cannot prove that a phosphoryl-enzyme is actually formed as an intermediate during the enzyme-catalyzed hydrolysis of phosphate esters.

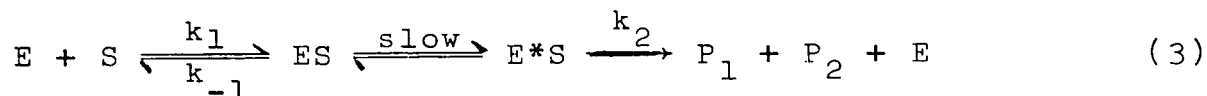
Similarly, the transient-phase kinetic studies briefly described earlier, although consistent with a phosphoryl-enzyme intermediate, allow for alternative interpretations (154). Furthermore, in these studies, using the E. coli enzyme, a rapid pre-steady-state liberation of colored product was observed only for  $\text{pH} < 7$ , which indicates

that the rate-determining step at alkaline pH is phosphorylation, rather than dephosphorylation. This result is in apparent disagreement with the observation that the enzyme catalyzes the hydrolysis of almost all substrates with equal ease at pH > 7. Since many of these substrates, such as 4-nitrophenyl phosphate and 2,4-dinitrophenyl phosphate, have widely differing leaving groups, their rates of hydrolysis should almost certainly differ if phosphorylation were the rate-determining step. Gutfreund et al. (136,138-9) have therefore proposed that a first-order conformational change of the enzyme-substrate complex, in which presumably the active site of the enzyme rearranges itself into a reactive form in response to a correct substrate, is the rate-determining step of the reaction. This conformational change, of course, takes place before phosphorylation, the complete mechanism being



in which  $E^*$  represents the reactive conformation of the enzyme. Unfortunately, this mechanism still does not explain why all substrates are hydrolyzed at about the same rate, unless one assumes that the rate of conversion of  $ES$  to  $E^*S$  is independent of the nature of  $S$ . Since the above mechanism requires that the conformational change be induced by the binding of the substrate, as in the induced-fit (155-161) or strain mechanism (161-3), such an assumption appears to be no more

reasonable than the previously unacceptable one that phosphorylation,  $ES \rightarrow ES' + ROH$ , is independent of R. In addition, it will later be shown that Scheme 2 does not lead to the correct kinetic behavior in the presence of competitive nucleophiles such as Tris or ethanolamine. Finally, even if a rate-determining conformational change were to be implicated in the enzyme catalyzed reaction, it would not necessarily follow that a phosphoryl-enzyme intermediate is also involved. Instead the mechanism could conceivably be



in which  $E^*S$ , the conformationally altered enzyme-substrate complex, breaks down with the simultaneous release of both reaction products.

Clearly, then, even in the case of the *E. coli* enzyme for which the most extensive data are available, the evidence presented above does not unequivocally demonstrate the participation of a phosphoryl-enzyme intermediate in the catalytic mechanism. Even if the existence of such an intermediate is assumed, one related question, fundamental to the understanding of the mechanism, still remains: which step of the reaction is the rate determining one, especially above pH 7? The situation is, of course, much less certain for alkaline phosphatases from other sources, since transient-phase studies with these enzymes are virtually non-existent,

and only the  $^{32}\text{P}$ -labeling experiments provide evidence pertinent to this problem.

The solution of this basic problem - the demonstration beyond any reasonable doubt of the involvement of a phosphoryl-enzyme intermediate in the alkaline phosphatase catalyzed reaction - necessitates the utilization of a different, indirect approach. One particularly convenient method - a steady-state kinetic one - involves the detailed investigation of the enzyme reaction in the presence of nucleophiles other than water. The added advantage of this method, it will be seen, is that not only does it show unequivocally whether or not a transient phosphoryl-enzyme intermediate exists, but, at least in theory, it also permits identification of the rate determining step of the reaction.

#### Added Nucleophile Studies

It has long been recognized (105,106) that when the alkaline phosphatase catalyzed hydrolysis of a typical phosphate monoester, ROX, is carried out in the presence of certain nucleophiles of the form R'OH, the following two reactions occur simultaneously:



Reaction (4), the hydrolysis reaction, may conveniently be visualized as the transfer of a phosphoryl group, X, from

the donor molecule ROX to  $H_2O$  which serves as the acceptor. Reaction (5), the transphosphorylation reaction therefore represents the transfer of this phosphoryl group to R'OH, the alternate acceptor; that is, R'OH competes with  $H_2O$  for the phosphoryl group of the substrate.

Alkaline phosphatase, Morton has pointed out (106), is by no means unique in this respect. In fact, a large number of hydrolytic enzymes are now known to exhibit the type of transferase activity shown in Eq. (5) - the ability to catalyze the transfer of a portion of the substrate, X, to acceptors other than  $H_2O$ . Some of these enzymes together with their typical donors and acceptors, are listed in Table 2, which is an updated version of a table published earlier by Morton (106).

Although all the earlier studies of the effect of competitive nucleophiles on the alkaline phosphatase catalyzed reaction were carried out with enzyme obtained from various mammalian sources, especially calf intestine (105-6,204,211-215), these were subsequently abandoned in favor of the more highly purified E. coli enzyme (152,154, 216-223). Some of these later studies are, in fact, detailed investigations involving the use of various substrates and nucleophiles and the measurement of the Michaelis parameters for the rates of formation of the different products as a function of nucleophile concentration to various degrees of precision. Without exception, however, these studies lead

TABLE 2

## Hydrolytic Enzymes with Associated Transferase Activity

Enzyme	Source	Typical Donor	Typical Acceptor	References
Ribonuclease	Pancreas	Cytidine-2':3'-Phosphate	Methanol	164-166
$\beta$ -Glycosidase	S. Purpurea L.	Phenyl- $\beta$ -Glycoside	Methanol	172
Lactase	Yeast	Lactose	Lactose	168
Invertase	Yeast	Sucrose	Sucrose	169
Myosin	Rabbit Muscle	ATP	[ $^{14}\text{C}$ ]- $\text{CH}_3\text{OH}$	170
Cholinesterases	e.g. Electric Eel	Sodium Acetate	Hydroxylamine	127, 171
$\beta$ -Galactosidase	E. Coli	Lactose	[ $^{14}\text{C}$ ]-Glycerol	172
Carboxyl-Esterase	Pig Liver	Methyl-Butyrate	Methanol	173
	Beef Liver	MPP	Glycine Methyl Ester	174
	Pig Liver	Phenyl Acetate	Methanol	175
Subtilisin	B. Subtilis	L-Tyrosine Ester	Various Alcohols; e.g. Methanol	176
Trypsin	Bovine Pancreas	Lysine Methyl Ester	Ethanol	177
			Methanol	178

Table 2 - continued

Enzyme	Source	Typical Donor	Typical Acceptor	References
Chymotrypsin	Bovine Pancreas	PNPA	Ethanol	179
		Methyl-Hippurate	Hydroxylamine	180
		Ethyl Hippurate	Hydroxylamine	181
		ATNA	Hydroxylamine	182
		Trans-Cinnamoyl- $\alpha$ -Chymotrypsin	Methanol	183
Ficin	Fig Tree Latex	Furoyl-Chymotrypsin	Ethanol	184
		Methyl Hippurate	Propan-2-ol	185
		PNP-Hippurate	Methanol	186
Papain	Papaya Latex	Methyl-Hippurate	[ $^{14}$ C]-Methanol	187
		BAEE	Methanol	188
		PNP-Hippurate	Methanol	186
		Ethyl-Hippurate	[ $^{14}$ C]-Ethanol	189
		PNP-Hippurate	Ethanol	189
		Trans-Cinnamoyl Papain	Various Amides e.g. Glycinamide	190

Table 2 - continued

Enzyme	Source	Typical Donor	Typical Acceptor	References
Papain	Papaya Latex	Trans-Cinnamoyl Papain	Di-,Tri- and Tetra- Glycinamide	191
Glutaminase	E.Coli	Glutamine	Hydroxylamine	192,193
Glucose-6- Phosphatase	Rat Liver Microsomes	Glucose-G-P Ppi	<sup>14</sup> C-Glucose Glucose	194-7 198-203
Acid Phosphatase	Human Prostate	Phenyl Phosphate, Phenolphthalein Diphosphate	Glycerol Ethanol	204,205
	Citrus Fruits	P-Nitrophenyl Phosphate	Methanol	206
	Malt	Phenyl Phosphate	Cytidine	207
	Human Prostate	Phenyl Phosphate	Various Alcohols, e.g. Butanediol	208,209
	Carrot	Phenyl Phosphate	Cytidine	210
Alkaline Phosphatase	Calf and other Mammalian Intestine	Phenyl Phosphate	Glucose	109,110 204,205
	Milk	Phenyl Phosphate	Glucose	204,205
	Calf Bone	<sup>32</sup> P-β-Glycero- phosphate	Serine, Threonine	213
	Calf Intestine	Phenolphthalein Diphosphate	Glycerol	214

Table 2 - concluded

Enzyme	Source	Typical Donor	Typical Acceptor	References
Alkaline Phosphatase	Calf Intestine	PNPP	Ethylene Glycol	215
	E.Coli	PNPP	Tris	152, 216, 217
		PNPP	Ethanolamine	152, 218, 219
		2,4-DNPP	Tris, Hydroxylamine	138
		Substituted Phenyl Phosphates	Tris	154
		OCPP	Tris, Glycerol	151
		PPi	Glucose	220
		NBOPPS	Ethanolamine	221
	E.Coli (CO <sub>2</sub> <sup>+</sup> enzyme)	OCPP, PNPP	Glycerol, Tris	141, 151 222-3

## Abbreviations Used:

PNPP	: p-nitrophenyl phosphate	ATNA	: Acetyl tyrosine p-nitroanilide
2,4-DNPP	: 2,4-dinitrophenyl phosphate	BAEE	: benzoyl arginine ethyl ester
OCPP	: o-carboxyphenyl phosphate	NBOPPS	: N-benzoyl-O-pyrophosphoserine
ATP	: adenosine triphosphate	PPi	: inorganic pyrophosphate
PNPA	: p-nitrophenyl acetate	MPP	: methyl β-phenyl propionate

to results apparently inconsistent with data obtained from stopped flow experiments. Although recently pointed out (144), these inconsistencies have not yet been explained in a completely satisfactory manner. The results obtained with the mammalian enzyme are of much more doubtful significance since they did not involve the measurement of true initial rates, the effect of nucleophile concentration on the actual Michaelis parameters was not studied, and few measurements were carried out at relatively low concentrations of nucleophile - the region in which the non-specific solvent effects are unimportant. Because of marked inhibition by orthophosphate (243), the  $K_m$  for many substrates being higher than  $K_i$ , and dependence of optimum pH on substrate concentration (146,224-223), time curves for hydrolysis of substrate by alkaline phosphatase are quite complex, and of limited kinetic value. It is therefore of paramount importance that only true initial rates be used in these mechanistic studies.

In view of the above discussed limitations of the previous studies, there still remain some important unresolved difficulties. The evidence outlined does not make it absolutely certain that there really is a phosphoryl-enzyme intermediate. It leaves unanswered the question of which step is rate-determining at pH values above about 7. The present chapter describes an alternative approach to the problem, involving the study of the kinetics of the reaction in the presence of nucleophiles other than water. It will

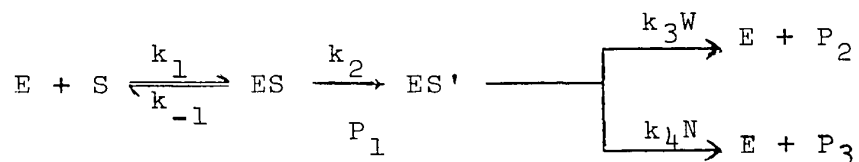
be seen that this technique demonstrates unequivocally the presence of a transient phosphoryl-enzyme intermediate, and that it also permits identification of the rate-determining step of the reaction. The main questions that the present experimental work was designed to answer are:

- (1) How does the nature of the substrate affect the ratio of transferase to hydrolytic activity?
- (2) How do the Michaelis parameters vary with the concentration of added nucleophile?

The answering of these questions leads to clear-cut mechanistic conclusions.

#### THEORETICAL PRINCIPLES

Alkaline phosphatase catalyzed hydrolysis of phosphate esters in the presence of competitive nucleophiles such as Tris, it is usually assumed, proceed via Mechanism I:



in which E is the free enzyme; S is the substrate of the form ROP, P being a phosphoryl group; ES is the Michaelis complex; while ES' is the covalent phosphoryl-enzyme intermediate; P<sub>1</sub> is the first product of the reaction, ROH; P<sub>2</sub> is the second product, phosphoric acid, produced by the hydrolysis of the phosphoryl-enzyme intermediate; P<sub>3</sub> is

is the product which results from the phosphorylation of the competitive nucleophile, N, by the phosphoryl-enzyme intermediate, for example, O-phosphoryl Tris if N is Tris.

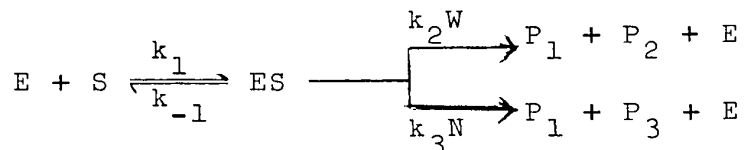
Implicit in this treatment are the following three assumptions:

(1) that the enzyme-catalyzed reaction does in fact proceed via a phosphoryl-enzyme intermediate;

(2) that the role of the nucleophile in the reaction is the same as that of water: it serves only as a kinetic acceptor; i.e., it reacts with the phosphoryl-enzyme intermediate to form product;

(3) that the enzyme does not contain a specific site for either H<sub>2</sub>O or the other nucleophile.

In the absence of a phosphoryl-enzyme intermediate the simplest scheme for the reaction carried out in the presence of competitive nucleophiles is Mechanism II:



According to this reaction scheme H<sub>2</sub>O and added nucleophile compete for the Michaelis complex; reaction with this complex results in its breakdown and simultaneous release of both reaction products P<sub>1</sub> and P<sub>2</sub> with H<sub>2</sub>O, and P<sub>1</sub> and P<sub>3</sub> when N is the reacting nucleophile.

Careful consideration of the kinetic consequences

of both these reaction mechanisms leads to the reassuring conclusion that it is readily possible to differentiate between them experimentally. For example, Mechanism I predicts that, regardless of which step of the reaction is rate determining, the ratio of the products  $P_3/P_2$  must be independent of the nature of the leaving group of the substrate, since according to this scheme it is no longer present when reaction with water or added nucleophile takes place, reaction instead occurring via a common phosphoryl-enzyme intermediate. On the other hand, if there is no common phosphoryl-enzyme intermediate and the reaction with water and added nucleophile occurs directly through the Michaelis complex containing the leaving group of the substrate, as in Mechanism II, the ratio of products,  $P_3/P_2$ , should depend on the structure of this leaving group. Therefore, by studying the enzyme catalyzed hydrolysis of a series of substrates with widely differing leaving groups in the presence of the same concentration of added nucleophile and measuring the ratio of the reaction products,  $P_3/P_2$ , it is possible to establish whether or not a phosphoryl-enzyme intermediate occurs during the reaction.

#### Steady-State Equations for Mechanism I

Application of the usual steady-state assumption to Mechanism I readily leads to the following rate equations:

$$v_1 = \frac{dP_1}{dt} = \frac{k_2(k'_3 + k_4N)}{k_2 + k'_3 + k_4N} \frac{E_0 S_0}{K_s(k'_3 + k_4N) + S_0} \quad (6)$$

$$v_2 = \frac{dP_2}{dt} = \frac{k_2 k'_3}{k_2 + k'_3 + k_4N} \frac{E_0 S_0}{K_s(k'_3 + k_4N) + S_0} \quad (7)$$

$$v_3 = \frac{dP_3}{dt} = \frac{k_2 k_4 N}{(k_2 + k'_3 + k_4N)} \frac{E_0 S_0}{K_s(k'_3 + k_4N) + S_0} \quad (8)$$

where  $v_i$  is the initial rate of formation of the  $i^{\text{th}}$  product,  $i = 1, 2$ , or  $3$ ;  $k'_3 = k_3 W$ ;  $K_s = (k_{-1} + k_2)/k_1$ ;  $E_0$  is the total concentration of enzyme; and  $S_0$  is the initial substrate concentration.

One of the above equations, it should be noted, is redundant: once any two of these equations are specified, the remaining one may be written down immediately, since the three equations are connected by the relationship

$$v_1 = v_2 + v_3 \quad (9)$$

It is also readily seen that Eqs. (6), (7), and (8) are Michaelis-Menten equations of the form

$$v_i = \frac{k_{ci} E_0 S_0}{K_m + S_0}, \quad i = 1, 2, 3 \quad (10)$$

in which  $k_{ci}$  is the "kcat" value for the  $i^{\text{th}}$  product.

Eq. (10) may also be written in the form

$$v_i = \frac{V_i S_o}{K_m + S_o}, \quad i = 1, 2, 3 \quad (11)$$

with 
$$V_i = k_{ci} E_o, \quad (12)$$

$V_i$  being the maximum rate of formation of the  $i^{\text{th}}$  product.

Inspection of Eqs. (6), (7), and (8) shows that the Michaelis constant,  $K_m$ , is identical in all three cases and is given by

$$K_m = \frac{K_s (k'_3 + k_4 N)}{k_2 + k'_3 + k_4 N} \quad (13)$$

Expressions for the catalytic rate constants,  $k_{ci}$ ,  $i = 1, 2, 3$ , which differ for different values of  $i$ , are given by the following equations:

$$k_{c1} = \frac{k_2 (k'_3 + k_4 N)}{k_2 + k'_3 + k_4 N} \quad (14)$$

$$k_{c2} = \frac{k_2 k'_3}{k_2 + k'_3 + k_4 N} \quad (15)$$

$$k_{c3} = \frac{k_2 k_4 N}{k_2 + k'_3 + k_4 N} \quad (16)$$

Again, it should be noted that

$$k_{c1} = k_{c2} + k_{c3} \quad (17)$$

It is often convenient to denote the rate of formation of phosphate divided by the rate of transphosphorylation by the symbol  $f$  (152,153,218,219); i.e.,

$$f = \frac{v_2}{v_3} \quad (18)$$

It can readily be seen that, at all substrate concentrations

$$f = \frac{k_{c2}}{k_{c3}} = \frac{v_2}{v_3} = \frac{k_3'}{k_4 N} \quad (19)$$

Another quantity sometimes used (105,126) is  $\beta$ , defined by the relation

$$\beta = k_4 / k_3' \quad (20)$$

A comparison of Eqs. (19) and (20) shows that

$$\beta = 1/(fN) \quad (21)$$

The kinetic behavior of alkaline phosphatase catalyzed reactions involving added nucleophiles should, according to Eqs. (13)-(16), depend only on the relative orders of magnitude of  $k_2$  and  $k_3'$ , provided  $N$  is chosen such that  $k_4 N \sim k_3'$ . It is experimentally convenient to work at a concentration  $N$  such that  $k_4 N \sim k_3'$  so that comparable amounts of  $P_2$  and  $P_3$  are obtained. The following three important special cases are therefore worthwhile considering in some detail:

Case Ia.  $ES \rightarrow ES' + P$ , is rate limiting; i.e.,

$k_2 \ll k_3' \sim k_4 N$ . The kinetic parameters reduce to

$$K_m = K_s = (k_{-1} + k_2)/k_1 \quad (22)$$

$$k_{c1} = k_2 \quad (23)$$

$$k_{c2} = \frac{k_2 k_3'}{k_3' + k_4 N} \quad (24)$$

$$k_{c3} = \frac{k_2 k_4 N}{k_3' + k_4 N} \quad (25)$$

Taking the reciprocals of Eqs. (24) and (25) yields the following two expressions:

$$\frac{1}{k_{c2}} = \frac{1}{k_2} + \frac{k_4}{k_2 k_3'} N \quad (26)$$

$$\frac{1}{k_{c3}} = \frac{1}{k_2} + \frac{k_3'}{k_2 k_4} \frac{1}{N} \quad (27)$$

The first two of these equations indicate that both the Michaelis constant,  $K_m$ , and the catalytic rate constant,  $k_{c1}$ , are independent of nucleophile concentration,  $N$ .  $K_m$  is in fact equal to  $K_s$ , defined by Eq. (22), while  $k_{c1}$  is equal to the rate-limiting constant of the reaction,  $k_2$ . The rate of formation of product  $P_1$ ,  $v_1$ , is, therefore, also independent of  $N$ :

$$v_1 = \frac{k_2 E_o S_o}{K_m + S_o} \quad (28)$$

The reciprocal of  $k_{c2}$ , Eq. (26) shows, is a linear function of  $N$ ; a plot of  $1/k_{c2}$  against  $N$  should yield a straight line of slope  $k_4/k_2k_3'$ , and a Y- intercept equal to  $1/k_2$ . The rate of formation of  $P_2$  is given by the expression

$$v_2 = \frac{\frac{k_2 k_3'}{k_3' + k_4 N} E_o S_o}{K_m + S_o} \quad (29)$$

It may be of some interest to note that Eq. (29) rearranges to

$$v_2 = \frac{\frac{k_2}{1 + (k_4/k_3')N} E_o S_o}{K_m + S_o} \quad (30)$$

In the absence of  $N$ , the rate equation becomes

$$v = \frac{k_2 E_o S_o}{K_m + S_o} \quad (31)$$

The effect of the nucleophile,  $N$ , on the rate of formation of  $P_2$ , these last two equations show, may be thought of as non-competitive inhibition, the term corresponding to  $K_i$ , the dissociation constant of the enzyme-inhibitor complex in the usual inhibition equation, here being equal to  $k_3'/k_4$ .

Finally, Eq. (25) indicates that a plot of  $k_{c3}$  against concentration of added nucleophile is hyperbolic and passes through the origin;  $k_{c3}$  increases with increasing  $N$  and asymptotically approaches the limiting value,  $k_2$ . The double reciprocal plot,  $k_{c3}^{-1}$  vs  $N^{-1}$  should, according to

Eq. (27), result in a straight line of slope  $k_3'/k_2k_4$  and a Y-intercept of  $1/k_2$ .

Case Ib. ES' reacting with W and N is rate limiting; i.e.,  $k_4N \sim k_3' \ll k_2$ . The kinetic parameters are now

$$K_m = K_s \frac{(k_3' + k_4N)}{k_2} \quad (32)$$

$$k_{c1} = k_3' + k_4N \quad (33)$$

$$k_{c2} = k_3' \quad (34)$$

$$k_{c3} = k_4N \quad (35)$$

Unlike the situation in the previous case,  $K_m$  is now a linear function of the concentration of added nucleophile. Slightly rearranging Eq. (32) one obtains an expression for  $K_m$  of the form

$$K_m = K_s \frac{k_3'}{k_2} + K_s \frac{k_4}{k_2} N \quad (36)$$

from which it is readily seen that a plot of  $K_m$  against N should give a straight line of slope  $K_s(k_4/k_2)$ , and a Y-intercept equal to  $K_s(k_3'/k_2)$ .

The catalytic rate constants,  $k_{c1}$ , and  $k_{c3}$ , are both linear functions of nucleophile concentration;  $k_{c2}$ , on the other hand is independent of N. However, because  $K_m$  is a function of N, the rate of formation of product  $P_2$  shows the following dependence on N:

$$v_2 = \frac{k_3' E_o S_o}{K_s \frac{k_3'}{k_2} + K_s \frac{k_4'}{k_2} N + S_o} \quad (37)$$

This last equation may also be written in the form

$$v_2 = \frac{k_3' E_o S_o}{\frac{k_3'}{k_2} + K_s \frac{k_3'}{k_2} \frac{k_4'}{k_3} N + S_o} \quad (38)$$

In the absence of N,

$$v_2 = \frac{k_3' E_o S_o}{K_s \frac{k_3'}{k_2} + S_o} \quad (39)$$

Inspection of Eqs. (38) and (39) shows that the effect of added nucleophile on the rate of formation of  $P_2$  is formally equivalent to that of a competitive inhibitor, the ratio  $k_3'/k_4'$  corresponding to the constant  $K_i$  in the equation for competitive inhibition.

Case Ic. The general case where no simplifications apply; i.e.,  $k_4' N \sim k_3' \sim k_2$ . Eqs. (13-16) cannot be further simplified so that all the Michaelis parameters are complex functions of added nucleophile concentration. For example, Eq. (13) shows that the Michaelis constant,  $K_m$ , increases hyperbolically with increasing concentration of added nucleophile from a minimum,  $K_s k_3' / (k_2 + k_3')$ , when  $N = 0$ , to the limiting value,  $K_s$ , which is approached asymptotically as N becomes very large compared to  $k_2$  and  $k_3'$ . Similarly, it is seen from Eq. (14) that the

catalytic rate constant,  $k_{c1}$ , also increases as the concentration of added nucleophile is increased: when  $N = 0$ ,  $k_{c1} = k_2 k_3' / (k_2 + k_3')$ , whereas for very large value of  $N$  such that  $k_4 N \gg k_2 + k_3'$ ,  $k_{c1}$  attains the limiting value,  $k_2$ . Although both  $K_m$  and  $k_{c1}$  vary with concentration of added nucleophile in a complex manner, the ratio of these two Michaelis parameters,  $k_{c1}/K_m$ , is independent of  $N$ :

$$\frac{k_{c1}}{K_m} = \frac{k_2}{K_s} \quad (40)$$

By taking the reciprocal of Eq. (15) one obtains the expression

$$\frac{1}{k_{c2}} = \frac{1}{k_2} + \frac{1}{k_3'} + \frac{k_4}{k_2 k_3'} N \quad (41)$$

from which it is readily seen that the reciprocal of  $k_{c2}$  is a linear function of  $N$ : a plot of  $1/k_{c2}$  against  $N$  should result in a straight line of slope  $k_4/k_2 k_3'$ , and a Y-intercept equal to  $(k_2 + k_3')/k_2 k_3'$ . The rate of formation of  $P_2$  is given by Eq. (7), which can be rearranged to

$$v_2 = \frac{\frac{k_2 k_3'}{k_2 + k_3'} E_0 S_0}{\left(1 + K_s \frac{k_3'}{k_2 + k_3'} \cdot \frac{1}{K_s} \cdot \frac{k_4 N}{k_3'}\right)} \quad (42)$$

$$= \frac{\frac{k_3'}{k_2 + k_3'} \left(1 + \frac{k_4}{k_3'} N\right)}{\left(1 + K_s \frac{k_3'}{k_2 + k_3'} \cdot \frac{1}{K_s} \cdot \frac{k_4 N}{k_3'}\right)} + S_0$$

In the absence of N,

$$v_2 = \frac{\frac{k_2 k_3'}{k_2 + k_3'} E_o S_o}{K_s \frac{k_3'}{k_2 + k_3'} + S_o} \quad (43)$$

The type of behavior described by Eq. (42), therefore, is formally equivalent to a mixed type of inhibition - partially competitive and partially non-competitive; the ratio  $k_3'/k_4$  here corresponds to the constant  $K_i$  which appears in the equation for this type of inhibition.

Eq. (16) shows that  $k_{c3}$  increases hyperbolically with increasing concentration of added nucleophile; the hyperbola passes through the origin -  $k_{c3} = 0$ , when  $N = 0$  - and asymptotically approaches the limiting value,  $k_2$ . Taking the reciprocal of Eq. (16) one obtains the expression

$$\frac{1}{k_{c3}} = \frac{1}{k_{c2}} + \frac{k_2 + k_3'}{k_2 k_4} \frac{1}{N} \quad (44)$$

which shows that the reciprocal of  $k_{c3}$  is a linear function of  $1/N$ : if  $1/k_{c3}$  is plotted against  $1/N$  a straight line of slope  $(k_2 + k_3')/k_2 k_4$  and a Y-intercept of  $1/k_{c2}$  is obtained.

It is of interest to note that when this mechanism applies it is possible to obtain the three constants  $k_2$ ,  $k_3'$  and  $k_4$  (but not to separate  $k_1$ ,  $k_{-1}$  and  $k_2$ ) by studying the effect of N on the Michaelis parameters. For example, by studying the effect of N on  $k_{c2}$  one can obtain  $k_2 k_3' / (k_2 + k_3')$

and  $k_4/k_2k_3'$ . Similarly, the variation of  $K_m$  with  $N$  gives  $K_s$  and  $K_s k_3'/(k_2+k_3')$ . From these four quantities  $k_2$ ,  $k_3'$  and  $k_4$  can be separated.

It should be apparent from the preceding discussion that the effect of added nucleophile on the various kinetic parameters pertaining to the enzyme catalyzed reaction depend in an entirely predictable way on the relative orders of magnitude of these parameters. Conversely, by following the variation of these parameters with concentration of added nucleophile it should therefore be possible to determine their relative orders of magnitude, if not their absolute values. This can probably be done most conveniently with reference to Table 3 or Figure 2. Table 3 classifies, according to the relative orders of magnitude of  $k_2$ ,  $k_3'$ , and  $k_4N$ , the effects of added nucleophile on the Michaelis parameters,  $k_c$  and  $K_m$ . Some of the more interesting situations considered in this table are also shown schematically in Figure 2.

The main results can be understood intuitively as follows. If  $ES'$  formation is rate-determining (Case Ia) the addition of  $N$  can have no effect on  $P_1$  formation; it will increase  $P_3$  formation but necessarily at the expense of  $P_2$  formation. If, however, the subsequent reactions of  $ES'$  are rate determining,  $ES'$  is reformed as fast as it is used up, so that  $P_2$  formation will be independent of  $N$ . Increasing

TABLE 3

The Effects of Added Nucleophile on the Michaelis Parameters according to Mechanism I

Conditions	Product Measured	$k_c$	$K_m$	$k_c/K_m$
$k_2 \ll k_3 \sim k_4 N$	$P_1$	$k_2$	$K_s$	$\frac{k_2}{K_s}$
	$P_2$	$\frac{k_2 k_3'}{k_3' + k_4 N}$	$K_s$	$\frac{k_2 k_3'}{k_3' + k_4 N} \frac{1}{K_s}$
	$P_3$	$\frac{k_2 k_4 N}{k_3' + k_4 N}$	$K_s$	$\frac{k_2 k_4 N}{k_3' + k_4 N} \frac{1}{K_s}$
$k_4 N \sim k_3' \ll k_2$	$P_1$	$k_3' + k_4 N$	$(k_3' + k_4 N) \frac{K_s}{k_2}$	$\frac{k_2}{K_s}$
	$P_2$	$k_3'$	$(k_3' + k_4 N) \frac{K_s}{k_2}$	$\frac{k_2 k_3'}{k_3' + k_4 N} \frac{1}{K_s}$
	$P_3$	$k_4 N$	$(k_3' + k_4 N) \frac{K_s}{k_2}$	$\frac{k_2 k_4 N}{k_3' + k_4 N} \frac{1}{K_s}$
$k_2 \sim k_3' \sim k_4 N$	$P_1$	$\frac{k_2 (k_3' + k_4 N)}{k_2 + k_3' + k_4 N}$	$\frac{(k_3' + k_4 N)}{k_2 + k_3' + k_4 N} K_s$	$\frac{k_2}{K_s}$
	$P_2$	$\frac{k_2 k_3'}{k_2 + k_3' + k_4 N}$	$\frac{(k_3' + k_4 N)}{k_2 + k_3' + k_4 N} K_s$	$\frac{k_2 k_3'}{k_3' + k_4 N} \frac{1}{K_s}$
	$P_3$	$\frac{k_2 k_4 N}{k_2 + k_3' + k_4 N}$	$\frac{(k_3' + k_4 N)}{k_2 + k_3' + k_4 N} K_s$	$\frac{k_2 k_4 N}{k_3' + k_4 N} \frac{1}{K_s}$

Figure 2

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  for various cases of Mechanism I, in which there is a second intermediate which can react with water W and alternative nucleophile N.

# CONDITIONS

PRODUCT MEASURED	$k_2 \ll k_3 \sim k_4 N$	$k_2 \sim k_3 \sim k_4 N$	$k_4 N \sim k_3 \ll k_2$
$P_1$			
$P_2$			
$P_3$			
$P_1, P_2, P_3$			

N will increase  $P_3$  formation and, by regenerating free enzyme, will increase  $P_1$  formation. When  $k_2 \sim k_3 \sim k_4 N$ , the addition of nucleophile will increase the overall rate of reaction until it reaches the limiting value of  $k_2$ , but since dephosphorylation is partially rate-determining in this case, will decrease the rate of formation of  $P_2$ .

### Steady-State Equations for Mechanism II

If the enzyme-catalyzed reaction does not proceed via a phosphoryl-enzyme intermediate, but instead reaction with water and added nucleophile occurs directly through the Michaelis complex, as in Mechanism II, it may readily be shown that the steady-state equations relating to this mechanism again lead to Eq. (10) with

$$k_{c1} = k_2' + k_3 N \quad (45)$$

$$k_{c2} = k_2' \quad (46)$$

$$k_{c3} = k_3 N \quad (47)$$

$$K_m = \frac{k_{-1} + k_2' + k_3 N}{k_1} \quad (48)$$

Eqs. (45)-(48) show that the  $k_{cat}$  values,  $k_{ci}$ ,  $i = 1, 2$ , or  $3$ , are related to  $N$  in the following manner:  $k_{c1}$  is a linear function of  $N$ ,  $k_{c2}$ , is independent of  $N$ , while  $k_{c3}$  is directly proportional to  $N$ . The Michaelis constant,  $K_m$ , should, according to Eq. (48), vary linearly with  $N$ : a plot of  $K_m$  against  $N$  should give a straight line

of slope  $k_3/k_1$  and a Y-intercept equal to  $K_s$ . However, when  $k_3 N \sim k_2' \ll k_{-1}$ , Eq. (48) reduces to

$$K_m = \frac{k_{-1}}{k_1} \quad (49)$$

in which case  $K_m$  should be independent of  $N$ . At the other extreme, when  $k_{-1} \ll k_2' \sim k_3 N$ , the expression for  $K_m$  becomes

$$K_m = \frac{k_2'}{k_1} + \frac{k_3}{k_1} N \quad (50)$$

so that if  $K_m$  is plotted against  $N$ , a straight line of slope  $k_3/k_1$  and a Y-intercept equal to  $k_2'/k_1$  should result.

The above equations show that if Mechanism II applies it is not, in general, possible to determine the relative orders of magnitude of  $k_{-1}$ ,  $k_2'$ , and  $k_3 N$  from the variation of the Michaelis parameters with added nucleophile concentration; it can, however, be decided, by studying the effect of added nucleophile on  $K_m$ , whether or not  $k_{-1} \gg k_2' \sim k_3 N$ : a necessary and sufficient condition for the above inequality to be satisfied, and hence for  $K_m$  to be a true equilibrium constant, is that  $K_m$  should be independent of concentration of added nucleophile.

The effects of added nucleophile on the Michaelis parameters are classified in Table 4 according to the relative orders of magnitude of  $k_{-1}$ ,  $k_2'$  and  $k_3 N$ . This classification is also shown schematically in Figure 3.

It has in the past often been implied that solely by studying the effect of added nucleophile on the Michaelis

TABLE 4

The Effects of Added Nucleophile on the Michaelis Parameters according to Mechanism II

Conditions	Product Measured	$k_c$	$K_m$	$k_c / K_m$
$k_{-1} \ll k_2' \sim k_3 N$	$P_1$	$k_2' + k_3 N$	$\frac{(k_2' + k_3 N)}{k_1}$	$k_1$
	$P_2$	$k_2'$	$\frac{(k_2' + k_3 N)}{k_1}$	$\frac{k_1 k_2'}{k_2' + k_3 N}$
	$P_3$	$k_3 N$	$\frac{(k_2' + k_3 N)}{k_1}$	$\frac{k_1 k_3 N}{k_2' + k_3 N}$
$k_{-1} \sim k_2' \sim k_3 N$	$P_1$	$k_2' + k_3 N$	$K_s + \frac{k_3}{k_1} N$	$\frac{k_1 (k_2' + k_3 N)}{k_{-1} + k_2' + k_3 N}$
	$P_2$	$k_2'$	$K_s + \frac{k_3}{k_1} N$	$\frac{k_1 k_2'}{k_{-1} + k_2' + k_3 N}$
	$P_3$	$k_3 N$	$K_s + \frac{k_3}{k_1} N$	$\frac{k_1 k_3 N}{k_{-1} + k_2' + k_3 N}$
$k_3 N \sim k_2' \ll k_{-1}$	$P_1$	$k_2' + k_3 N$	$\frac{k_{-1}}{k_1}$	$\frac{k_1 (k_2' + k_3 N)}{k_{-1}}$
	$P_2$	$k_2'$	$\frac{k_{-1}}{k_1}$	$\frac{k_1 k_2'}{k_{-1}}$
	$P_3$	$k_3 N$	$\frac{k_{-1}}{k_1}$	$\frac{k_1 k_3 N}{k_{-1}}$

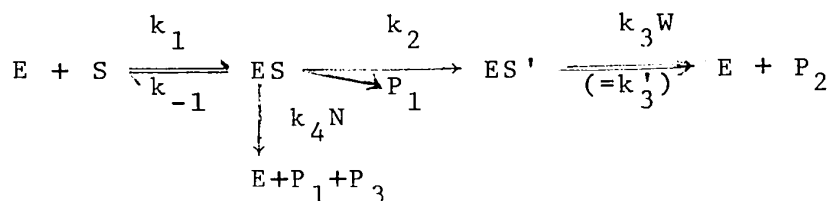
Figure 3

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  for various special cases of Mechanism II, in which there is no second intermediate; the Michaelis complex reacts with W and N.

PRODUCT MEASURED	CONDITIONS		
	$k_{-1} \ll k_2' \sim k_3 N$	$k_{-1} \sim k_2' \sim k_3 N$	$k_3 N \sim k_2' \ll k_{-1}$
$P_1$			
$P_2$			
$P_3$			
$P_1, P_2, P_3$			

parameters it is possible to distinguish between the 2-intermediate scheme - Mechanism I-and Mechanism II, the 1-intermediate scheme. However, by comparing Figures 2 and 3 it can be seen that this experimental procedure cannot always differentiate between the above two mechanisms. Suppose, for example, it is found that both  $k_{c1}$  and  $K_m$  are linear functions of  $N$ , while  $k_{c2}$  is entirely independent of  $N$ , while  $k_{c2}$  is entirely independent of nucleophile concentration. Although consistent with a 2-intermediate scheme in which  $k_4 N \sim k_3 \ll k_2$ , this behavior is also that predicted by Mechanism II, the 1-intermediate scheme, whenever  $k_{-1}$  is not much larger than  $k_2' \sim k_3 N$ . To determine which of these two schemes best describes the enzyme-catalyzed reaction it is therefore usually necessary to apply another criterion, for example whether or not the ratio of the reaction products  $P_2/P_3$  is independent of the nature of the leaving group of the substrate.

Mechanism III is one in which the role of the nucleophile  $N$  is different from that of water, in that  $N$  reacts with  $ES$  and  $W$  with  $ES'$ :



The steady-state solution again leads to an equation of the Michaelis-Menten form (10) with

$$k_{c1} = \frac{k'_3 (k_2 + k_4 N)}{k_2 + k'_3} \quad (51)$$

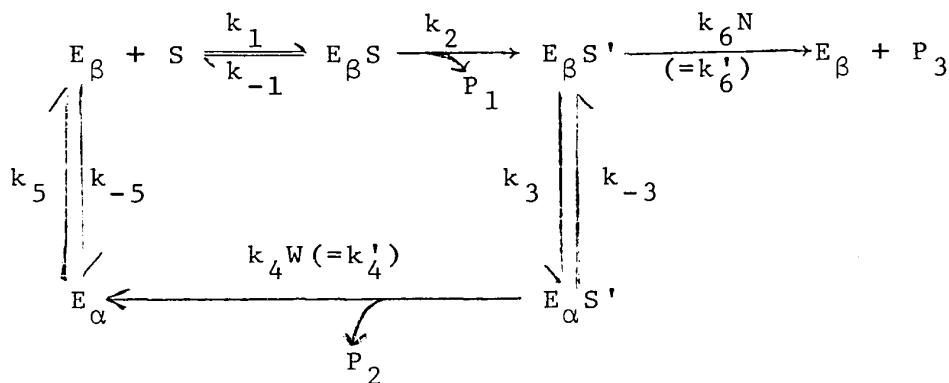
$$k_{c2} = \frac{k_2 k'_3}{k_2 + k'_3} \quad (52)$$

$$k_{c3} = \frac{k'_3 k_4 N}{k_2 + k'_3} \quad (53)$$

$$K_m = \frac{k'_3}{k_2 + k'_3} \frac{(k_{-1} + k_2 + k_4 N)}{k_1} \quad (54)$$

In the event that  $k_{-1} \gg k_2 + k_4 N$  this mechanism leads to the conclusion that  $K_m$  is independent of  $N$ . The mechanism predicts, on this assumption, the results found with alkaline phosphatase (see Results and Discussion) but it cannot explain the fact that  $v_2/v_3$  is independent of the leaving group, since according to this scheme reaction with added nucleophile does not take place via a common intermediate, but, rather, through the Michaelis-Menten complex which still contains the leaving group of the substrate.

Mechanism IV involves two forms of the enzyme,  $E_\alpha$  and  $E_\beta$ , in conformational equilibrium:



This scheme has been proposed by Reid and Wilson (144,243). The first-order conformational change between  $E_{\alpha}$  and  $E_{\beta}$  is regarded as the rate-determining step in the reaction. The substrate S reacts only with  $E_{\beta}$ ; the phosphoryl-enzyme  $E_{\beta}S'$  reacts with N but not with water, while  $E_{\alpha}S'$  reacts only with water. This scheme with  $N=0$  reduces to one proposed by Fernley and Walker (303) to explain the effects of varying pH, ionic strength and temperature on the kinetic parameters for calf-intestinal alkaline phosphatase.

An important feature of Mechanism IV is that it explains lack of specificity of an enzyme (with respect to the leaving group  $P_1$ ) without the assumption that the breakdown of  $ES'$  is rate determining; the  $V_{max}$  values will be the same for widely different leaving groups because the rate controlling step is the conformation change of  $E_{\alpha}$  into  $E_{\beta}$ . Mechanism IV also predicts that the maximum rate of formation of  $P_1$  increases with increasing of N; thus, the added N reacts with  $E_{\beta}S'$  to form  $E_{\beta}$  and so bypasses the slow conversion of  $E_{\alpha}$  into  $E_{\beta}$ . Presumably  $k_2$  is very large;  $E_{\beta}S'$  is therefore formed so rapidly that the supply of it is rapidly replenished, and hence the formation of  $P_2$  is not affected by N.

The steady-state equations leads to

$$k_{c1} = \frac{k_2 \left( 1 + \frac{k_6^N K_{E_{\beta}S'}}{k_4'} \right)}{\frac{k_2}{k_5} + \left( 1 + \frac{k_6^N K_{E_{\beta}S'}}{k_4'} \right) + \frac{k_2}{k_4'} \left( 1 + K_{E_{\beta}S'} \right)} \quad (55)$$

$$k_{c2} = \frac{k_2}{\frac{k_2}{k_5} + \left(1 + \frac{k_6^N K_{E\beta S'}}{k'_4}\right) + \frac{k_2}{k'_4} (1 + K_{E\beta S'})} \quad (56)$$

$$k_{c3} = \frac{k_2 \frac{k_6^N K_{E\beta S'}}{k'_4}}{\frac{k_2}{k_5} + \left(1 + \frac{k_6^N K_{E\beta S'}}{k'_4}\right) + \frac{k_2}{k'_4} (1 + K_{E\beta S'})} \quad (57)$$

$$K_m = \frac{K_s \left(1 + \frac{k_{-5}}{k_5}\right) \left(1 + \frac{k_6^N K_{E\beta S'}}{k}\right)}{\frac{k_2}{k_5} + \left(1 + \frac{k_6^N K_{E\beta S'}}{k'_4}\right) + \frac{k_2}{k'_4} (1 + K_{E\beta S'})} \quad (58)$$

where

$$K_{E\beta S'} = \frac{k'_4 + k_{-3}}{k_3} \quad (59)$$

$$K_s = \frac{k_{-1} + k_2}{k_1} \quad (60)$$

The ratio  $k_{c1}/k_{c2}$  is given by

$$\frac{k_{c1}}{k_{c2}} = 1 + \frac{k_6}{k'_4} K_{E\beta S'}^N \quad (61)$$

For alkaline phosphatase Reid and Wilson have suggested that the following conditions apply:

$$k_{-5} \gg k_5 \quad (62)$$

$$k_2 \gg k_5 \quad (63)$$

$$\frac{k_2}{k_5} \gg \frac{k_2}{k'_4} \left(1 + \frac{k'_4 + k_3}{k_3}\right) \quad (64)$$

$$\frac{k_2}{k_5} \gg 1 + \frac{k_6 K_E \beta S'}{k'_4} \cdot N \quad (65)$$

The equations then reduce to

$$k_{c1} = k_5 \left(1 + \frac{k_6 K_E \beta S'}{k'_4} \cdot N\right) \quad (66)$$

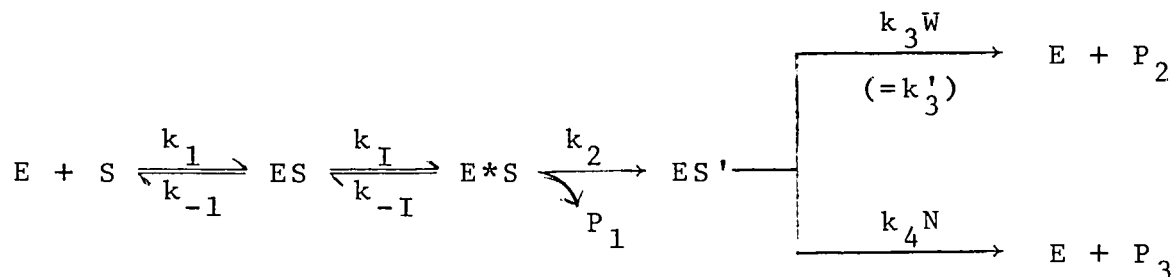
$$k_{c2} = k_5 \quad (67)$$

$$k_{c3} = \frac{k_5 k_6 K_E \beta P}{k'_4} \cdot N \quad (68)$$

$$K_m = \frac{k_{-5}}{k_2} K_s \left(1 + \frac{k_6 K_E \beta S'}{k'_4} \cdot N\right) \quad (69)$$

The variations of the kinetic parameters with N, for this mechanism, are shown in Figure 4.

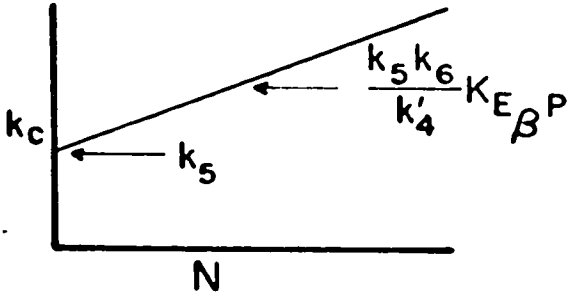
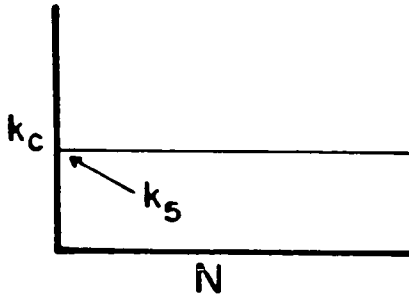
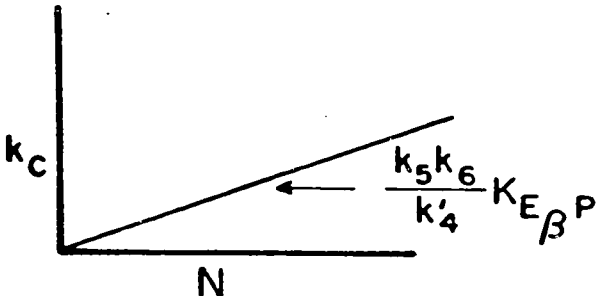
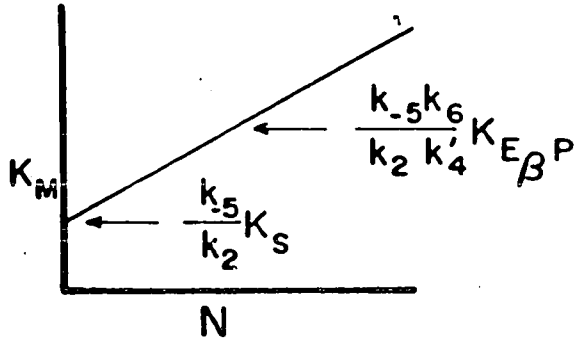
Mechanism V also involves a conformational change:



Such a scheme was proposed by Trentham and Gutfreund (138); it has the particular virtue of accounting for the spectral

Figure 4

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  for Mechanism IV, proposed by Reid and Wilson(144 ); there are two forms of the enzyme,  $E_\alpha$  and  $E_\beta$  in conformational equilibrium.

PRODUCT MEASURED	CONDITIONS
	$k_{-5} \gg k_5; k_2 \gg k_5; \text{ and } k_4 k_{+3}/k_{-3} \gg k_5$
$P_1$	
$P_2$	
$P_3$	
$P_1, P_2, P_3$	

changes observed when the enzyme interacts with certain reversible competitive inhibitors containing suitably located chromophores (135). Application of the steady-state treatment leads to an equation of the Michaelis-Menten form with

$$k_{c1} = \frac{(k_3' + k_4 N)}{1 + \left[ \frac{k_3' + k_4 N}{k_2} \right] + \left[ \frac{k_3' + k_4 N}{k_2} \right] \left[ \frac{k_{-I} + k_2}{k_1} \right]} \quad (70)$$

$$k_{c2} = \frac{k_3'}{1 + \left[ \frac{k_3' + k_4 N}{k_2} \right] + \left[ \frac{k_3' + k_4 N}{k_2} \right] \left[ \frac{k_{-I} + k_2}{k_1} \right]} \quad (71)$$

$$k_{c3} = \frac{k_4 N}{1 + \left[ \frac{k_3' + k_4 N}{k_2} \right] + \left[ \frac{k_3' + k_4 N}{k_2} \right] \left[ \frac{k_{-I} + k_2}{k_1} \right]} \quad (72)$$

$$K_m = \frac{\left[ \left[ \frac{k_{-I} + k_1}{k_1} \right] \left[ \frac{k_{-I} + k_2}{k_I} \right] + \frac{k_I}{k_1} \right] \left[ \frac{k_3' + k_4 N}{k_2} \right]}{1 + \left[ \frac{k_3' + k_4 N}{k_2} \right] + \left[ \frac{k_3' + k_4 N}{k_2} \right] \left[ \frac{k_{-I} + k_2}{k_1} \right]} \quad (73)$$

If  $k_2$  is very much larger than both  $k_3'$  and  $k_4 N$ , and  $(k_{-I} + k_2)/k_1$  is sufficiently small that

$$\left[ \frac{k_{-I} + k_2}{k_I} \right] \left[ \frac{k_3' + k_4 N}{k_2} \right] \ll 1 \quad (74)$$

then Eqs. (70) to (73) reduce to

$$k_{c1} = k_3' + k_4 N \quad (75)$$

$$k_{c2} = k_3' \quad (76)$$

$$k_{c3} = k_4 N \quad (77)$$

$$K_m = \left[ \left( \frac{k_{-1} + k_I}{k_1} \right) \left( \frac{k_{-1} + k_2}{k_1} \right) + \frac{k_I}{k_1} \right] \left( \frac{k_3' + k_4 N}{k_2} \right) \quad (78)$$

Under these conditions, therefore,  $K_m$  and the rates of formation of  $P_1$  and  $P_3$  will be linear in  $N_1$  and the rate of formation of  $P_2$  independent of  $N$ . This conclusion is the same as that for Mechanism I with  $k_2 \gg k_3 \sim k_4 N$ .

If, on the other hand, it is assumed that  $k_2$  is much smaller than both  $k_3'$  and  $k_4 N$  and that the ratio  $(k_{-1} + k_2)/k_1$  is sufficiently large that

$$\left( \frac{k_3' + k_4 N}{k_2} \right) \left( \frac{k_{-1} + k_2}{k_1} \right) \gg 1 \quad (79)$$

it then follows that

$$k_{c1} = \frac{k_2}{1 + \left( \frac{k_{-1} + k_2}{k_I} \right)} \quad (80)$$

$$k_{c2} = \frac{k_3'}{k_3' + k_4 N} \cdot \frac{k_2}{1 + \frac{k_{-1} + k_2}{k_I}} \quad (81)$$

$$k_{c3} = \frac{k_4 N}{k_3' + k_4 N} \cdot \frac{k_2}{1 + \frac{k_{-1} + k_2}{k_I}} \quad (82)$$

$$K_m = \frac{\left( \frac{k_{-6} + k_1}{k_1} \right) \left( \frac{k_{-1} + k_2}{k_1} \right) + \frac{k_I}{k_1}}{1 + \frac{k_{-1} + k_2}{k_I}} \quad (83)$$

$K_m$  and  $k_{c1}$  will then be independent of  $N$ , while the reciprocals of  $k_{c2}$  and  $k_{c3}$  will vary linearly with  $N$ .

Figure 5 summarizes the conclusions for the two special cases considered above. The quantities  $\rho$  and  $c$  are defined by Eqs. (84) and (85):

$$\rho = \left( \frac{k_{-1} + k_I}{k_1} \right) \left( \frac{k_{-1} + k_2}{k_I} \right) + \frac{k_I}{k_1} \quad (84)$$

$$c = \frac{k_2}{1 + \left( \frac{k_{-1} + k_2}{k_I} \right)} \quad (85)$$

Mechanism VI is a modification of the Trentham-Gutfreund mechanism (Mechanism V) in which it is longer assumed that  $ES \rightarrow E^*S$  is rate controlling; there are now two forms of  $ES'$ , namely  $E^*S'$  and  $ES'$ , the interconversion of which is one of the rate-determining steps. This mechanism is

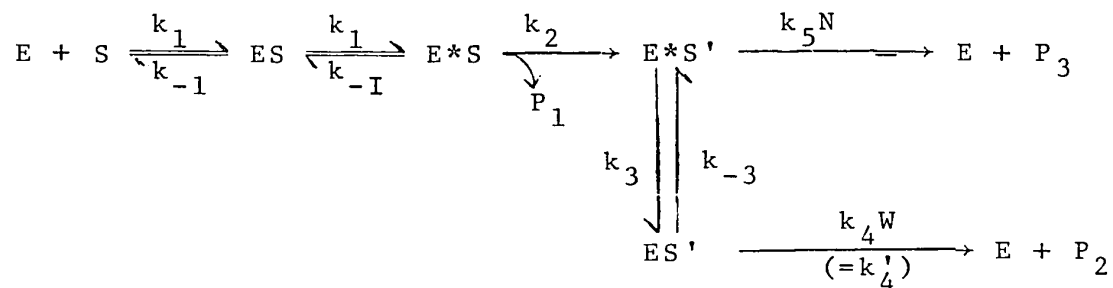
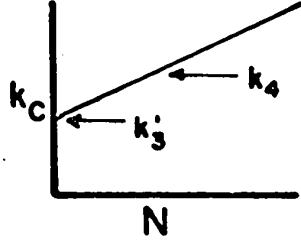
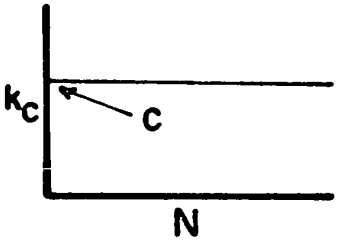
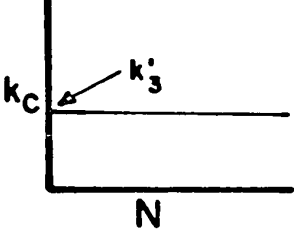
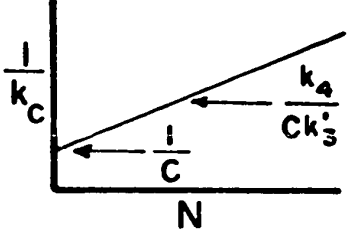
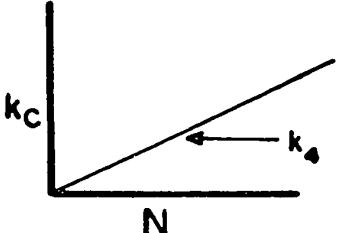
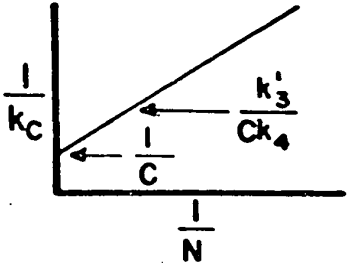
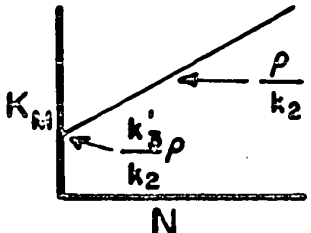
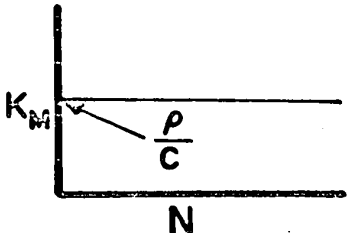


Figure 5

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  for two special cases of Mechanism V, proposed by Trentham and Gutfreund(138); in this mechanism the Michaelis complex undergoes a rate-controlling conformational change before it splits off  $P_1$ .

PRODUCT MEASURED	CONDITIONS	
	$k_2 \gg (k'_3 + k'_4)$ $\& \left( \frac{k_{-1} + k_2}{k_1} \right) \left( \frac{k'_3 + k'_4}{k_2} \right) \ll 1$	$k_2 \ll (k'_3 + k'_4)$ $\& \left( \frac{k_{-1} + k_2}{k_1} \right) \left( \frac{k'_3 + k'_4}{k_2} \right) \gg 1$
$P_1$		
$P_2$		
$P_3$		
$P_1, P_2, P_3$		

It is assumed that E\*S' reacts with N but not with water, while ES' reacts only with water. This scheme can explain the fact that in some systems (e.g., E.coli alkaline phosphatase) the rate is the same irrespective of the leaving group (P<sub>1</sub>) of the substrate, without having to assume that the elimination of this product is fast compared with that of P<sub>2</sub> and P<sub>3</sub>; instead the rate-controlling step may be the conversion of E\*S' into ES'.

This scheme can explain an increase with N of the maximum rate of P<sub>1</sub> production, and an independence of the maximum rate of P<sub>2</sub> production on N. The argument is similar to that for Mechanism IV: added N reacts with E\*S' and bypasses the slow step E\*S' → ES', which must occur for reaction to take place in the absence of N. If E\*S → E\*S' is faster than the subsequent steps, E\*S' will be formed so rapidly that the supply of it will be replenished as it is used up in the reaction with water; the rate of the reaction with water, to form P<sub>2</sub>, will therefore not be affected by added N.

The steady-state equations again lead to a Michaelis-Menten equation with

$$k_{c1} = \frac{k'_4 + K_4 k_5 N}{1 - (K_2 + 1) \frac{k_{-3}}{k_2} + K_4 [1 + K'_3 (1 + K_2)]} \quad (86)$$

$$k_{c2} = \frac{k'_4}{1 - (K_2 + 1) \frac{k_{-3}}{k_2} + K_4 [1 + K'_3 (1 + K_2)]} \quad (87)$$

$$k_{c3} = \frac{K_4 k_5 N}{1 - (K_2 + 1) \frac{k_{-3}}{k_2} + K_4 [1 + K_3' (1 + K_2)]} \quad (88)$$

$$K_m = \frac{\left( K_1 K_2 - \frac{k_{-1}}{k_1} \right) \left( K_3' K_4 - \frac{k_{-3}}{k_2} \right)}{1 - (K_2 + 1) \frac{k_{-3}}{k_2} + K_4 [1 + K_3' (1 + K_2)]} \quad (89)$$

where the constants are

$$K_1 = \frac{k_{-1} + k_I}{k_1} ; \quad K_2 = \frac{k_{-1} + k_2}{k_1} \quad (90)$$

$$K_3' + \frac{k_3 + k_5 N}{k_2} ; \quad K_4 = \frac{k_{-3} + k_4'}{k_3} \quad (91)$$

(Note that  $K_1$ ,  $K_2$  and  $K_4$  are independent of  $N$  and  $K_3'$  linear in  $N$ ).

A set of conditions that leads to the correct dependence of the Michaelis parameters on  $N$  alkaline for phosphatase (see following paper) is

$$K_1 K_2 K_3' K_4 - \frac{k_{-1}}{k_1} K_2 K_3' \ll \frac{k_{-1} k_{-3}}{k_1 k_2} - \frac{k_{-3}}{k_2} K_1 K_2 \quad (92)$$

and

$$K_4 K_3' (1 + K_2) \ll 1 \quad (93)$$

These conditions lead to

$$k_{c1} = k_4' + \left( \frac{k_{-3} + k_4'}{k_3} \right) k_5 N \quad (94)$$

$$k_{c2} = k_4' \quad (95)$$

$$k_{c3} = \left( \frac{k_{-3} + k'_4}{k_3} \right) k_5 N \quad (96)$$

$$K_m = \frac{k_{-1}}{k_1} \frac{k_{-3}}{k_2} \quad (97)$$

The corresponding plots are shown in Figure 6.

The above equations are of the correct form to explain the behavior with alkaline phosphatase, but require that in the absence of N the breakdown of E\*S' (dephosphorylation) is rate-determining. The experimental evidence, however, is against this, so that additional possibilities have to be explored. One of these involves removing the restriction that  $K_4$  is small compared with unity; when this is done the following are the expressions obtained:

$$k_{c1} = \frac{k'_4 + \left( \frac{k_{-3} + k'_4}{k_3} \right) k_5 N}{1 + \left( \frac{k_{-3} + k'_4}{k_3} \right)} \quad (98)$$

$$k_{c2} = \frac{k'_4}{1 + \left( \frac{k_{-3} + k'_4}{k_3} \right)} \quad (99)$$

$$k_{c3} = \frac{\left( \frac{k_{-3} + k'_4}{k_3} \right) k_5 N}{1 + \left( \frac{k_{-3} + k'_4}{k_3} \right)} \quad (100)$$

Figure 6

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  for three special cases of Mechanism VI; this is a modification of the Trentham-Gutfreund mechanism (Mechanism V) in which there are two forms of the intermediate  $ES'$ , one of which reacts with  $W$  and the other with  $N$ . This mechanism appears to apply to the alkaline phosphatase reactions.

		CONDITIONS		
PRODUCT MEASURED		$K_2, K'_3, K \ll 1$ & $(1+K_2)k_3 \ll 1$ $k_2$	$K_2, K'_3 \ll 1$ & $(1+K_2)k_3 \ll 1$ $k_2$	$K_2 \ll 1$ & $K_3 \ll 1$
$P_1$				
$P_2$				
$P_3$				
$P_1, P_2, P_3$				

$$K_m = \frac{\frac{k_{-1}}{k_1} \frac{k_{-3}}{k_2}}{1 + \left( \frac{k_{-3} + k'_4}{k_3} \right)} \quad (101)$$

The plots corresponding to this case, with the slopes and intercepts, are shown in the next-to-right column in Figure 6. In the absence of N equations (88) to (99) reduce to

$$k_c = \frac{k'_4}{1 + \frac{k_{-3} + k'_4}{k_3}} \quad (102)$$

and no longer predict that when N=0 the breakdown of E\*S' is rate determining.

Another situation that gives the correct behavior for alkaline phosphatase arises if it is simply assumed that  $K_2$  and  $K_3$  are much less than unity; the equations are now

$$k_{c1} = \frac{k'_4 + \left( \frac{k_{-3} + k'_4}{k_3} \right) k_5 N}{1 - (1 + K_2) \frac{k_{-3}}{k_2} + \frac{k_{-3} + k'_4}{k_3}} \quad (103)$$

$$k_{c2} = \frac{k'_4}{1 - (1 + K_2) \frac{k_{-3}}{k_2} + \frac{k_{-3} + k'_4}{k_3}} \quad (104)$$

$$k_{c3} = \frac{\left(\frac{k_{-3} + k'_4}{k_3}\right) k_5 N}{1 - (1 + K_2) \frac{k_{-3}}{k_2} + \frac{k_{-3} + k'_4}{k_3}} \quad (105)$$

$$K_m = \frac{\frac{k_{-1}}{k_1} \frac{k_{-3}}{k_2}}{1 - (1 + K_2) \frac{k_{-3}}{k_2} + \frac{k_{-3} + k'_4}{k_3}} \quad (106)$$

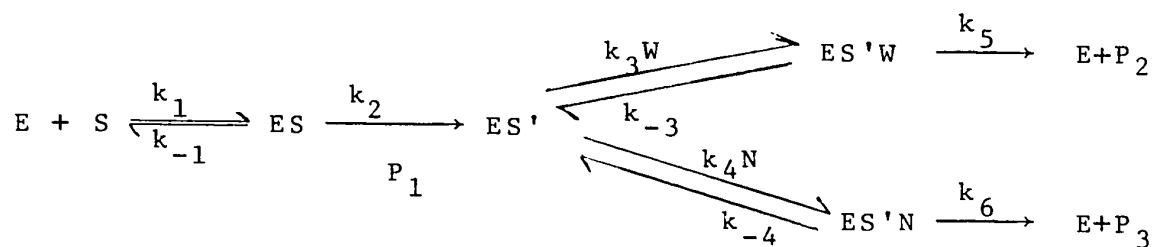
The corresponding plots are shown in the last column of Figure 6; the quantity P is

$$P = 1 - (K_2 + 1) \frac{k_{-3}}{k_2} + \frac{k_{-3} + k'_4}{k_3} \quad (107)$$

Inherent in all of the mechanisms considered so far is the assumption that the enzyme does not have a specific site for either water or the added nucleophile. However, it has sometimes been suggested (see Appendix I) that such sites do in fact exist and that the steps of the reaction involving these sites may be kinetically important. The mechanisms now to be considered, therefore, involve such specific sites.

Mechanisms Involving Specific Binding Sites for Water and the Added Nucleophile

Mechanism VII is one in which there is a single site for both W and N:



The steady-state treatment leads to an equation of the Michaelis-Menten form with

$$k_{c1} = \frac{\frac{k_5 k_3 W}{k_5 + k_{-3}} + \frac{k_6 k_4 N}{k_6 + k_{-4}}}{1 + \frac{k_3 W}{k_5 + k_{-3}} + \frac{k_4 N}{k_6 + k_{-4}} + \frac{1}{k_2} \left( \frac{k_5 k_3 W}{k_5 + k_{-3}} + \frac{k_6 k_4 N}{k_6 + k_{-4}} \right)} \quad (108)$$

$$k_{c2} = \frac{\frac{k_5 k_3 W}{k_5 + k_{-3}}}{1 + \frac{k_3 W}{k_5 + k_{-3}} + \frac{k_4 N}{k_6 + k_{-4}} + \frac{1}{k_2} \left( \frac{k_5 k_3 W}{k_5 + k_{-3}} + \frac{k_6 k_4 N}{k_6 + k_{-4}} \right)} \quad (109)$$

$$k_{c3} = \frac{\frac{k_6 k_4 N}{k_6 + k_{-4}}}{1 + \frac{k_3 W}{k_5 + k_{-3}} + \frac{k_4 N}{k_6 + k_{-4}} + \frac{1}{k_2} \left( \frac{k_5 k_3 W}{k_5 + k_{-3}} + \frac{k_6 k_4 N}{k_6 + k_{-4}} \right)} \quad (110)$$

$$K_m = \frac{\frac{K_5}{k_2} \left( \frac{k_5 k_3 W}{k_5 + k_{-3}} + \frac{k_6 k_4 N}{k_6 + k_{-4}} \right)}{1 + \frac{k_3 W}{k_5 + k_{-3}} + \frac{k_4 N}{k_6 + k_{-4}} + \frac{1}{k_2} \left( \frac{k_5 k_3 W}{k_5 + k_{-3}} + \frac{k_6 k_4 N}{k_6 + k_{-4}} \right)} \quad (111)$$

A special case of this is when  $k_{-3} \ll k_5$  and  $k_{-4} \ll k_6$  - i.e., when the rates of transformation of  $ES'W$

and ES'N to  $P_2$  and  $P_3$  are such that negligible amounts of ES'W and ES'N are present. The equations are of the same form as those obtained for Mechanism I, in which no W and N sites have been postulated.

A consideration of Eq. (111) shows that  $K_m$  can only be independent of N in the following two cases:

Case VIIa when

$$\frac{1}{k_2} \left( \frac{k_3 k_5 W}{k_5 + k_{-3}} + \frac{k_4 k_6 N}{k_6 + k_{-4}} \right) \gg 1 + \frac{k_3 W}{k_5 + k_{-3}} + \frac{k_4 W}{k_6 + k_{-4}} \quad (112)$$

the behavior is as represented in the first column of Figure 7, where

$$\gamma = \frac{k_3 W}{k_5 + k_{-3}} \quad (113)$$

and

$$\delta = \frac{k_4}{k_6 + k_{-4}} \quad (114)$$

It will be seen that  $k_{c1}$  is independent of N, but that  $k_{c2}$  and  $k_{c3}$  vary with N.

Case VIIb If, instead, it is assumed that

$$\frac{k_3 k_5 W}{k_5 + k_{-3}} \gg \frac{k_4 k_6 N}{k_6 + k_{-4}} \quad (115)$$

and also

Figure 7

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  for two special cases of Mechanism VII (Cases VIIa and VIIc). Mechanism VII is the simple two-intermediate mechanism in which there are binding sites for W and N.

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		CONDITIONS	
PRODUCT MEASURED		$\frac{1}{k_2}(k_5\gamma + k_6\delta N) \gg 1 + \gamma + \delta N$	$\frac{1}{k_2}(k_5\gamma + k_6\delta N) + \delta N \ll 1 + \gamma$
$P_1$			
$P_2$			
$P_3$			
$P_1, P_2, P_3$			

$$1 + \frac{k_3^W}{k_5 + k_{-3}} \gg \frac{k_4^N}{k_6 + k_{-4}} + \frac{1}{k_2} \left( \frac{k_3 k_5^W}{k_5 + k_{-3}} \right) \quad (116)$$

the behavior is as shown in the first column of Figure 8, where  $\gamma$  and  $\delta$  are as defined above. Now  $k_{c1}$  and  $k_{c2}$  are independent of  $N$ , and  $k_{c3}$  dependent on  $N$ .

Cases in which  $K_m$  is dependent on  $N$  are the two following:

Case VIIc This case is shown in the last column of Figure 7.

Case VIId This case is shown in the last column of Figure 8.

Case VIIe Another special case of interest is when  $ES'$  is in true equilibrium with both  $ES'W$  and  $ES'N$ , i.e.,  $k_5 \ll k_{-3}$  are  $k_6 \ll k_{-4}$ . The equations are now

$$k_{c1} = \frac{k_2 \left( k_5 + k_6 \frac{K_W}{W} \frac{N}{K_N} \right)}{k_2 \left( 1 + \frac{K_W}{W} + \frac{K_W}{W} \frac{N}{K_N} \right) + k_5 + k_6 \frac{K_W}{W} \frac{N}{K_N}} \quad (117)$$

$$k_{c2} = \frac{k_2 k_5}{k_2 \left( 1 + \frac{K_W}{W} + \frac{K_W}{W} \frac{N}{K_N} \right) + k_5 + k_6 \frac{K_W}{W} \frac{N}{K_N}} \quad (118)$$

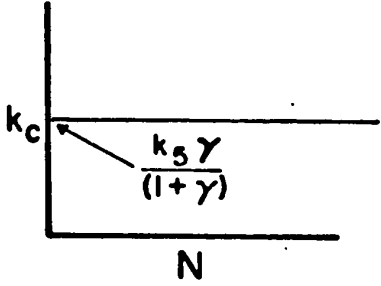
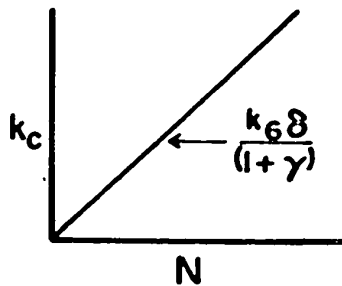
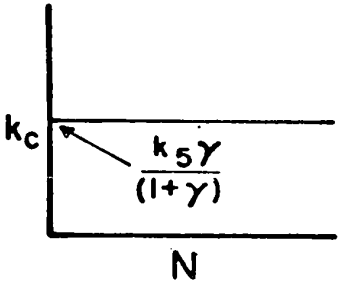
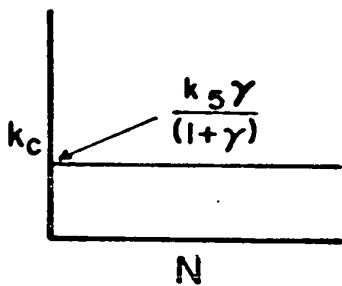
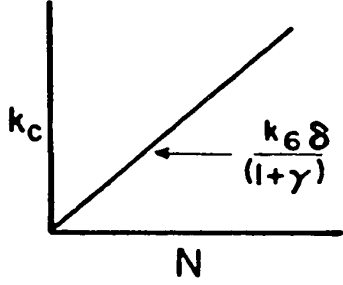
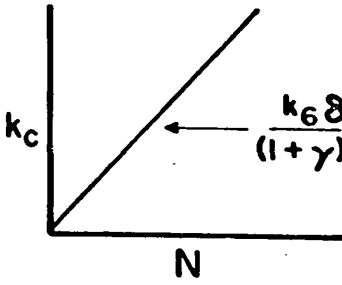
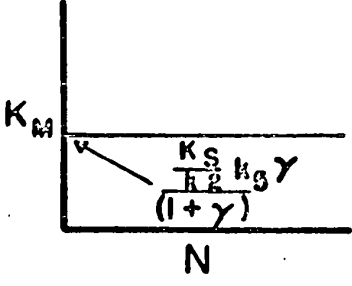
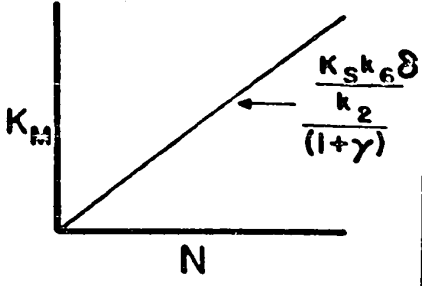
$$k_{c3} = \frac{k_2 k_5 \frac{K_W}{W} \frac{N}{K_N}}{k_2 \left( 1 + \frac{K_W}{W} + \frac{K_W}{W} \frac{N}{K_N} \right) + k_5 + k_6 \frac{K_W}{W} \frac{N}{K_N}} \quad (119)$$

Figure 8

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  for two other special cases of Mechanism VII (Cases VIIb and VIIId).

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PRODUCT MEASURED	CONDITIONS	
	$k_5 \gamma \gg k_6 \delta N$ $\delta(1+\gamma) \gg \delta N + \left(\frac{k_5}{k_2}\right)\gamma$	$k_6 \delta N \gg k_5 \gamma$ $\delta \left(1 + \frac{k_6}{k_2}\right) \delta N \ll 1 + \gamma$
$P_1$		
$P_2$		
$P_3$		
$P_1, P_2, P_3$		

$$K_m = \frac{K_s \left( k_5 + k_6 \frac{K_W}{W} \frac{N}{K_N} \right)}{k_2 \left( 1 + \frac{K_W}{W} + \frac{K_W}{W} \frac{N}{K_N} \right) + k_5 + k_6 \frac{K_W}{W} \frac{N}{K_N}} \quad (120)$$

where

$$K_W = \frac{ES' \cdot W}{ES'W} = \frac{k_{-3}}{k_3} \quad (121)$$

and

$$K_N = \frac{ES' \cdot N}{ES'N} = \frac{k_{-4}}{k_4} \quad (122)$$

Equations (117) to (120) are equivalent to equations obtained previously by Seydoux and Yon (178) for this special case.

Figures 7 and 8 apply to this case also, the quantities  $\gamma$  and  $\delta$  now reducing to

$$\gamma = \frac{W}{K_W} \quad (123)$$

and

$$\delta = \frac{1}{K_N} \quad (124)$$

As Seydoux and Yon have pointed out, when both  $K_W/W$  and  $K_N N/WK_N$  are much less than unity the equations reduce to

$$k_{c1} = \frac{k_2 (k_5 + k_6' N)}{k_2 + k_5 + k_6' N} \quad (125)$$

$$k_{c2} = \frac{k_2 k_5}{k_2 + k_5 + k_6' N} \quad (126)$$

$$k_{c3} = \frac{k_2 k_6' N}{k_2 + k_5 + k_6' N} \quad (127)$$

and

$$K_m = K_s \left( \frac{k_5 + k'_6 N}{k_2 + k_5 + k'_6 N} \right) \quad (128)$$

where

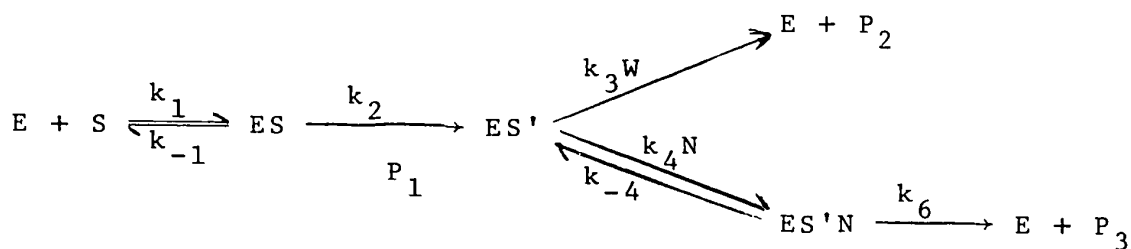
$$k'_6 = k_6 \frac{K_W}{W} \frac{1}{K_N} \quad (129)$$

This last equation shows that  $k'_6$  is inversely proportional to  $W$ , which in practice usually does not vary greatly as  $N$  is varied.

The equations for Mechanism VII show that, in contrast to the earlier schemes in which no specific nucleophile binding site was considered, the mechanism predicts that the apparent reactivities of nucleophiles will not necessarily be the same as their intrinsic reactivities; their affinity for the binding site is also an important factor.

It is to be noted that Eqs. (108) to (111) are of the same form as those obtained for Mechanism I. Figure 2 therefore illustrates the behavior predicted by this special case.

Case VIIf is when the existence of a specific water binding site is kinetically irrelevant; i.e., when  $k_5$  is much greater than both  $k_3 W$  and  $k_{-3}$ . This case is equivalent to the mechanism



Eqs. (108) to (111) now reduce to

$$k_{c1} = \frac{k_3 W + k_6 \delta N}{1 + \delta N + \frac{1}{k_2} (k_3 W + k_6 \delta N)} \quad (130)$$

$$k_{c2} = \frac{k_3 W}{1 + \delta N + \frac{1}{k_2} (k_3 W + k_6 \delta N)} \quad (131)$$

$$k_{c3} = \frac{k_6 \delta N}{1 + \delta N + \frac{1}{k_2} (k_3 W + k_6 \delta N)} \quad (132)$$

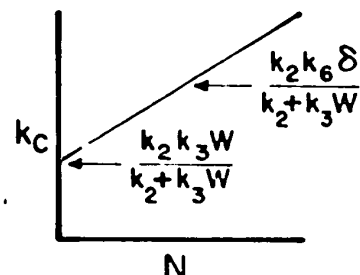
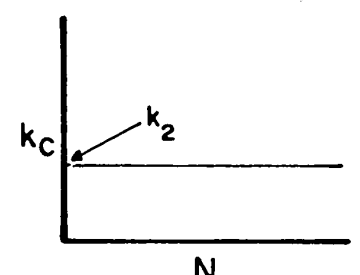
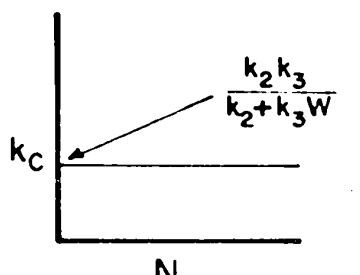
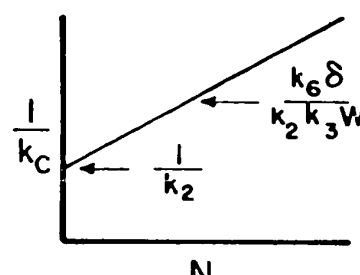
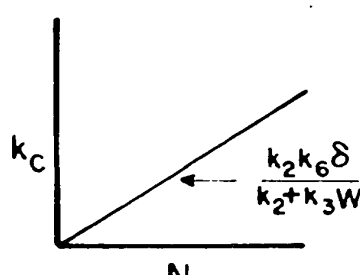
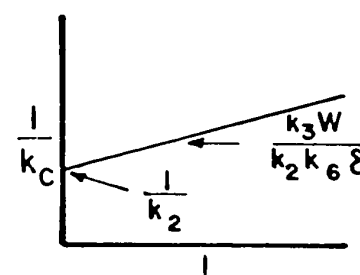
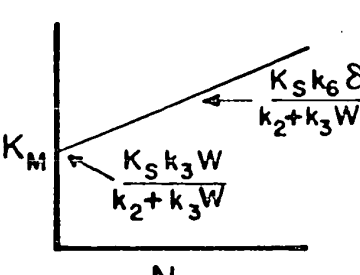
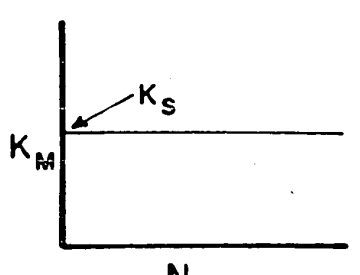
$$K_m = \frac{K_s \frac{k_6}{k_2} \delta N}{1 + \delta N + \frac{1}{k_2} (k_3 W + k_6 \delta N)} \quad (133)$$

where  $\delta$  is defined by Eq. (114). Some special cases are shown in Figures 9 and 10.

Case VIIg Alternatively,  $k_6$  may be very large compared with  $k_4 N$  and  $k_{-4}$ ; i.e.,  $ES'N$  passes into  $E + P_3$  as fast as it is formed. This case is kinetically equivalent to a mechanism

Figure 9

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  for two other special cases of Mechanism VII in which the existence of the specific water binding site is kinetically irrelevant (VIIIf).

PRODUCT MEASURED	CONDITIONS	
	$(1 + \frac{k_6}{k_2}) \delta N \ll 1$	$\frac{1}{k_2} (k_3 W + k_6 \delta N) \gg 1 + \delta N$
$P_1$		
$P_2$		
$P_3$		
$P_1, P_2, P_3$		

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Figure 10

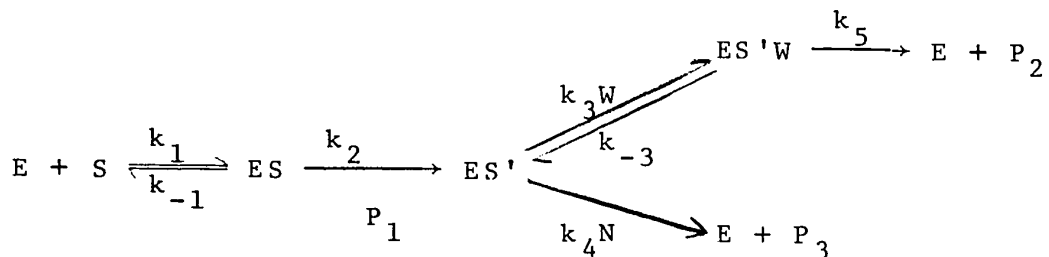
Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  for two other subcases of VIIf.

PRODUCT MEASURED	CONDITIONS	
	$k_3 W \gg k_6 \delta N$ & $\delta N \ll \frac{k_3 W}{k_2}$	$k_6 \delta N \gg k_3 W$ & $(1 + \frac{k_6}{k_2}) \delta N \gg 1$
$P_1$		
$P_2$		
$P_3$		
$P_1, P_2, P_3$		

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in which there is no specific binding site for the alternative nucleophile N:



The steady-state treatment now gives

$$k_{c1} = \frac{k_5 \gamma + k_4 N}{1 + \gamma + \frac{1}{k_2} (k_5 \gamma + k_4 N)} \quad (134)$$

$$k_{c2} = \frac{k_5 \gamma}{1 + \gamma + \frac{1}{k_2} (k_5 \gamma + k_4 N)} \quad (135)$$

$$k_{c3} = \frac{k_4 N}{1 + \gamma + \frac{1}{k_2} (k_5 \gamma + k_4 N)} \quad (136)$$

$$K_m = \frac{K_s \frac{k_5}{k_2} \gamma}{1 + \gamma + \frac{1}{k_2} (k_5 \gamma + k_4 N)} \quad (137)$$

where  $\gamma$  is defined by Eq. (112). The patterns of behavior given by four special subcases of this are shown in Figures 11 and 12.

Figure 11

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  for two subcases of VIIg, in which the specific binding site for N is kinetically irrelevant.

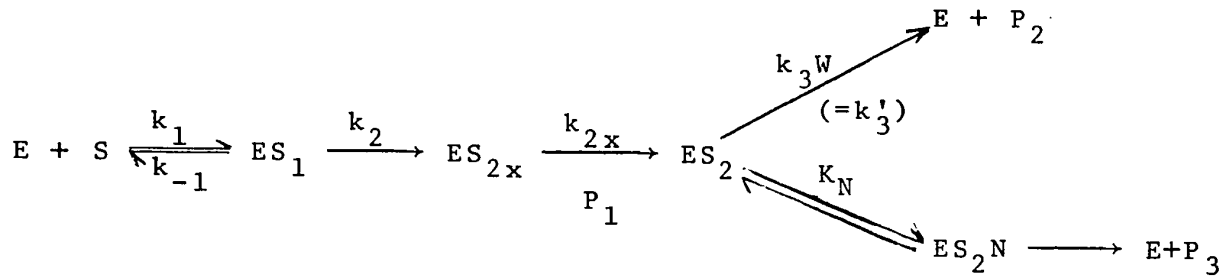
PRODUCT MEASURED	CONDITIONS	
	$\frac{1}{k_2} (k_5\gamma + k_4N) \gg 1 + \gamma$	$k_4N \ll k_5\gamma$
$P_1$		
$P_2$		
$P_3$		
$P_1, P_2, P_3$		

Figure 12

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  for two other subcases of VIIg.

PRODUCT MEASURED	CONDITIONS	
	$\frac{k_4 N}{k_2} \ll 1 + \left(1 + \frac{k_5}{k_2}\right) \gamma$	$\frac{1}{k_2} (k_5 \gamma + k_4 N) \ll 1$
$P_1$		
$P_2$		
$P_3$		
$P_1, P_2, P_3$		

Mechanism VIII involves one additional kinetically important step - the release of the alcohol portion of the substrate from the surface of the enzyme molecule:



where  $ES_1$  is the non-covalent enzyme-substrate complex,  $ES_{2x}$  is the covalent acyl- or phosphoryl-enzyme intermediate with the leaving group of the original substrate still bound to it, and  $ES_2$  is the same intermediate without the leaving group of the substrate. This scheme was proposed by Henry and Kirsch (189) to account for the effects of methanol and ethanol on the kinetic parameters of the papain-catalyzed hydrolysis of the ethyl and p-nitrophenyl esters of hippuric acid. Application of the steady-state treatment leads to an equation of the Michaelis-Menten form with

$$k_{c1} = \frac{k_2 k_{2x} \left( k_3^W + \frac{k_4}{K_N} N \right)}{k_2 k_{2x} + (k_{2x} + k_2) \left( k_3^W + \frac{k_4}{K_N} N \right)} \quad (138)$$

$$k_{c2} = \frac{k_2 k_{2x} k_3^W}{k_2 k_{2x} + (k_{2x} + k_2) \left( k_3^W + \frac{k_4}{K_N} N \right)} \quad (139)$$

$$k_{c3} = \frac{k_2 k_{2x} \frac{k_4}{K_N} N}{k_2 k_{2x} + (k_{2x} + k_2) \left( k_3 W + \frac{k_4}{K_N} N \right)} \quad (140)$$

$$K_m = \frac{k_2 k_{2x} \left( k_3 W + \frac{k_4}{K_N} N \right)}{k_2 k_{2x} + (k_{2x} + k_2) \left( k_3 W + \frac{k_4}{K_N} N \right)} \quad (141)$$

An inspection of Eq. (141) shows that  $K_m$  will be independent of  $N$  only for the following two cases:

Case VIIIa When  $k_4 N / K_N \ll k_3 W$  Eqs. (138) - (141) reduce to

$$k_{c1} = \frac{k_2 k_{2x} k_3 W}{k_2 k_{2x} + (k_{2x} + k_2) k_3 W} \quad (142)$$

$$k_{c2} = \frac{k_2 k_{2x} k_3 W}{k_2 k_{2x} + (k_{2x} + k_2) k_3 W} \quad (143)$$

$$k_{c3} = \frac{k_2 k_{2x} \frac{k_4}{K_N} N}{k_2 k_{2x} + (k_{2x} + k_2) k_3 W} \quad (144)$$

and

$$K_m = \frac{k_{2x} k_3 W K_s}{k_2 k_{2x} + (k_{2x} + k_2) k_3 W} \quad (145)$$

i.e.,  $k_{c1}$ ,  $k_{c2}$ , and  $K_m$  will all be entirely independent of  $N$ , while  $k_{c3}$  will be directly proportional to  $N$ .

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Case VIIIb When

$$k_2 k_{2x} \ll (k_{2x} + k_2) \left( k_3 W + \frac{k_4 N}{K_N} \right)$$

Eqs. (132) - (141) simplify to

$$k_{c1} = \frac{k_2 k_{2x}}{k_2 + k_{2x}} \quad (146)$$

$$k_{c2} = \frac{k_2 k_{2x} k_3 W}{(k_2 + k_{2x}) \left( k_3 W + \frac{k_4 N}{K_N} \right)} \quad (147)$$

$$k_{c3} = \frac{k_2 k_{2x} \frac{k_4 N}{K_N}}{(k_2 + k_{2x}) \left( k_3 W + \frac{k_4 N}{K_N} \right)} \quad (148)$$

and

$$K_m = \frac{k_{2x} K_s}{k_2 k_{2x}} \quad (149)$$

i.e., both  $k_{c1}$  and  $K_m$  are now independent of  $N$ ;  $k_{c2}$  and  $k_{c3}$ , however, vary with  $N$ : The reciprocals of  $k_{c2}$  and  $k_{c3}$  are linear functions of  $N$ , and  $1/N$ , respectively.

Case VIIIc If, instead, it is assumed that

$$k_2 k_{2x} \gg (k_2 + k_{2x}) \left( k_3 W + \frac{k_4 N}{K_N} \right)$$

the kinetic parameters reduce to

$$k_{c1} = k_3 W + \frac{k_4}{K_N} N \quad (150)$$

$$k_{c2} = k_3 W \quad (151)$$

$$k_{c3} = \frac{k_4}{K_N} N \quad (152)$$

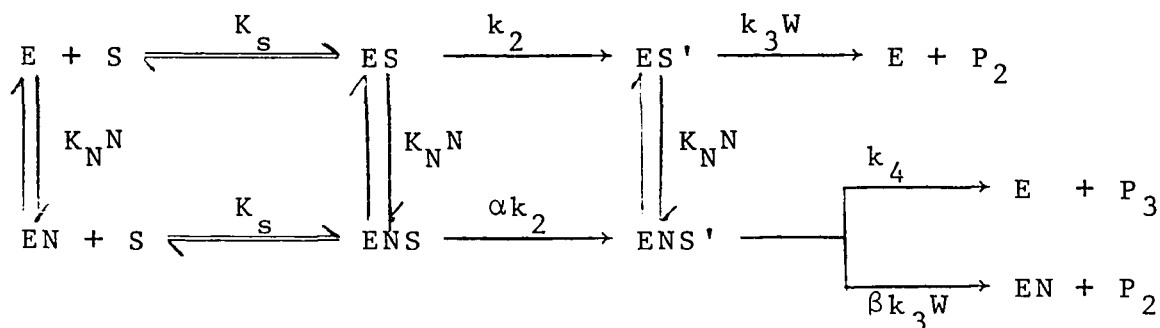
and

$$K_m = \frac{K_s}{k_2} \left( k_3 W + \frac{k_4}{K_N} N \right) \quad (153)$$

Eqs. (138)-(141) predict that  $k_{c2}$  will be independent of  $N$ ,  $k_{c3}$  will be directly proportional to  $N$ , while both  $k_{c1}$  and  $K_m$  will be linear functions of  $N$ .

The variations of the kinetic parameters with  $N$  predicted by the above three cases of Mechanism VIII are shown schematically in Figure 13.

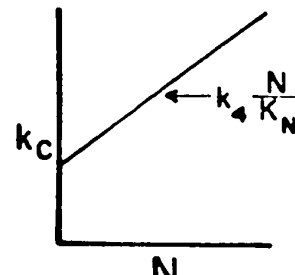
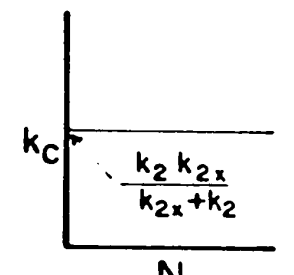
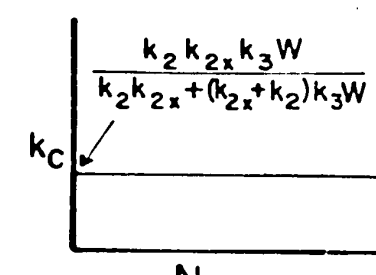
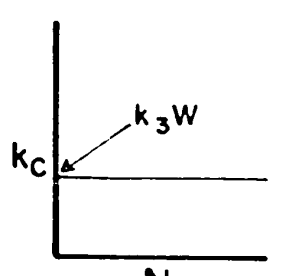
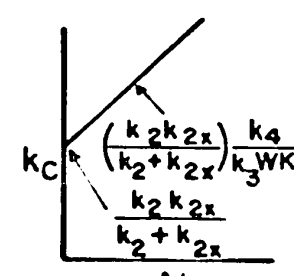
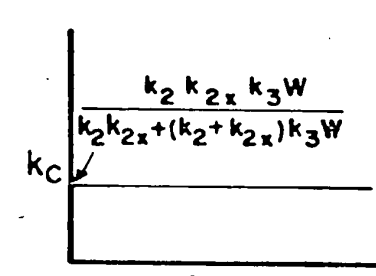
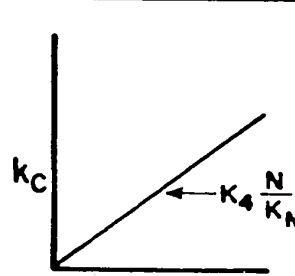
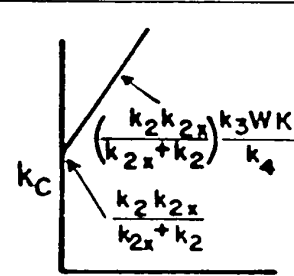
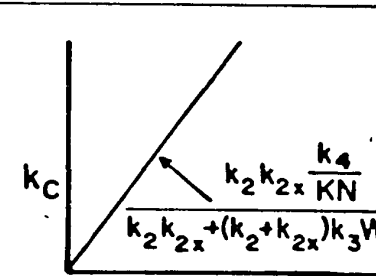
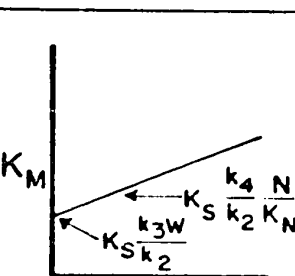
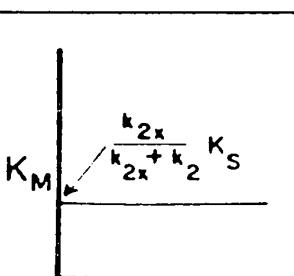
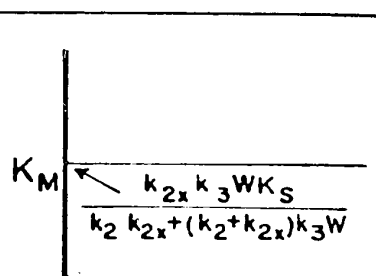
Mechanism IX is one in which the added nucleophile binds to the free enzyme, enzyme-substrate complex, as well as to the acyl-enzyme:



where  $K_s$  and  $K_N$  are the dissociation constants for substrate and nucleophile from the enzyme complexes;  $\alpha$  is a factor to account for any changes in the rate of acylation

Figure 13

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  against  $N$  for  
three special cases of Mechanism VIII.

		CONDITIONS		
PRODUCT MEASURED				
	$k_2 k_{2x} \gg$ $(k_{2x} + k_2) \left( k_3 W + \frac{k_4 N}{K_N} \right)$	$k_2 k_{2x} \ll$ $(k_{2x} + k_2) \left( k_3 W + \frac{k_4 N}{K_N} \right)$	$\frac{k_4}{K_N} \ll k_3 W$	
$P_1$				
$P_2$				
$P_3$				
$P_1, P_2, P_3$				

due to the added nucleophile; and  $\beta$  is a factor to account for any attack by water on the acyl-enzyme when the nucleophile is bound to the enzyme. This mechanism was originally proposed by Fink and Bender (244) to account for the effects of added alcohols and amines of widely varying structure on the reactions of two specific substrates - the p-nitrophenyl esters of N-acetyl-L-tryptophan and N-CBz-L-lysine- with papain.

The steady-state treatment again leads to an equation of the Michaelis-Menten form with

$$k_{c1} = \frac{k_2 \left(1 + \frac{\alpha N}{K_N}\right) \left(k_3 W + \frac{N}{K_N} [k_4 + \beta k_3 W]\right)}{\left(1 + \frac{N}{K_N}\right) \left[k_3 W + \frac{N}{K_N} (k_4 + \beta k_3 W)\right] + k_2 \left(1 + \frac{\alpha N}{K_N}\right)} \quad (154)$$

$$k_{c2} = \frac{\left(\beta + \frac{K_N}{N}\right) k_2 k_3 W \left(1 + \frac{\alpha N}{K_N}\right)}{\frac{K_N}{N} \left(1 + \frac{N}{K_N}\right) \left[k_3 W + \frac{N}{K_N} (k_4 + \beta k_3 W)\right] + k_2 \left(1 + \frac{\alpha N}{K_N}\right)} \quad (155)$$

$$k_{c3} = \frac{k_2 k_4 \left(1 + \frac{\alpha N}{K_N}\right)}{\frac{K_N}{N} \left(1 + \frac{N}{K_N}\right) \left[k_3 W + \frac{N}{K_N} (k_4 + \beta k_3 W)\right] + k_2 \left(1 + \frac{\alpha N}{K_N}\right)} \quad (156)$$

and

$$K_m = \frac{K_s \left(k_3 W + \frac{N}{K_N} [k_4 + \beta k_3 W]\right)}{\left(k_3 W + \frac{N}{K_N} [k_4 + \beta k_3 W]\right) + k_2 \left(1 + \frac{\alpha N}{K_N}\right)} \quad (157)$$

The ratio of rate constants  $k_{c2}/k_{c3}$  is

$$\frac{k_{c2}}{k_{c3}} = \beta \frac{k_3 W}{k_4} + \frac{k_3 W}{k_4} \frac{K_N}{N} \quad (158)$$

Although the above expressions for  $k_c$  and  $K_m$  have been simplified by the omission of a specific binding site for water, the existence of such a site could readily be taken into account, if necessary, by replacing  $W$  by  $W/K_W$ , where  $K_W$  is the dissociation constant for water from the acyl-enzyme-water complex.

Eqs. (154) - (157) show that the Michaelis parameters will, in general, be complex functions of  $N$ . A relatively simple variation of  $k_c$  and  $K_m$  with  $N$  will, however, be observed for the following two cases:

Case IXa When  $k_2 \gg k_3 W + \frac{k_4}{K_N} N$  and at the same time it is also true that  $\frac{N}{K_N} \ll 1$ ,  $\alpha \frac{N}{K_N} \ll 1$ , and  $\beta \gg \frac{K_N}{N}$ , then the expressions for the Michaelis parameters simplify to

$$k_{c1} = k_3 W + \frac{k_4}{K_N} N \quad (159)$$

$$k_{c2} = k_3 W \quad (160)$$

$$k_{c3} = \frac{k_4}{K_N} N \quad (161)$$

and

$$K_m = \frac{K_S}{k_2} \left( k_3 W + \frac{k_4}{K_N} N \right) \quad (162)$$

i.e.,  $k_{c1}$ ,  $k_{c3}$ , and  $K_m$  will vary linearly with  $N$ , while  $k_{c2}$  will be independent of  $N$ .

Case IXb When  $k_3W \gg k_2 \frac{k_4}{K_N} N$ ;  $N/K_N \ll 1$ ;  $\alpha N/K_N \ll 1$ ;  
and  $\beta \ll K_N/N$ , the Michaelis parameters reduce to

$$k_{c1} = k_2 \quad (163)$$

$$k_{c2} = k_2 \quad (164)$$

$$k_{c3} = \frac{k_2 k_4}{k_3 W} \frac{1}{K_N} N \quad (165)$$

and

$$K_m = K_s \quad (166)$$

i.e.,  $k_{c1}$ ,  $k_{c2}$ , and  $K_m$  are all independent of  $N$ , while  $k_{c3}$  is directly proportional to  $N$ .

The kinetic behavior in the presence of  $N$  predicted by the two special cases of Mechanism IX considered above is shown schematically in Figure 14. Although numerous other subcases of this mechanism are possible, none of the predict that  $k_{c2}$  and  $K_m$  will be independent of  $N$ , while  $k_{c1}$  and  $k_{c3}$  will vary linearly with  $N$ .

Figure 14

Plots of  $k_{c1}$ ,  $k_{c2}$ ,  $k_{c3}$  and  $K_m$  against  $N$  for  
two special cases of Mechanism IX.

PRODUCT MEASURED	CONDITIONS	
	$k_2 \gg k_3W + \frac{k_4}{K_N} N$ $\frac{N}{K_N} \ll 1; \alpha \frac{N}{K_N} \ll 1$ $\beta \gg \frac{K_N}{N}$	$k_3W \gg k_2 + \frac{k_4}{K_N} N$ $\frac{N}{K_N} \ll 1, \alpha \frac{N}{K_N} \ll 1$ $\beta \ll \frac{K_N}{N}; k_3W \gg k_2$
$P_1$		
$P_2$		
$P_3$		
$P_1, P_2, P_3$		

## EXPERIMENTAL

The purpose of these experiments is twofold:

(1) to determine whether or not a phosphoryl-enzyme intermediate occurs during the enzyme catalyzed reaction and (2) to evaluate the relative orders of magnitude of some, if not all, of the individual rate constants pertaining to the reaction. As pointed out in the theoretical section this may be accomplished by studying the kinetic behavior of the enzyme catalyzed reaction in the presence of an appropriate added nucleophile, in particular by (1) measuring the ratio of transferase to hydrolytic activity for a series of substrates with different leaving groups and (2) studying the variation of the Michaelis parameters with concentration of added nucleophile.

### Choice of Experimental Conditions

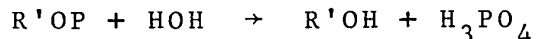
The success or failure of the above outlined experimental approach revolves to a large extent about the proper choice of competitive nucleophile. One important reason for this is that in the preceding theoretical treatment the effect of added nucleophile on the rate of the enzyme-catalyzed reaction was assumed to be due entirely to competition between added nucleophile and water for the phosphoryl-enzyme intermediate (or the Michaelis-Menten complex). However, it is well-known that the addition of an

organic solvent to the reaction mixture may also give rise to various specific and non-specific solvent effects that may considerably alter the rate of the reaction (245-255). Specifically, apart from functioning as a competitive nucleophile, the added organic solvent may (1) change the dielectric constant of the reaction mixture (2) lower the concentration of water, one of the reactants (3) competitively inhibit the enzyme (4) irreversibly or reversibly denature the enzyme (5) alter the overall tertiary structure of the enzyme or at least the conformation about its active site (6) cause aggregation of substrate or enzyme (7) shift the pK values of the buffers and substrates in the reaction mixture (8) shift the pK values of the essential ionizable groups of the enzyme, thereby causing a shift in the activity - pH profile. Since the magnitude of these solvent effects in general increases with increasing concentration of added organic solvent, they may usually be avoided by working at sufficiently low solvent concentration. This of course is feasible only if the added nucleophile is much more reactive than water: 1M or less of added nucleophile must be able to react with phosphoryl-enzyme as rapidly as the 55M H<sub>2</sub>O also present in the reaction mixture. The most reactive nucleophile, however, is by no means always the most appropriate choice. Hydroxylamine, for example, because it is a highly reactive analog of water, has often been used as a competitive nucleophile in kinetic studies of enzyme-catalyzed reactions

(183,256-261). Unfortunately, hydroxylamine often participates in various side reactions, thereby giving rise to results which are difficult to interpret since they appear to be inconsistent with both Mechanisms I and II and (183,256-262).

Another objection to the use of hydroxylamine comes from non-enzymic studies which indicate that this particular substance is a very special nucleophile, in that it reacts as a bifunctional catalyst in the hydroxaminolysis of esters at pH 7.0 (262).

An important factor which must be taken into consideration in choosing an appropriate nucleophile for competition experiments is the nature of the transphosphorylation product which will be formed. If the method is to be successful, the transphosphorylation product R'OP, should be a much poorer substrate than ROP, the starting substrate. If R'OP were itself a good substrate relative to ROP, it would be hydrolyzed to a considerable extent as it formed according to the following reaction



and thereby lower the ratio of the products R'OP/P<sub>i</sub>, and give rise to erroneous conclusions about the reaction mechanism.

The results of previous experiments with the E.coli enzyme (152-4,217) indicate that both Tris and ethanolamine are suitable choices of competitive nucleophiles. Considerable

transphosphorylation occurs in the presence of these two nucleophiles: the expected transphosphorylation products, O-phosphoryl Tris and O-phosphorylethanolamine have been separated, characterized, and quantitatively measured by means of column chromatography (152-3) and high voltage electrophoretic techniques (217). Although these nucleophiles are very efficient phosphoryl acceptors, the resulting products are extremely poor substrates compared to typical phosphate esters, ROP: they are not hydrolyzed to any large extent during the hydrolysis of ROP. Consequently, the ratio of products (R'OP)/(P<sub>i</sub>) should remain essentially constant throughout the course of the reaction.

All reaction mixtures were adjusted to an ionic strength  $I = 1.0$  by the addition of NaCl. This relatively high ionic strength was chosen in order to (1) obtain the maximum rate and the maximum linearity during the course of the reaction (2) minimize the relative importance of the solvent effects arising from the addition of competitive nucleophile and (3) eliminate the considerable substrate activation observed at low ionic strength, for example,  $I = 0.1$ , which gives rise to pronounced deviations at high substrate concentrations. Although maintaining the reaction mixtures at  $I = 1.0$  necessitated varying the NaCl concentration from 0.67 to 1.0M, depending on the amount of added nucleophile present, this had no effect on the rate of the reaction, since the rate above 0.2M NaCl is virtually independent of NaCl concentration.

A pH of 10.0 was selected for studying the variation of  $V_{\max}$  and  $K_m$  with concentration of added nucleophile because these parameters can be accurately determined at that pH:  $K_m$  is relatively large (approximately 1mM), while  $V_{\max}$  is at its highest value. Furthermore, by carrying out the determination at pH 10.0 which is in the plateau region of the  $V_{\max}$ -pH profile the possible complication that varying concentrations of added nucleophile may cause a change in the activity of the hydrogen ion not detected on the pH meter is completely eliminated.

#### Materials

All solutions were prepared from doubly distilled deionized water.

#### Substrates

The p-nitrophenyl phosphate used was a Calbiochem Grade A reagent (Code No. 4876) obtained in the form of the sodium salt. It was stored in the dark in a vacuum dessicator at 0°C. Even under these conditions the water content varied measurably with time. The actual concentration of p-nitrophenyl phosphate in a given stock solution was therefore determined from the maximum amount of p-nitrophenol that could be obtained upon its complete hydrolysis according to a modification of the method previously used by Chanely and Feageson (263): a portion of the stock solution was heated

in 0.1M acetate buffer, pH 4.0, at 100° for 2-4 hr so that complete hydrolysis was achieved. Several aliquots of the resulting solution were then diluted with 10% Na<sub>2</sub>CO<sub>3</sub>, 0.1N in NaOH. The concentration of p-nitrophenol in these solutions were then determined spectrophotometrically at 400 mμ. The initial concentration of free p-nitrophenol in the stock solution of substrate was determined in a similar manner - by diluting a portion of the solution with 10% Na<sub>2</sub>CO<sub>3</sub>, 0.1N in NaOH, and reading the absorbance at 400 mμ. The initial amount of free p-nitrophenol in the stock solutions of substrate immediately after preparation in all cases amounted to less than 0.05%, although a higher percentage of free p-nitrophenol - up to 0.08% - was obtained with stock solutions which were one day old.

Disodium phenyl phosphate was purchased from Sigma Chemical Co. (Code No. P7751). To eliminate the marked inhibitory effects of inorganic phosphate present as an impurity this compound was further purified according to the procedure described by Salomon et al. (264), the only modification being that a Radiometer automatic titrator (Model TT1C) was used to adjust the pH to the required value during the various steps of the purification. The amount of phenyl phosphate in both the starting material and the end product was determined by hydrolyzing a portion of the material employing the method used to hydrolyze p-nitrophenyl phosphate, appropriately diluting an aliquot of the reaction mixture in

0.15N NaOH, and reading the absorbance at 290 m $\mu$ . No free phenol or inorganic phosphate could be detected in the purified material, which was stable when stored in a vacuum dessicator at 0°.

#### Added Nucleophiles

Tris (hydroxymethyl aminomethane) was obtained from the Sigma Chemical Co. (Code No. T1503), while the ethanolamine used was purchased from B.D.H. Chemicals (Code No. 28306). Both of these chemicals were of the highest grade available commercially - the Tris, according to the manufacturer, was approximately 99.9% pure, while the ethanolamine was reported to be at least 99% pure - and were used without further purification.

#### Enzyme

A partially purified, salt free, lyophilized preparation of chicken intestinal alkaline phosphatase purchased from the Worthington Biochemical Corp. (Code No. PC9KB) was used throughout these studies. Stock solutions of enzyme were prepared by weighing out 10-15 mg of material in a 5 ml volumetric flask on a semi-micro balance. The flask was allowed to equilibrate for 5 minutes in an ice-water bath. Ice-cold water was then added dropwise and with gentle stirring to bring the enzyme into solution. The solution was then removed from the ice-water bath and accurately made up to the

mark at  $20^{\circ}$ . The concentration of protein in the solution was determined in three different ways: (1) gravimetrically - from the amount of material weighed out in preparing the solution, (2) spectrophotometrically - by measuring the absorbance of the solution at 278  $\mu$ , using a specific absorptivity of  $0.74 \text{ cm}^{-1} \text{ g}^{-1} \text{ ml}$  and (3) colorimetrically - by the method of Lowry et al. (265) as modified by Legget-Bailey (266). When the important precaution of equilibrating the enzyme in the balance for 15 minutes before weighing was taken, all three methods gave values which agreed to within 3%.

#### Other Reagents

The amidol (2,4-diaminophenol) used in the colorimetric assay for inorganic phosphate was purchased from Eastman Organic Chemicals. The other chemicals required for this assay - sodium metabisulfite, ammonium molybdate, and trichloroacetic acid - were B.D.H. products (Code Numbers 30180, 10028, and 30490, respectively). The HCl, NaOH, and NaCl used to adjust the pH and ionic strength of the buffer solutions were obtained from Fisher Scientific. All these chemicals were analytical grade reagents, and were used without further purification.

#### Kinetic Procedure

Measurements of pH accurate to 0.005 pH units were obtained with a Radiometer model TTT1a pH meter fitted

with a model PHA630 scale expander, using a G202B glass electrode and a K401 saturated calomel reference electrode, both electrodes having been previously calibrated with two standard buffers of accurately known pH and temperature dependence (267). The pH of each assay mixture was measured before and after the reaction, and the pH of the assay mixture during the course of the reaction was taken as the arithmetic mean of these two measurements which, in general, differed by no more than 0.05 pH units.

Spectrophotometric measurements were made with a Perkin Elmer model 350 double beam recording spectrophotometer equipped with water-jacketted quartz cuvettes with an optical path length of 1 cm. The cell compartment of the spectrophotometer and the jacketted cuvettes are shown schematically in Figure 15. In order to maintain the temperature of the system constant to within  $\pm 0.1^{\circ}$  these cuvettes were connected to a temperature bath, circulation pump, and temperature regulator adjusted to operate at  $25^{\circ}$ .

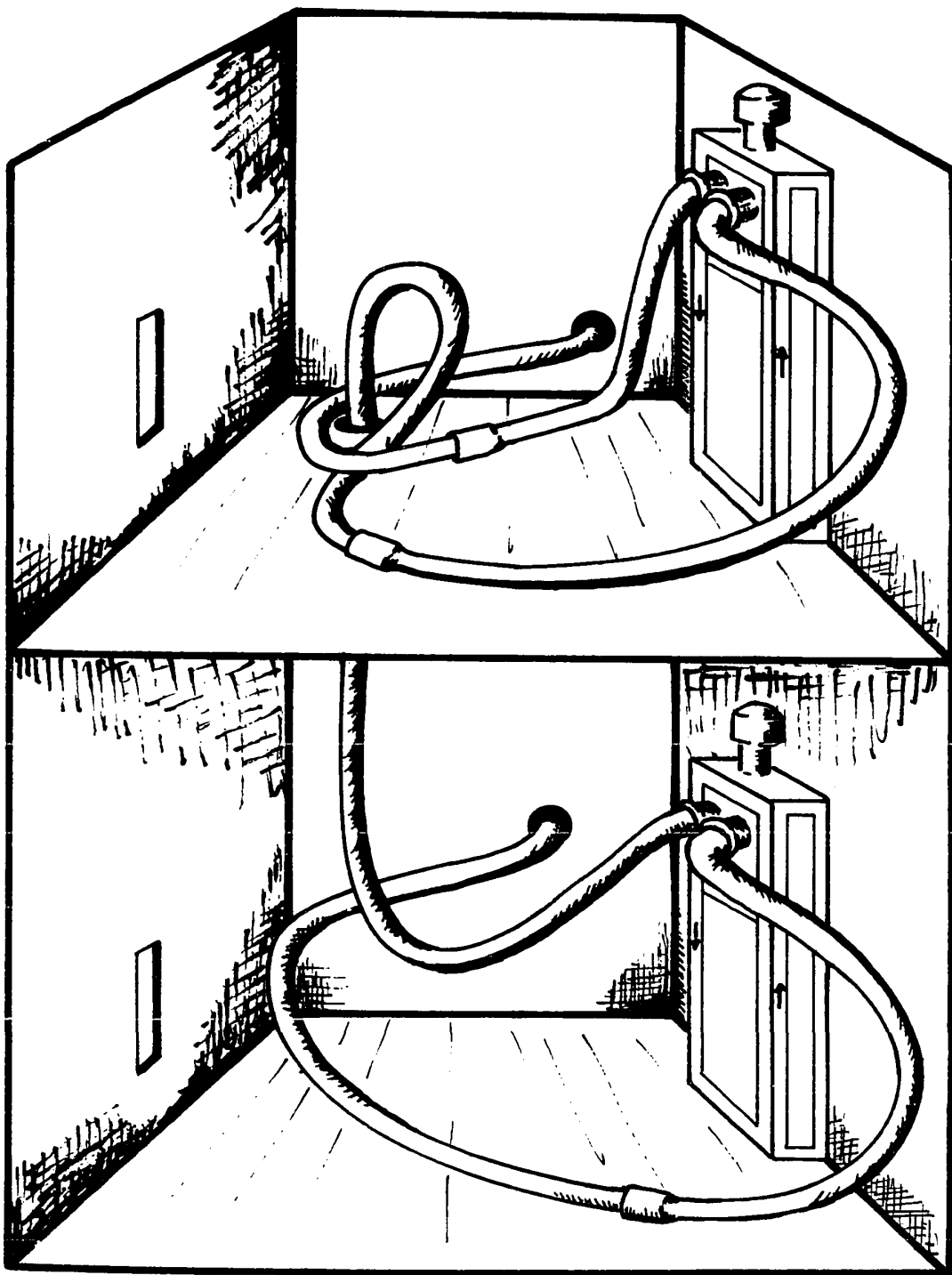
The rate of enzyme catalyzed hydrolysis of p-nitrophenyl phosphate was determined by following the rate of liberation of p-nitrophenol. The total concentration of p-nitrophenol in both the ionic and the undissociated form,  $C_T$ , was determined spectrophotometrically at 400 m $\mu$  using the modified form of Beer's law

Figure 15

Schematic diagram of the sample and reference cell compartments of the spectrophotometer.

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$$C_T = \left[ \frac{\left( 1 + \frac{(H)}{K_a} \right)}{\epsilon_{A^-} + \frac{(H)}{K_a} \epsilon_{HA}} \right] A_T \quad (167)$$

in which  $A_T$  is the total absorbance at 400  $m\mu$ ;  $\epsilon_{A^-}$  is the molar absorptivity of the p-nitrophenolate ion (at 400  $m\mu$ ), and  $\epsilon_{HA}$  is that of the undissociated p-nitrophenol;  $K_a$  is the dissociation constant of p-nitrophenol.

The above equation, which takes into account the variation of the apparent molar absorptivity of p-nitrophenol with pH, may be rewritten in the form

$$C_T = A_T / \tilde{\epsilon}_{APP} \quad (168)$$

in which  $\tilde{\epsilon}_{APP}$ , the apparent molar absorptivity - a constant at a given pH - is given by the following expression:

$$\tilde{\epsilon}_{APP} = \frac{\left( \epsilon_{A^-} + \frac{(H)}{K_a} \epsilon_{HA} \right)}{\left( 1 + \frac{(H)}{K_a} \right)} \quad (169)$$

Apparent molar absorptivities were calculated for different pH values from Eq. (268), values for  $\epsilon_{A^-}$ ,  $\epsilon_{HA}$ , and  $K_a$  having been previously determined in the usual manner (263) by carrying out preliminary experiments with standard solutions of p-nitrophenol. The change in apparent molar absorptivity with ionic strength was also taken into account by determining the dissociation constant of p-nitrophenol at both  $I = 0.1$ , and  $I = 1.0$ , the two ionic strengths used in this

study; pKa values of  $7.13 \pm .02$  ( $I = 0.1$ ), and  $7.04 \pm .03$  ( $I = 1.0$ ) were obtained.

As a further check on the calculated values of  $\tilde{\epsilon}_{APP}$ , standard solutions of p-nitrophenol were prepared at several pH values, and linear plots of  $A_T$  against  $C_T$  were obtained. The slopes of these linear plots were in good agreement with corresponding values of  $\tilde{\epsilon}_{APP}$  calculated using Eq. (169).

It is readily seen from Eq. (168) that the initial rate of hydrolysis of p-nitrophenyl phosphate is given by the expression

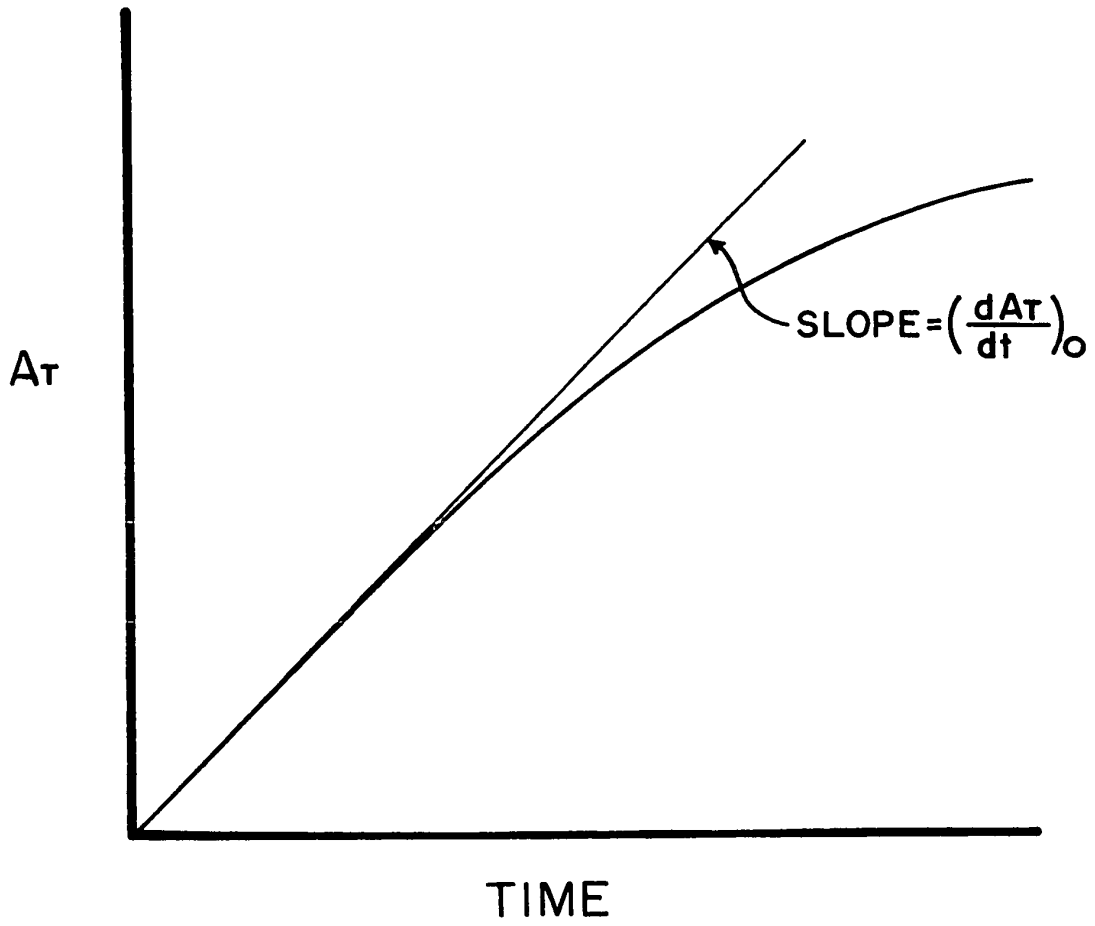
$$v = \frac{dC_T}{dt} = \frac{1}{\tilde{\epsilon}_{APP}} \left( \frac{dA_T}{dt} \right)_0 \quad (170)$$

where  $(dA_T/dt)_0$  is the initial slope of the  $A_T$  vs time curve recorded automatically on the spectrophotometer, as shown in Figure 16.

The enzyme catalyzed hydrolysis of p-nitrophenyl phosphate was initiated by the addition of 10-25  $\mu$ l of the enzyme stock solution to the reaction cuvet, the exact amount being adjusted to give a reaction rate which could be conveniently followed on the spectrophotometer. The plots, in general, were linear for the first 15-20 minutes of the reaction, which allowed accurate initial slopes to be measured. To further increase the accuracy, the reactions were always followed beyond the region of linearity, sometimes to 100% completion.

Figure 16

Determination of the initial rate of reaction  
from a plot of absorbance against time.



The rate of liberation of phenol during the enzyme catalyzed hydrolysis of phenyl phosphate was determined spectrophotometrically using the above described equipment but with the following modifications in experimental procedure: the reaction was carried out in a 50 ml reaction vessel immersed in a thermostated water bath operated at 25° . The reaction was initiated by the addition of 0.2 - 0.4 ml of enzyme stock solution to the reaction vessel; aliquots of the reaction mixture were removed every 30 sec for the first 10-15 minutes, rapidly diluted in ice-cold 2N NaOH to stop the enzyme catalyzed reaction, and brought to a suitable volume with doubly distilled water. The absorbance of each solution was then read at 290 mμ. The values obtained were plotted against time and initial rates were obtained from the linear portion of the curves in the above described manner. A molar absorptivity of  $(2.52 \pm .04) \times 10^3 \text{ cm}^{-1} \text{ l mole}^{-1}$  determined from absorbance measurements with standard phenol solutions was assumed for the phenolate ion in 0.1N NaOH. This value, which was used to convert the measured absorbances into moles of phenol per liter of solution, was in excellent agreement with that obtained by Salomon et al. (264) under identical conditions.

The rate of formation of inorganic phosphate during the course of the enzyme catalyzed hydrolysis of substrate (p-nitrophenyl phosphate or phenyl phosphate) was determined employing the following experimental procedure: the reaction

was started by adding 0.2 ml of the stock solution of enzyme to the assay mixture kept in a 50 ml reaction vessel immersed in a thermostated water bath operated at 25°C. After appropriate time intervals, which varied from 1 minute during the early stages of the reaction to 10 minutes during the later stages, 2 ml aliquots of the reaction mixture were rapidly added to test tubes containing 2 ml of 30% trichloroacetic acid, previously equilibrated in an ice-water bath, to stop the reaction. The strongly acidic reaction mixture in each test tube was then diluted with 10 ml of doubly distilled water and stored in the ice-water bath. In order to obtain a true reaction blank 2 ml of the assay mixture were removed from the reaction vessel before addition of enzyme, added to a test tube containing 2 ml of 30% ice-cold trichloroacetic acid, diluted with 10 ml water, and the stored in the ice-water bath. Immediately after the reaction was completed, the contents of the test tubes kept in the ice-water bath were analyzed for inorganic phosphate. This was done colorimetrically using a modification of Allen's method (269) which involves the addition of an excess of ammonium molybdate to the phosphate sample, reducing the resultant phosphomolybdate complex with a mixture of 2,4-diaminophenol and sodium metabisulfite, and measuring the absorbance of 680 m $\mu$  after full color development. Specifically, the test tube containing the reaction blank and the first four test tubes were removed from the ice-water

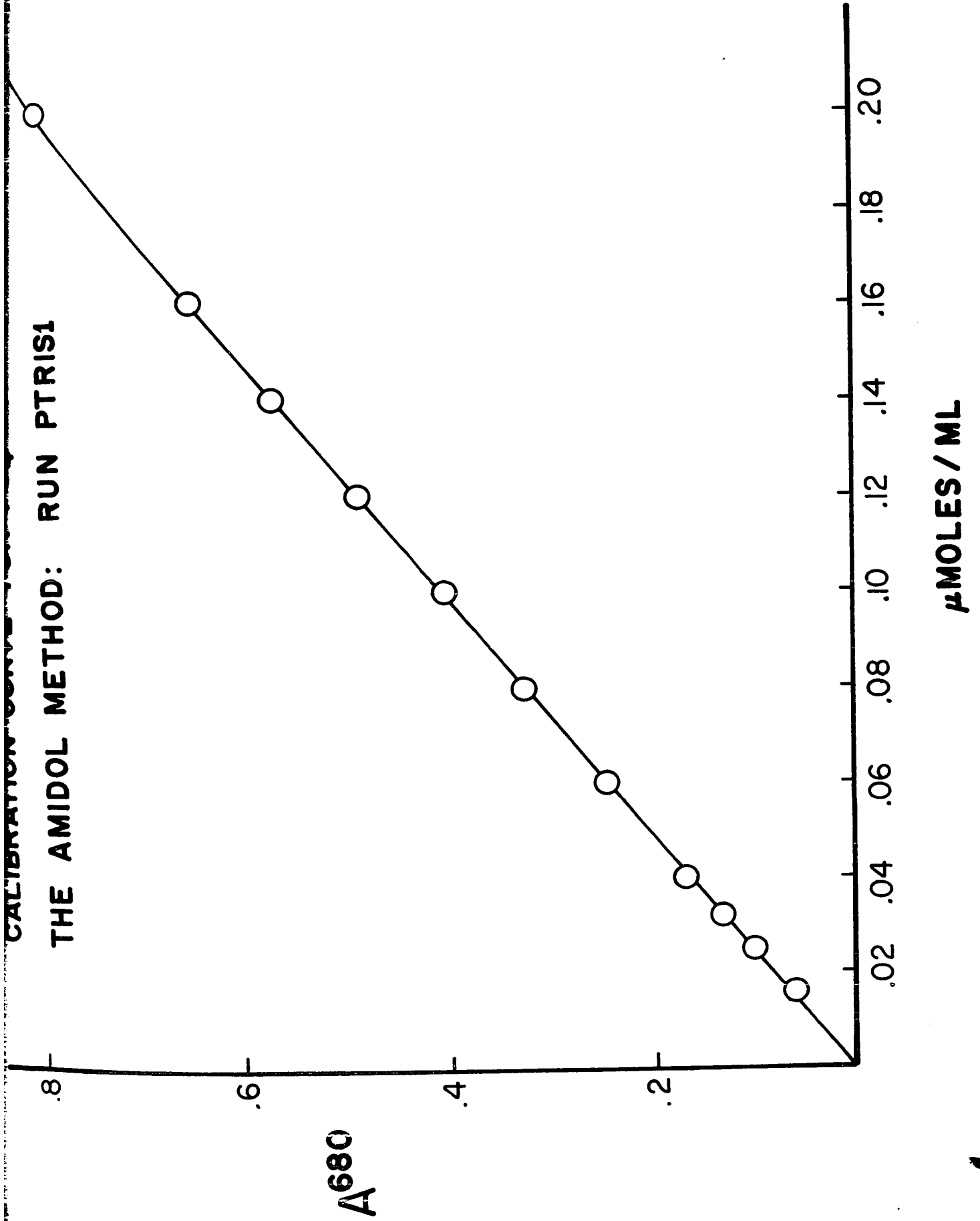
bath and brought to room temperature. One ml of ammonium molybdate solution (8.3g/100 ml) was then added to each test tube which was then mixed vigorously on a vortex mixer. A 2 ml aliquot of the ammidol mixture (1.0g 2,4-diaminophenol and 20.0g sodium metabisulfite in 100 ml of water) was then added to each test tube and thoroughly mixed, the exact time of addition being noted in each case. Exactly twenty minutes after the addition of ammidol to it, the contents of the test tube were diluted to 25 ml, and the absorbance was read at 680 m $\mu$ . Absorbance values were converted into concentrations ( $\mu$  moles of inorganic phosphate per ml) using a molar absorptivity of  $(4.03 \pm .03) \text{ cm}^{-1} \text{ ml-}\mu\text{mole}^{-1}$  - a value which was determined from absorbance measurements with standard inorganic phosphate solutions (Figure 17).

Preliminary experiments in which inorganic phosphate was determined by other methods - Dryer's method (270) and the recently described method of Itaya and Ui (271) - showed that although both these methods were more sensitive ones, they were much less convenient to use and led to less reliable results. The intensity of the color produced, in all three methods, is strongly dependent upon pH, temperature, and time. However, even when these parameters are carefully controlled, it is only with Allen's method that one obtains accurately linear and at the same time highly reproducible plots of absorbance against concentration. With the other two methods, standard solutions of inorganic phosphate give

Figure 17

Typical plot of absorbance at 680 m $\mu$  against concentration of inorganic phosphate obtained with Allen's method.

**CALIBRATION CURVE**  
**THE AMIDOL METHOD: RUN PTRIS1**



absorbances which do not follow Beer's law; nor are the curves obtained accurately reproducible. As a result, when either of these two methods is used it is necessary to prepare a separate set of phosphate standards and read their absorbances before each experiment. If Allen's method is used, this becomes unnecessary: a least squares analysis of the experimental data indicates that plots of  $A^{680}$ , the absorbance at 680  $\mu$ , against phosphate concentration obey Beer's law to within 0.4% provided  $A^{680}$  is less than 0.85 absorbance units, while the slopes obtained from these plots are reproducible to better than 1%.

Provided the pH of the solution, the time allowed for color development, and the temperature are carefully controlled, reliable results can readily be obtained using Allen's method. The above three parameters were controlled by adjusting the pH of each solution to 1.50 before reading its absorbance, maintaining the temperature during color development at 25°C, as described earlier, and taking all absorbance measurements about twenty minutes after the addition of the ammidol reagent. This time interval was chosen on the basis of preliminary experiments in which the Allen method was carried out on a series of standard phosphate solutions, except that the absorbance of each solution at 680  $\mu$  was followed for a period of 2-3 hours, starting about 1 min after the addition of ammidol reagent to the solution. Typically, the absorbance values increased very rapidly

during the first few minutes in what appeared to be a linear fashion, then at a much slower rate, finally leveling off completely. In general, no increase in absorbance could be detected 10-35 minutes after the addition of ammidol. After this time interval the absorbance began to drift upward once again, slowly at first, but always with an increasing rate. The % drift after 1 hr was usually less than 2% of the absorbance value obtained at the plateau, but sometimes up to 6% after 1.5 hr.

#### Analysis of Results

Values for the slopes and intercepts of all the linear plots obtained in these studies were calculated by the method of least squares (272), the actual computations being carried out on an I.B.M. 360/65 digital computer. Values of  $K_m$  and  $V_m$  were obtained by statistically fitting the experimental data directly to the Michaelis-Menten equation according to the method developed by Bliss and James ( 274 ), or, alternatively, by using a modified version of the computer program written by Cleland (273). This approach yielded more accurate estimates of the Michaelis parameters, together with some estimate of their reliability.

## RESULTS

Typical time course curves for the alkaline phosphates catalyzed hydrolysis of 5mM p-nitrophenyl phosphate in the presence of 1.0M Tris at pH 8.0,  $I = 1.0$  (adjusted with 5M NaCl) and  $T = 25.0^{\circ}$  are shown in Figure 18, while Figure 19 shows the time course curves typically obtained when 5mM phenyl phosphate are hydrolyzed under identical conditions. The upper curve in each figure shows the amount of p-nitrophenol (Figure 18) or phenol (Figure 19) produced as a function of time. The lower curves in both Figures 18 and 19 show the concentration of inorganic phosphate at various times during the course of the reaction.

The concentration of O-phosphoryl Tris at any time,  $t$ , during the reaction, as Eq. 9 indicates, is given by the difference between the upper curve and the lower one at  $t$ .

Although the enzyme catalyzed rate of hydrolysis of phenyl phosphate is significantly higher than that for p-nitrophenyl phosphate, the time course curves obtained with both substrates nevertheless have very similar shapes: after a short initial non-linear lag period, which is observed only in the presence of high concentrations of Tris or ethanolamine, the rate (of formation of phosphate, p-nitrophenol, or phenol) remains linear for approximately 30 minutes. Following this linear period the rate begins to decrease noticeably,

Figure 18

Typical time-course curves for the alkaline-phosphatase catalyzed hydrolysis of 5 mM p-nitrophenyl phosphate in 1.0M Tris at pH 8.0, I = 1.0M, T = 25.0°C.

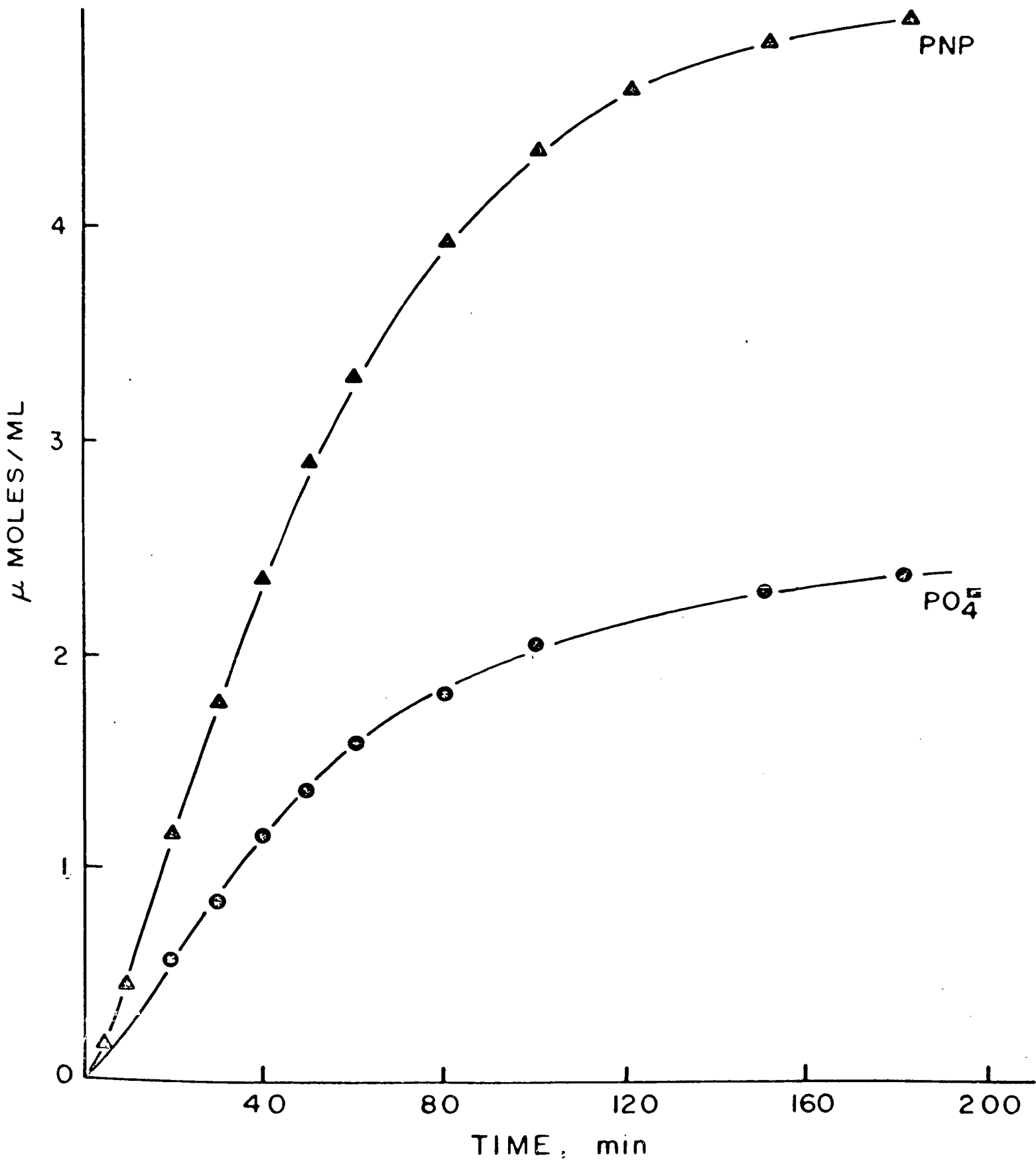
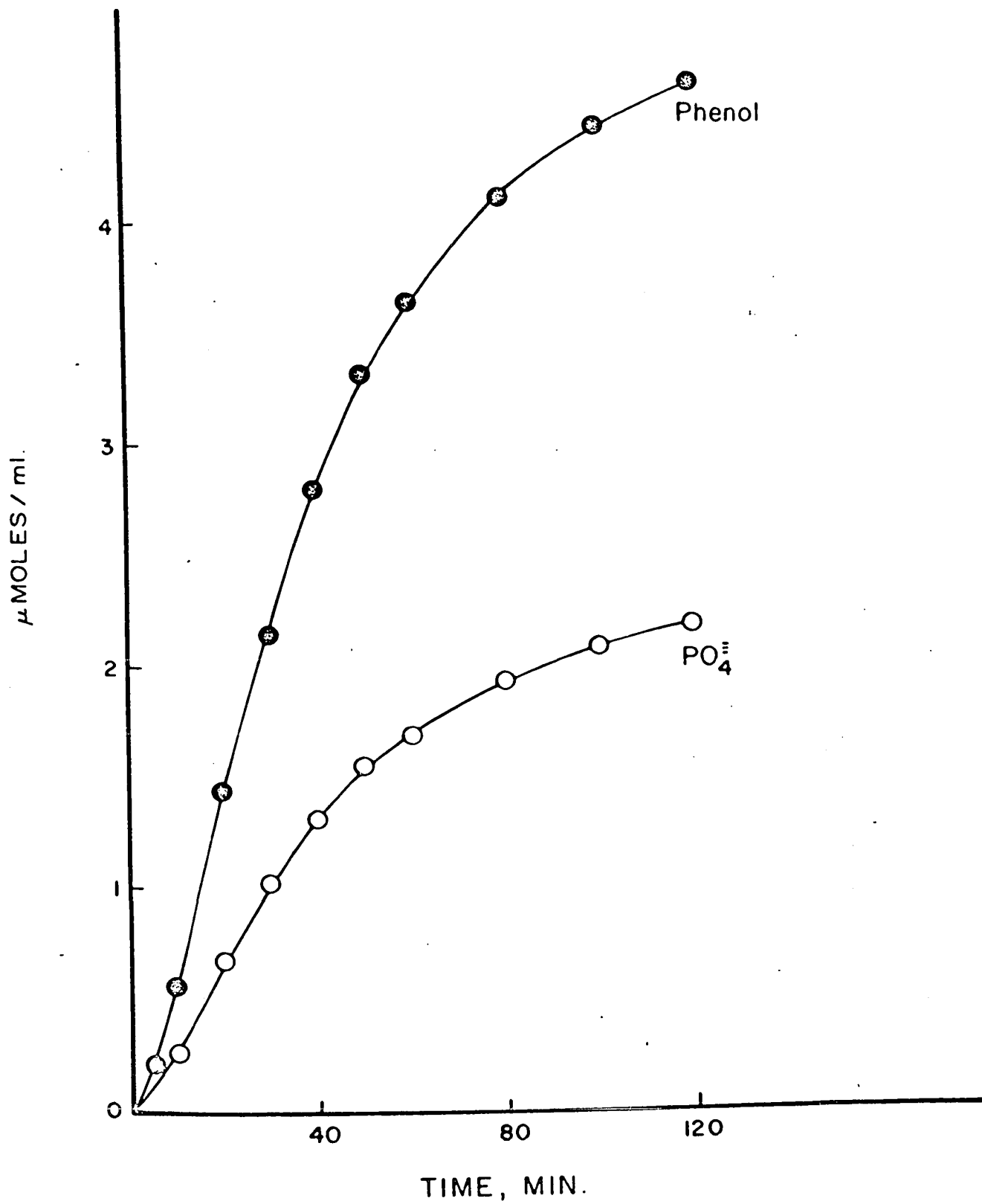


Figure 19

Typical time-course curves for the alkaline-phosphatase catalyzed hydrolysis of 5 mM phenyl phosphate in 1.0M Tris at pH 8.0, I = 1.0M, T = 25.0°C.



slowing down to about 1/8 of the initial rate after 90% completion.

The data corresponding to Figures 18 and 19 are shown in Tables 5 and 6, respectively. The last column in either table gives the ratio of products at various times during the reaction. Even though the rate decreases considerably with time, this ratio remains essentially constant, i.e.,

$$\frac{(PNP)}{(PO_4)} = R_{PNP} \quad (171)$$

and

$$\frac{(PHP)}{(PO_4)} = R_{PHP} \quad (172)$$

where both  $R_{PNP}$  and  $R_{PHP}$  are time independent constants. Eq. (171) therefore predicts that if the concentration of p-nitrophenol is plotted against the concentration of inorganic phosphate at the same time a straight line passing through the origin and with a slope equal to  $R_{PNP}$  should be obtained. Similarly a plot of concentration phenol against concentration of inorganic phosphate should, according to Eq. (172), also result in a straight line which passes through the origin but with a slope equal to  $R_{PHP}$ .

Plots of this type are shown in Figures 20 and 21. The straight lines drawn through the experimental points were obtained by the method of least squares (272), in the manner described previously. Both straight lines pass

TABLE 5

Product Ratios for the Hydrolysis of p-Nitrophenyl  
Phosphate, with Tris as added Nucleophile

1.0M Tris                      pH 8.0                      I = 1.0M                      T = 25.0°C			
5 mM p-nitrophenyl phosphate			
Time min.	PNP μmoles/ml	Phosphate μmoles/ml	(PNP)/(PO <sub>4</sub> ) Ratio
5	0.171	-	-
10	0.466	-	-
20	1.16	0.570	2.09
30	1.78	0.850	2.10
40	2.36	1.16	2.12
50	2.90	1.37	2.12
60	3.31	1.59	2.08
80	3.95	1.83	2.15
100	4.36	2.06	2.12
120	4.66	-	-
150	4.88	2.31	2.11
180	5.00	2.40	2.08
		Avg.	2.10±.04

TABLE 6

Product Ratios for the Hydrolysis of Phenyl Phosphate,  
with Tris as added Nucleophile

1.0M Tris			
pH 8.0			
I = 1.0M			
T = 25.0°C			
5 mM phenyl phosphate			
Time min.	Phenyl Phosphate μmoles/ml	Phosphate μmoles/ml	(PhP)/(PO <sub>4</sub> ) Ratio
5	0.210	-	-
10	0.564	0.262	2.15
20	1.44	0.676	2.13
30	2.15	1.02	2.11
40	2.80	1.31	2.14
50	3.32	1.55	2.14
60	3.64	1.69	2.15
80	4.12	1.93	2.13
100	4.45	2.09	2.13
120	4.66	2.19	2.13
		Avg.	2.13 ± .02

Figure 20

The concentration of p-nitrophenol versus the concentration of phosphate during the course of hydrolysis of p-nitrophenyl phosphate in the presence of 1M Tris. The reaction was carried out at 25.0°C, pH 8.0, and I = 1.0M (adjusted with 4N HCl and 5N NaCl).

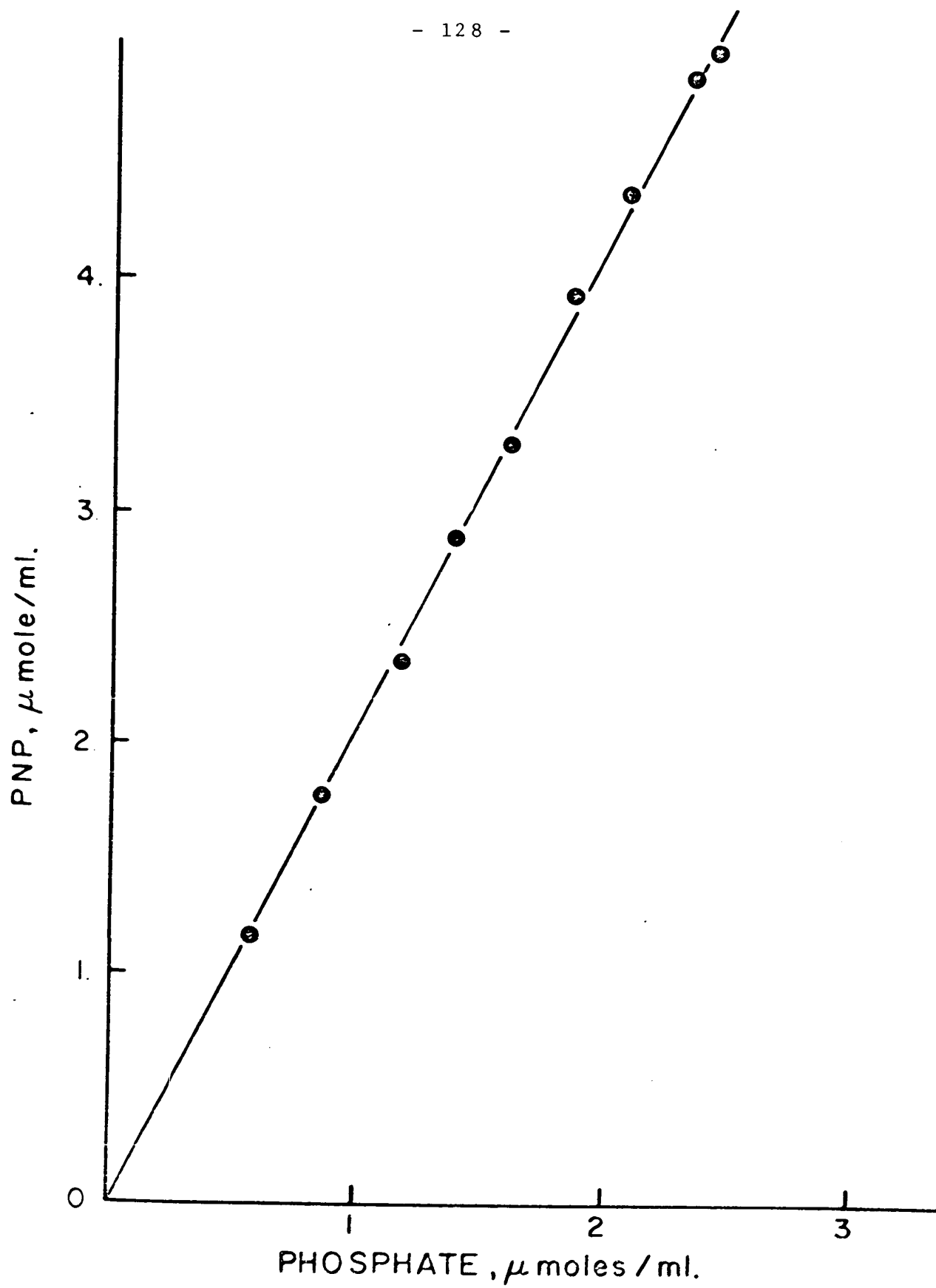
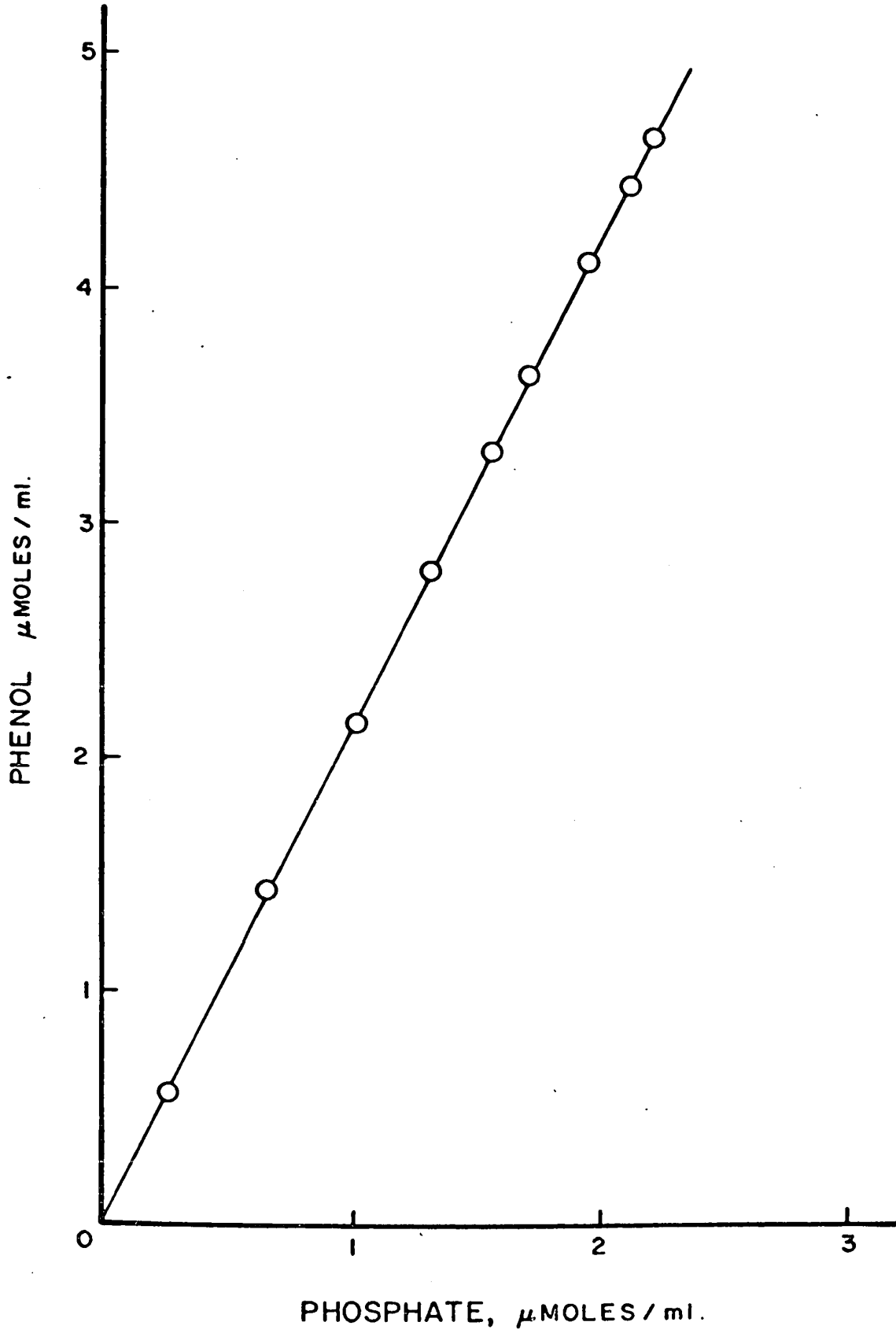


Figure 21

The concentration of phenol versus the concentration of phosphate during the course of hydrolysis of 5 mM phenyl phosphate in the presence of 1M Tris. The reaction was carried out at 25.0°C, pH 8.0 and I = 1.0M (adjusted with 4N HCl and 5N NaCl).



through the origin as expected, and appear to have identical slopes. The results of the least squares analysis corroborate this latter conclusion: the slope of the first straight line,  $R_{\text{PNP}}$ , is equal to  $2.10 \pm .04$ , while that of the second,  $R_{\text{PHP}}$  has a value of  $2.13 \pm .02$ . The difference in the two slopes is clearly within the small experimental error, i.e.,

$$R_{\text{PNP}} = R_{\text{PHP}} = R \quad (173)$$

The ratio of products,  $P_1/P_2$ , formed during the alkaline phosphatase catalyzed hydrolysis of a phosphate ester in the presence of Tris therefore seems to be independent of the nature of the leaving group of the ester. Since

$$\frac{P_3}{P_2} = \frac{P_1}{P_2} - 1 \quad (174)$$

i.e.,

$$\frac{P_3}{P_2} = R - 1 \quad (175)$$

the results also indicate that the ratio of products, O-phosphoryl Tris: inorganic phosphate, formed during the reaction is independent of the nature of the leaving group of the substrate.

These important results were verified by studying the enzyme catalyzed hydrolysis of the same two substrates under identical conditions but in the presence of a different added nucleophile - 1M ethanolamine. The results of these experiments are summarized in Tables 7-8, and are also shown

TABLE 7

Product Ratios for the Hydrolysis of p-Nitrophenyl  
Phosphate, with Ethanolamine as Added Nucleophile

---

1.0M Ethanolamine	pH 8.0	I = 1.0M	T = 25.0°C
5 mM p-Nitrophenyl phosphate			
Time min.	PNP μmoles/ml	Phosphate μmoles/ml	(PNP)/(PO <sub>4</sub> ) Ratio
10	0.338	0.220	1.54
20	0.863	0.564	1.53
30	1.29	0.820	1.57
40	1.72	1.14	1.51
50	2.03	1.33	1.53
60	2.30	1.47	1.56
80	2.69	1.79	1.50
100	2.99	2.01	1.49
150	3.43	2.24	1.53
180	3.59	2.38	1.51
		Avg.	1.53±.025

---

TABLE 8

Product Ratios for the Hydrolysis of Phenyl Phosphate,  
with Ethanolamine as added Nucleophile

1.0M Ethanolamine      pH 8.0      I = 1.0M      T = 25.0°C			
5 mM Phenyl phosphate			
Time Min.	Phenyl Phosphate μmoles/ml	Phosphate μmoles/ml	(PhP)/(PO <sub>4</sub> ) Ratio
10	0.407	0.259	1.57
20	1.04	0.670	1.55
30	1.62	1.02	1.59
40	2.01	1.31	1.53
50	2.44	1.54	1.58
60	2.57	1.68	1.53
80	3.01	1.93	1.56
100	3.19	2.07	1.54
120	3.39	2.20	1.54
		Avg.	1.55±0.22

graphically in Figures 22-25. It can be seen from Tables 7 and 8 that  $R_{\text{PNP}} = 1.53 \pm .025$ , while  $R_{\text{PHP}} = 1.55 \pm .022$ . Within the experimental error, then,  $R_{\text{PNP}} = R_{\text{PHP}}$ . The ratio of products formed during the reaction,  $P_1/P_2$ , and hence  $P_3/P_2$ , as in the previous case where Tris was the added nucleophile, is independent of the nature of the leaving group on the substrate. The magnitude of these ratios, however, clearly does depend on the chemical nature of the added nucleophile.

The rate of formation of p-nitrophenol and phosphate was also studied as a function of Tris concentration. In these experiments the reaction mixtures contained 5mM p-nitrophenyl phosphate and 0.010-0.70M Tris adjusted to pH 10.0 and an ionic strength of 1.0 with 4N HCl and 5N NaCl respectively. The temperature was maintained at 25°C. The results of these experiments are shown in Table 9 and Figure 26.

Figure 26 shows that the rate of formation of inorganic phosphate at high substrate concentrations ( $S \gg K_m$ ) is completely independent of the concentration of Tris. The rate of formation of p-nitrophenol which is equal to the rate of disappearance of substrate, on the other hand, increases linearly with Tris concentration up to concentrations of about 0.6M: all the rates obtained at Tris concentrations below 0.6M can be fitted to a straight line by the method of least squares with standard errors of 3.8 and 1.2% in the slope and intercept, respectively. The value obtained for

Figure 22

Typical time-course curves for the alkaline phosphatase catalyzed hydrolysis of 5 mM p-nitrophenyl phosphate in 1.0M ethanolamine at pH 8.0, I = 1.0M and T = 25.0°C.

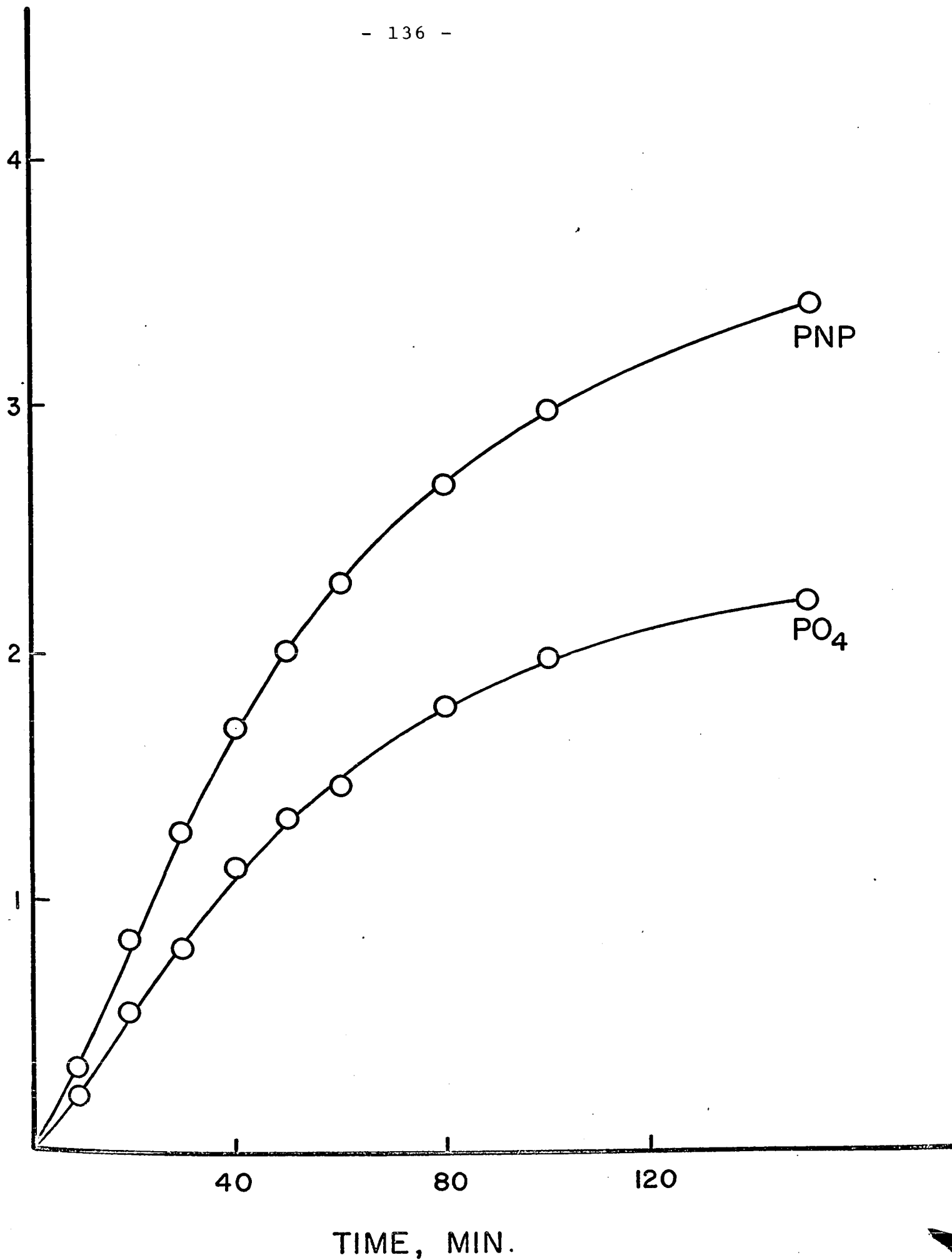


Figure 23

Typical time-course curves for the alkaline phosphatase catalyzed hydrolysis of 5mM phenyl phosphate in 1.0M ethanolamine at pH 8.0, I = 1.0M and T = 25.0°C.

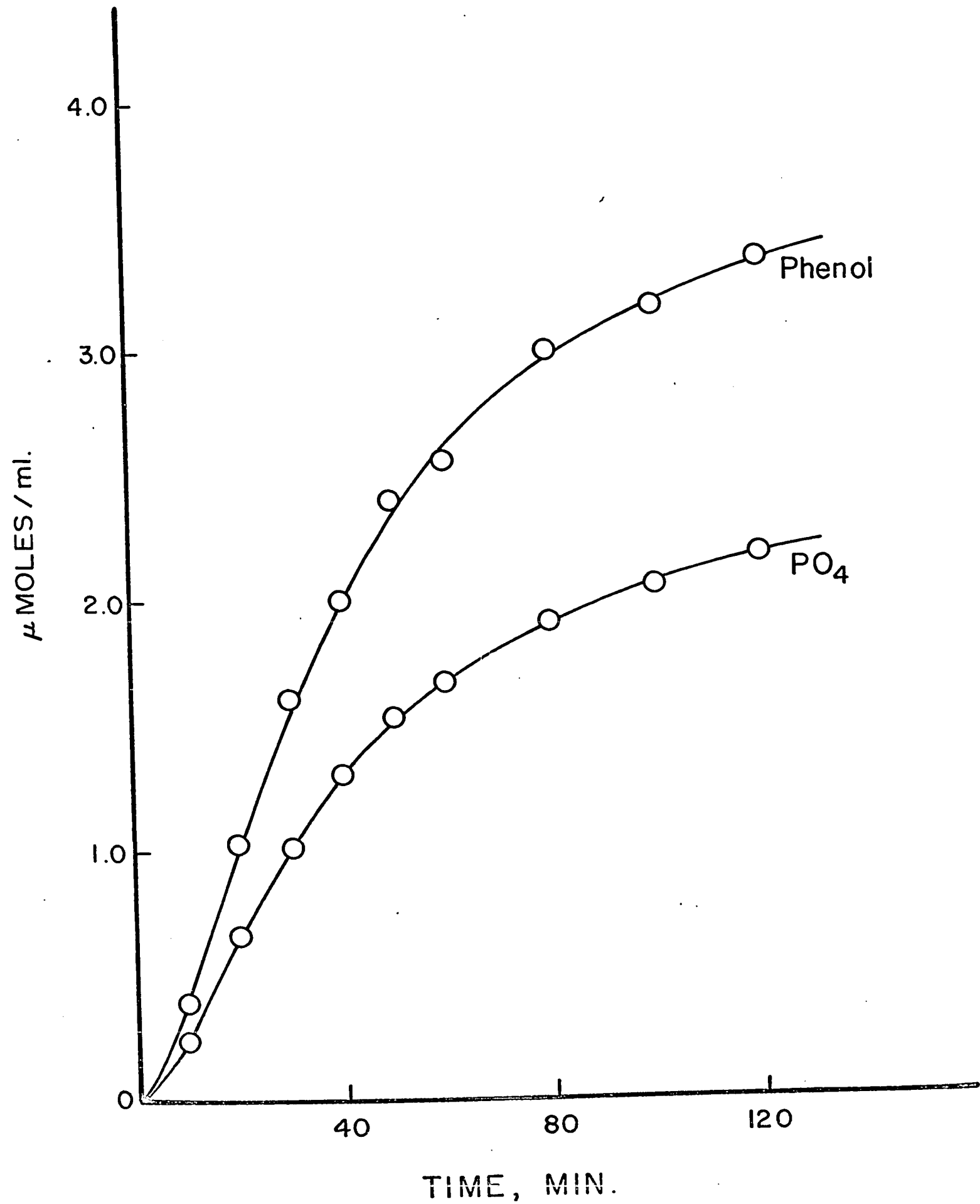


Figure 24

The concentration of p-nitrophenyl versus the concentration of phosphate during the course of hydrolysis of 5 mM p-nitrophenyl phosphate in the presence of 1M ethanolamine. The reaction was carried out at 25.0°C, pH 8.0 and I = 1.0M (adjusted with 4N HCl and 5N NaCl).

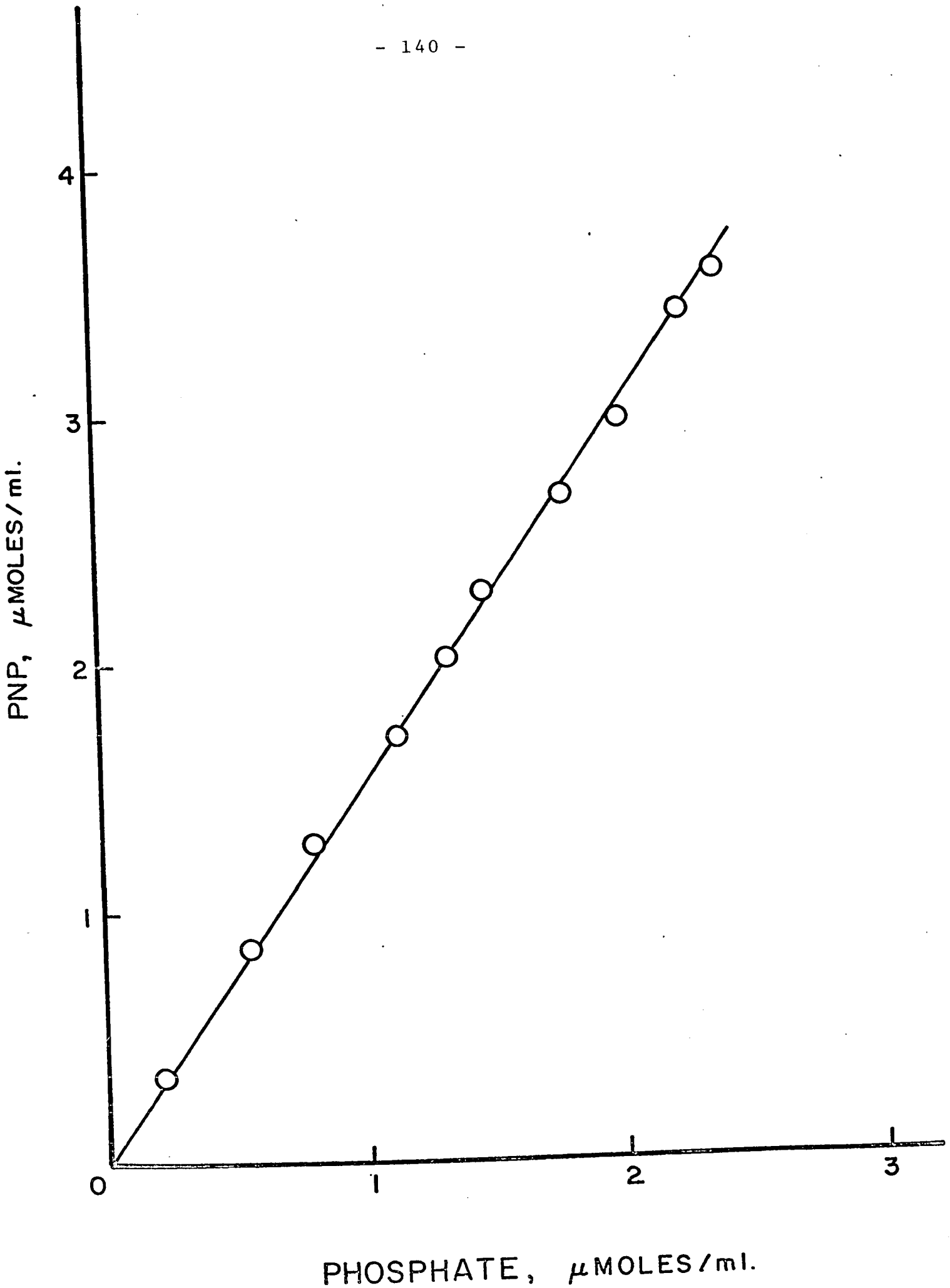
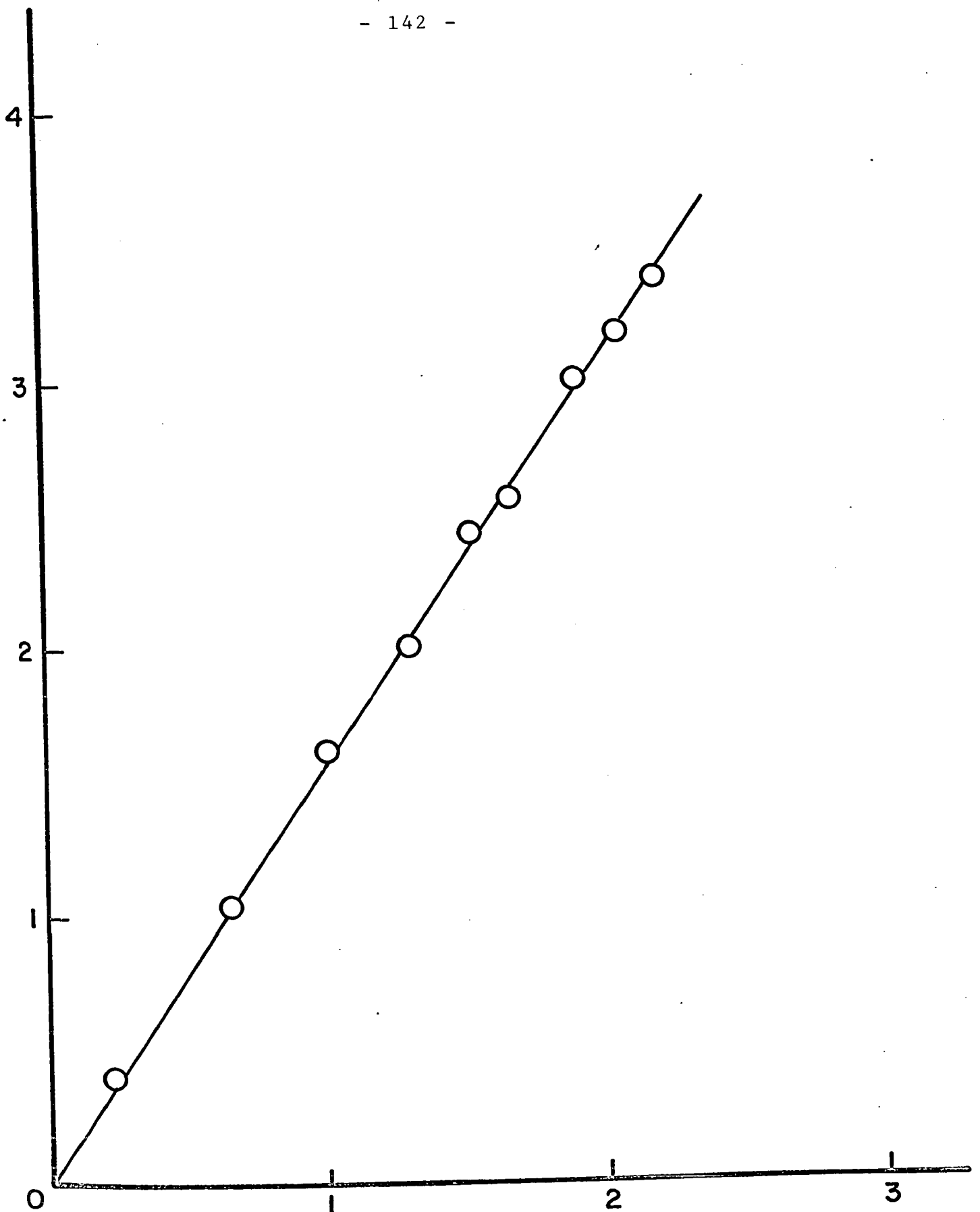


Figure 25

The concentration of phenol versus the concentration of phosphate during the course of hydrolysis of 5 mM phenyl phosphate in the presence of 1M ethanolamine. The reaction was carried out at 25.0°C, pH 8.0 and I = 1.0M (adjusted with 4N HCl and 5N NaCl).



PHOSPHATE,  $\mu$ MOLES / ml.

TABLE 9

Rates of Formation of PNP and Phosphate at pH 10.0, I=1.0, and T=25.° as a Function of Tris Concentration<sup>1</sup>

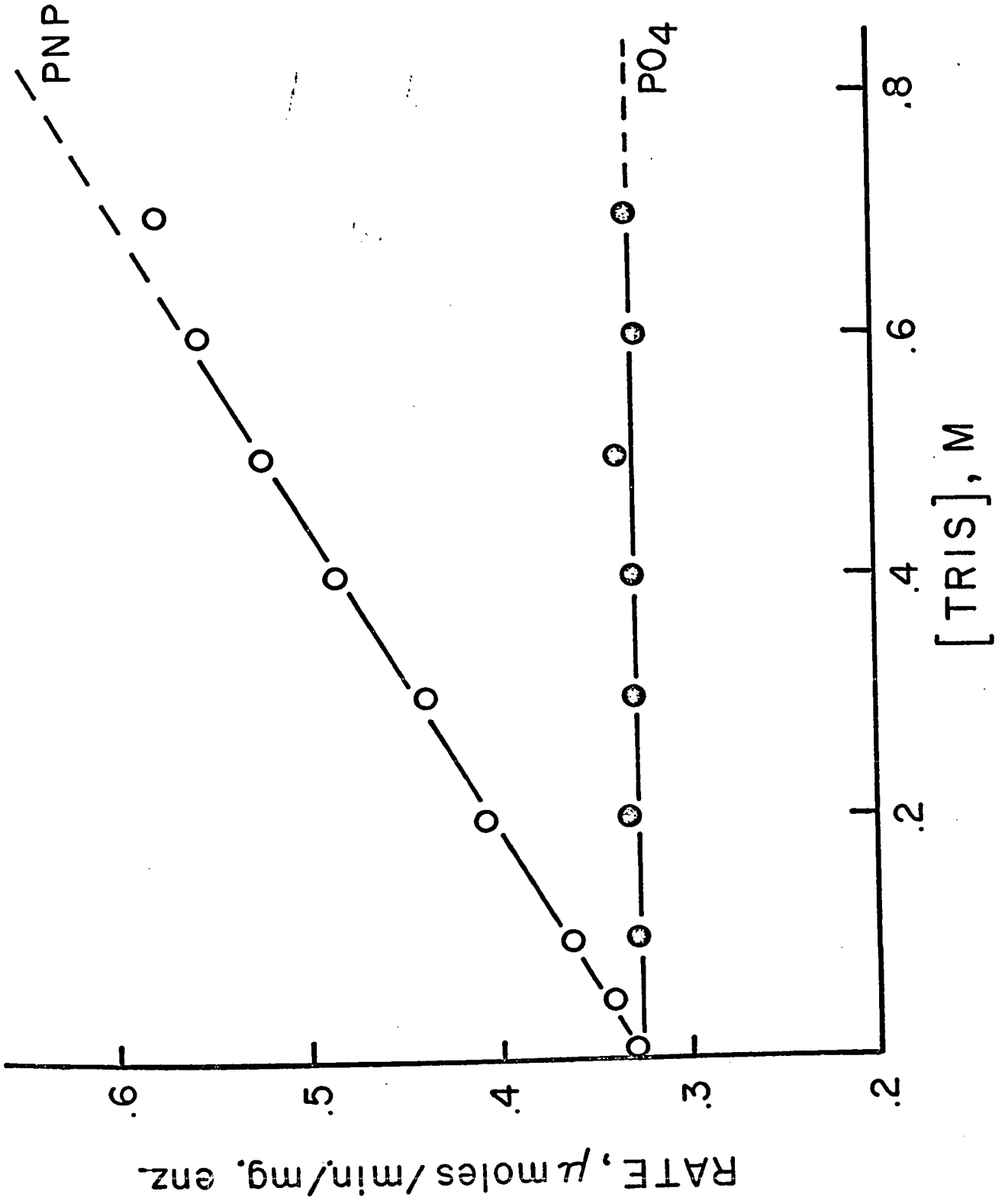
Tris (M)	Rate (μmoles/min/mg Enz.)		R <sup>2</sup>
	PNP	Phosphate	
0.010	0.330	0.326	
0.10	0.364	0.326	1.12
0.20	0.406	0.332	1.22
0.30	0.436	0.326	1.34
0.40	0.484	0.327	1.48
0.50	0.520	0.338	1.54
0.60	0.566	0.322	1.70
0.70	0.575	0.326	1.76
	Avg.	0.328±.005	

<sup>1</sup>The initial concentration of PNPP was 5.0 mM

<sup>2</sup>R = (rate of formation of PNP)/(rate of formation of phosphate)

Figure 26

Rates of formation of p-nitrophenol and of phosphate as functions of Tris concentration, for the hydrolysis of 5 mM p-nitrophenyl phosphate.  $T = 25.0^{\circ}\text{C}$ ,  $\text{pH } 10.0$ ,  $I = 1.0\text{M}$ .



the intercept,  $0.329 \pm .004$   $\mu\text{moles}/\text{min}/\text{mg}$  enzyme, is essentially identical to the average value obtained for the rate of formation of inorganic phosphate at various Tris concentrations ( $0.328 \pm .005$   $\mu\text{moles}/\text{min}/\text{mg}$  enzyme), which shows that in the absence of Tris the rate of formation of p-nitrophenol is equal to the rate of formation of phosphate, as required under steady state conditions.

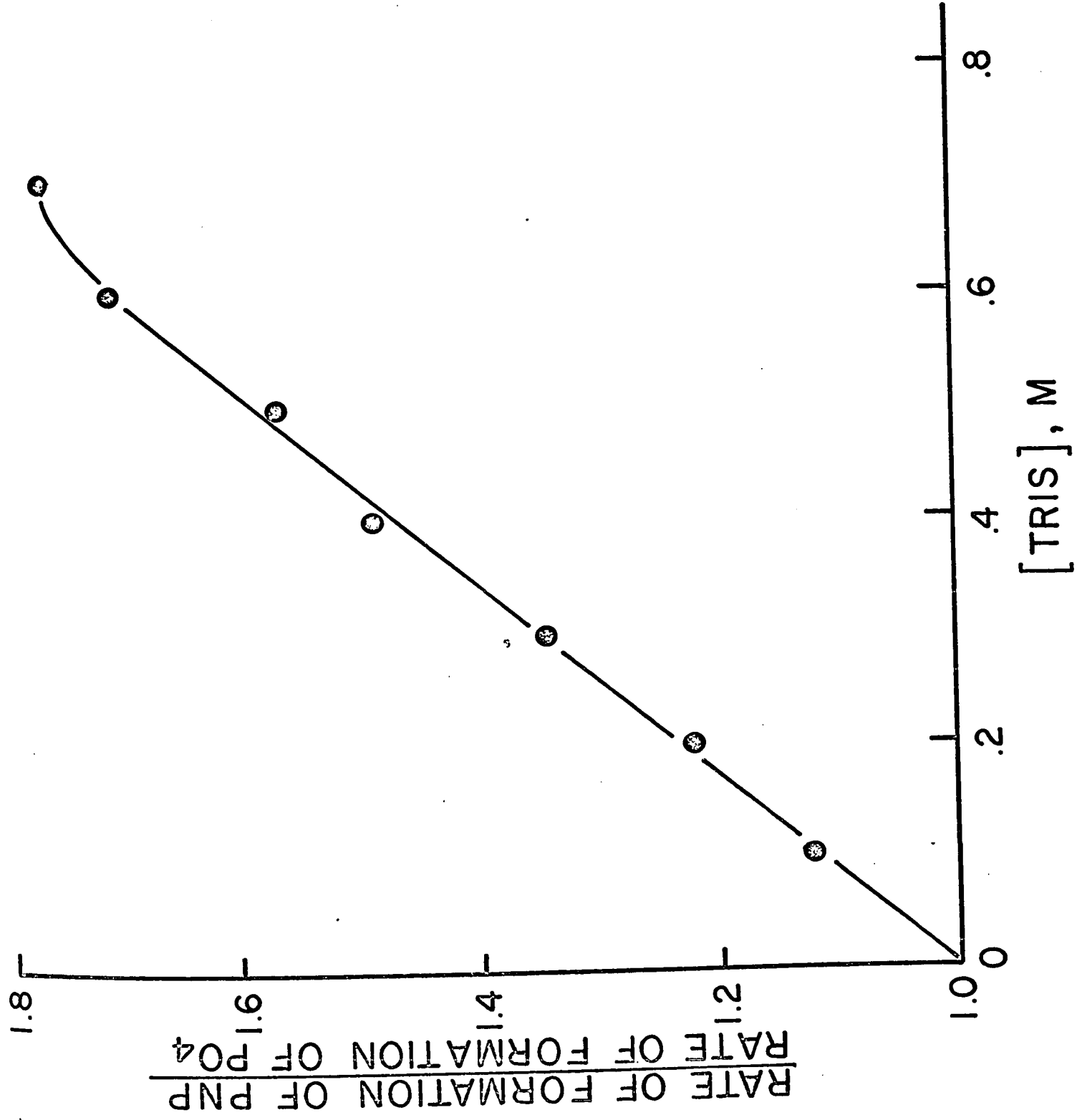
The rate of formation of O-phosphoryl Tris (i.e., the rate of transphosphorylation) which is calculated by subtracting the rate of formation of inorganic phosphate from the rate of disappearance of substrate is also a linear function of Tris concentration: if the rate of transphosphorylation is plotted against the concentration of Tris a straight line of zero intercept is obtained. (This latter plot, not shown in Figure 26, can readily be constructed from the data given in Table 9).

Figure 27 shows the data of Table 9 plotted in a different manner: the quantity R, defined as the ratio of the rate of formation of p-nitrophenol to the rate of formation of phosphate, is plotted as a function of Tris concentration. A straight line with an intercept equal to  $1.00 \pm .012$  (1.2%) is obtained.

The enzyme catalyzed hydrolysis of p-nitrophenyl phosphate was also studied as a function of substrate concentration at five different concentrations of Tris in order to determine the dependence of the Michaelis parameters on

Figure 27

The ratio of the rate of formation of  
p-nitrophenol to the rate of formation of  
phosphate versus the concentration of Tris.  
T = 25.0°C, pH 10.0, I = 1.0M.



the concentration of added nucleophile. All reactions were carried out at pH 10.0,  $I = 1.0$ , and  $T = 25.0^{\circ}\text{C}$ . The results obtained are given in Tables 10-14. Figure 28 shows that the data obtained at a given concentration of Tris obey Michaelis-Menten kinetics: if the reciprocal of the rate is plotted against the reciprocal of the substrate concentration a straight line of slope  $K_m/V$ , and with Y and X intercepts equal to  $1/V$  and  $-K_m$ , respectively, is obtained. The straight lines obtained for the various Tris concentrations all seem to intersect at one point on the  $1/(S)$  axis, indicating that the Michaelis parameter,  $K_m$ , is independent of Tris concentration. The Y intercepts, on the other hand, decrease with increasing concentration of added nucleophile, which indicates that  $V_{\text{max}}$  increases with increasing concentration of added nucleophile.

These last two tentative conclusions were verified by statistically fitting the data obtained at each Tris concentration to the Michaelis-Menten equation in the manner described previously. The values obtained in this way for  $V_{\text{max}}$  and  $K_m$  are listed together with their standard errors in Table 15 from which it is seen the  $K_m$  does not vary to any significant degree with varying concentration of added nucleophile. The relatively small variation which is observed is well within the expected magnitude of the experimental error. Furthermore, the average value calculated for  $K_m$  -  $0.971 \pm .03$  mM - is essentially identical to that obtained

TABLE 10

Hydrolysis of p-Nitrophenyl Phosphate in 0.010M

Tris at pH 10.0, I = 1.0 and T = 25.0°

(PNPP) mM	Rate $\mu\text{moles}/\text{min}/\text{mg Enz.}$	1/Rate
1.983	0.339	2.95
0.992	0.255	3.92
0.496	0.172	5.81
0.198	0.0862	11.6
0.149	0.0658	15.2

$$K_m = 0.966 \pm 0.015 \text{ mM}$$

$$V_{\text{max}} = 0.504 \pm 0.004 \mu\text{moles}/\text{min}/\text{mg Enz.}$$

TABLE 11

Hydrolysis of p-Nitrophenyl Phosphate in 0.050M  
Tris at pH 10.0, I = 1.0 and T = 25.0°

(PNPP) mM	Rate μmoles/min/mg Enz.	1/Rate
1.983	0.374	2.67
0.992	0.275	3.64
0.496	0.188	5.32
0.198	0.906	11.0
0.0992	0.528	18.9

$$K_m = 1.01 \pm 0.04 \text{ mM}$$

$$V_{\max} = 0.562 \pm 0.012 \text{ } \mu\text{moles/min/mg Enz.}$$

TABLE 12

Hydrolysis of p-Nitrophenyl Phosphate in 0.20M  
Tris at pH 10.0, I = 1.0 and T = 25.0°

(PNPP) mM	Rate μmoles/min/mg Enz.	1/Rate
2.012	0.484	2.07
1.006	0.359	2.79
0.503	0.244	4.10
0.201	0.125	8.00
0.101	0.0683	14.6

$$K_m = 0.975 \pm 0.033 \text{ mM}$$

$$V_{\max} = 0.716 \pm 0.011 \text{ μmoles/min/mg Enz.}$$

TABLE 13

Hydrolysis of p-Nitrophenyl Phosphate in 0.30M  
Tris at pH 10.0, I = 1.0 and T = 25.0°

(PNPP) mM	Rate μmoles/min/mg Enz.	1/Rate
2.012	0.568	1.76
1.006	0.423	2.36
.503	0.287	3.48
.201	0.150	6.67
.101	0.0775	12.9

$$K_m = 0.963 \pm .037 \text{ mM}$$

$$V_{\max} = 0.837 \pm .015 \text{ } \mu\text{moles/min/mg Enz.}$$

TABLE 14

Hydrolysis of p-Nitrophenyl Phosphate in 0.50M  
Tris at pH 10.0, I = 1.0 and T = 25.0°

(PNPP) mM	Rate $\mu\text{moles}/\text{min}/\text{mg Enz.}$	1/Rate
2.012	0.730	1.37
1.006	0.559	1.79
0.503	0.374	2.67
0.201	0.190	5.26
0.149	0.144	6.94

$$K_m = 0.936 \pm 0.018 \text{ mM}$$

$$V_{\text{max}} = 1.08 \pm 0.010 \mu\text{moles}/\text{min}/\text{mg Enz.}$$

Figure 28

Lineweaver-Burk plots for the hydrolysis  
of p-nitrophenyl phosphate at five different  
Tris concentrations.  $T = 25.0^{\circ}\text{C}$ ,  $\text{pH } 10.0$ ,  
 $I = 1.0\text{M}$ .

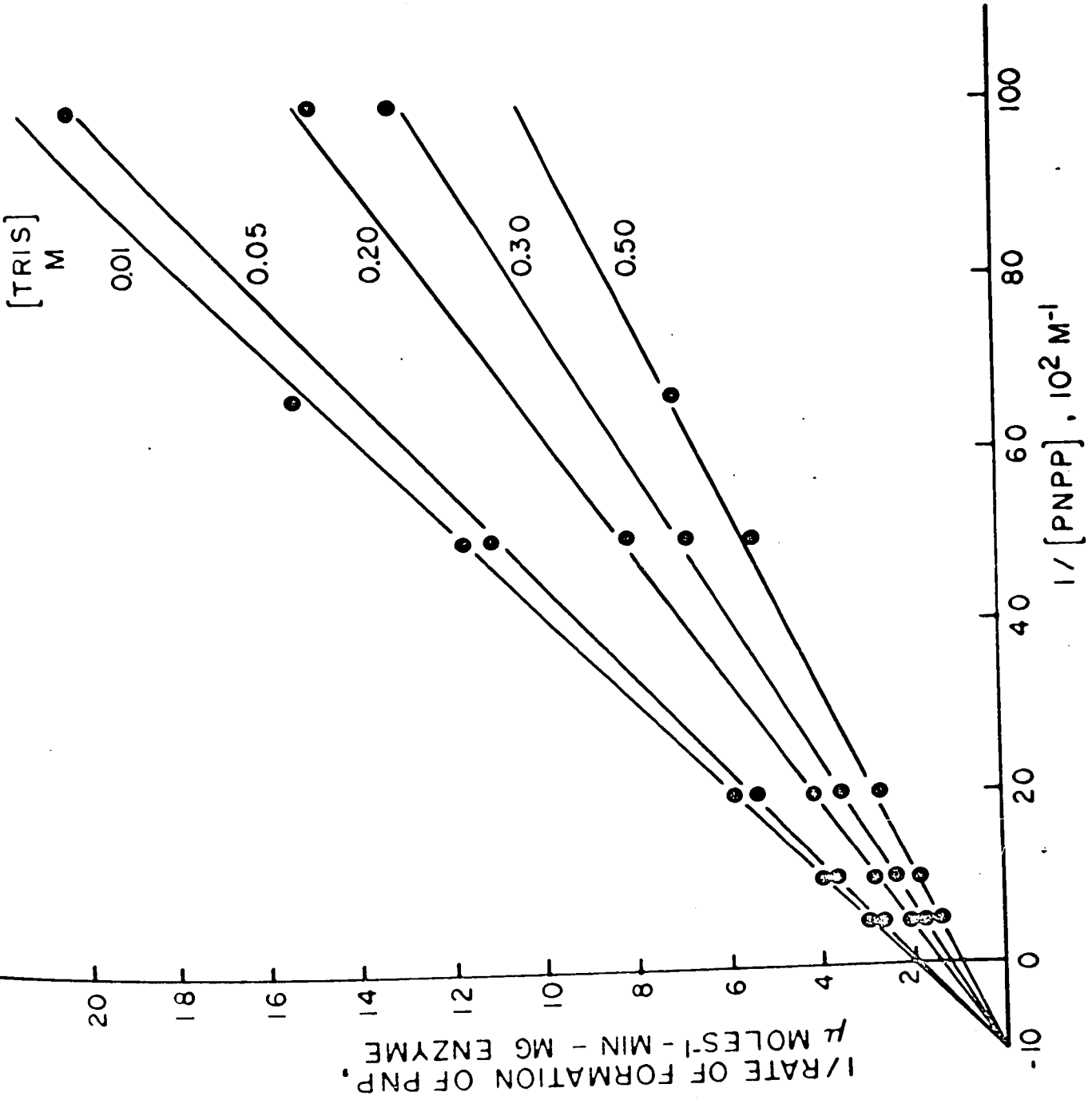


TABLE 15

Kinetic Parameters for the Hydrolysis of  
p-Nitrophenyl Phosphate in the Presence of Tris

pH 10.0		I = 1.0M	T = 25.0°C
[Tris] M	Range of [PNPP] mM	K <sub>m</sub> mM	V <sub>max</sub> <sup>*</sup> μmoles/min/mg Enz.
0.010	0.15-1.98	0.966±.015	0.504±.004
0.050	0.10-1.98	1.01 ±.040	0.562±.012
0.20	0.10-2.01	0.975±.033	0.716±.011
0.30	0.10-2.01	0.963±.037	0.837±.015
0.50	0.15-2.01	0.936±.018	1.08 ±.010

Avg. 0.970±.027

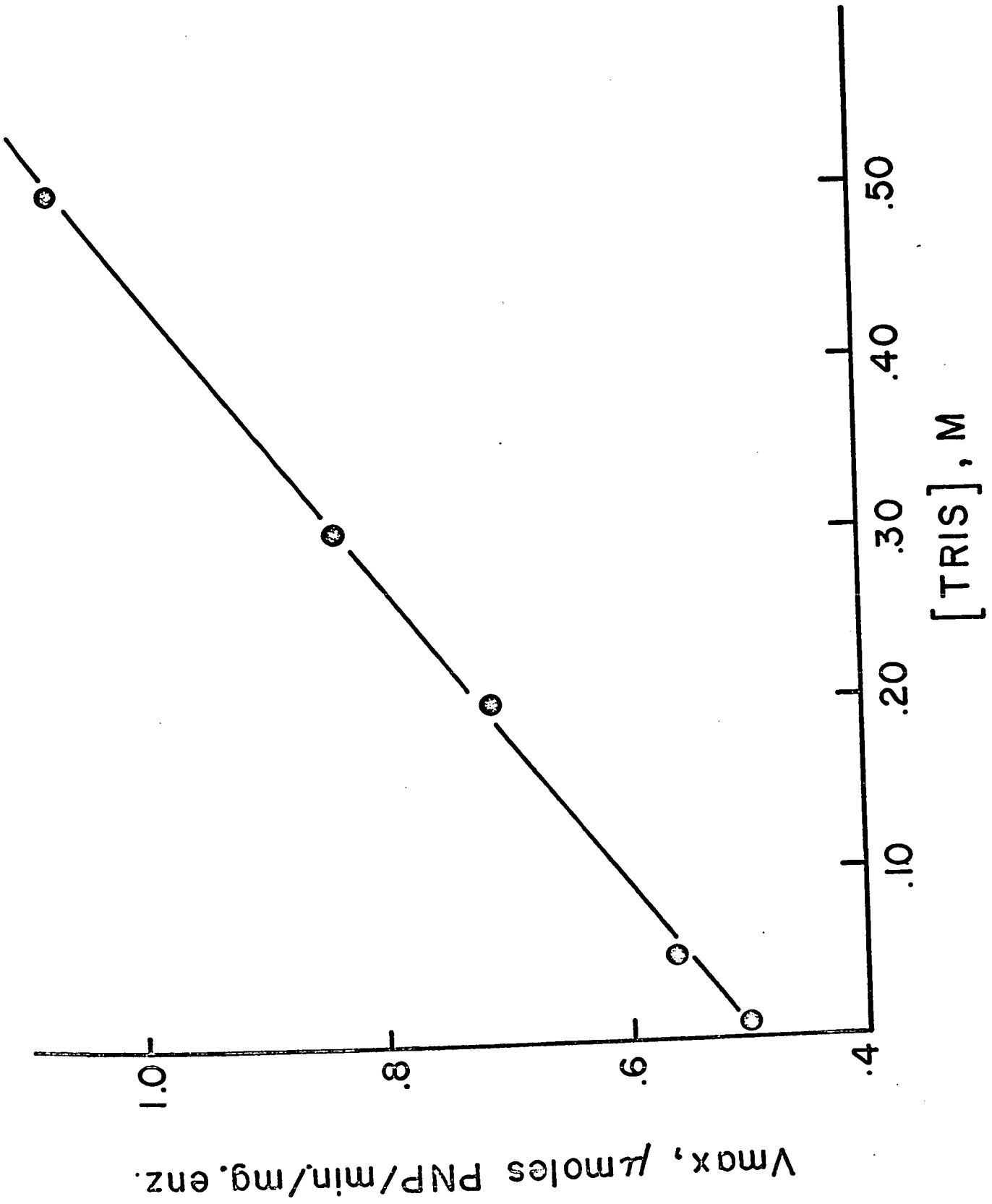
$$*V_{\max} = (1.12 \pm .04)N + (0.504 \pm .009)$$

under identical conditions but in the absence of added nucleophile.

Figure 29 shows the  $V_{\max}$  values listed in Table 15 plotted against the corresponding concentrations of Tris. The straight line drawn through the data was obtained by the method of least squares, and has standard errors of 3.8 and 1.8% in its slope and intercept, respectively. The maximum rate of formation of p-nitrophenol (and hence the maximum rate of disappearance of substrate) increases linearly with increasing concentrations of Tris at least for concentrations of Tris below 0.5M.

Figure 29

$V_{\max}$  plotted as a function of concentration  
of added Tris.  $T = 25.0^{\circ}\text{C}$ ,  $\text{pH } 10.0$ ,  $I = 1.0\text{M}$ .



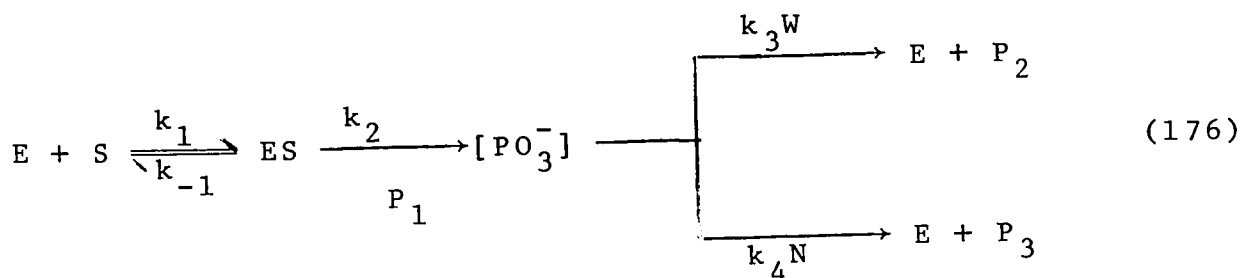
## DISCUSSION

One important conclusion of the present work is that when two very different phosphate esters are hydrolyzed by the enzyme in the presence of the competitive nucleophile Tris, the ratio of the rates of production of the products O-phosphoryl-Tris and inorganic phosphate is the same ( $\sim 2.1$ ) for both substrates. Similarly with ethanolamine the ratio was the same ( $\sim 1.5$ ) for both substrates. These results agree with those obtained by Barrett, Butler and Wilson (154) for the E.coli enzyme. These results are consistent with Mechanism I, and are difficult to explain in terms of Mechanism II, in which the enzyme-substrate addition complex is directly converted into products.

Since the two substrates used have dissimilar reactivities - the non-enzymic hydrolysis of the p-nitrophenyl ester is 2.09 times as rapid as that of the corresponding phenyl compound (276) - it is highly unlikely that they would give a constant ratio of rates unless a common reaction intermediate is involved. In addition, the two competitive nucleophiles employed, Tris and ethanolamine, differ greatly in both structure and reactivity from the  $H_2O$  with which they compete: 1M Tris, for example, was shown to be more effective as a phosphoryl group acceptor than 55M  $H_2O$ . The constant ratio of rates obtained - despite the large differences in reactivity between the two substrates and also between water

and both added nucleophiles - therefore strongly suggests that a common intermediate occurs during the alkaline phosphatase catalyzed hydrolysis of phosphate esters.

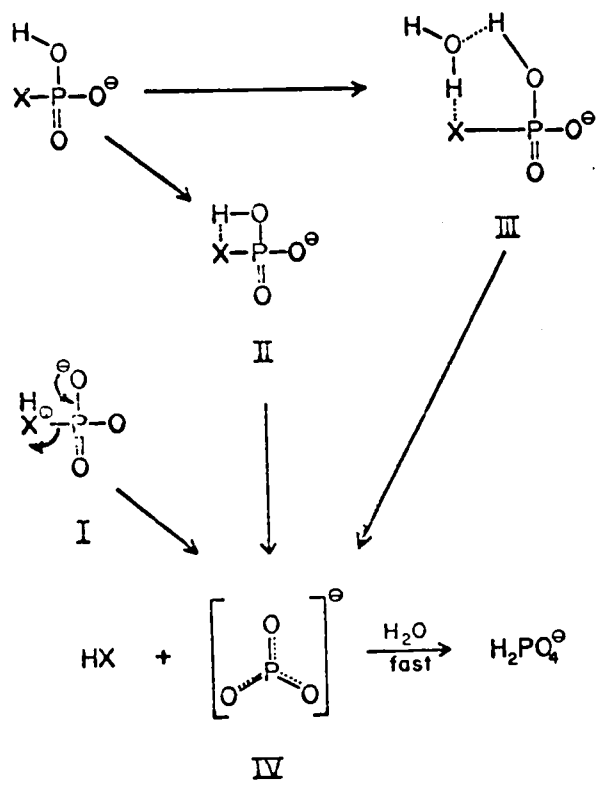
Although studies with competitive nucleophiles cannot, by themselves, reveal the identity of this common reaction intermediate, they do eliminate certain, otherwise reasonable, possibilities. For example, considerable evidence has accumulated that the non-enzymic hydrolysis of the monoanion of phosphate esters proceeds through the formation of a monomeric metaphosphate ion intermediate, which is then hydrolyzed to give inorganic phosphate, as shown in Figure 30 (276 - 281). The corresponding enzyme catalyzed reaction, it would therefore seem reasonable to assume, also proceeds via this hypothetical metaphosphate ion according to the following sequence of steps:



However, as Barrett et al. have pointed out (154), the postulated metaphosphate ion is an unstable, highly reactive species, whose formation is the rate determining step of the reaction. The rate of hydrolysis of substrate - equal to the rate of formation of  $P_1$  - should not under these conditions be affected by substances which speed up the solvolysis

Figure 30

Mechanism for the non-enzymic hydrolysis  
of the monoanion of phosphate esters



of the metaphosphate ion. Since added nucleophiles, such as Tris or ethanolamine, do, however, increase the rate of formation of  $P_1$ , Scheme 176 must be incorrect: the highly reactive metaphosphate species cannot be the common intermediate which occurs during the enzyme catalyzed hydrolysis of phosphate esters. This conclusion, it should be noted, is in no way altered if the formation of the metaphosphate ion is assumed to be a fast step in the overall reaction, since it would then be a transient, kinetically irrelevant species, incapable of influencing the rate of formation of products.

Active site labeling studies with compounds such as  $^{32}P$  - inorganic phosphate have provided some evidence for the existence of a phosphoryl-enzyme intermediate (113-125). Although these studies have conclusively demonstrated that a phosphoprotein, containing a phosphoryl group covalently attached to a uniquely reactive serine OH residue at its active site, is formed when the enzyme is incubated at  $pH < 6$  with low concentrations of  $^{32}P$ -inorganic phosphate or glucose-6-phosphate, by themselves they provide no evidence whatsoever for the involvement of a similar phosphoprotein in the enzyme catalyzed hydrolysis of phosphate esters. However, since studies with competitive nucleophiles show that a common intermediate is in fact involved, it seems reasonable to assume that this intermediate is the phosphoryl-enzyme species implicated in the  $^{32}P$ -phosphate labeling studies.

More convincing evidence in favor of this conclusion is available in the case of the E.coli enzyme. Using a rapid quenching technique in conjunction with scintillation counting Reid et al. (282) studied the phosphoprotein formed upon reacting the enzyme with  $^{32}\text{P}$ -labeled orthophosphate at  $\text{pH} < 8$ . By measuring the covalent labeling at different concentrations of orthophosphate they were able to determine the equilibrium constants for the dissociation of the enzyme-phosphate addition complex and for the hydrolysis of the covalent phosphoprotein. These same two quantities were also evaluated kinetically (219) - by studying the effect of ethanolamine and inorganic phosphate on the kinetics of the enzyme catalyzed reaction. The results of these two entirely different approaches were in good agreement: the properties of the kinetically deduced phosphoryl-enzyme intermediate were found to be essentially identical to those of the phosphoprotein obtained in  $^{32}\text{P}$ -orthophosphate labeling studies, indicating that the two species are in fact the same.

Similar experiments should, of course, be carried out with alkaline phosphatases from other sources, since only by means of this approach can it be established beyond a reasonable doubt whether or not the involvement of a phosphoryl-enzyme intermediate is a general property of all alkaline phosphatase catalyzed reactions.

The present results on the Michaelis parameters are not, however, consistent with Mechanism I as it stands. It has been seen that the maximum rate of formation of p-nitrophenol increases linearly with Tris concentration, whereas the rate of formation of inorganic phosphate, and the  $K_m$  value, are independent of Tris concentrations (cf. Figures 3-5). Reference to the figures shows that for Mechanism I the predicted behavior is as follows, depending on which step is rate-determining:

- (1) Phosphorylation rate-determining. The maximum rate of formation of p-nitrophenol should be independent of added nucleophile.
- (2) Dephosphorylation rate-determining. The Michaelis constant  $K_m$  should increase linearly with increasing nucleophile concentration.

Failure of the results to conform with these predictions shows that Mechanism I must be abandoned or seriously modified.

Although not previously pointed out, a similar difficulty is also encountered in the case of the E.coli enzyme. The maximum rate of formation of p-nitrophenol is a linear function of the concentration of added Tris or ethanolamine (152-3), implying that  $k_3$  is rate determining. Inconsistent with this conclusion, however, is the further observation that the Michaelis constant is entirely independent of Tris concentration (217). The situation is even further complicated by the observation that the virtual independence

of the rate of the enzyme catalyzed reaction of the leaving group of the substrate (145-151) suggests that  $k_3$  is rate determining, whereas the results of transient phase kinetic studies (130,138-140), as noted previously, lead to the exact opposite conclusion - that at  $\text{pH} > 7$  it is  $k_2$  which is rate determining.

Mechanism I is thus inconsistent with the evidence for both the chicken intestinal and E.coli enzymes. Alternative mechanisms have been explored in the theoretical section of this chapter, and are now considered with reference to the present experimental results.

Examination of Figures 2-14 indicates that there is only one mechanism which can explain the facts that  $P_1$  and  $P_3$  production are linear in  $N$ , and  $P_2$  production and  $K_m$  are independent of  $N$  and at the same time is consistent with other observed behavior\*: this is Mechanism VI, which is a modification of the Trentham-Gutfreund mechanism. In Mechanism VI there are two conformers of the phosphorylated enzyme,  $E^*S'$  and  $ES'$ , the former only can react with  $N$  and the latter only with water. Neglect of this factor does not lead to agreement with the experimental results. No subcases of the other mechanisms can lead to the observed behavior with added nucleophiles, and at the same time be consistent with other observed behavior.\*

---

\* For example, Mechanisms II and III can explain the behavior with added nucleophiles, but cannot explain the fact that the ratio of the rates of formation of  $P_2$  and  $P_3$  is independent of the leaving group of the substrate.

Nevertheless, Neumann (217) has suggested that the results can be reconciled with Mechanism I in the special case that  $k_2 \gg k'_3 \sim k_4 N$ . It will be seen from Figure 2 that that case, while predicting the correct behavior for  $k_{c1}$ ,  $k_{c2}$  and  $k_{c3}$ , requires that  $K_m$  should vary linearly with  $N$ , in conflict with the evidence. However, Neumann argues (217) that over the range of nucleophile concentrations employed the expected increase in  $K_m$  is smaller than the standard error in this parameter. That this argument cannot be substantiated is indicated by the following calculations.

From Eq. (13) it follows that  $K_m$  in the absence of  $N$ , i.e.,  $K_m^0$ , is given by

$$K_m^0 = \frac{K_s k'_3}{k_2} \quad (177)$$

so that

$$\frac{K_m}{K_m^0} = \frac{k'_3 + k_4 N}{k'_3} \quad (178)$$

The quantity  $k'_3$  is the intercept of the plot of  $k_c$  against  $N$  (cf. Figure 2), and  $k_4$  is the slope. The values so obtained for p-nitrophenyl phosphate, in the presence of Tris at pH 7.8,  $I = 1.0M$  and  $T = 25.0^\circ$  are  $k_4 \approx 5.0 \mu\text{moles min}^{-1} \text{mg}^{-1}/M$  and  $k'_3 = 0.5 \mu\text{moles min}^{-1} \text{mg}^{-1}$ ; when  $N = 0.50M$ , therefore, Eq. (178) leads to

$$\frac{K_m}{K_m^0} \approx 6.0 \quad (179)$$

which is well outside the experimental uncertainty. It is therefore concluded that Mechanism I, Case Ib, cannot be reconciled with the E.coli studies.

A somewhat similar type of argument has been involved by Reid and Wilson (144) in favor of Mechanism IV which they proposed. They state that this mechanism does predict that  $K_m$  should be independent of N since the factor

$$1 + \frac{k_6 K_N S'}{k_4'}$$

(cf. Eq. (69)) is 'more or less' independent of N. This argument again cannot be substantiated, as is shown as follows.

From Eq. (69) the Michaelis constant in the absence of N is

$$K_m^0 = \frac{k_{-5}}{k_2} K_s \quad (180)$$

so that

$$\frac{K_m}{K_m^0} = 1 + \frac{k_6}{k_4'} K_{E\beta} S' N \quad (181)$$

$$= \frac{k_{c1}}{k_{c2}} \quad (182)$$

(cf. Eq. (66) and (67)). According to Neumann (213), for the E.coli enzyme, when N = 0.5M Tris, pH = 7.8, I = 1.0M and T = 25.0°, the ratio  $k_{c1}/k_{c2}$  is ~6.0 for p-nitrophenyl

phosphate\*. Such a ratio of  $K_m/K_m^0$  is well outside the experimental uncertainty.

Additional evidence against the mechanism of Reid and Wilson has been provided by the three following observations of Halford (284):

(1) Identical rates of transient product formation with different substrates.

(2) The unaltered amplitude of this transient phase after preincubation with  $P_i$ .

(3) The binding of 2-hydroxy-5-nitrobenzylphosphonate to the enzyme at pH 8.0,  $I = 1.0M$ ; Reid and Wilson's mechanism predicts the wrong dependence of the relaxation time on the substrate and enzyme concentrations.

It is therefore concluded that Mechanism IV cannot explain Reid and Wilson's results.

Similar arguments relate to the mechanism proposed by Trentham and Gutfreund (our Mechanism V). For this mechanism

$$\frac{K_m}{K_m^0} = 1 + \frac{k_4 N}{k_3} \quad (183)$$

and again the results show that the mechanism predicts a variation of  $K_m$  with  $N$  that is well outside the experimental uncertainty.

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\* This value of 6.0 was calculated from Neumann's plot of  $V_{max}$  for the formation of p-nitrophenol against  $N$ .  $V_{max}$  for the formation of phosphate at any  $N$ , according to the mechanism of Reid and Wilson, is equal to  $V_{max}$  for the formation of p-nitrophenol at  $N=0$ .

It can similarly be shown that the other mechanisms are inconsistent with the present results. Thus for Mechanism I,  $K_m/K_m^0$  is given by Eq. (178).

For p-nitrophenyl phosphate in the presence of Tris at pH 10.0, I = 1.0M and T = 25.0°C the constants are

$$k_4 = 1.12$$

$$k'_3 = 0.504$$

and it then follows that  $K_m/K_m^0$  at N = 0.5M should be 2.11; this is well outside the standard error of 2.8%. A similar argument applies to other mechanisms.

APPENDIX I TO CHAPTER II

EVIDENCE FOR THE INVOLVEMENT OF A SPECIFIC SITE  
FOR WATER AND ADDED NUCLEOPHILES IN  
REACTIONS CATALYZED BY VARIOUS HYDROLASES

The last three mechanisms considered involved the specific binding of water and the added nucleophile to the enzyme molecule during catalysis. Although it has not yet been established that these two leaving group acceptors do in fact act from a specific site rather than directly from solution, there have been several promising approaches to this problem, the most successful of these to date being the ones listed in Table 16.

The most common approach, by far, involves studying the transferase activity of the enzyme in the presence of various water analogs. Such an approach was in fact first applied by Morton (106) to various phosphohydrolases, including purified intestinal alkaline phosphatase. Morton found that in reactions catalyzed by the latter enzyme in the presence of either glycerol or glucose (1) there was a marked specificity in the nature of the products formed, (2) the percentage transphosphorylation at first increased with increasing concentration of added nucleophile, but eventually levelled off, and (3) the amount of transphosphorylation also depended on the nature of the added

TABLE 16

Evidence for the Involvement of a Specific Site for Water  
(and Added Nucleophiles) in Reactions Catalyzed by Various Hydrolases

Enzyme	Source	Type of Evidence	Details	Ref.
Myosin	Rabbit Muscle	Selectivity toward Nucleophiles	S = ATP; N = [ <sup>14</sup> C]-MeOH Products measured by isotropic procedures	170
5'- Nucleotidase	Snake Venom	Specificity for H <sub>2</sub> O	S = ADP; N = various alcohols, e.g. glycerol	285
Glutaminase	<u>E. coli</u>	Effect of added N on Kinetic Parameters and Product Distri- bution	S = glutamine; N = NH <sub>2</sub> OH	192, 193
Esterase	Beef Liver	Effect of N on Kinetic Parameters and Product Distri- bution	S = methyl-β-proprionate MP-d-L, MP-ℓ-L N = IleOMe, GlyOMe	174
Chymotrysin	Pig Liver	Effect of N on Kinetic Parameters and Product Distri- bution	S = phenyl acetate, and OMeEtAc N = methanol	175
		Effect of N on Kinetic Parameters and Product Distri- bution	S = Ac-L-Phe[ <sup>14</sup> C]OMe N = MeOH	286

Table 16 continued

Effect of N on Rate of Deacylation	Studied deacylation of furoyl-chymotrypsin N = series of alcohols (e.g. MeOH) and amines (e.g. n-hexylamine)	184
Crystallographic Study	2.5 Å resolution of indoleacryloyl- $\alpha$ -Cht	294
Crystallographic Study	2.5 Å resolution of Tosyl- $\alpha$ -Cht	295
Effect of N on Kinetic Parameters and Product Distribution	S = BAEE, BAME, TSAME, and APAME;	178
Effect of N on Rate of Deacylation of Cinnamoyl-Papain	N = MeOH and EtOH	190
Selectivity toward added N	N = H <sub>2</sub> O, MeOH and BzOH	191
Action of Diastereoisomeric Peptides of alanine	N = tetra-, tri-, di-, and monoglycine amides	191
Effect of N on Kinetic Parameters and Product Distribution	S = D, L-alanine oligopeptides of known stereochemistry	288, 289
Effect of N on Kinetic Parameters and Product Distribution	S = EtH, and PNPH;	189
	N = ethanol	

Trypsin

Papain

Table 16 concluded

Effect of N on Kinetic Parameters and Product Distribution	S = PNP-N-Ac- $\beta$ -Trp N = MeOH, 1-propanol, 2-propanol, 1-butanol, and 1-pentanol	244
Effect of Solvents on Rate of Deacylation of -acryloyl-Papains	Studied deacylation of furylacryloyl, indolyl acryloyl, and trans-cinnamoyl-papains in non-reacting organic solvents	287
Effect of N on Kinetic Parameters	S = ZGlyPe and p-SGlyPN N = TrpNH <sub>2</sub> and glycynamide	292

ABBREVIATIONS USED

ATP	adenosine triphosphate
ADP	adenosine diphosphate
FDP	fructose 1,6-diphosphate
CP	cytidine phosphate (i.e., 2':3'-phosphate)
MP-d-L	methyl 3-phenyl D-lactate
MP-l-L	methyl 3-phenyl-L-lactate
IleOMe	L-isoleucine methyl ester
GlyOMe	glycine methyl ester
OMeEtAc	methoxyl ethyl acetate
Ac-l-Phe[ <sup>14</sup> C]OMe	acetyl-L-phenylalanine [ <sup>14</sup> C] methoxyl ester
BAME	α-N-benzoyl-L-arginine methyl ester
BAEE	α-N-benzoyl-L-arginine ethyl ester
TSAME	α-N-toluene-sulfonyl-L-arginine methyl ester
APAME	α-N-acetyl-L-phenylalanine methyl ester
EtH	ethyl hippurate
PNPH	p-nitrophenyl hippurate
ZGlyPN	carbobenzoyglycine-p-nitrophenyl ester
ZGlyPE	carbobenzoyglycine phenyl ester
TrpNH <sub>2</sub>	L-tryptophanamide

nucleophile. In marked contrast, other water analogs such as sucrose were not phosphorylated by the enzyme. Furthermore, an increase in the concentration of sucrose caused a decrease in the rate of the enzyme-catalyzed hydrolysis of the substrate. The above observations, Morton pointed out, may be readily interpreted if the existence of a specific water and added nucleophile binding site is assumed; however, alternative explanations involving a consideration of the various non-specific solvent and steric effects are also available.

As Table 16 shows, the same type of approach has also been used to elucidate the specific role of water in reactions catalyzed by various other hydrolases including 5'-nucleosidase (285), beef and pig liver esterase (174,175), trypsin (178), papain (189,244), and glutaminase (192,193).

The rather serious limitation of Morton's approach—that it is experimentally feasible only when the added nucleophile is considerably more reactive than water—may often be overcome by making use of a radioactively labeled nucleophile and analyzing for the resulting transphosphorylation product by means of the isotope dilution method. Koshland and Herr, who introduced this approach (170), applied it first to the study of the specific role of water in the myosin-catalyzed hydrolysis of ATP. They found that water was more reactive than methanol in the enzyme-catalyzed reaction by a factor of  $10^3$ , in striking contrast to the

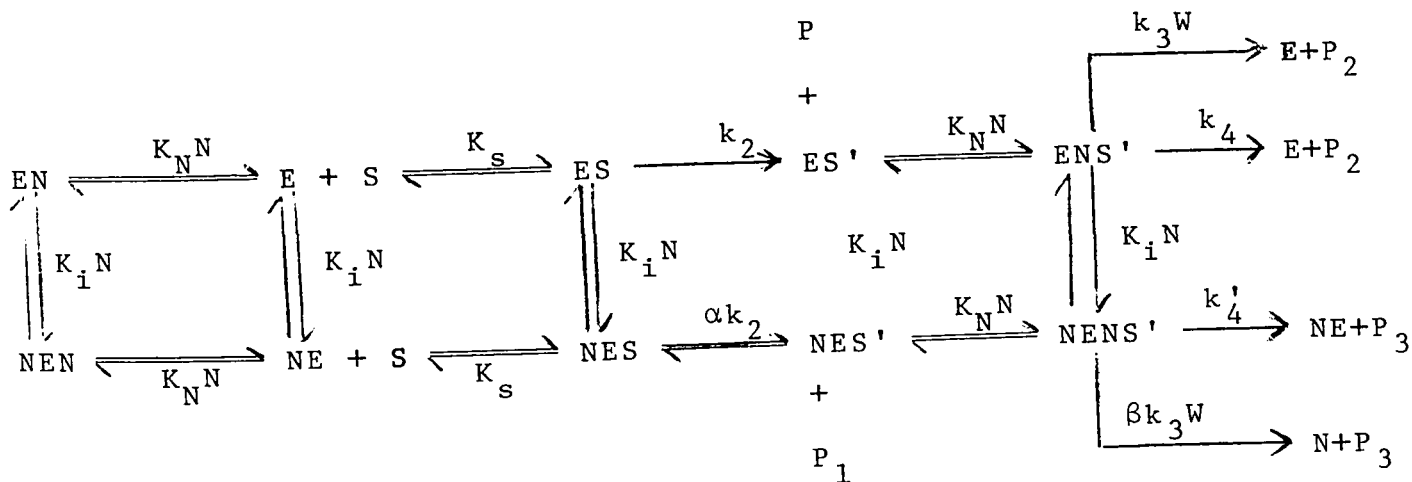
non-enzymic reaction. The conclusion arrived at was that myosin probably contains a specific site for water on its surface, although other explanations consistent with the above results do exist. One possibility, for example, is that the site for ATP is easily accessible to water, but not to methanol as a result of strong steric hindrance.

The same approach was later used by Bender and Glasson (286) to demonstrate the involvement of a specific binding site for water and the added nucleophile in  $\alpha$ -chymotrypsin-catalyzed reactions.

Another important modification of Morton's approach involves studying the effect of added nucleophiles on the covalent intermediate formed during the enzyme-catalyzed reaction (184,287). Brubacher and Bender (190), for example, studied the selectivity of the non-specific acyl-enzyme, trans-cinnamoyl-papain, toward added nucleophiles. The absence of an obvious correlation between the basicity of the nucleophile and its effect on the rate of deacylation of the acyl-enzyme led them to conclude that the added nucleophile and, by analogy, the water molecule, must bind to the enzyme before taking part in deacylation. In fact, the results of the systematic added-nucleophile study recently carried out by Fink and Bender (244), using specific substrates and alcohols and amines of widely varying structure, indicate that the binding of the added nucleophile to papain occurs even prior to acyl-enzyme formation: apparently, the

added nucleophile binds to the free enzyme and to the enzyme-substrate complex in addition to the acyl-enzyme (Mechanism IX).

Unfortunately, even Mechanism IX, Fink and Bender were careful to point out, is in all probability also a gross oversimplification of the actual reaction mechanism, since it can by no means accommodate all the relevant experimental data. On the basis of competition experiments between 2-propanol and 1-pentanol, and from a consideration of the exacting steric requirements in the active center, Fink and Bender concluded that there are two different ways in which added nucleophiles can bind to papain. One mode of binding overlaps that of the substrate leaving group, while the other interferes with substrate binding, as depicted below:



$K_N$  is the dissociation constant for the nucleophile bound to the  $P_1$  site, and  $K_i$ , that for the nucleophile bound to the other site.

The problem with this last mechanism is that it cannot be adequately tested by kinetic methods, since it postulates the existence of certain kinetically unimportant species. Nevertheless, kinetic studies have provided some evidence for multiple binding sites on the surface of the enzyme (288-291) and for the non-productive mode of binding (292,293) predicted by the mechanism.

More detailed information about these binding sites and the mode of attachment of water and added nucleophiles to them will almost certainly come from X-ray crystallographic studies. In fact, Steitz, Henderson and Blow (294, 295) have already used this approach to obtain useful information about the way the water molecule which takes part in the deacylation step is attached to  $\alpha$ -chymotrypsin during the reaction. Their results showed that one particular water molecule - apparently the same one which participates in deacylation - is firmly bound to the enzyme in a very specific manner: hydrogen-bonded both to the carbonyl oxygen of the acyl group and to the imidazole of His-57.

Specific Nucleophile and Water Binding Sites for Alkaline Phosphatase

Alkaline phosphatase exhibits marked specificity toward different added nucleophiles (22,217). Ethanolamine, which contains both an amino and a hydroxyl group, is a good phosphoryl group acceptor. Since phosphorylation occurs at the hydroxyl group, substances such as ethylamine, glycine, cysteamine, and ethylenediamine, in which this group is either absent or has been replaced by an amino group, are not acceptors. However, compounds such as methanol or ethanol, which contain this one functional group only, are not acceptors either. A good acceptor, apparently, must also contain a second hydroxyl or amino group. These groups, this last observation suggests, are required to properly bind the acceptor molecule to the surface of the enzyme, which it appears, contains a specific nucleophile binding site.

The existence of such a site is also suggested by the further observation that the distance between the two functional groups on the molecule and their stereochemistry are of critical importance in determining its phosphotransferase activity. The two groups, apparently, cannot be separated by more than three carbon atoms: 4-aminobutanol, for example, is completely inactive. Further evidence in favor of a specific nucleophile binding site may, perhaps, be adduced from the fact that dihydroxy alcohols generally decrease the rate of formation of  $P_1$ , whereas the corresponding amino alcohols increase the rate.

Also directly relevant to the present discussion is the discovery by Tait and Vallee (222) that certain organic and inorganic modifications of the E.coli enzyme affect the hydrolase and phosphotransferase activities differently. One important inorganic modification consisted of completely replacing the Zn(II) content of the enzyme by Co(II). Although the Co(II) enzyme obtained in this way was an active hydrolase, it had no measurable phosphotransferase activity. Reaction of the zinc enzyme with a 22-fold molar excess of N-bromosuccinimide more than doubled the phosphotransferase activity, but only slightly increased the hydrolase activity. In contrast, exposure of the cobalt enzyme to a fifty-fold excess of this reagent increased the hydrolase activity three-fold and, at the same time, remarkably, also generated measurable phosphotransferase activity.

These findings can readily be accommodated by the hypothesis that phosphotransferase activity requires the specific binding of the acceptor molecule to the enzyme. Certain inorganic and organic modifications, it is not unreasonable to assume, may alter its ability to bind the acceptor and possibly also the water molecule, thereby changing its phosphotransferase activity and, in some cases, the phosphotransferase to hydrolase ratio. According to this hypothesis, the reason the cobalt enzyme does not exhibit any measurable phosphotransferase activity at

pH 8.0 (even though the stopped-flow data obtained by Gottesman et al. (141) indicate that dephosphorylation is rate determining at that pH) is that the metal atom is involved in the binding of the acceptor molecule to the enzyme. The substitution of Co(II) for Zn(II) will therefore interfere with the proper binding of the acceptor to the enzyme, abolishing its phosphotransferase activity. Alternatively, however, it is possible, as Tait and Vallee have suggested, that substituting Co(II) for Zn(II) in the enzyme drastically alters the environment about its active site, making the organic acceptor molecule completely inaccessible to the phosphorylated serine hydroxyl group, thereby eliminating all phosphotransferase activity.

Finally, it is worth mentioning the possibility that the reason the phosphotransferase activities of nucleophiles such as Tris and ethanolamine differ is that they are actually involved in different reaction mechanisms. Reactions carried out in the presence of Tris, it was shown, do follow Mechanism VI. However, reactions carried out in the presence of higher molecular weight nucleophiles, such as ethanolamine, may proceed via Mechanism IX - Bender's reaction scheme. Since Mechanism IX is kinetically distinguishable from the previous reaction mechanism, studying the detailed kinetic behavior of the enzyme-catalyzed reaction in the presence of some higher molecular weight acceptors, especially dihydroxy alcohols, may result in more concrete evidence for the

involvement of a specific nucleophile binding site in the reaction. If such a site is found to exist, then the effect of systematically varying the structure of the added nucleophile should reflect the specificity of this site, and should thereby provide a better understanding of the enzyme-catalyzed reaction.

### CHAPTER III

## THE INFLUENCE OF pH ON THE STEADY-STATE KINETICS OF REACTIONS CATALYZED BY ALKALINE PHOSPHATASE

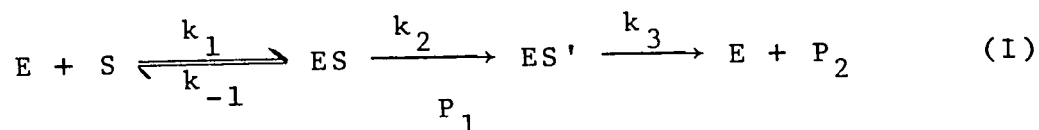
### INTRODUCTION

#### Theoretical Principles

Establishing the mechanism of an enzyme-catalyzed reaction requires a knowledge of the amino acid residues involved. At least part of this information may be obtained from a systematic study of the effects of pH on the kinetic parameters of the reaction. Such a study allows one to evaluate the dissociation constants of certain ionizing groups, at or near the active center of the enzyme, which participate directly or indirectly in catalysis. Not all the participating groups, however, will necessarily be revealed by this kinetic approach; only those groups whose change in state of ionization has some effect on the rate of reaction - i.e., groups which ionize in the initial state and whose ionization affects the ease of formation of the activated state - will be revealed.

It was demonstrated in the previous chapter that reactions catalyzed by alkaline phosphatases proceed via a phosphoryl-enzyme intermediate. In this respect, it was pointed out, alkaline phosphatase resembles many other

enzymes such as the phosphotransferases, many proteolytic enzymes (e.g., trypsin and chymotrypsin), and many other esterases (e.g., acetylcholinesterase). The hydrolytic, phosphoryl-, or acyl- transfer reactions catalyzed by these enzymes apparently all proceed through phosphoryl- or acyl-enzyme intermediates according to the general scheme.



Here ES is the enzyme-substrate addition complex, and ES' is the phosphoryl- or analogous acyl-enzyme intermediate; P<sub>1</sub> is the first product, the alcohol moiety, ROH, of the substrate S, and P<sub>2</sub> is the second product, such as inorganic phosphate.

Application of the steady-state treatment to Scheme I leads to an equation of the Michaelis-Menten form

$$v = \frac{\tilde{k}_c E_o S_o}{\tilde{K}_m + S_o} \quad (1)$$

where E<sub>o</sub> and S<sub>o</sub> are the total enzyme and substrate concentrations and  $\tilde{k}_c$  and  $\tilde{K}_m$  are constants at a given pH; the symbol ~ indicates that they are usually pH-dependent quantities. At high substrate concentrations (S >>  $\tilde{K}_m$ ) the pH dependence of the overall rate is that of  $\tilde{k}_c$ , while at low substrate concentrations (S <<  $\tilde{K}_m$ ) the pH dependence is that of  $\tilde{k}_c/\tilde{K}_m$ .

The Michaelis parameters,  $\tilde{k}_c$  and  $\tilde{K}_m$ , are given by

$$\tilde{k}_c = \frac{\tilde{k}_2 \tilde{k}_3}{\tilde{k}_2 + \tilde{k}_3} \quad (2)$$

and

$$\tilde{K}_m = \left( \frac{\tilde{k}_3}{\tilde{k}_2 + \tilde{k}_3} \right) \tilde{K}_s \quad (3)$$

where

$$\tilde{K}_s = (\tilde{k}_{-1} + \tilde{k}_2) / \tilde{k}_1 \quad (4)$$

Some of the kinetic consequences of this two-intermediate scheme have been considered by Zerner and Bender (296). They pointed out that in this scheme  $\tilde{k}_c$  and  $\tilde{K}_m$  depend on a number of non-separable individual rate constants. The interpretation of these Michaelis parameters may therefore be fundamentally different from, and not as well-defined as, that associated with those of the classical Michaelis-Menten scheme. In particular, the physical significance of  $\tilde{k}_c$  and  $\tilde{K}_m$  will depend on the relative values of the two individual rate constants,  $k_2$  and  $k_3$ .

Krupka and Laidler (297) have discussed the pH dependence of the overall rates in terms of the nature of the ionizations of E, ES, and ES', taking into consideration the various possible rate-determining steps. Webb (298) has extended this treatment to cases in which the substrates or

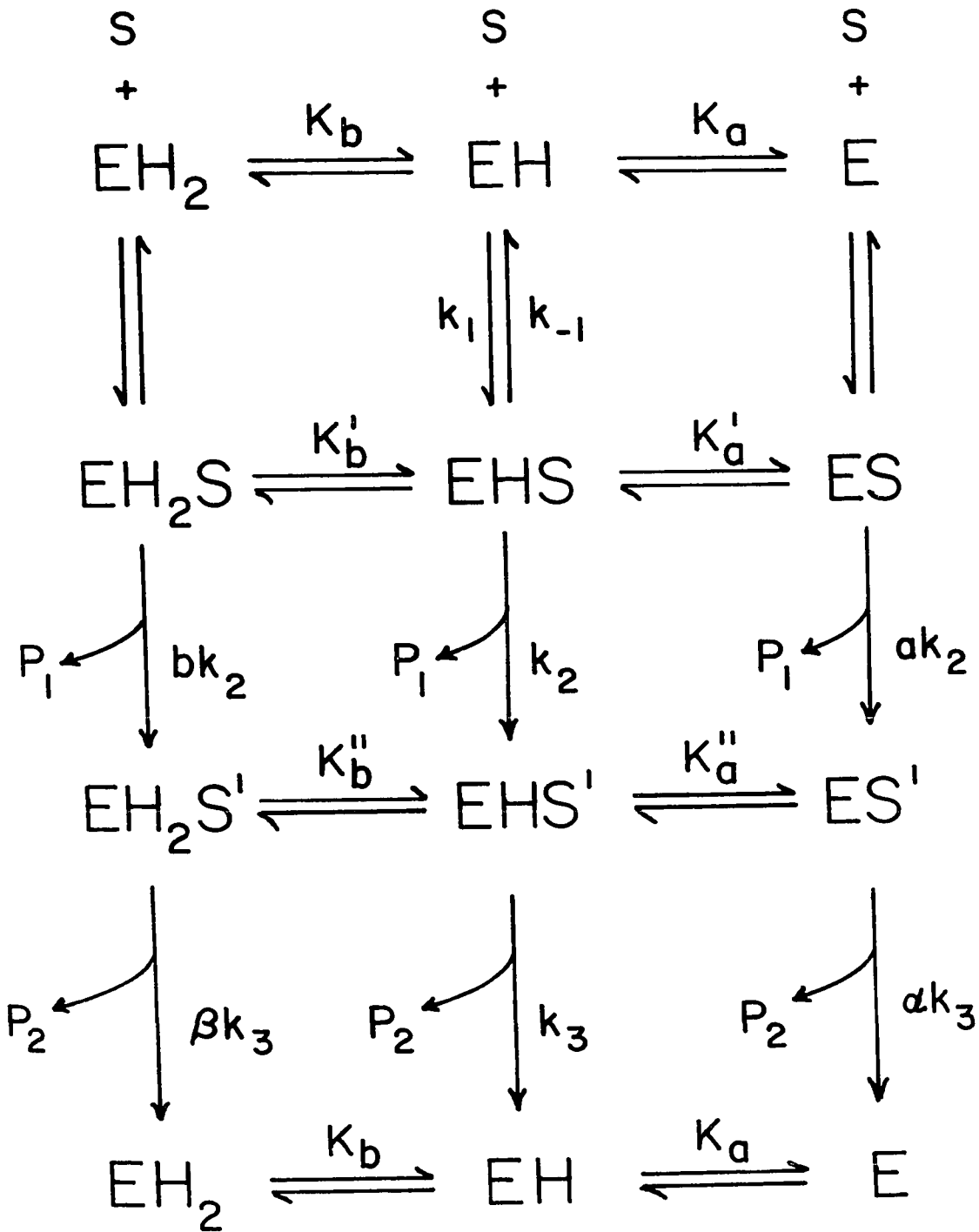
inhibitors are charged. More recently, Kaplan and Laidler (299) have developed equations showing the pH dependence of the Michaelis parameters in a form which distinguishes between essential and non-essential ionizing groups.

### General Reaction Scheme

A general reaction mechanism involving two ionizing groups is shown in Figure 31. In this scheme (which will be referred to as Scheme II)  $\text{EH}_2$ ,  $\text{EH}$ , and  $\text{E}$  are the three different ionization states for the active center.  $K_a$  and  $K_b$  are both dissociation constants for the free enzyme, and represent the ionization constants of an acid and a basic group, respectively, while  $K'_a$ ,  $K'_b$ ,  $K''_a$ , and  $K''_b$  are the ionization constants of the same groups in the Michaelis complex and the covalent phosphoryl- or acyl-enzyme, respectively. The processes of breakdown of the Michaelis complex and of the covalent intermediate are shown to be irreversible, since under initial conditions the concentrations of  $\text{P}_1$  and  $\text{P}_2$  are so small that the reverse reactions may be neglected. An important feature of this scheme, which was first written down in this form by Kaplan and Laidler (299), is that it allows for the breakdown of different ionization states of both the Michaelis-Menten complex and the second intermediate: the constants  $a$ ,  $b$ ,  $\alpha$ , and  $\beta$  may take on any value between zero and unity. Because of this feature Scheme II readily distinguishes between essential and non-essential ionizing

Figure 31

A general scheme for pH dependence in which the formation of a second intermediate is considered.



groups. If, for example,  $a = 1$ , then that ionizing group is not essential for activity; on the other hand, if  $a = 0$ , it is essential and a fall-off in the rate constant for the formation of  $ES'$  will be observed at low pH.

An approximate steady-state treatment, which assumes that the horizontal reactions, representing ionizations, are much more rapid than the vertical ones, which represent chemical transformations, lead to the following expressions for the kinetic parameters:

$$\tilde{k}_c = \frac{k_2}{1 + \frac{K'_a}{H} + \frac{H}{K'_b}} + \frac{k_2}{k_3} \left[ \frac{1 + \frac{K''_a}{H} + \frac{H}{K''_b}}{1 + \alpha \frac{K''_a}{H} + \beta \frac{H}{K''_b}} \right] \quad (5)$$

$$\tilde{K}_m = \frac{K_s \left[ \frac{1 + \frac{K_a}{H} + \frac{H}{K_b}}{1 + \frac{K'_a}{H} + \frac{H}{K'_b}} \right]}{1 + \frac{K'_a}{H} + \frac{H}{K'_b}} + \frac{k_2}{k_3} \left[ \frac{1 + \frac{K''_a}{H} + \frac{H}{K''_b}}{1 + \alpha \frac{K''_a}{H} + \beta \frac{H}{K''_b}} \right] \quad (6)$$

and

$$\frac{\tilde{k}_c}{\tilde{K}_m} = \frac{k_2}{K_s} \left[ \frac{1 + a \frac{K'_a}{H} + b \frac{H}{K'_b}}{1 + \frac{K_a}{H} + \frac{H}{K_b}} \right] \quad (7)$$

The pH Dependence of  $\tilde{k}_c/\tilde{K}_m$

When  $S_o \ll K_m$ , Eq. (1) reduces to

$$v = \frac{\tilde{k}_c}{\tilde{K}_m} E_o S_o \quad (8)$$

i.e., at low substrate concentrations the pH dependence of the overall rate is that of the ratio  $\tilde{k}_c/\tilde{K}_m$  - the second order rate constant for the process  $E + S \rightarrow ES$ . The way in which this ratio varies with pH is shown in Eq. (7). The following three cases, previously discussed by Kaplan and Laidler (1999), are of special interest:

Case I. The enzyme-substrate complex, ES, ionizes in the same way as does the free enzyme, E (i.e.,  $K'_a = K_a$ , and  $K'_b = K_b$ ) and  $a = b = 1$ . This is the case of the non-essential ionizing groups. Even if these groups are involved in subsequent reactions (e.g., in the breakdown of ES') they will not be revealed from the pH dependence of  $\tilde{k}_c/\tilde{K}_m$ .

Case II. If  $a = b = 0$  the pH dependence of  $\tilde{k}_c/\tilde{K}_m$  will reveal both  $K_a$  and  $K_b$  for the ionization of the free enzyme. If  $a = 0$ , but  $b \neq 0$ ,  $\tilde{k}_c/\tilde{K}_m$  will be pH dependent on the basic side, and the results will reveal  $K_a$ ; on the other hand, if  $b = 0$ , but  $a \neq 0$ , the pH dependence, which will under these conditions occur on the acid side, will reveal  $K_b$ .

Case III. If either  $K'_a = 0$  or  $K'_b = \infty$  (i.e., the corresponding group is not free to ionize in the Michaelis complex), the pH variation of  $\tilde{k}_c/\tilde{K}_m$  will then reveal either  $K_a$  or  $K_b$  for the ionization of the free enzyme. This is the case of an ionizing group which is essential to the binding of the substrate.

From the above discussion it follows that when  $K_a$  and  $K_b$  values are obtained from the pH dependence of  $\tilde{k}_c/\tilde{K}_m$  the corresponding groups not only ionize in the free enzyme but are also essential to the subsequent reaction of the enzyme-substrate complex - the formation of the covalent intermediate  $ES'$ . These same groups may also be involved in a subsequent stage of the reaction, e.g., breakdown of  $ES'$  into free enzyme and  $P_2$ , but this must be demonstrated in some other way, for example, by examining the pH profile of  $\tilde{k}_c$  if  $k_3$  is rate-limiting (see discussion below). However, regardless of which step of the reaction is rate-limiting, a study of the variation of  $\tilde{k}_c/\tilde{K}_m$  with pH will reveal the ionization groups involved in the process  $ES \rightarrow ES'$ .

### The pH Dependence of $\tilde{k}_c$

When  $S_o \gg K_m$ , Eq. (1) reduces to

$$v = \tilde{k}_c E_o \tag{9}$$

i.e., at high substrate concentrations the pH dependence of the overall rate is that of  $\tilde{k}_c$ . The way in which this

constant varies with pH is given by Eq. (5). This equation indicates that the physical significance of  $k_c$ , and, hence, the interpretation of its pH profile depends on the value of the ratio  $k_2/k_3$ . The following two special cases are of particular importance.

Case I. The second stage of the reaction,  $ES \rightarrow ES'$  is rate limiting (i.e.,  $k_2 \ll k_3$ ). Eq. (5) then reduces to

$$\tilde{k}_c = \frac{k_2}{\left(1 + \frac{K'_a}{H} + \frac{H}{K'_b}\right)} \quad (9)$$

provided  $a = b = 0$  (i.e., the ionizing groups are essential). The pH profile of  $k_c$  will reveal only those groups involved in stage 2 of the reaction - i.e., the pK values corresponding to the ionization of the Michaelis complex ( $pK'_a$  and  $pK'_b$ ).

Case II. If, on the other hand, stage 3 of the reaction,  $ES' \rightarrow E + P_2$ , is rate-limiting (i.e.,  $k_2 \gg k_3$ ), then Eq. (5) reduces, instead, to

$$\tilde{k}_c = \frac{k_3}{1 + \frac{K''_a}{H} + \frac{H}{K''_b}} \quad (10)$$

again provided that  $a = b = 0$  (i.e., provided the ionizing groups are essential). The pH profile of  $\tilde{k}_c$  will, under these circumstances, reveal only those groups involved in the third stage of the reaction - i.e., those pK values

corresponding to ionization of the covalent intermediate ( $pK''_a$  and  $pK''_b$ ).

By studying the pH variation of  $\tilde{k}_c$ , first using a substrate for which  $k_2 \ll k_3$  and also one for which  $k_2 \gg k_3$ , it should be possible, in principle, to determine four dissociation constants:  $K'_a$ ,  $K'_b$ ,  $K''_a$ , and  $K''_b$ .

### The pH Dependence of $\tilde{K}_m$

A comparison of Eqs. (5), (6), and (7) shows that  $\tilde{K}_m$  has a more complex pH dependence than either  $\tilde{k}_c$  or  $\tilde{k}_c/\tilde{K}_m$ :  $\tilde{K}_m$  depends on the ionization constants of the free enzyme and either those of the Michaelis-complex ( $k_2 \ll k_3$ ) or those of the covalent intermediate ( $k_2 \gg k_3$ ), or both ( $k_2 \sim k_3$ ). Since

$$\tilde{K}_m = \tilde{k}_c / (\tilde{k}_c / \tilde{K}_m) \quad (11)$$

or

$$p\tilde{K}_m = \log_{10}(\tilde{k}_c / \tilde{K}_m) - \log_{10}\tilde{k}_c \quad (12)$$

where  $p\tilde{K}_m$  is defined by Eq. (13):

$$p\tilde{K}_m = - \log_{10}\tilde{K}_m \quad (13)$$

the four pK values which can be determined from the pH profiles of  $\tilde{k}_c/\tilde{K}_m$  (i.e.,  $K'_a$  and  $K'_b$ ) and of  $\tilde{k}_c$  (i.e.,  $K''_a$  and  $K''_b$ , or  $K'_a$  and  $K''_a$  and  $K'_b$  and  $K''_b$ ) may, in theory, all be obtained from the variation of  $\tilde{K}_m$  with pH. In practice, however, the pH profile of  $\tilde{K}_m$  is often found to be less complex than the profiles

of  $\tilde{k}_c$  and  $\tilde{k}_c/\tilde{K}_m$ . The reason for this is that groups which ionize as strongly in the free enzyme as in the ES or ES' intermediate will not be detected in the  $\tilde{K}_m$  versus pH profile, since their effects on  $K_m$  will completely cancel out. If, for example,  $pK'_b = pK_b$ , the pH profile of  $\tilde{K}_m$  will show no fall-off at low pH. Only those groups associated with the active site whose ionizations in ES or ES' differ from those of the free enzyme will be revealed.

### Previous pH Studies

Studies of the effects of pH on the alkaline phosphatase-catalyzed reaction have been carried out with enzyme obtained from a wide variety of sources. Fernley in a recent review (300) has summarized some of the results obtained with mammalian enzyme. The large differences observed with the various enzyme preparations are, to a large extent, misleading: since the studies were not carried out under identical conditions the differences may not necessarily be due to intrinsic differences in the catalytic properties of the preparation, but, rather, to differences in ionic strength, nature and concentration of substrate, buffering and activating ions, and, possibly, even to differences in the assay techniques employed. These factors very often markedly affect the overall rate of the reactions, and alter its pH dependence (224). In fact, one characteristic feature of alkaline phosphatase is the upward shift in pH

optimum with increasing substrate concentration. Such a shift can readily be explained with reference to the pH dependence of  $\tilde{k}_c$  and  $\tilde{K}_m$  (see the following discussion); significant deviations from the predicted behavior do, however, occur at high substrate concentrations.

### The Patterns of pH Behavior

A wide variety of patterns of pH behavior have been observed with the different alkaline phosphatase. The variations in the dependence of  $\tilde{k}_c$  and  $\tilde{K}_m$  on pH are shown in Figure 32. Examples of each of these different types of pH dependence are given in Table 17, which summarizes and classifies the previous pH studies carried out with the various alkaline phosphatases. The lower case letters a, b, ..., k, which appear in this table correspond to those in Figure 32.

### The $pK_m$ versus pH Profiles

The following features of the  $pK_m$  versus pH profiles depicted in Figure 32 are worth noting:

- (1) There exists a range of pH in the alkaline region over which  $pK_m$  decreases with increasing pH. Plots of  $pK_m$  against pH in this region are sometimes found to have slopes which differ significantly from the theoretical value of -1 (302-4).

TABLE 17  
Effects of pH on the Kinetic Parameters of Reactions Catalyzed  
by Various Alkaline Phosphatases

Source of Enzyme	Substrate	Buffer System	Temp °C	pH Dependence		Ref
				k <sub>c</sub>	K <sub>m</sub>	
Calf Intestinal Mucosa	PhP	0.25M Veronal or Ethanolamine	38	-	a*	229
	CPP	0.067M Ethanol-amine; 0.0025M Mg	38	h	a	301
	PNPP	0.01M Tris or Ethanolamine	24.4	f	b	302
Bovine Synovial Fluid	MUFP	0.05M Ammediol I = 0.02, 0.2	20	g	c	303
	PNPP	0.10M Ammediol	37	f	b	304
Rat Kidney	PNPP	0.2M Tris	25	f	b	305
Pig Kidney	PNPP	0.05M Veronal; Mg Added	38	-	e	306
Bovine Milk	CPP, PhP	0.067 Ethanol-amine; 0.0025M Mg	38	f	a	301
Human Liver	PNPP	0.1M Ammediol or Tris	38	f	d	307

\* a, b, c, . . . ., etc. refer to the pH profiles in Figure 32

Table 17 - continued

Source of Enzyme	Substrate	Buffer System	Temp °C	Dependence	Ref
				k <sub>c</sub> k <sub>m</sub>	
Human Liver	PP <sub>i</sub>	0.1M Ammediol or Tris	38	f    d	307
Human Bone	PP <sub>i</sub>	0.1M Ammediol or Tris	38	i    d	307
	PNPP	0.1M Ammediol or Tris	38	h    a	307
Human Intestine	PNPP	0.1M Ammediol or Tris	38	f    a	307
	PP <sub>i</sub>	0.1M Ammediol or Tris	38	f    k	307
Human	PhP	0.05M Na <sub>2</sub> CO <sub>3</sub>	37	-    k	309
Rat Intestine	PhP	0.05M Na <sub>2</sub> CO <sub>3</sub>	37	i    d	308
	PNPP, DNPP	0.05M Tris	25	f    -	138
E. Coli	PNPP	0.10M Tris; I = 0.10, 1.0	25	f    -	218
	PNPP	0.01M Tris or Ethanolamine; Mg	25	f    c	225, 310
	PNPP	Barbital or Na <sub>2</sub> CO <sub>3</sub> , I = 0.27	25	f    a	311

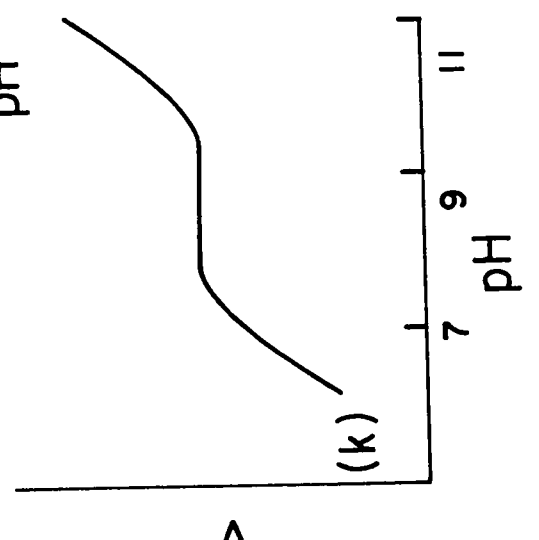
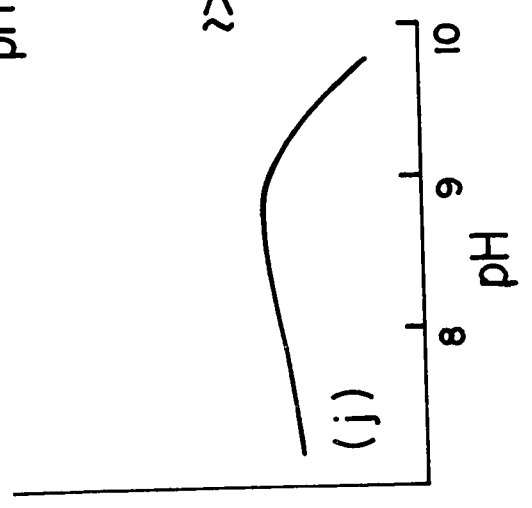
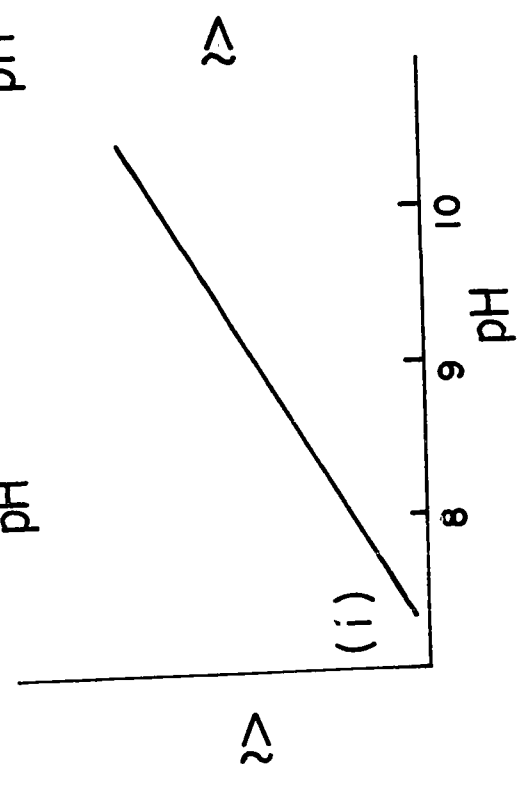
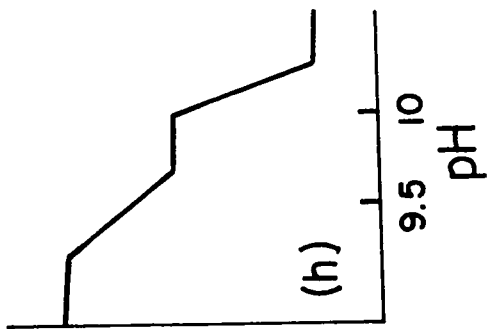
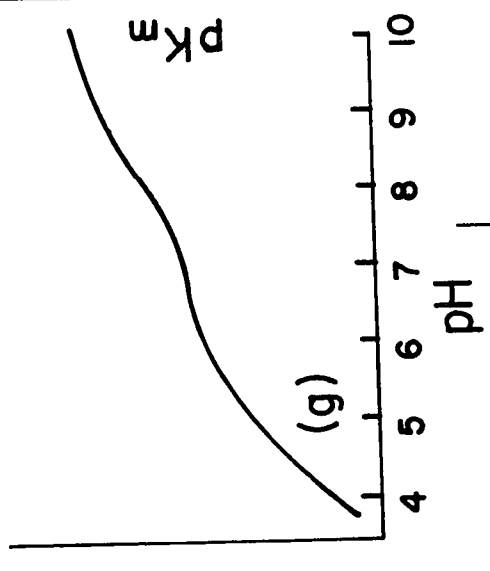
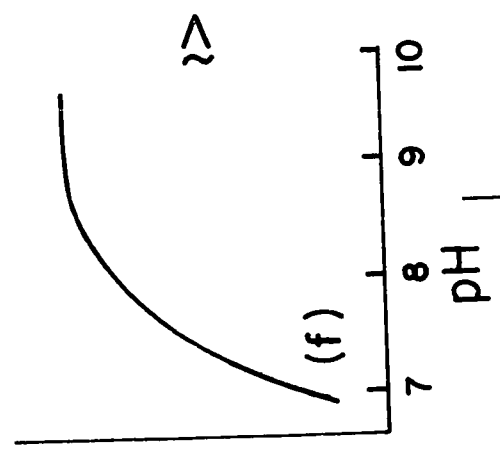
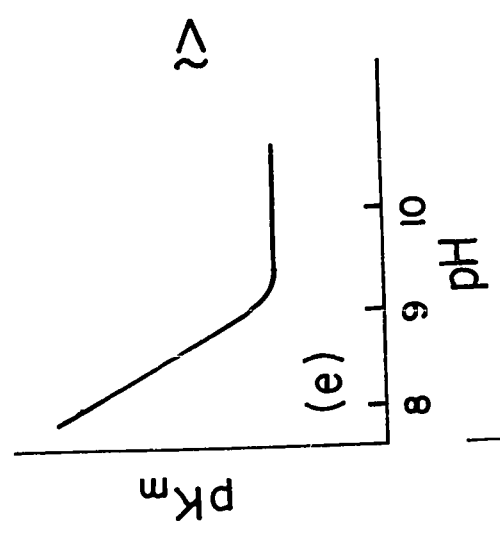
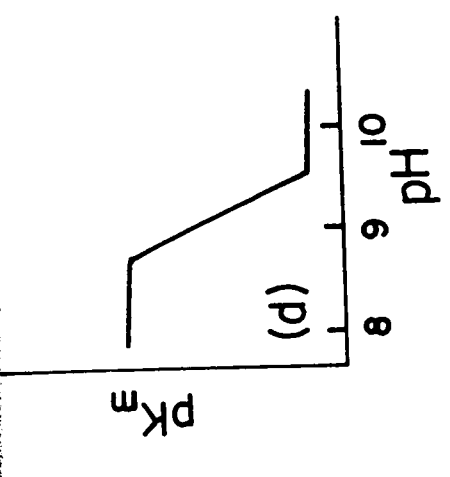
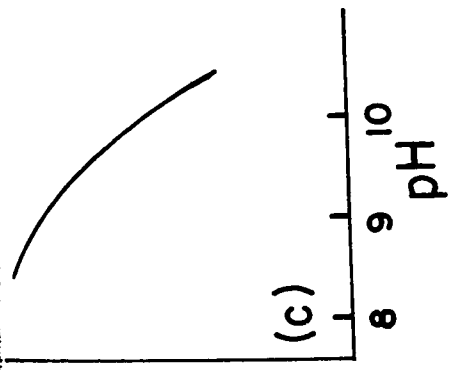
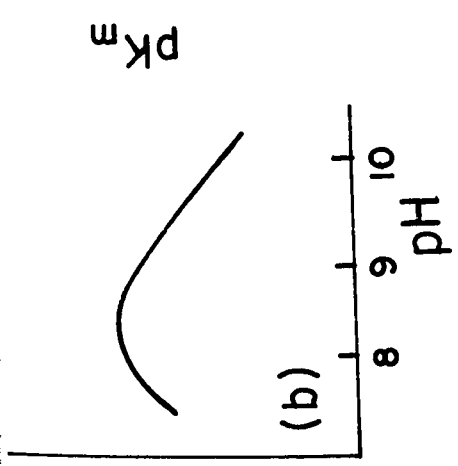
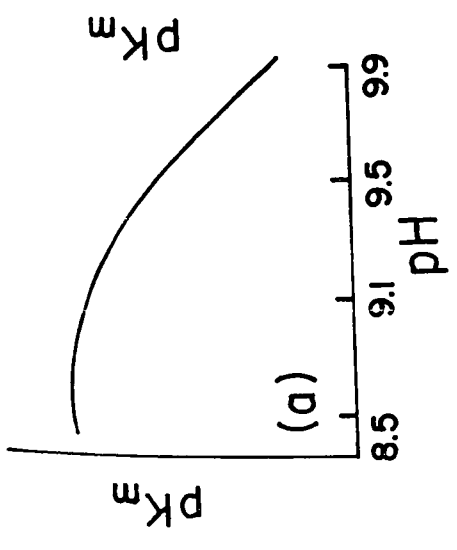
1957

Table 17 - concluded

Source of Enzyme	Substrate	Buffer System	Temp °C	pH Dependence	Ref
E.Coli	CPP	0.01M Tris, 0.4M NaCl	25	f -	151
E.Coli (Co <sup>2+</sup> )	CPP	0.01M Tris, 0.4M NaCl	25	f -	151
	CPP	0.02M Veronal or 0.01M Tris, 0.5M NaCl	25	- b	151
	PNPP, DNPP	0.01M MES or Tris or Glycine, I = 1	25	j a	141
Neurospora Crassa	PNPP	Na-maleate, Tris, or Na <sub>2</sub> CO <sub>3</sub>	37	g c	312

Figure 32

Patterns of pH Behavior Observed with the Various  
Alkaline Phosphatases.



(2) There exists a  $pH_{max}$ , at which  $pK_m$  is at a maximum (e.g., see Figure 3b); this maximum, however, may occur at  $pH < 7$ , and may therefore be impossible to observe (when  $pK_m$  continues to increase below  $pH 7$  it almost invariably is too large to be measured at  $pH_{max}$ ). Below  $pH_{max}$ ,  $pK_m$  either remains constant (229) or decreases with decreasing  $pH$  (302).

(3) The  $pH$  region over which  $pK_m$  decreases with increasing  $pH$  is sometimes followed at higher  $pH$  values by a region over which  $pK_m$  is essentially  $pH$ -independent (e.g., Figure 3d and Figure 3e). The  $pH$  at which this horizontal portion of the  $pK_m$ - $pH$  profile begins depends very much on the type of phosphatase used (cf. Figure 3d and Figure 3e).

### The $\tilde{V}$ versus $pH$ Profiles

Features of the  $\tilde{V}$  versus  $pH$  profiles shown in Figure 3 which are particularly worth noting are the following:

(1) A plot of  $\tilde{V}$  against  $pH$  is usually sigmoid in shape:  $\tilde{V}$  generally increases with increasing  $pH$  and approaches a limiting value at high  $pH$ . The acid limb of the  $\log \tilde{V}$ - $pH$  profile, however, often has a slope which differs significantly from the theoretical value of +1 (302-3).

(2) The  $pH$  at which  $\tilde{V}$  first reaches a maximum depend on the type of preparation used. In fact, with some phosphatases (301) this maximum is not attained even at  $pH 11$ .

(3) When the  $\tilde{V}$ -pH profile is studied at sufficiently low pH, a plateau is observed (e.g., Figure 3g or Figure 3j). The pH region over which this plateau occurs depends on the source of the enzyme: with calf-intestinal phosphatase the plateau starts at about pH 7 (303); in contrast, a broad plateau between pH 7-9 is found with both the phosphatase from *Neurospora crassa* (312) and the Co(II) enzyme of *E. coli* (141).

Dependence of Optimum pH on Substrate Concentration

The pH optimum of a reaction proceeding via Scheme II,  $pH_o$ , is defined by

$$H_o = \left[ \frac{K_a + \frac{S_o}{K_s} \left( K'_a + \frac{k_2}{k_3} K''_a \right)}{\frac{1}{K_b} + \frac{S_o}{K_s} \left( \frac{1}{K'_b} + \frac{k_2}{k_3} \frac{1}{K''_b} \right)} \right]^{1/2} \quad (13)$$

Eq. (13) is obtained by substituting the expressions for  $\tilde{V}$  and  $\tilde{K}_m$  - Eqs. (5) and (6) - into the Michaelis-Menten equation - Eq. (1), then setting the first derivative of the equation which results equal to zero, and solving for H.

The observed rise in  $pH_o$  with increasing  $S_o$ , which is a well-established characteristic of the various alkaline phosphatases ( 229-240 ), is wholly consistent with Eq. (13) in view of the observed pH dependence of the Michaelis parameters: the sigmoid  $\tilde{V}$  versus pH profile indicates that

(1) either  $K'_b$  or  $K''_b$  or both are not infinite and that (2) either  $K'_a$  or  $K''_a$  or both are equal to zero. It can readily be verified\* that according to Eq. (13) if either  $K'_b$  or  $K''_b \neq \infty$ , but  $K'_a = K''_a = 0$ , then  $pH_o$  will rise with increasing  $S_o$ . At high substrate concentrations (i.e.,  $S_o \gg \tilde{K}_m$ ), it should be pointed out, the shape of the pH-activity profile of alkaline phosphatase is to a large extent determined, not by the pH dependence of  $\tilde{V}$  and  $\tilde{K}_m$ , but, rather, by the strong inhibition from excess substrate (229,232,233, 239).

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\*There appear to be several typographical errors in Reference (297) in the section dealing with  $pH_o$ . This section should read as follows:

"Eq. (13) indicates that  $pH_o$  will be independent of  $S_o$  if

$$(1) K'_a = K''_a = 0 \quad \text{and} \quad K'_b = K''_b = \infty,$$

or if

$$(2) (K'_a + \frac{k_2}{k_3} K''_a) \frac{1}{K_a} = (\frac{1}{K'_b} + \frac{k_2}{k_3} \frac{1}{K''_b}) K_b$$

If  $K'_b = K''_b = \infty$ , but either  $K'_a$  or  $K''_a$  or both  $\neq 0$ ,  $pH_o$  will decrease with increasing  $S_o$ , while if  $K'_a = K''_a = 0$ , but either  $K'_b$  or  $K''_b$  or both are not infinite, it will instead rise with increasing  $S_o$ ."

## EXPERIMENTAL

### Choice of Experimental Conditions

The present investigation was undertaken to obtain a consistent set of pH profiles for chicken-intestinal phosphatase and to compare them with the ones obtained previously with the enzyme from other sources. Although the rise in  $\text{pH}_0$  with increasing  $S_0$  has already been confirmed for chicken-intestinal phosphatase (232-3), more detailed kinetic studies have not yet been carried out with this enzyme.

Because of the very small values of  $\tilde{K}_m$  involved, most of the previous kinetic studies of the alkaline phosphatases have been carried out, at least in part, under the conditions that  $S_0 > \tilde{K}_m$  where, as a result of either strong substrate inhibition (232-4,239) or substrate activation (148,223,315), the Michaelis-Menten equation is no longer obeyed. Special care was therefore taken in the present study to determine the Michaelis parameters under the conditions that  $S_0 < \tilde{K}_m$ . The choice of p-nitrophenyl phosphate as substrate together with the use of the scale expansion attachment of the spectrophotometer, when necessary, made this possible. An added advantage was that the time course of the reaction could be followed continuously with the spectrophotometer so that true initial rates could readily be measured. Because of strong inhibition by products,

particularly inorganic phosphate (140,146-7), it is absolutely essential that only true initial rates be used in determining the Michaelis parameters.

The choice of buffer was also a matter of considerable importance. It was pointed out in the previous chapter (Chapter II) that certain amines with suitably located hydroxyl groups, such as Tris and ethanolamine, noticeably enhance the alkaline phosphatase - catalyzed hydrolysis of substrate. Compounds such as these could therefore not be used as buffers in the present study. Substances such as borate and carbonate which inhibit the enzyme (316-18) should, if possible, also be avoided. Preliminary experiments indicated that low concentrations of barbital (5,5-diethyl barbituric acid) neither activated nor inhibited the alkaline phosphatase catalyzed reaction. This substance was therefore the buffer used between pH 7.0-9.6. For this range of pH a concentration 0.010M was found to give adequate buffering, especially for the measurement of initial rates. The buffer used above pH 9.6 was, of necessity, sodium carbonate. Since this substance is known to act as a competitive inhibitor, leaving  $\tilde{V}$  unaltered but markedly increasing  $\tilde{K}_m$ , the  $\tilde{V}/\tilde{K}_m$  values above pH 9.6 were not included in the pH profile of  $\tilde{V}/\tilde{K}_m$ , nor were they considered in the computation of the dissociation constants of the free enzyme,  $pK_a$  and  $pK_b$ .

To avoid the variety of complications arising from the presence of  $Mg^{2+}$  (317,319-23), this ion was excluded from all the reaction mixtures. Complications arising from the variation of  $\tilde{V}$  and  $\tilde{K}_m$  with ionic strength (52,252) were also avoided. This was achieved by carrying out all the reactions at a constant ionic strength of 0.1M. (The reaction mixtures were adjusted to this ionic strength before the start of the reaction by the addition of the appropriate amount of sodium chloride.)

### Materials

The enzyme used was a salt-free lyophilized preparation of chicken intestinal alkaline phosphatase (Code No. PC8CA) purchased from the Worthington Biochemical Corporation. Stock solutions of the enzyme were prepared by weighing out 10-15 mg in a 5 ml volumetric flask; after equilibration for 5 minutes in an ice-water bath, ice-cold water was slowly added with gentle stirring. The solution was then removed from the bath and made up to the mark at 20°C. The protein concentration was determined in three different ways: (1) gravimetrically, (2) spectrophotometrically at 278 m $\mu$  (specific molar absorptivity = 0.74 ml  $cm^{-1} g^{-1}$ ), and (3) colorimetrically, by the method of Lowry et al. (265) as modified by Legget-Bailey (266). All three methods gave values within 3%.

The p-nitrophenyl phosphate used as substrate was a Calbiochem Grade A reagent (Code No. 4876) obtained in the form of the sodium salt. It was stored in a vacuum desiccator in the dark at 0°C. The concentration of substrate was found to be most reliably determined from the amount of p-nitrophenol produced on complete hydrolysis, following a modification of the method used by Chanley and Feagson (263). Details of this method have been described in Chapter II, p.102. The unhydrolyzed substrate was found to be essentially free from p-nitrophenol (<0.05%).

Barbital (5,5-diethyl-barbituric acid) was obtained from Sigma Chemical Co. (Code No. B-0375), and sodium carbonate from B.D.H. Chemicals (Code No. 10240); both were of high purity and were used without further purification. The HCl, NaOH and NaCl used to adjust the pH and ionic strength were analytical grade reagents (purity >99.9%), and were also used without further purification.

All solutions were prepared in doubly-distilled deionized water.

### Kinetic Procedure

The apparatus used was that described in Chapter II, p.104-9. The following is a summary of that description.

pH measurements accurate to 0.005 pH units were obtained with a Radiometer pH meter, model TTT1a, fitted with a model PHA630 scale expander. A G202B glass electrode and

a K401 saturated calomel electrode were used, both having been calibrated against standard buffers. The pH was measured before and after each reaction, the variation being always less than 0.02 pH units.

Spectrophotometric measurements were made with a Perkin Elmer model 350 double-beam recording spectrophotometer, which was fitted with water-jacketted quartz cuvettes having an optical path length of 1 cm. The temperature was maintained at  $25.0 \pm 0.1^{\circ}\text{C}$  by connecting the cuvettes to a water bath equipped with a sensitive temperature regulator.

The hydrolysis rate of p-nitrophenyl phosphate was determined by the rate of formation of p-nitrophenol, measured at 400 m $\mu$ . The total concentration,  $C_T$ , of the ionic and undissociated forms is given by the modified Beer's law

$$C_T = A_T / \tilde{\epsilon}_{\text{app}} \quad (14)$$

where  $A_T$  is the total absorbance at 400 m $\mu$  and  $\tilde{\epsilon}_{\text{app}}$ , the apparent molar absorptivity, is given by

$$\tilde{\epsilon}_{\text{app}} = \frac{\epsilon_{A^-} + \frac{H}{K_a} \epsilon_{\text{HA}}}{1 + \frac{H}{K_a}} \quad (15)$$

Here  $\epsilon_{A^-}$  and  $\epsilon_{\text{HA}}$  are the molar absorptivities (at 400 m $\mu$ ) of the anion and the neutral p-nitrophenol, and  $K_a$  is the acid dissociation constant. Values of  $\epsilon_{A^-}$ ,  $\epsilon_{\text{HA}}$ , and  $K_a$  were determined by direct measurements with p-nitrophenol at

at various pH values and at I = 0.1 and 1.0M. At I = 0.1M,  $\epsilon_{A^-}$  and  $\epsilon_{HA}$  were found to be 18,330 and 81.4 M<sup>-1</sup> cm<sup>-1</sup>, respectively, while the pK<sub>a</sub> was 7.13. It follows from Eq. (14) that the initial rate of hydrolysis of p-nitrophenyl phosphate is

$$v = \left( \frac{dC_T}{dt} \right)_0 = \frac{1}{\epsilon_{app}} \left( \frac{dA_T}{dt} \right)_0 \quad (16)$$

where  $(dA_T/dt)_0$  is the initial slope of the A<sub>T</sub> versus time plot.

Before the start of a typical run the reaction and reference cuvettes (final volume 1.4 ml) both contained the same concentration of substrate, 0.010M in buffer, adjusted to the desired pH and an ionic strength of 0.10M. After a waiting period of five minutes which allowed the system to come to temperature equilibrium, the enzyme-catalyzed hydrolysis of substrate was initiated by the addition of 10-25  $\mu$ l of the enzyme stock solution to the reaction cuvette.

The plots of A<sub>T</sub> against time were usually linear for the first 5-10 minutes so that accurate initial rates could be determined. To further increase the accuracy, the reactions were always followed beyond the linear region, sometimes to 100% completion.

## Analysis of Experimental Results

### A. Determination of $\tilde{V}$ and $\tilde{K}_m$

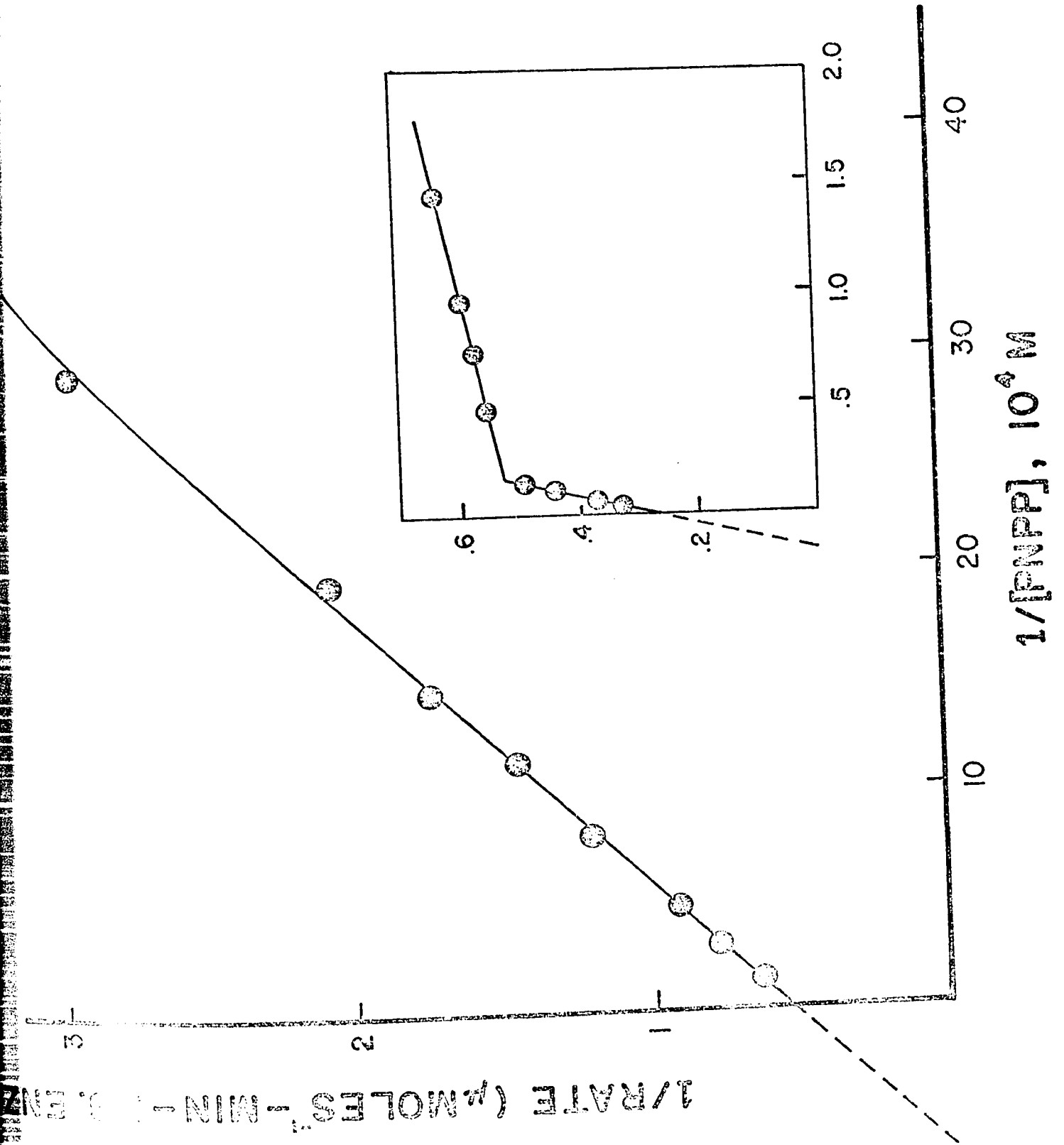
The reciprocals of the initial rates were first plotted against the reciprocals of the corresponding substrate concentrations. A typical plot is shown in Figure 33. The most striking feature of this double reciprocal plot (324) is that it contains two linear regions: (1) a high substrate concentration region ( $S > 8 \times 10^{-4} M$ ) and (2) a low substrate concentration region ( $S < 2 \times 10^{-4} M$ ); the change in slope takes place over a relatively narrow range of substrate concentrations.

Similarly shaped biphasic double reciprocal plots have been observed with alkaline phosphatases from several other sources (148,230-1,315) and also with several other enzymes (325-7). Simpson and Vallee (315) have recently studied this phenomenon of substrate activation at high substrate concentrations in some detail, and have concluded that, at least in the case of the E.coli enzyme, it reflects the binding of a second molecule of substrate to a second catalytic site, and, more specifically, negative homotropic binding (158,328).

Values of  $\tilde{V}$  and  $\tilde{K}_m$  were obtained by statistically fitting the experimental data directly to the Michaelis-Menten equation (Eq. (1)) according to the method developed by Bliss and James (274-5), or by using a modified version

Figure 33

Lineweaver-Burk plot for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate in 0.010M barbital.  $T = 25.0^{\circ}\text{C}$ , pH 9.05,  $I = 0.10\text{M}$ .



of the computer program written by Cleland ( 273 ). In both cases the actual calculations were carried out on an I.B.M. 360/65 digital computer. In this way it was possible to obtain accurate, non-biased estimates of  $\tilde{V}$  and  $\tilde{K}_m$  together with an estimate of their reliability. Only the data of the low substrate concentration region were used in the calculation of these parameters.

Determination of Ionization Constants from the Variation of the Kinetic Parameters with pH

A. Graphical Procedures

Dixon's Method

Ionization constants in enzyme systems are usually obtained from plots of the common logarithms of the kinetic parameters against pH (297,329-32). The procedure employed has been described by Laidler (332) with reference to a plot of  $\log_{10} (\tilde{k}_c/\tilde{K}_m)$  against pH for the case in which  $a = b = 0$  (Figure 34). For this case Eq. (7) reduces to

$$\frac{\tilde{k}_c}{K_m} = \frac{k_2}{K_s} \left( \frac{1}{1 + \frac{K_a}{H} + \frac{H}{K_b}} \right) \quad (17)$$

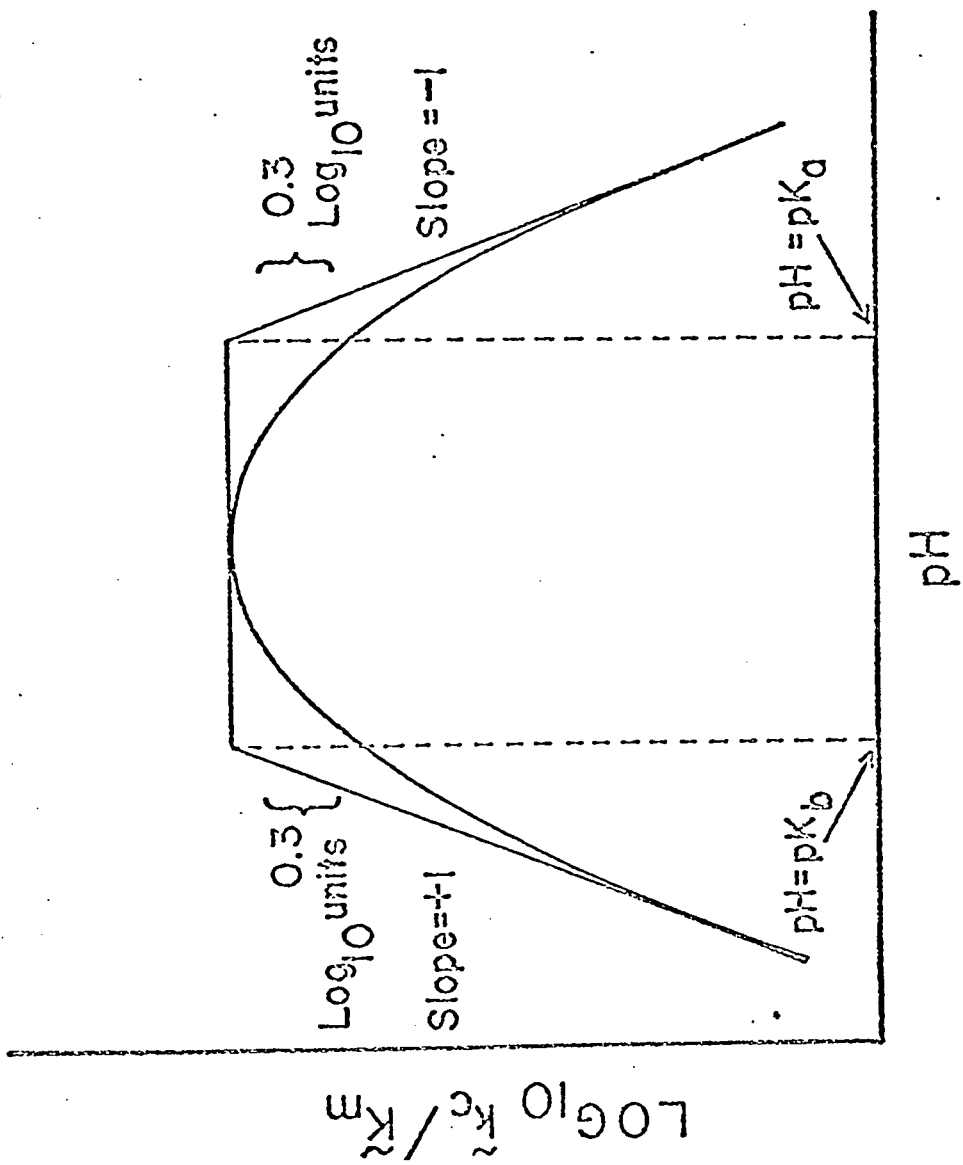
At sufficiently low pH

$$\frac{H}{K_b} \gg 1 + \frac{K_a}{H}$$

so that

Figure 34

Schematic plot of  $\log_{10}(k_c/K_m)$  against pH for systems to which Eq. (17) applies.



$$\frac{\tilde{k}_c}{\tilde{K}_m} = \frac{k_2}{K_s} \frac{K_b}{H} \quad (18)$$

or

$$\log_{10} \left( \frac{\tilde{k}_c}{\tilde{K}_m} \right) = \log_{10} \left( \frac{k_2 K_b}{K_s} \right) + \text{pH} \quad (19)$$

i.e., at sufficiently low pH values the slope of a plot of  $\log_{10}(\tilde{k}_c/\tilde{K}_m)$  against pH is equal to +1, as shown in Figure 5. As the pH is raised a region is reached in which

$$\frac{K_a}{H} + \frac{H}{K_b} \ll 1.$$

Eq. (17) then reduces to

$$\frac{\tilde{k}_c}{\tilde{K}_m} = \frac{k_2}{K_s} \quad (20)$$

or

$$\log_{10} \left( \frac{\tilde{k}_c}{\tilde{K}_m} \right) = \log_{10} \left( \frac{k_2}{K_s} \right) \quad (21)$$

The slope of the  $\log_{10}(\tilde{k}_c/\tilde{K}_m)$  - pH plot in this region is therefore zero. Eq. (18) is the equation for the left hand limb of the plot, and Eq. (20) that for the horizontal region; these two lines therefore intersect when

$$\frac{k_2}{K_s} \frac{K_b}{H} = \frac{k_2}{K_s} \quad (22)$$

i.e., when

$$H = K_b \quad (23)$$

or

$$\text{pH} = \text{pK}_b \quad (24)$$

It can similarly be shown that the point of intersection on the alkaline side corresponds to

$$\text{pH} = \text{pK}_a \quad (25)$$

The plot of  $\log_{10} (\tilde{k}_c / \tilde{K}_m)$  against pH does not actually show sharp changes in slope. The reason for this is that between the low pH region of slope +1 ( $H/K_b \gg (1+K_a/H)$ ) and the region at higher pH in which the slope is zero ( $K_a/H + H/K_b \ll 1$ ) is a region in which  $K_a/H \ll 1$  but  $H/K_b$  is of the same order of magnitude as unity. The value of  $\tilde{k}_c / \tilde{K}_m$  in this region is given by

$$\frac{\tilde{k}_c}{\tilde{K}_m} = \frac{k_2}{K_s} \left( \frac{1}{1 + \frac{H}{K_b}} \right) \quad (26)$$

or

$$\log_{10} \left( \frac{\tilde{k}_c}{\tilde{K}_m} \right) = \log_{10} \left( \frac{k_2}{K_s} \right) - \log_{10} \left( 1 + \frac{H}{K_b} \right) \quad (27)$$

At the inflection point, when  $H = K_b$ , Eq. (27) becomes

$$\log_{10} \left( \frac{\tilde{k}_c}{\tilde{K}_m} \right) = \log_{10} \left( \frac{k_2}{K_s} \right) - \log_{10} 2 \quad (28)$$

The curve at  $H = K_b$  therefore lies  $\log_{10} 2$ , or 0.303, below the point of intersection of the two straight lines described by Eqs. (19) and (21).

Similar arguments apply to plots of  $\log_{10} \tilde{k}_c$  and  $p\tilde{K}_m$  against pH. In both these cases, it was pointed out, the interpretation of the pH profile depends on the relative magnitudes of  $k_2$  and  $k_3$ . For example, if  $k_2 \ll k_3$  and  $a=b=0$ , Eq. (9) applies;  $\log_{10} \tilde{k}_c$  is then given by

$$\log_{10} \tilde{k}_c = \log_{10} k_2 - \log_{10} \left( 1 + \frac{K'_a}{H} + \frac{H}{K'_b} \right) \quad (29)$$

The inflection points of a plot of  $\log_{10} k_c$  against pH will reveal  $pK'_a$  and  $pK'_b$ . If  $a = 1$ ,  $b = 0$ , only  $pK'_b$  will be revealed, while if  $a = 0$ ,  $b = 1$ , only  $pK'_a$  will be revealed.

If, on the other hand,  $k_3 \ll k_2$  and  $a = b = 0$ , then Eq. (10) applies; in this case  $\log_{10} k_c$  will be given by

$$\log_{10} \tilde{k}_c = \log_{10} k_3 - \log \left( 1 + \frac{K''_a}{H} + \frac{H}{K''_b} \right) \quad (30)$$

and the inflection points will then reveal  $pK''_a$  and  $pK''_b$ .

Another plot often made is that of  $p\tilde{K}_m$  against pH. Since  $\tilde{K}_m$  has a more complex pH dependence than either  $\tilde{k}_c$  or  $\tilde{k}_c/\tilde{K}_m$  many patterns of behavior can be observed with this plot. These have been discussed in detail by Dixon (329). Reference to Eq. (12), however, indicates that a plot of  $p\tilde{K}_m$  is the difference between plots of  $\log_{10} (\tilde{k}_c/\tilde{K}_m)$  and  $\log_{10} \tilde{k}_c$ . As a result, the pH profile of  $p\tilde{K}_m$  is often found to be less complex than the profiles of either  $\log_{10} k_c$  or  $\log_{10} (\tilde{k}_c/\tilde{K}_m)$  or of both. Suppose, for example, that  $k_2 \ll k_3$ ,  $a = 1$ ,  $b = 0$ ,  $K'_b = K_b$  (i.e., the basic group dissociates as readily

in the Michaelis complex as in the free enzyme), and  $K'_a = 0$  (the acidic group is not free to dissociate in the Michaelis complex). The pH dependence of  $\log_{10} (\tilde{k}_c/\tilde{K}_m)$  is then given by Eq. (17), and the expression for  $\log_{10} \tilde{k}_c$  and  $p\tilde{K}_m$  become

$$\log_{10} \tilde{k}_c = \log_{10} k_2 - \log_{10} \left(1 + \frac{H}{K_b}\right) \quad (31)$$

and

$$p\tilde{K}_m = -\log_{10} K_s + \log_{10} \frac{1 + \frac{H}{K'_b}}{1 + \frac{K_a}{H} + \frac{H}{K_b}} \quad (32)$$

The pH profile of  $\log_{10} (\tilde{k}_c/\tilde{K}_m)$  will show a fall-off in rate at both low and high pH and will therefore reveal both  $pK_a$  and  $pK_b$ , while  $\log_{10} \tilde{k}_c$  will be pH dependent on the basic side and the results will reveal  $pK'_b$ . The pH dependence of  $p\tilde{K}_m$ , however, will occur only on the acid side and will reveal only  $pK_a$ .

The above procedure of determining  $pK_s$  is applicable only when the ionizations of the dissociating groups are well separated (i.e., by several units of pH). In such a case fairly reliable estimates of the  $pK_s$  may also be obtained by plotting the kinetic parameter itself against pH and noting the pHs at which it has half its maximum value. If, instead, the common logarithm of the parameter is plotted against pH, the  $pK_s$  may be read off as the pHs at which the parameter falls 0.303 units below its maximum value. When

the ionizations are not well-separated this method fails. This can readily be demonstrated with reference to Figure 6, which is a plot of  $\tilde{V}/\tilde{K}_m$  against pH when  $pK_b = 7.0$ ,  $pK_a = 8.0$ , and  $a = b = 0$ . The two pHs at which  $\tilde{V}/\tilde{K}_m$  falls to one half its maximum,  $pH_1$  and  $pH_2$ , are 6.65 and 8.33. The same incorrect values of  $pK_b$  and  $pK_a$  are obtained from a plot of  $\log_{10} (\tilde{V}/\tilde{K}_m)$  against pH (Figure 36).

### Method of Alberty and Massey

Alberty and Massey (333) have developed a method for determining pK values when the ionizations are not well separated. This method will be described with reference to the plot of  $\tilde{V}/\tilde{K}_m$  shown in Figure 35. For this case the pH dependence of  $\tilde{V}/\tilde{K}_m$  is given by Eq. (17).

The relationship between the pH of optimum activity,  $pH_o$ , and the dissociation constants  $pK_b$  and  $pK_a$  may be obtained by setting the first derivative of Eq. (17) equal to zero. This yields

$$H_o^2 = K_a K_b \quad (33)$$

or

$$pH_o = (pK_a + pK_b)/2 \quad (34)$$

The value of  $\tilde{V}/\tilde{K}_m$  at  $pH_o$  is

$$\left[ \frac{\tilde{V}}{\tilde{K}_m} \right]_o = \frac{k_2 E_o}{1 + 2 \left( \frac{K_a}{K_b} \right)^{1/2}} \quad (35)$$

Figure 35

Plot of  $(V/K_m)$  against pH when  $pK_b = 7.0$ ,  
 $pK_a = 8.0$ , and  $a = b = 0$ .

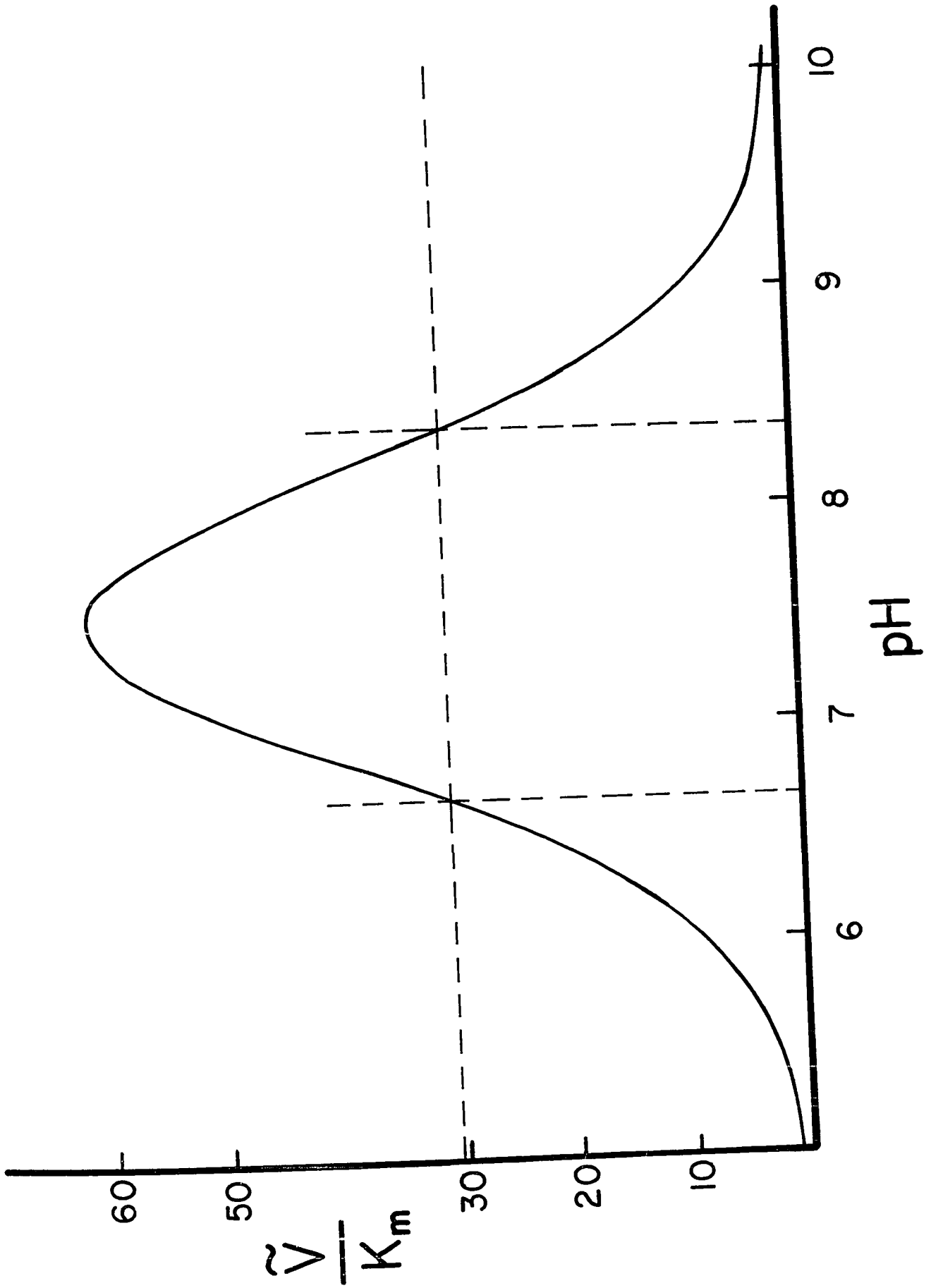
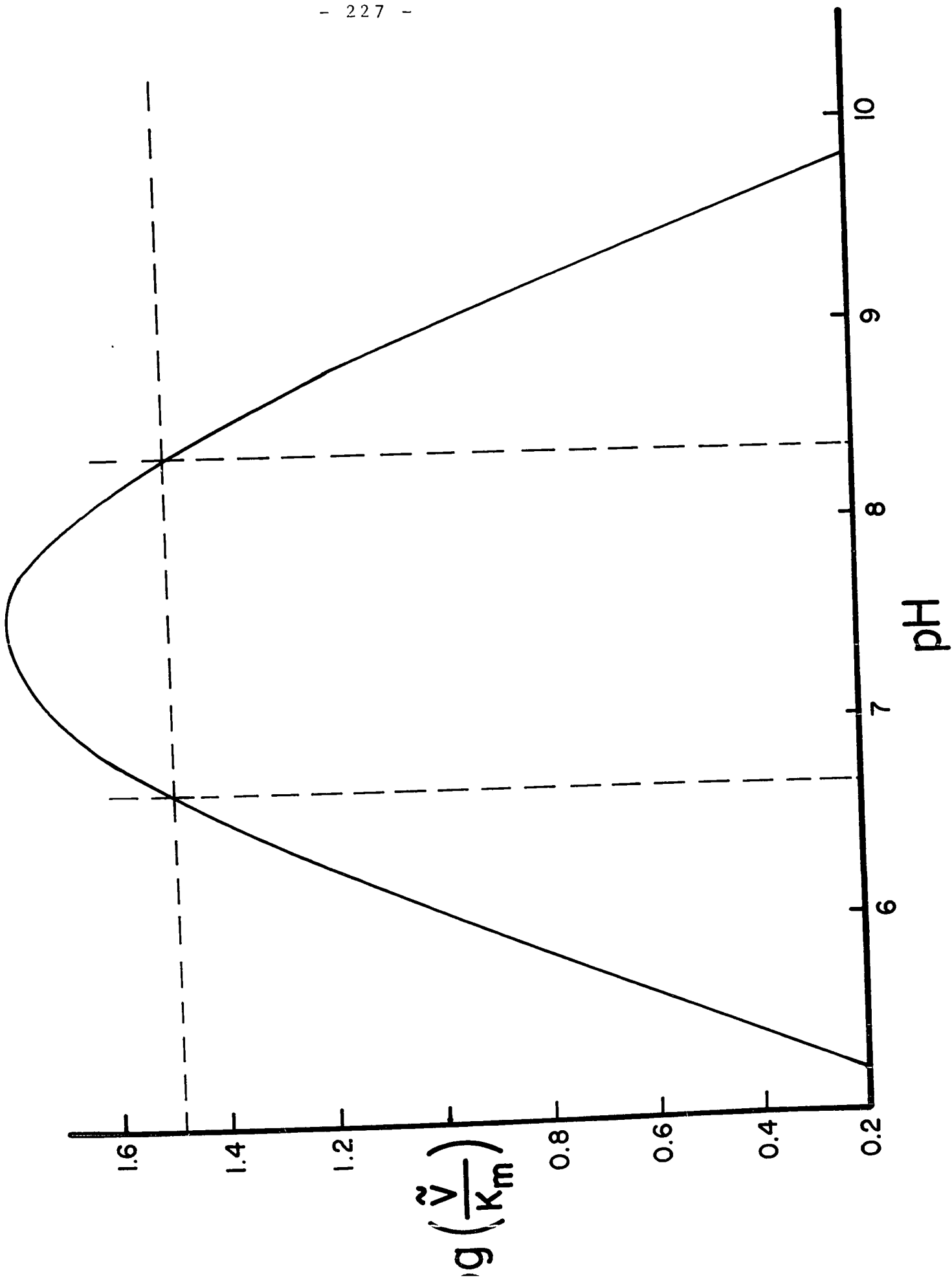


Figure 36

Plot of  $\log_{10} (V/K_m)$  against pH when  $pK_b = 7.0$ ,  
 $pK_a = 8.0$ , and  $a = b = 0$ .



and the pHs at which  $\tilde{V}/\tilde{K}_m$  is half this maximum value may be obtained by putting  $\tilde{V}/\tilde{K}_m$  equal to half  $(\tilde{V}/\tilde{K}_m)_o$ , i.e.,

$$\left(\frac{\tilde{V}}{\tilde{K}_m}\right) = \frac{k_2 E_o (HK_b)}{K_b H + K_a K_b + H^2} + \frac{k_2 E_o}{2 + 4\left(\frac{K_a}{K_b}\right)^{1/2}} \quad (36)$$

which simplifies to

$$H^2 - (K_b + 4H_o)H + H_o^2 = 0 \quad (37)$$

Eq. (37) has two real roots,  $H_1$  and  $H_2$ , corresponding to the hydrogen ion concentrations at which  $(\tilde{V}/\tilde{K}_m)/(\tilde{V}/\tilde{K}_m)_o = 0.5$  on the acid and basic side of the pH profile; the sum of the roots is given by the expression in brackets, i.e.,

$$H_1 + H_2 = K_b + 4H_o \quad (38)$$

or

$$K_b = (H_1 + H_2) - 4H_o \quad (39)$$

The ionization constant  $K_b$  can be evaluated from Eq. (39) even when the  $pK_s$  are not well separated ( $H_1$ ,  $H_2$  and  $H_o$  can always be obtained graphically).  $K_a$  may then be calculated from Eq. (33) which rearranges to

$$K_a = H_o^2 / K_b \quad (40)$$

Exactly the same equations can be used to determine  $pK_b$  and  $pK_a$  from a plot of  $\log_{10} (\tilde{V}/\tilde{K}_m)$  (Figure 36).

Applying this method to the pH profile shown in Figure 6 we find that  $H_1 = 2.239 \times 10^{-7}$ ,  $H_2 = 4.732 \times 10^{-9}$ ,

and  $H_o = 3.162 \times 10^{-8}$ , so that  $K_b = (H_1 + H_2) - 4H_o = 1.022 \times 10^{-7}$  ( $pK_b = 6.99$ ) and  $K_a = 10^{-15.00} / (1.022 \times 10^{-7}) = 9.790 \times 10^{-9}$  ( $pK_a = 8.01$ ), in excellent agreement with the true  $pK_b$  and  $pK_a$  values of 7.00 and 8.00, respectively.

## B. Statistical Curve-Fitting Methods

### Bilinear Regression Analysis

Eq. (17) may be rearranged to

$$\frac{\tilde{K}_m}{k_c} = \frac{K_s}{k_2} + \frac{K_s}{k_2} \frac{H}{K_b} + \frac{K_s}{k_2} \frac{K_a}{H} \quad (41)$$

which when expressed in terms of  $\tilde{V} (= \tilde{k}_c E_o)$  becomes

$$\frac{\tilde{K}_m}{\tilde{V}} = \frac{K_s}{k_2 E_o} + \frac{K_s}{k_2 E_o} \frac{H}{K_b} + \frac{K_s}{k_2 E_o} \frac{K_a}{H} \quad (42)$$

Eq. (42) is a bilinear equation of the form

$$y = a + b_1 x_1 + b_2 x_2, \quad (43)$$

the determining variables being  $x_1 = H$ ,  $x_2 = 1/H$ ;  $y = \tilde{K}_m / \tilde{V}$ ,  $a = K_s / k_2 E_o$ ,  $b_1 = a / K_b$ , and  $b_2 = a K_a$ . Values of  $a$ ,  $b_1$ ,  $b_2$ ,  $K_a$ , and  $K_b$  which best fit the data to Eq. (42) may therefore be obtained by fitting a weighted bilinear regression of  $\tilde{V} / K_m$  on  $H$  and  $1/H$  using the method of Wilkinson (272). A digital computer program has been written in Fortran IV for the IBM 360/65 computer system to facilitate the performance of the lengthy calculations involved. In Wilkinson's method the appropriate weights initially used are the reciprocals of

the internal variances of the  $\tilde{V}/\tilde{K}_m$  values. However, when several estimates of  $\tilde{V}/\tilde{K}_m$  are available at a given pH, it is preferable to use the inter-experimental variance associated with these as the weighting factor at that pH.

### Reiterative Least Squares Fit Technique

Eq. (42) may also be written as

$$y = aZ \quad (44)$$

where

$$Z = 1 + \frac{H}{K_b} + \frac{K_a}{H} \quad (45)$$

The deviation of the experimental value of the parameter  $y_i$  from the one calculated from Eq. (44) is given by

$$D_i = (y_i - aZ_i) \quad (46)$$

so that

$$\sum_{i=1}^N D_i^2 = \sum_{i=1}^N (y_i - aZ_i)^2 \quad (47)$$

Differentiating with respect to  $a$ ,

$$\frac{\partial}{\partial a} \sum_{i=1}^N D_i^2 = \sum_{i=1}^N 2(y_i - aZ_i)(-Z) \quad (48)$$

To satisfy the least-squares criterion for best fit - that the sum of the squares of the deviations be minimized - Eq. (48) is put equal to zero; i.e.,

$$\sum_{i=1}^N (y_i - aZ_i)Z_i = 0 \quad (49)$$

which upon solving for a result in the following equation:

$$a = \frac{\sum_{i=1}^N Z_i Y_i}{\sum_{i=1}^N Z_i^2} \quad (50)$$

A digital computer program involving multiple iterations with different values of  $K_b$  and  $K_a$  was written to calculate the best value of  $a$  by this method. An approximate value of  $a$  was first calculated by allowing  $K_a$  and  $K_b$  to assume values over a predetermined range, using an interval of 0.1 between successive pK values, computing  $\sum_{i=1}^N D_i^2$  for each combination of  $K_a$  and  $K_b$ , and determining which combination gave the smallest value of  $\sum_{i=1}^N D_i^2$ . The range of values  $K_a$  and  $K_b$  were allowed to take on and the interval between values was then narrowed and, once again, the combination of  $K_a$  and  $K_b$  which resulted in the smallest  $\sum_{i=1}^N D_i^2$  was determined. This process was repeated until two successive iterations gave essentially the same value of  $a$ .

### Simple Least Squares Fit

The variation of either  $\tilde{k}_c$  or  $\tilde{k}_c/\tilde{K}_m$  with pH often involves only one ionization constant. For example, if  $k_2 \ll k_3$ ,  $a = b = 0$ , and  $K'_a = 0$ , the pH dependence of  $\tilde{V}$  will be given by

$$\tilde{V} = \frac{k_2 E_o}{\left(1 + \frac{H}{K'_b}\right)} \quad (51)$$

To achieve a linear plot it is convenient to rearrange Eq. (51) to

$$\tilde{V} = k_2 E_o - \frac{1}{K_b'} (\tilde{V}H) \quad (52)$$

If  $\tilde{V}$  is plotted against  $\tilde{V}H$  a straight line having a slope equal to  $-1/K_b'$  and a Y-intercept of  $k_2 E_o$  will be obtained.

Alternatively, Eq. (51) may be transformed into

$$\frac{1}{\tilde{V}} = \frac{1}{k_2 E_o} + \frac{1}{k_2 E_o K_b} (H) \quad (53)$$

When the reciprocal of  $\tilde{V}$  is plotted against the hydrogen ion concentration a straight line of slope  $1/k_2 E_o K_b$  and having an intercept equal to  $1/k_2 E_o$  will result.

Values of  $k_2 E_o$  and  $K_b$  may therefore readily be obtained by statistically fitting the data to either Eq. (52) or (53) by standard linear least-squares regression technique (272).

## RESULTS

### pH Dependence of Kinetic Parameters

Typical Lineweaver-Burk plots for the alkaline phosphatase catalyzed hydrolysis of p-nitrophenyl phosphate are shown in Figures 37-40 all these plots are entirely linear over the tenfold concentration range between  $0.2-2K_m$  which was studied. The values of  $\tilde{V}$ ,  $\tilde{K}_m$ , and  $\tilde{V}/K_m$  obtained at the various pHs studied and the standard errors associated with these parameters are listed in Table 18. The pH dependencies of these parameters are shown graphically in Figures 41-46. The kinetic parameters are average values based on the results of three to five runs covering the full range of substrate concentrations ( $0.2 - 2K_m$ ). The standard errors reported (calculated as the square roots of the inter-experimental variances) show that the kinetic parameters can be considered reliable to  $\pm 5\%$ .

### pH Dependence of $\tilde{V}/\tilde{K}_m$

The pH profile of  $\log_{10} \tilde{V}/\tilde{K}_m$  is shown in Figure 41. Such a plot, it was explained earlier, reveals the ionization constants of kinetically important groups on the free enzyme and substrate which are also essential to the subsequent reaction of the enzyme-substrate complex. Since the pKs of the substrate, p-nitrophenyl phosphate, lie well outside the

Figure 37

Lineweaver-Burk plots for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate on the acid side of the pH optimum. The reactions were carried out in 0.010M barbital at  $T = 25.0^{\circ}\text{C}$  and  $I = 0.10\text{M}$  (adjusted with 5.0M NaCl).

1/RATE ( $\mu$ MOLES<sup>-1</sup>-MIN-MG, ENZYME)

100  
80  
60  
40  
20

pH 7.2

pH 7.6

pH 8.0

1/[PNPP], 10<sup>4</sup>M

10 20 30 40 50 60 70

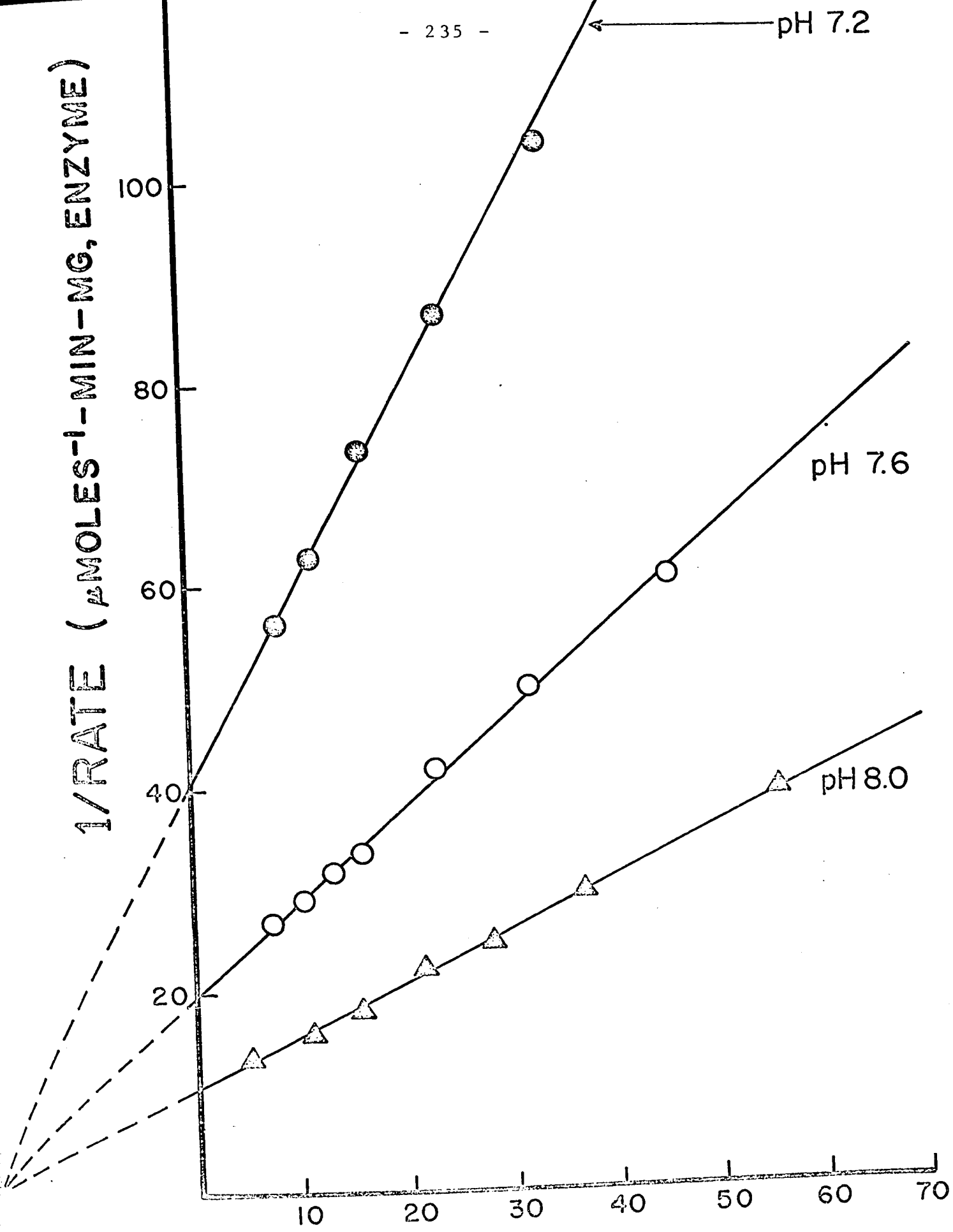


Figure 38

Lineweaver-Burk plots for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate on the acid side of the pH optimum (continued).

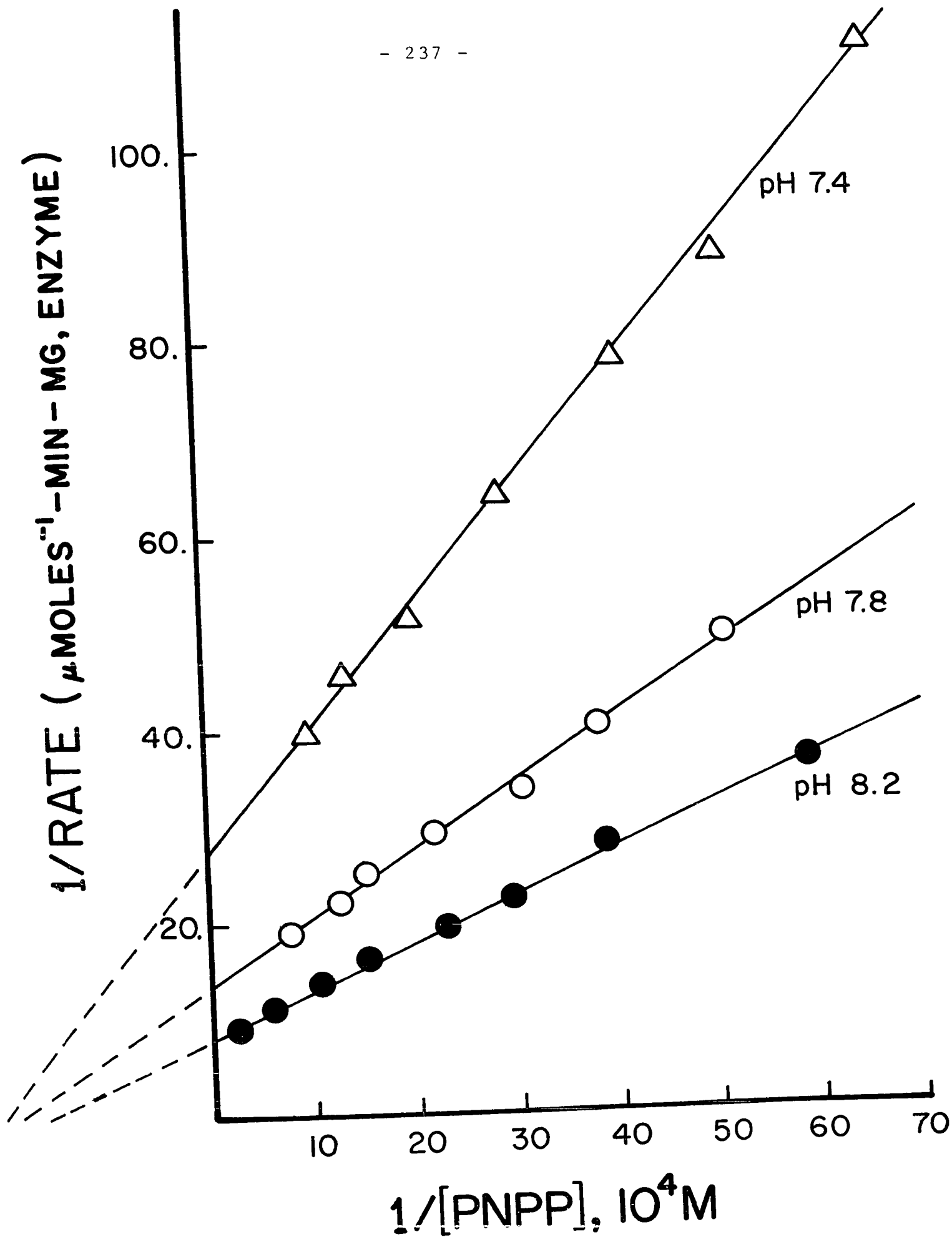


Figure 39

Lineweaver-Burk plots for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate on the basic side of the pH optimum. The reactions were carried out in 0.010M barbital at 25.0°C and I = 0.10M (adjusted with 5.0M NaCl).

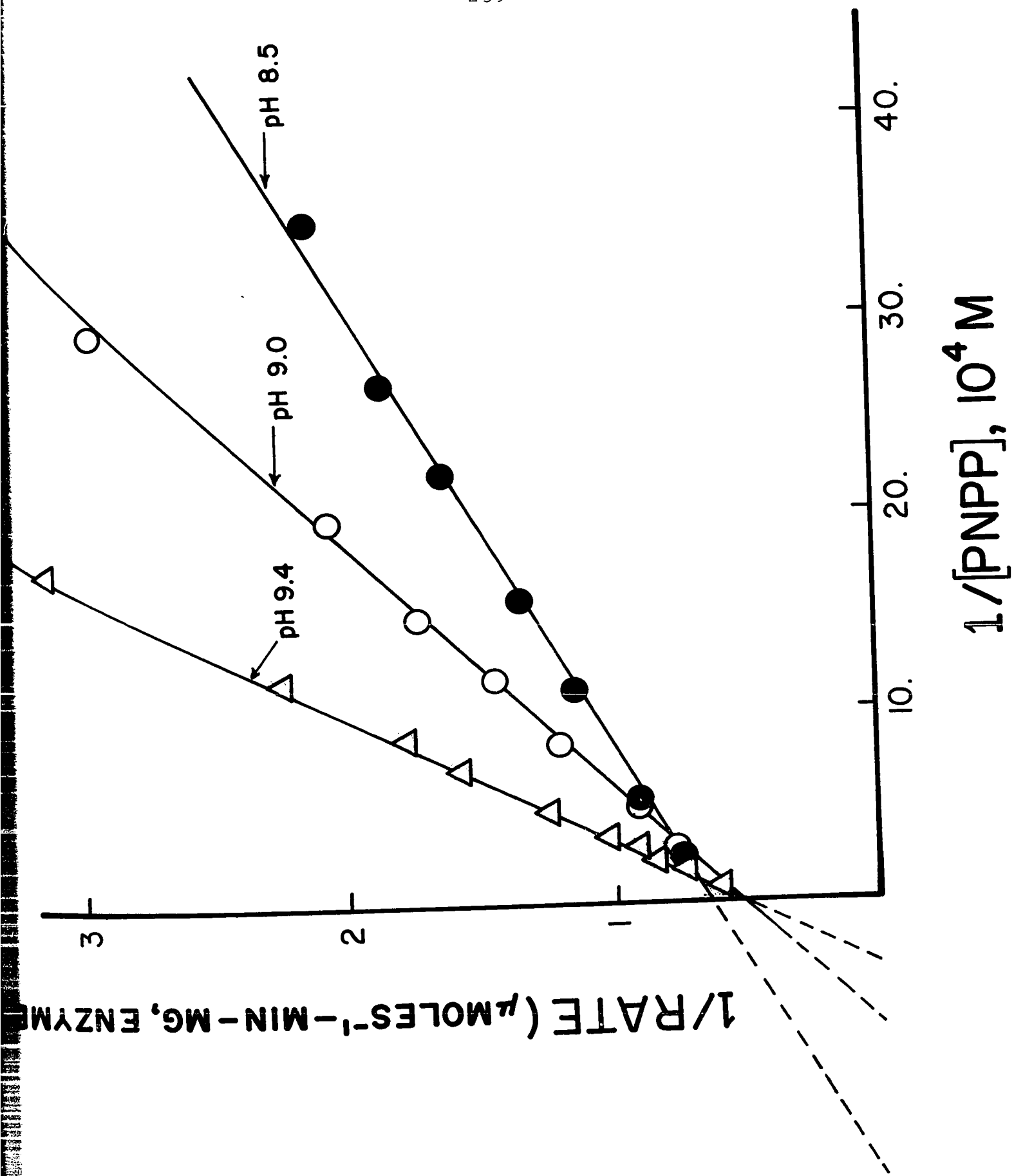


Figure 40

Lineweaver-Burk plots for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate on the basic side of the pH optimum (continued).

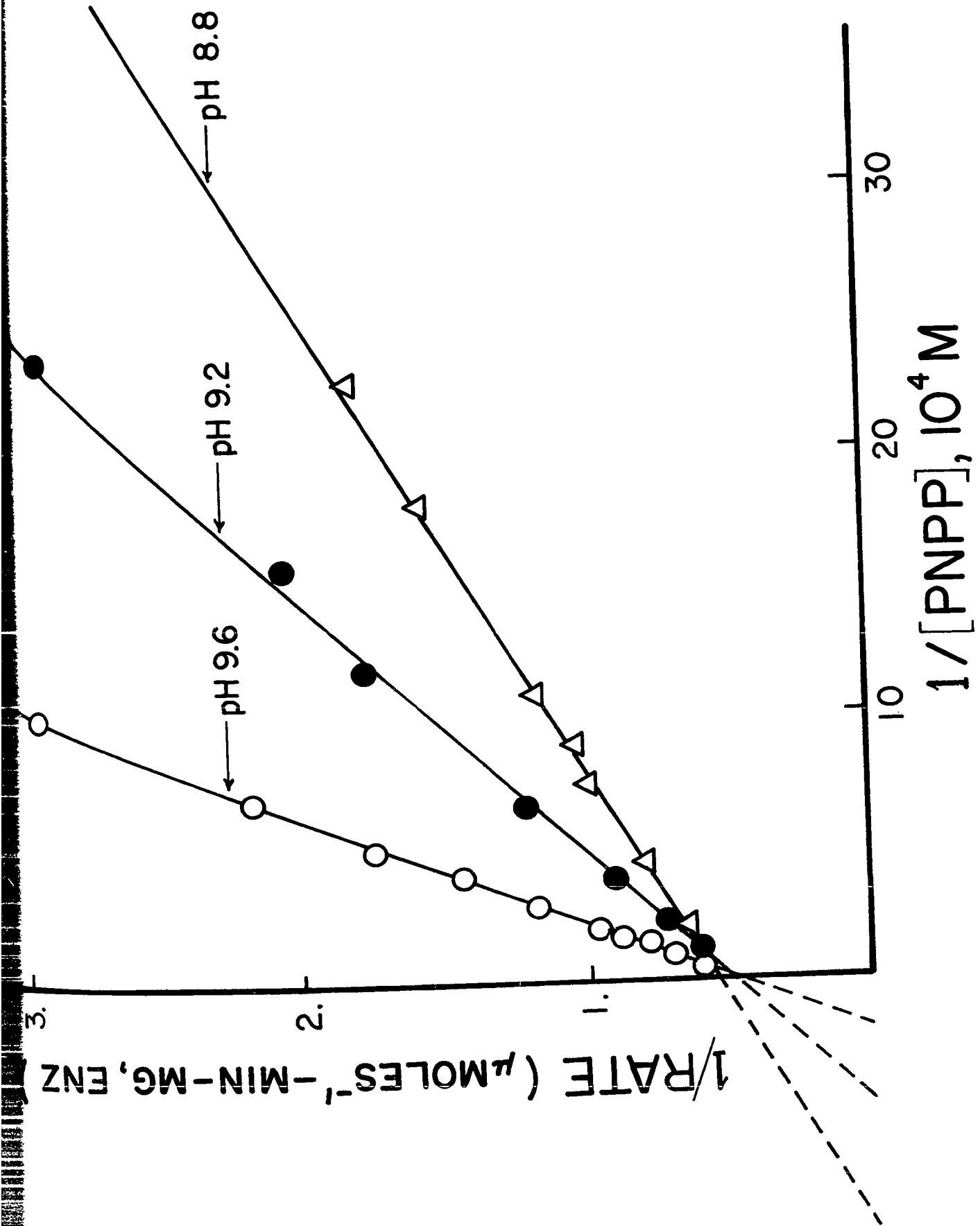


TABLE 18  
 Kinetic Parameters for the Alkaline Phosphatase-catalyzed Hydrolysis  
 of PNPP at 25.0°C and I = 0.10

pH	$V \times 10^3$ ( $\mu\text{moles}/\text{min}/\text{mg Enz.}$ )	$K_m$ ( $\mu\text{moles}/\text{liter}$ )	$(V/K_m) \times 10^3$ (liters/min/mg Enz.)
7.20	24.7±0.5	4.74±0.21	5.21±0.14
7.40	37.5±0.8	4.64±0.22	8.10±0.21
7.60	51.4±0.9	4.79±0.19	10.7 ±0.26
7.80	73.1±1.6	5.07±0.25	14.4 ±0.40
8.00	98.4±1.7	5.02±0.20	19.6 ±0.49
8.20	120. ±1.2	5.75±0.15	20.9 ±0.37
8.50	158. ±3.2	7.28±0.36	21.7 ±0.72
8.80	183. ±2.6	10.5 ±0.35	17.4 ±0.37
9.05	194. ±3.7	16.0 ±0.71	12.2 ±0.35
9.20	205. ±2.9	21.9 ±0.76	9.37±0.21
9.40	208. ±3.0	33.0 ±1.1	6.29±0.12
9.60	213. ±2.6	52.5 ±1.4	4.06±0.07
7.00	14.9±0.8	-	-

Figure 41

Variation of  $\log_{10} V/K_m$  with pH for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate in 0.010M barbital at 25.0°C and  $I = 0.10M$  (adjusted with 5.0M NaCl).

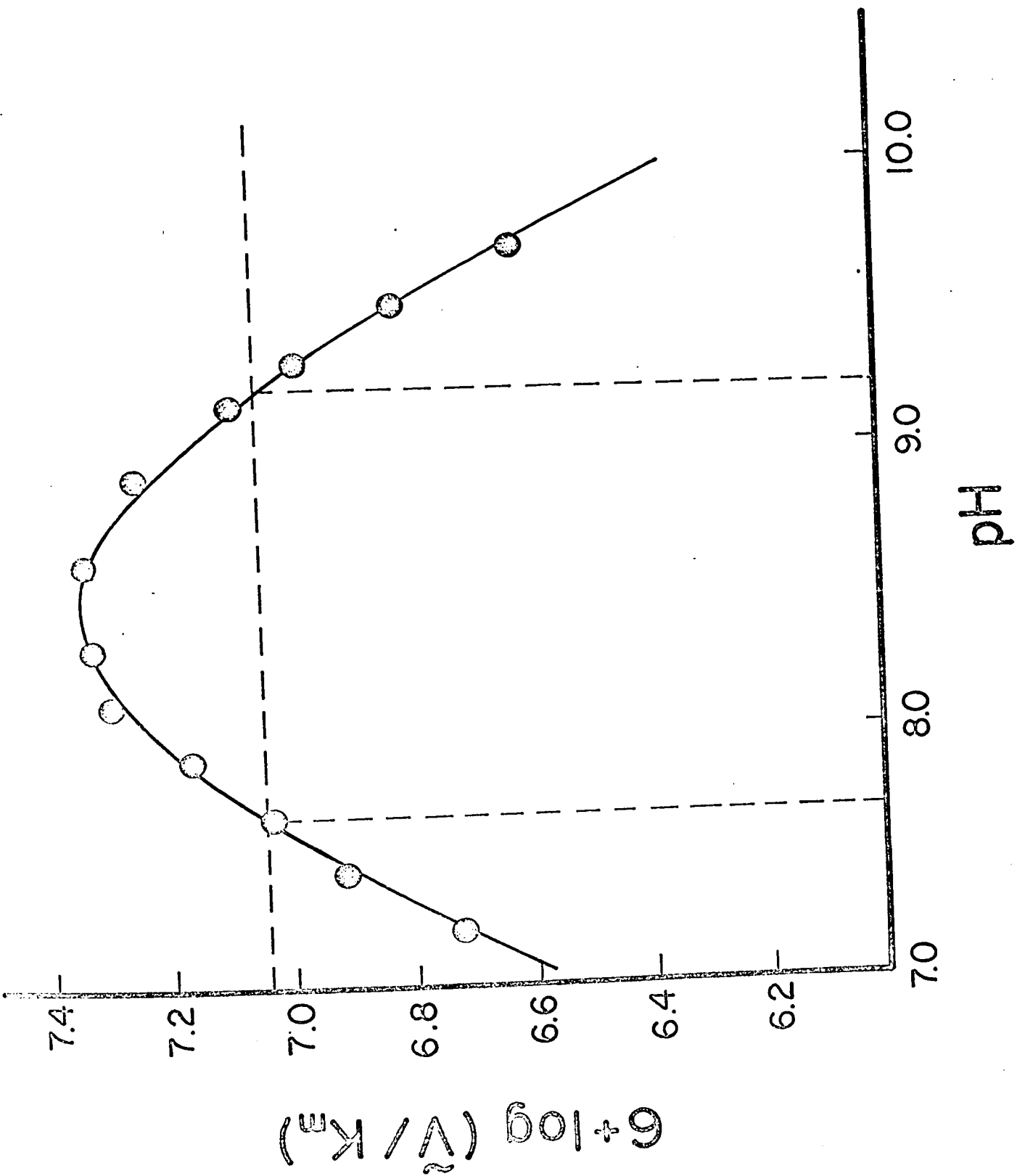


Figure 42

Variation of  $\log_{10} V$  with pH for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate in 0.010M barbital at 25.0°C and  $I = 0.10M$  (adjusted with 5.0M NaCl).

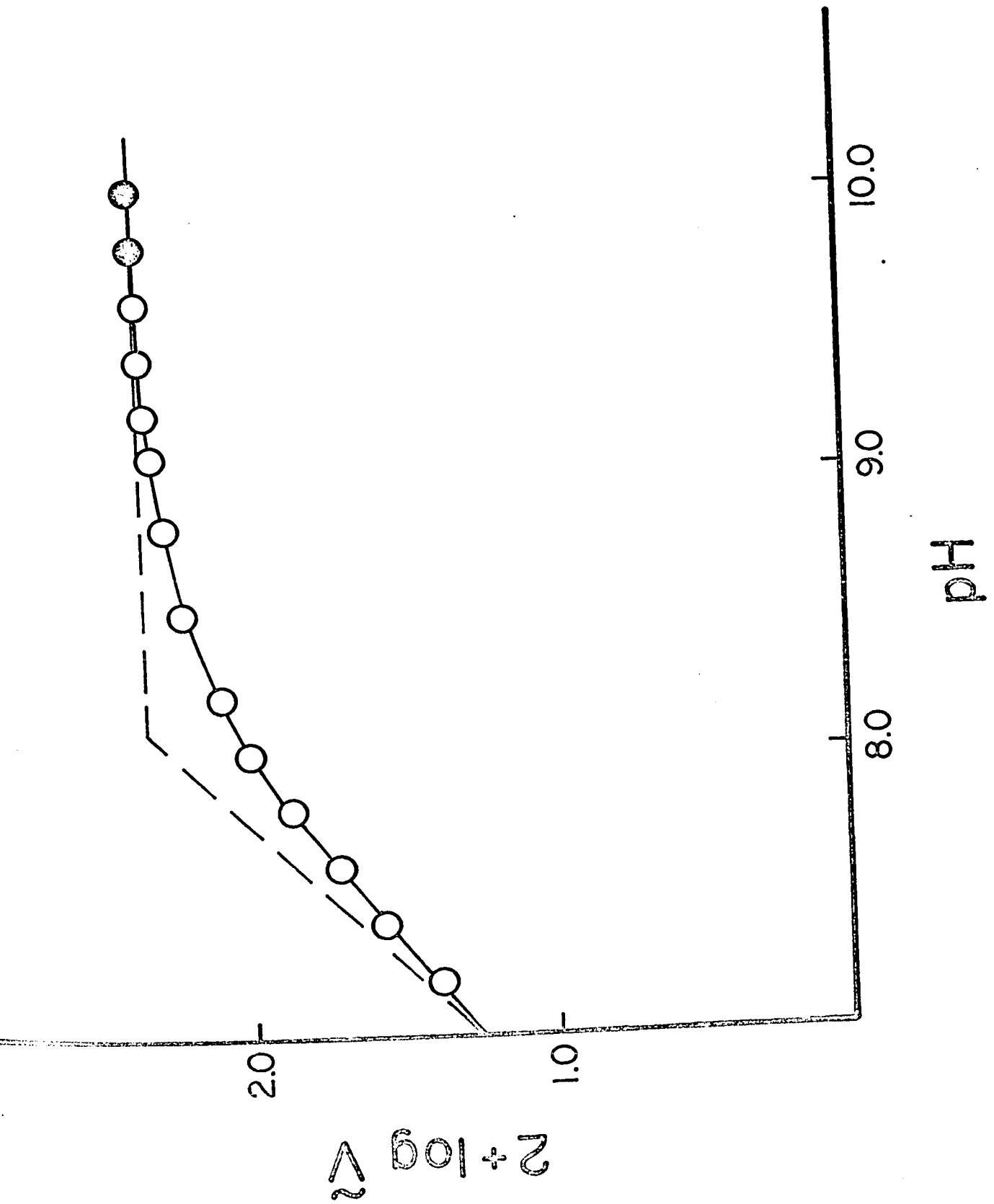


Figure 43

Variation of  $pK_m$  with pH for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate in 0.010M barbital at 25.0°C and  $I = 0.10M$  (adjusted with 5.0M NaCl).

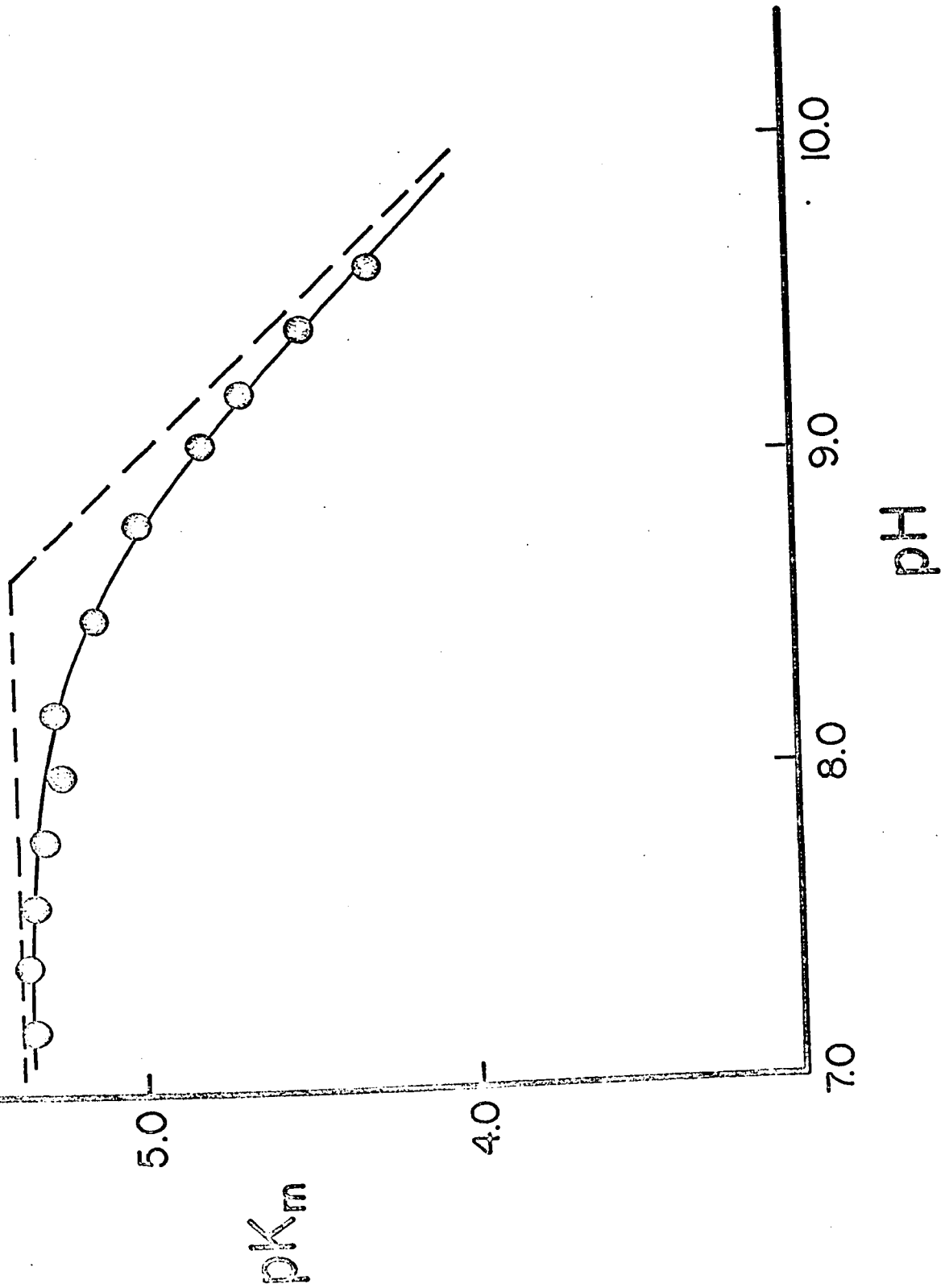


Figure 44

Variation of  $K_m/V$  with pH for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate in 0.010M barbital at 25.0°C and I = 0.10M (adjusted with 5.0M NaCl).

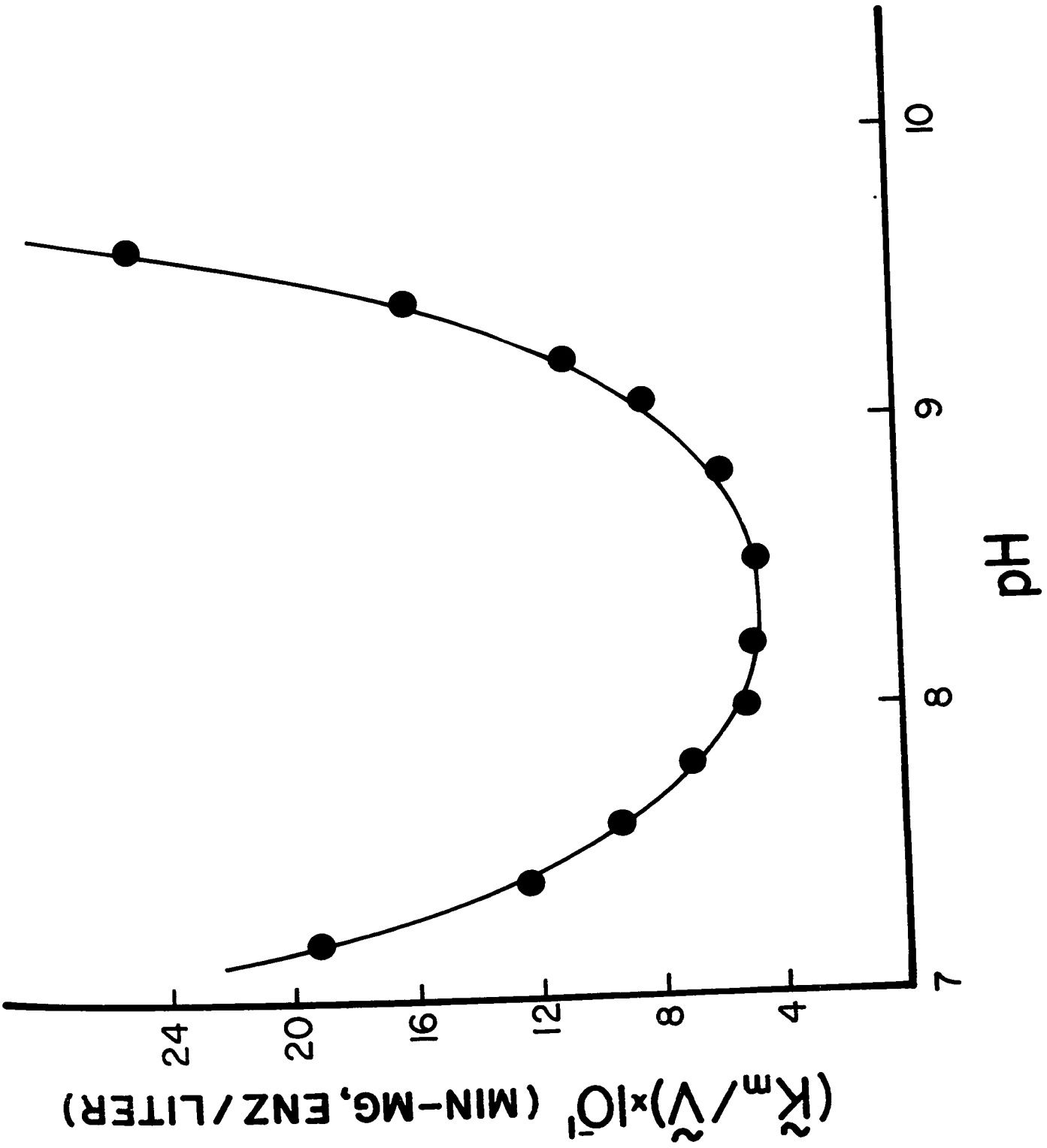


Figure 45

Variation of V with pH for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate in 0.010M barbital at 25.0°C and I = 0.10M (adjusted with 5.0M NaCl).

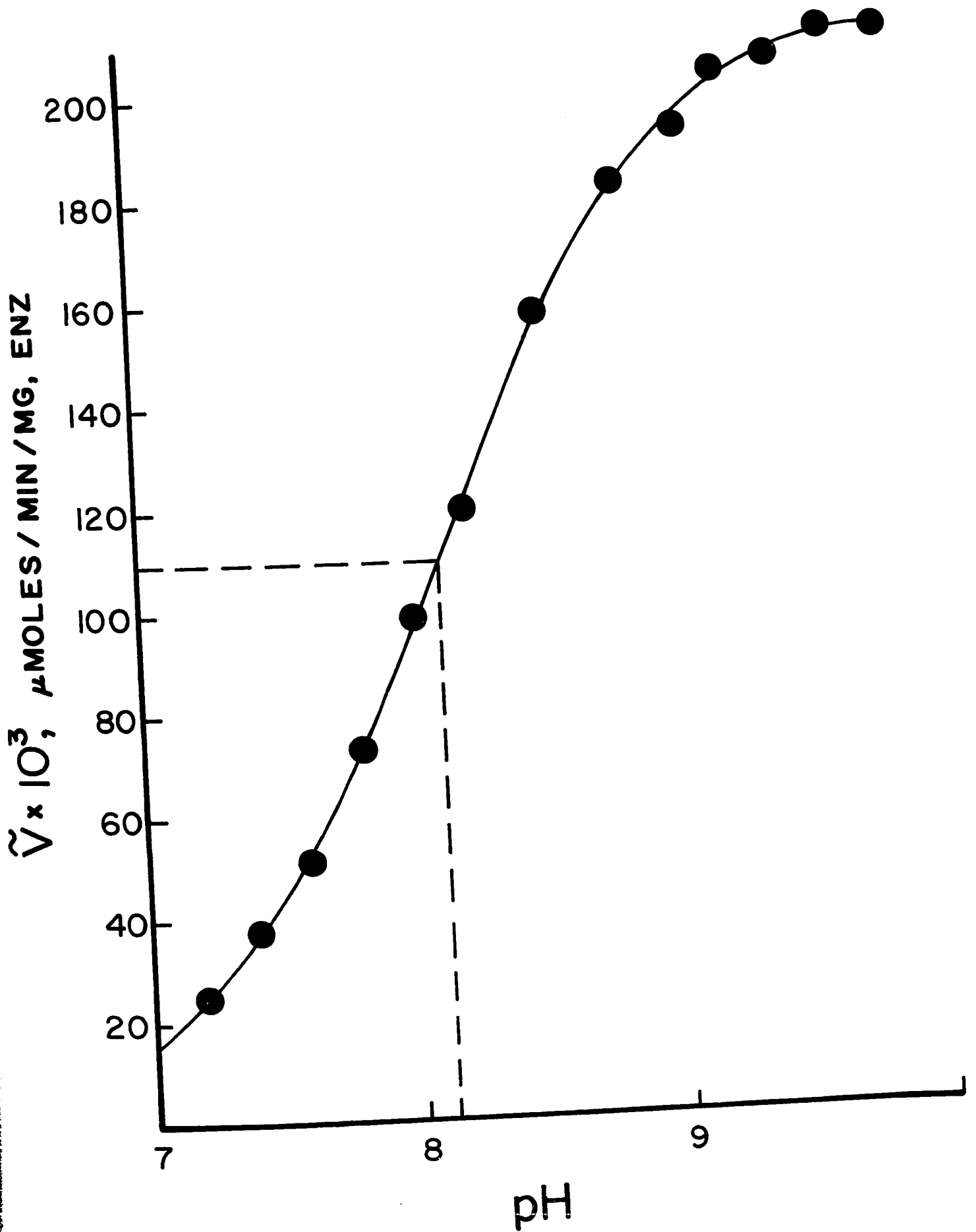
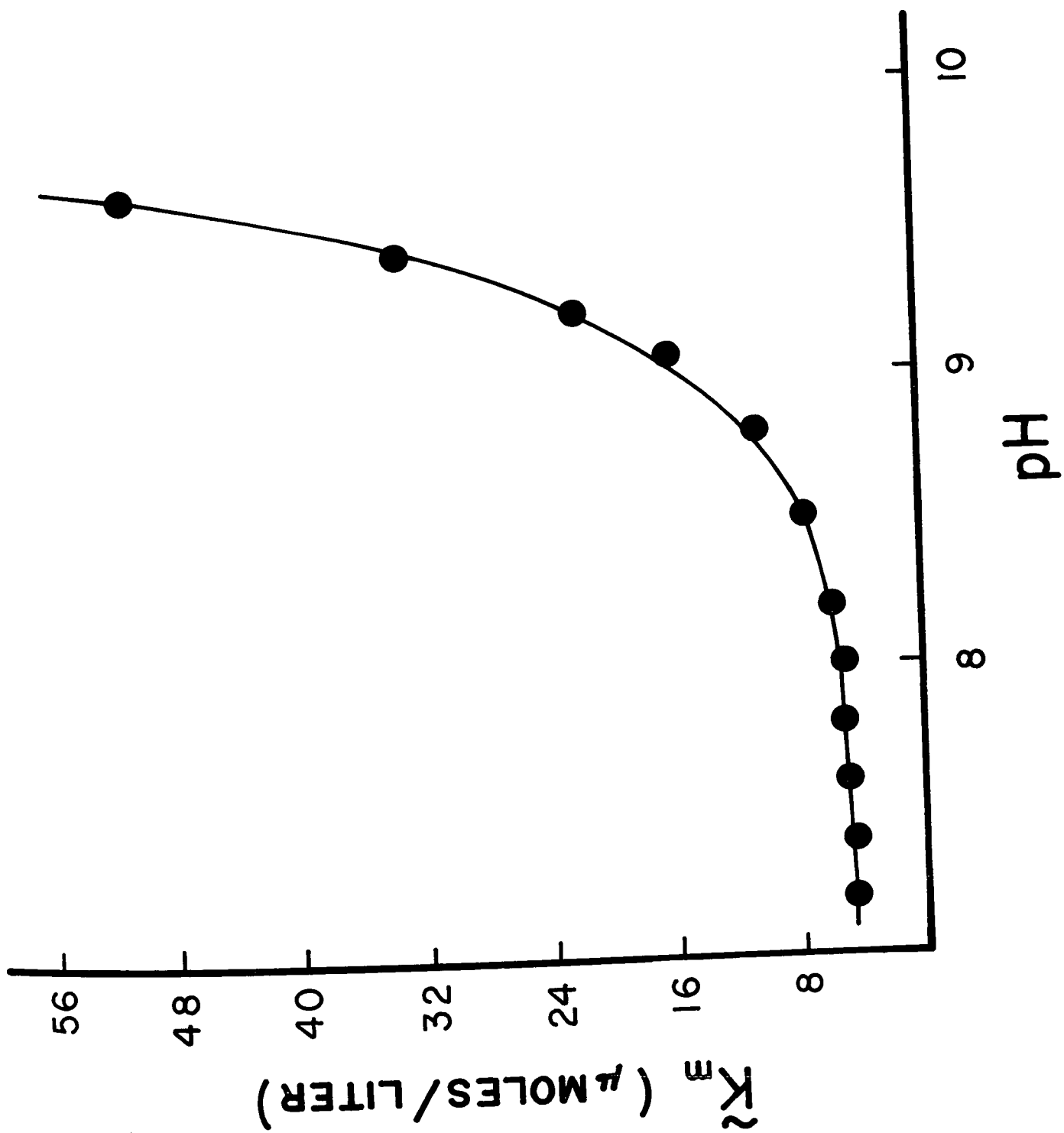


Figure 46

Variation of  $K_m$  with pH for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate in 0.010M barbital at 25.0°C and  $I = 0.10M$  (adjusted with 5.0M NaCl).



pH range investigated - Jaffe et al. have reported pKs of 1.24 and 6.23 (334-5) - the observed bell-shaped form of the curve therefore indicates that the free enzyme contains two ionizing groups, a basic group ( $pK_b = 8.1$ ) and an acidic group ( $pK_a = 8.6$ ), both of which in some way participate in the phosphorylation step of the reaction. These groups may also be involved in the dephosphorylation step, but this cannot, of course, be determined from the pH dependence of  $\tilde{V}/K_m$ .

Since the two  $pK_s$  in the free enzyme are not well-separated ( $pH_2 - pH_1 < 1.5$ ), they cannot be evaluated by Dixon's method (Figure 5). Very reliable values of  $pK_b$  and  $pK_a$  can, however, be obtained using the method of Albery and Massey (333). Applying this method to the data of Figure 12, it is found that  $H_1 = 10^{-7.60} = 2.512 \times 10^{-8}$ ,  $H_2 = 10^{-9.11} = 7.762 \times 10^{-10}$ , and  $H_0 = 10^{-8.35} = 4.467 \times 10^{-9}$ , so that  $K_b = 8.028 \times 10^{-9}$ , i.e.,  $pK_b = 8.09$ , and  $pK_a = 2pH_0 - pK_b = 8.61$ .

The statistical curve-fitting methods give similar values for  $pK_b$  and  $pK_a$ . An equation consistent with the pH dependence of  $\tilde{V}/\tilde{K}_m$  shown in Figure 12 is

$$\frac{\tilde{V}}{K_m} = \frac{k_2 E_0}{K_s} \left( \frac{1}{1 + \frac{H}{K_b} + \frac{K_a}{H}} \right) \quad (54)$$

which rearranges to Eq. (42). Two methods of fitting the data to Eq. (42) - one involving a bilinear regression

analysis and the other, a reiterative least-squares fit - have already been described. Both these methods give exactly the same results:  $pK_b = 8.13 \pm .03$ ,  $pK_a = 8.55 \pm .03$ , and  $K_s/k_2E_o = (2.01 \pm .16)$  min-mg enzyme/l. The percentage errors in  $K_a$ ,  $K_b$ , and  $a$  (calculated from the internal variances) were about 8%.

Figures 44 and 47 show how closely the data fit the theoretical curve obtained by the two statistical curve fitting methods. Figure 44 is a plot of  $\tilde{K}_m/\tilde{V}$  against pH. The points shown are those obtained experimentally; the curve drawn through the points was calculated from Eq. (42) using the values for  $K_a$ ,  $K_b$ , and  $a$  obtained from the bilinear regression analysis. Figure 47 is a plot of  $\tilde{K}_m/\tilde{V}$  against  $Z(=1 + H/K_b + K_a/H)$ . The points shown and the straight line which passes through them and through the origin were calculated using the values for  $K_a$ ,  $K_b$ , and  $a$  derived from the reiterative fit program.

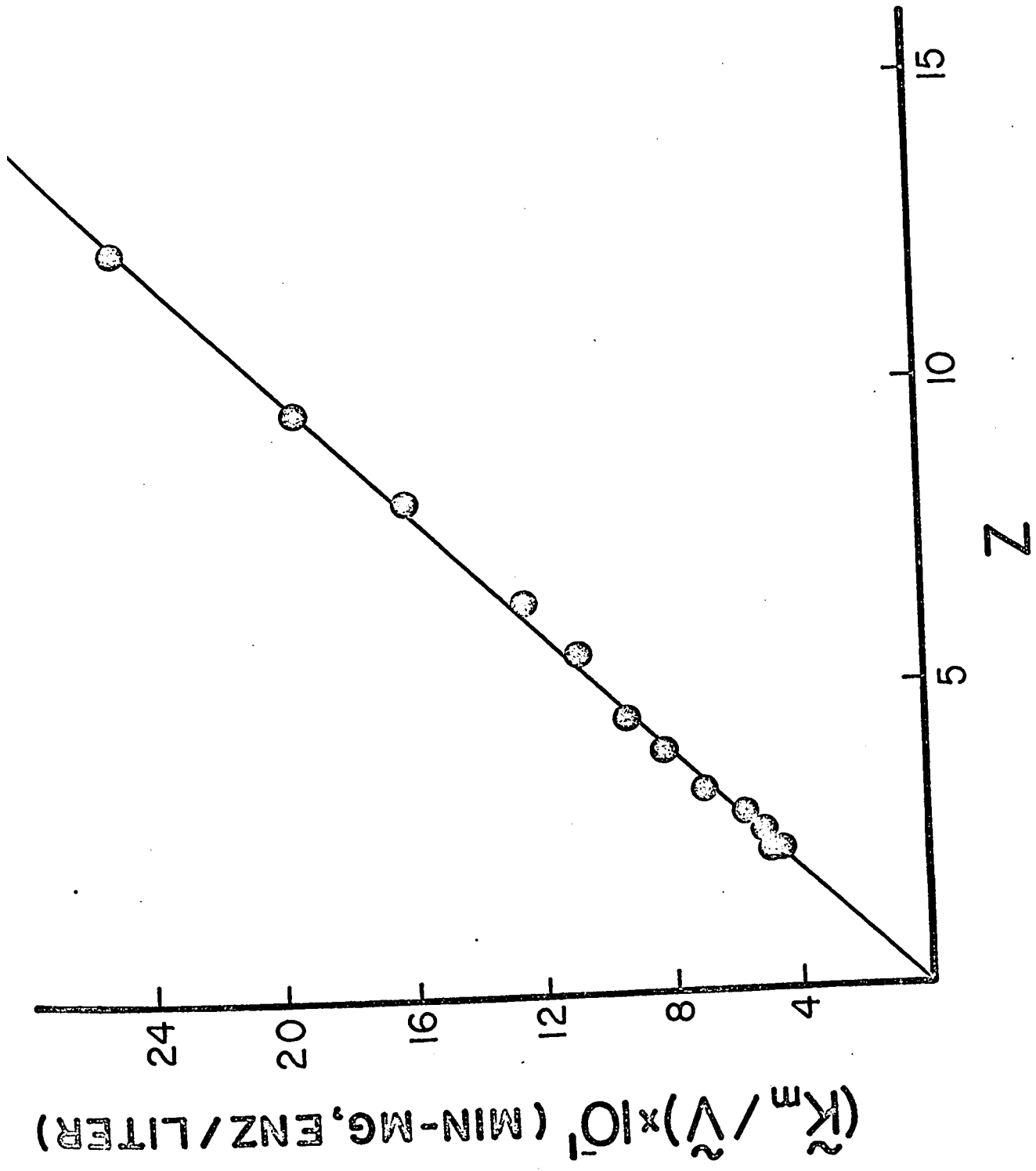
#### pH Dependence of $\tilde{V}$

The pH profile of  $\log_{10} \tilde{V}$  is shown in Figure 42. Since  $k_2 \ll k_3$  for alkaline phosphatase at  $pH > 7$ , the sigmoid shape of the curve shown that a basic group ( $pK'_b = 8.1$ ), which ionizes in the enzyme-substrate complex, is involved in the phosphorylation step of the reaction.

Since no fall-off in  $\log_{10} \tilde{V}$  is observed at high pH only one ionizing group is implicated; a good estimate of

Figure 47

Plot of  $K_m/V$  against  $Z(=1 + H/K_b + K_a/H)$  for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate in 0.010M barbital at 25.0°C and  $I = 0.10M$  (adjusted with 5.0M NaCl).



its  $pK$  may therefore be obtained by Dixon's method, as indicated in Figure 42. The dashed lines shown in this figure have slopes of +1 and 0, respectively, and they intersect 0.303 units above the experimental plot; the pH at their point of intersection, 8.08, therefore corresponds to  $pK'_b$ . A similar value of  $pK'_b$  can be obtained from the plot of  $\tilde{V}$  against pH shown in Figure 45. The value obtained - the pH at which  $\tilde{V}$  falls to one half its maximum value - is 8.10.

An equation consistent with the observed pH dependence of  $\tilde{V}$  is

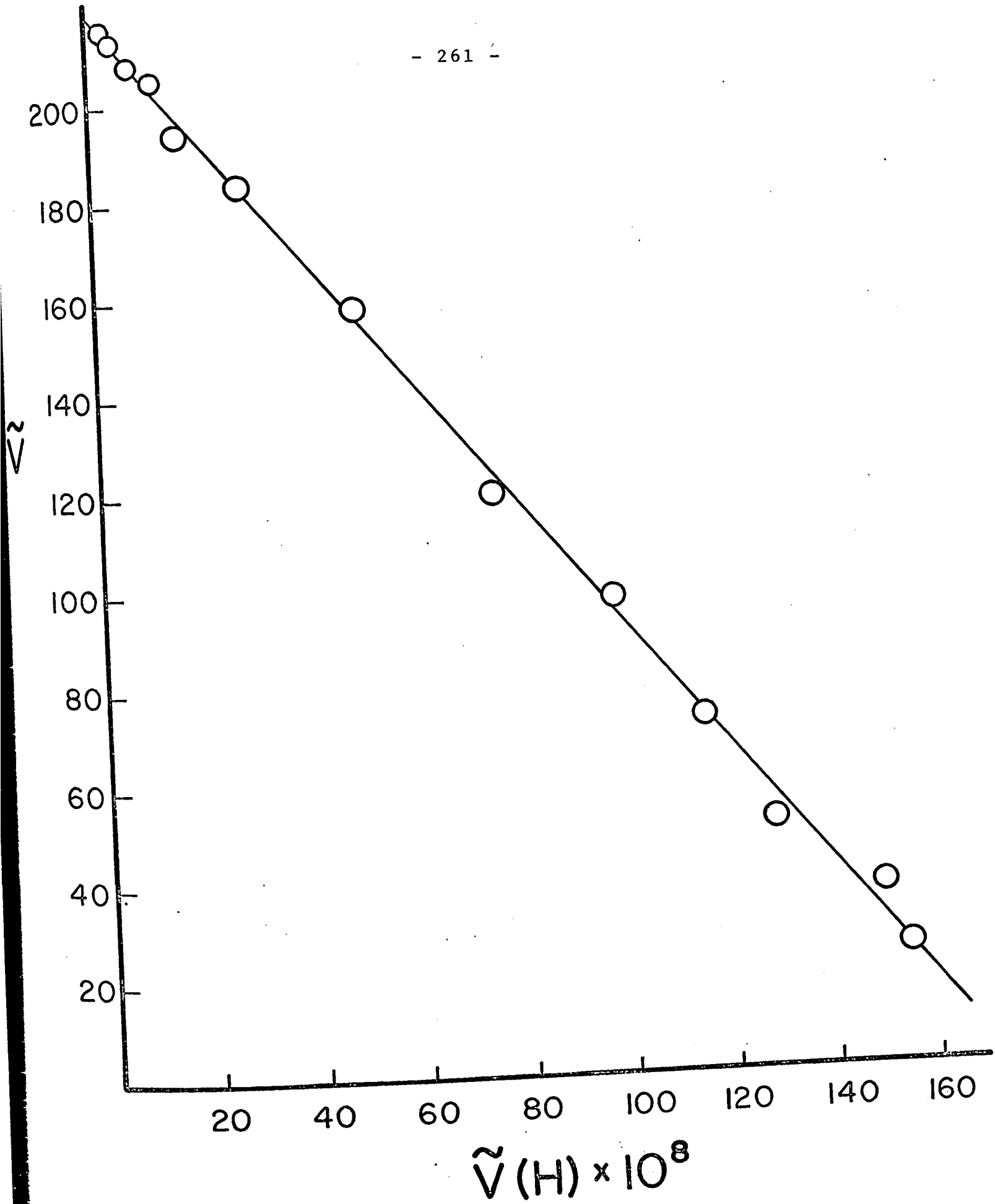
$$\tilde{V} = \left( \frac{k_2 E_o}{1 + \frac{H}{K'_b}} \right) \quad (51)$$

This equation, it was previously pointed out, predicts that a plot of  $\tilde{V}$  against  $\tilde{V}(H)$  should result in a straight line having a slope of  $-1/K'_b$  and an intercept equal to  $k_2 E_o$ . Such a plot is shown in Figure 48. The straight line drawn through the experimental points was obtained by statistically fitting the data to Eq. (52) by the method of least squares; the results of this analysis indicate that  $pK'_b = 8.10 \pm .03$  and  $k_2 E_o = (0.2187 \pm .0065) \mu\text{moles}/\text{min}/\text{mg}$  enzyme.

The value obtained for  $\tilde{V}$  at pH 7.0 was not included in the determination of  $pK'_b$ . Although the agreement between the observed values of  $\tilde{V}$  and those predicted by Eq. (51) assuming a  $pK'_b$  of 8.10 was excellent above pH 7.0, the value

Figure 48

Plot of  $V$  against  $V(H)$  for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate in 0.010M barbital at 25.0°C and  $I = 0.10M$  (adjusted with 5.0M NaCl). The rates are expressed as  $\mu\text{moles}/\text{min}/\text{mg}$  enzyme.



obtained at pH 7.0 deviated very significantly from the theoretical one. Similar deviations have in fact been previously observed with phosphatases from other sources (300,302-3,311). The results of transient-phase studies with these enzymes (130,132,300) suggest that these deviations may reflect a change in the rate-limiting step of the reaction: the assumption that  $k_2 \ll k_3$  may no longer be true at pH < 7.

### pH Dependence of $p\tilde{K}_m$

The pH profile of  $p\tilde{K}_m$  is shown in Figure 43. Over the entire pH range studied  $p\tilde{K}_m$  increases with decreasing pH. It is therefore difficult to obtain a very reliable estimate of the pK of the ionizing group responsible for the fall-off in  $p\tilde{K}_m$  at alkaline pH using Dixon's method; however, the approximate value obtained, 8.64, is in reasonable agreement with the  $pK_a$  value of 8.55 obtained from the pH profile of  $\tilde{V}/K_m$ . The two values should of course be identical, since the ionizing group detected from the variation of  $p\tilde{K}_m$  with pH is in fact the acidic group in the free enzyme. This can be readily verified by examining the equation which gives the pH dependence of  $\tilde{K}_m$ . As pointed out previously,

$$\tilde{K}_m = \tilde{V} / (\tilde{V} / \tilde{K}_m) \quad (55)$$

or

$$p\tilde{K}_m = \log_{10} \frac{\tilde{V}}{K_m} - \log_{10} \tilde{V} \quad (56)$$

(cf. Eqs. (11) and (12)).

Substituting the expressions for  $\tilde{V}$  and  $\tilde{V}/K_m$  (Eqs. (51) and (54) into Eqs. (55) and (56) results in the following expressions for  $\tilde{K}_m$  and  $p\tilde{K}_m$ :

$$\tilde{K}_m = K_s \frac{\left(1 + \frac{H}{K_b} + \frac{K_a}{H}\right)}{\left(1 + \frac{H}{K'_b}\right)} \quad (57)$$

and

$$p\tilde{K}_m = -\log_{10} K_s + \log_{10} \frac{\left(1 + \frac{H}{K'_b}\right)}{\left(1 + \frac{H}{K_b} + \frac{K_a}{H}\right)} \quad (58)$$

At sufficiently high pH Eq. (58) reduces to

$$p\tilde{K}_m = -\log_{10} K_s + \log_{10} \frac{H}{K_a}, \quad (59)$$

while at sufficiently low pH Eq. (58) becomes

$$p\tilde{K}_m = -\log_{10} K_s \quad (60)$$

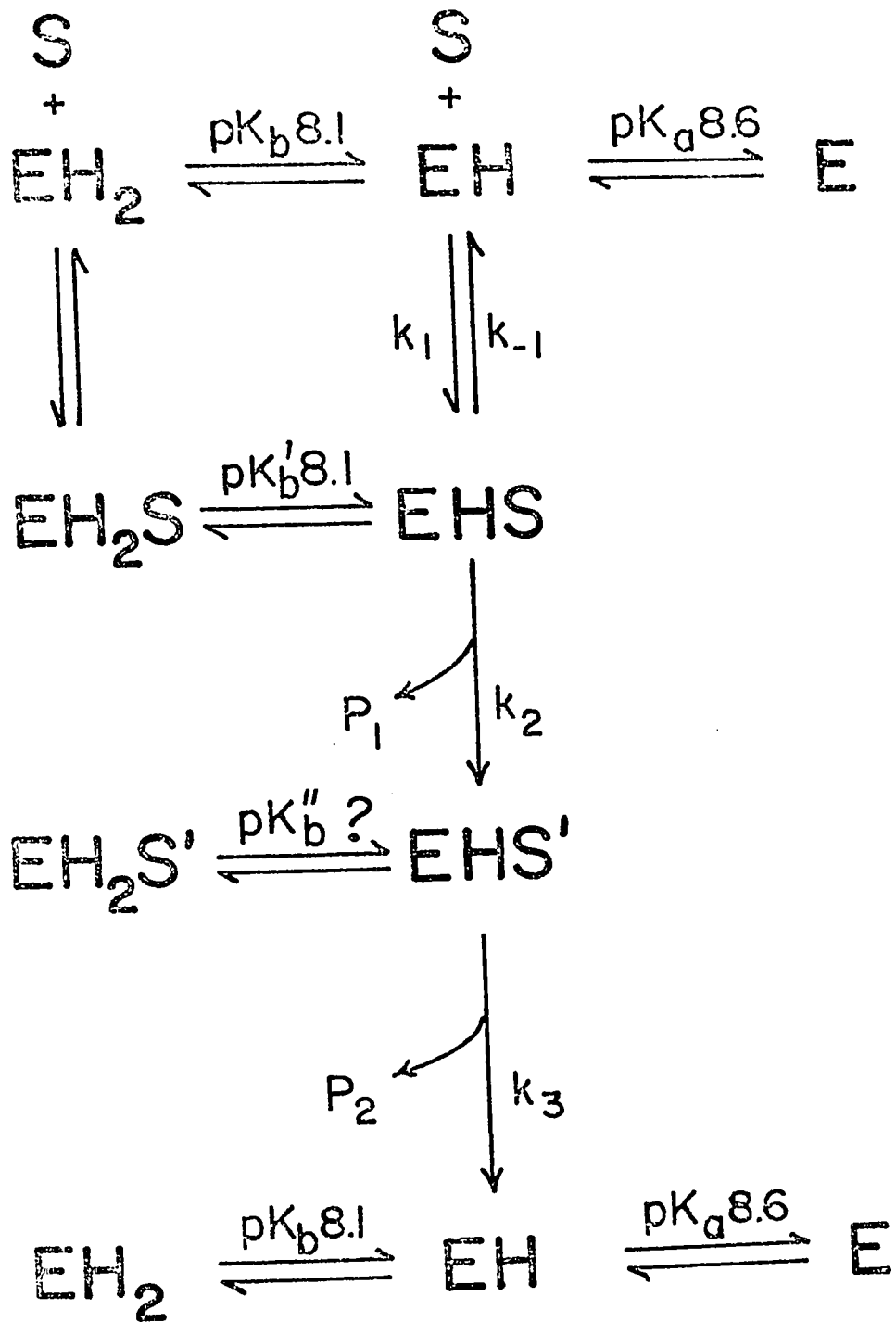
It can readily be shown that the two straight lines described by Eqs. (59) and (60) have slopes of -1 and 0, respectively, and that they intersect at  $pH = pK_a$ .

## DISCUSSION

The values obtained for the dissociation constants of the acidic and basic groups involved in the hydrolysis of p-nitrophenyl phosphate with chicken intestinal phosphatase are summarized in Scheme III (Figure 49). Scheme III is the simplest reaction scheme which explains the pH dependence of reactions catalyzed by this enzyme (Figures 12-17) and by phosphatases from several other sources including the calf intestinal (302-3) and the E.coli (225,311) enzyme, and which is at the same time also consistent with other experimental results; it is a special case of Scheme II, the general scheme for pH dependence discussed earlier (Figure 2): the general scheme reduces to Scheme III when  $k_2 \ll k_3$ ,  $a = b = 0$ ,  $\alpha = \beta = 0$ , and  $K'_a = K''_a = 0$ . It is of interest to note that an identical reaction mechanism has previously been postulated to account for the pH dependence of reactions catalyzed by trypsin and chymotrypsin (336). Indeed, as will be pointed out later (see discussion below), there are many important similarities between the catalytic action of these two proteolytic enzymes and that of alkaline phosphatase. The relationship between Scheme III and the catalytic action of alkaline phosphatase is most conveniently discussed by considering first the ionizations of the acidic group and then those involving the basic group.

Figure 49

Scheme III, the simplest reaction scheme  
which explains the pH dependence of  
reactions catalyzed by alkaline phosphatase.



### Ionizations of the Basic Group

The pH profile of  $\log_{10} \tilde{V}/K_m$  (Figure 41) shows a fall-off in rate on the acid side corresponding to the ionization of a basic group having a  $pK_b$  of 8.13. The fact that this group is revealed in the  $\log_{10} \tilde{V}/\tilde{K}_m$  plot indicates not only that it is free to ionize in the free enzyme, but also that it is involved in the phosphorylation step; phosphorylation can occur if the group is in the unprotonated form. Scheme III therefore shows no arrows between  $\text{EH}_2\text{S}$  and  $\text{EH}_2\text{S}'$ .

Uncertainty about the relative orders of magnitude of  $k_2$  and  $k_3$  has, until recently, resulted in a considerable amount of confusion concerning the interpretation of the pH profile of  $\log_{10} \tilde{V}$ . Lazdunski et al. (151), for example, have asserted that dephosphorylation is very much slower than phosphorylation (i.e.,  $k_3 \ll k_2$ ) over the entire pH range, and have therefore concluded that the ionizing group revealed in the plot of  $\log_{10} \tilde{V}$  against pH is involved in dephosphorylation. These conclusions were based on the assumption that marked phosphotransferase activity (reflected by an increase in the rate of formation of  $\text{P}_1$ ) will be observed in the presence of added nucleophiles such as Tris or ethanolamine only if  $k_3 > k_2$ . However, it was demonstrated in the previous chapter that this assumption is not necessarily correct. Furthermore, the results of transient phase experi-

ments (129-32,300) have conclusively shown that dephosphorylation is not rate limiting above pH 7.0. The pH profile of  $\log_{10} \tilde{V}$  (Figure 41) will therefore provide information about the phosphorylation process.\* The decrease in  $\log_{10} \tilde{V}$  on the acid side corresponds to the ionization of a basic group having a  $pK'_b$  of 8.10. The fact that there is a fall-off in rate at low pH shows that this group is essential to phosphorylation, which can occur only if the group is in the unprotonated form - a conclusion identical to the one arrived at earlier by examining the pH profile of  $\log_{10} \tilde{V}/K_m$ . The observation that the pK value obtained from the plot of  $\log_{10} \tilde{V}$  against pH is essentially the same as the one derived from the pH profile of  $\log_{10} \tilde{V}/\tilde{K}_m$  implies that the ionization of the basic group having this pK is completely unaffected by the combination of the enzyme with the substrate: the group is as free to ionize in the enzyme-substrate complex as in the free enzyme. This indicates that the basic group, although it does play a role in the phosphorylation process, is not involved in the binding of the substrate. It is for this reason that Scheme III shows arrows not only between EH and EHS but also between  $EH_2$  and  $EH_2S$ .

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\* Although the actual catalytic mechanism involves three steps between the formation of the Michaelis-Menten complex and dephosphorylation - (1) a substrate induced conformation change preceding phosphorylation,  $ES \rightleftharpoons E^*S$ , (2) the phosphorylation step,  $E^*S \rightarrow E^*S' + P_1$ , and (3) a conformational change following it,  $E^*S' \rightleftharpoons E\bar{S}'$ , these are all considered part of the phosphorylation process in the present discussion;  $k_2$  is not a simple rate constant: it refers to the whole phosphorylation process, not just  $E^*S \rightarrow E^*S + P_1$ .

It is worth noting that Lazdunski and Lazdunski (225,310) have reported that  $pK_b = 7.1$ , while  $pK'_b = 7.4$  for the E.coli enzyme. The fact that the  $pK$  is 0.3 of a unit higher in the enzyme-substrate complex than in the free enzyme, they concluded, indicates that the basic group also plays an important role in the binding of the substrate. However, since the value for  $pK_b$  reported by Lazdunski and Lazdunski was obtained by Dixon's method which, it was seen, leads to erroneous results when the two  $pK_s$  are not well-separated ( $pK_a = 8.6$  for the E.coli enzyme), the observed shift in  $pK$  is almost certainly insignificant. Indeed, the value of  $pK_b$  obtained by the method of Alberty and Massey is 7.4, which is identical to the value reported for  $pK'_b$ . The shift of 0.13 units of  $pK$  reported by Krishnaswamy and Kenkare (311) similarly disappears when  $pK_b$  is recalculated by the method of Alberty and Massey.

Since  $k_2 \ll k_3$  the present investigation has provided no information about the pH dependence of the dephosphorylation step. Such information could of course be obtained by carrying out a pH study with a substrate for which dephosphorylation is rate limiting. Unfortunately, no such substrate is known for chicken intestinal phosphatase. However, the transient phase experiments of Gottesman et al. (141) with Co(II) alkaline phosphatase from E.coli suggest that between pH 5 and 12 dephosphorylation is the rate limiting step in the hydrolysis of both p-nitrophenyl and 2,4-dinitro-

phenyl phosphate by this enzyme. The fall-off in  $\log_{10} \tilde{V}$  which they observed at low pH therefore indicates that the basic group is also involved in the dephosphorylation step; dephosphorylation can occur only if this group is in the unprotonated form.

#### Ionizations of the Acidic Group

The pH profile of  $\log_{10} \tilde{V}/K_m$  (Figure 41) also shows a fall-off in rate at high pH, corresponding to the ionization of an acidic group having a  $pK_a$  of 8.55. The fact that this group is revealed in the  $\log_{10} \tilde{V}/\tilde{K}_m$  plot indicates not only that it is free to ionize in the free enzyme, but also that it is involved in the phosphorylation step; phosphorylation can occur only if the group is in the protonated form.

The pH profile of  $\log_{10} \tilde{V}$ , it was seen, provides additional information about the phosphorylation process. The observation that there is no decrease in  $\log_{10} \tilde{V}$  at high pH indicates that the acidic group is no longer free to ionize in the enzyme-substrate complex ( $pK'_a = -\infty$ ); it is attached to the substrate, either through ionic or hydrogen bonding, in such a way that it is unable to lose its proton at high pH.

The pH profile of  $\log_{10} \tilde{V}$  obtained with Co(II) phosphatase from E.coli shows no fall-off in rate at high pH. Since dephosphorylation appears to be rate limiting for

this enzyme, this observation implies that the acidic group is also not free to ionize in the phosphoryl-enzyme; it is probably involved in binding the phosphoryl group to the enzyme during dephosphorylation.

#### Identification of the Two Essential Ionizing Groups

Since the identification of the components of the active site is of fundamental importance to the detailed understanding of the catalytic mechanism, attempts are usually made to assign the pK values obtained in pH studies to specific amino acid residues. Although the validity of identifying the amino acid residues involved in catalysis on the basis of kinetically derived pK values has often been questioned (337-42) because of (1) the possibility that the pK of the group in the enzyme may be altered drastically by electrostatic effects of neighboring groups (337-8 ), (2) the uncertainty which may be introduced by the influence of equilibrium steps preceding or following the rate-limiting step (339-41), and (3) the existence of alternative ionization pathways, especially in systems in which the two pK values are close together (341), it is usually found that the measured pK values do not differ by more than one unit from those found in model systems (338). Differences of this size are usually not large enough to seriously affect the identification of the amino acid residue involved.

The identity of neither of the two groups implicated in the catalytic mechanism of alkaline phosphatase has yet been established. It is therefore of interest to consider which amino acid residues have pKs consistent with those obtained from the pH dependence of the enzyme catalyzed reaction. It is convenient to first consider the identity of the basic group and then that of the acidic group.

#### Identification of the Basic Group

The basic group has a pK of 8.1 in chicken intestinal phosphatase; the pK of the corresponding group in the E.coli enzyme is 7.4 (225,311). The same amino acid residue may therefore not be involved in both enzymes. It has been suggested (225,311) that the pK values of 7.4 corresponds to the ionization of an imidazole group of histidine located at the active site of the E.coli enzyme. Although the pK of imidazole in histidine is considerably lower - 6.15 (343-4), pKs of about 7 have been found for this group in protein molecules (299,336). Indeed, the observed pK would be expected to be considerably larger than 6.15 if it were due to several imidazole groups on histidine residues adjacent to each other. Under these conditions, the protonation of one imidazole group would prevent the protonation of the others. The assignment of the pK<sub>b</sub> value to the imidazole group of histidine is further supported by the heat of ionization observed for this group - 6.5 kcal/mole

225,310), which is in good agreement with the values of 6.5-7.5 kcal/mole usually found for the heat of ionization of imidazole in other protein molecules (343-4). Furthermore, a recent study of the effects of organic solvents on the kinetic parameters of the E.coli phosphatase catalyzed reaction (311) has indicated that the group having a pK of 7.4 is cationic in nature, which is also consistent with the hypothesis that this group is the imidazole group of histidine.

Few chemical modification studies have confirmed the involvement of a histidine residue in the catalytic mechanism of E.coli alkaline phosphatase. However, this is not especially surprising since most of the chemical methods at present available for the identification of this amino acid residue are relatively unsatisfactory (345-6). Plotch and Lukton (347), for example, found that the photooxidation of E.coli alkaline phosphatase does not diminish its catalytic activity, even though the imidazole of chymotrypsin is readily photooxidized under identical conditions. However, Tait and Vallee (222) later found that the apoenzyme on similar treatment does lose its capacity to bind  $Zn^{2+}$ ; amino acid analyses indicated that approximately three histidine residues were modified. These results suggest that the  $Zn^{2+}$  is bound to the three histidine residues in the holoenzyme, thereby protecting them from photooxidation. It has not yet been established that the histidine residue postulated to parti-

cipate in catalysis is one of the three bound to the zinc ion.

More recently, Csopak and Folsch (348) found that substrate analogs such as mono- and dichloroacetyl- $\beta$ -glycerophosphate irreversibly inactivate the E.coli enzyme. Substrates such as glucose-6-phosphate and the reversible inhibitor inorganic phosphate protect the enzyme from this irreversible inactivation. The absorption spectrum of the inactivated enzyme differed from that of the native one in the 250 m $\mu$  region, implicating the formation of acetyl-imidazole; amino acid analyses on the inactivated enzyme showed that only one histidine was modified per molecule of enzyme. These results taken together suggest the presence of an essential histidine residue at or near the active site of the enzyme.

Similar chemical modifications have not been carried out with alkaline phosphatases from other sources. However, in view of the high value of  $pK_b$  observed ( $pK_b$  is 8.1 in chicken intestinal phosphatase, and even higher  $pK$  values have been reported for other intestinal enzymes (229,301-4,308), it seems unlikely that this  $pK$  can be assigned to the ionization of an imidazole group of histidine in these enzymes.

A more reasonable choice is the terminal amino group, which in model peptides has a  $pK$  of about 7.8 and is also cationic. The heat of ionization of the basic group in

calf-intestinal phosphatase is about 6.5 kcal/mole (302), while that of a terminal  $\alpha$ -amino group is considerably higher - 10 to 13 kcal/mole in model compounds (231). However, as Lindley has pointed out (349), when two ionizing groups are adjacent to each other each will exhibit a heat of ionization which is intermediate between the values normally found for either of them. The presence of a charged carboxyl group ( $\Delta H = \pm 1.5$  kcal/mole) close to an  $\alpha$ -amino group would therefore lower its heat of ionization to the value observed - 6.5 kcal/mole. Indeed, in such a case; because of electrostatic effects, the pK value of the  $\alpha$ -amino group should be significantly higher than that in model compounds. This, of course, agrees well with the results obtained with intestinal phosphatases.

The results of chemical modification studies (226,350-2) seem to confirm the assignment of pK<sub>b</sub> to an  $\alpha$ -amino group. Fishman and Ghosh (351-2), for example, found that various amino groups reagents, especially acetic anhydride, almost completely inactivate rat intestinal phosphatase. Unfortunately, amino acid analyses were not carried out, so that the number of amino groups modified was not determined; nor was it established that other side chains of the enzyme were unaffected by the amino group reagents.

It has sometimes been suggested that the basic group may be a thiol group of cysteine (226,302,351-2). The observed pK and heat of ionization are certainly consistent

with this assignment; in model compounds and other enzymes sulfhydryl groups usually have  $pK_s$  between 8 and 9 and heats of ionization of about 7 kcal/mole (338,343-4). Indeed, thiol group reagents such as iodoacetamide, iodosobenzoate, p-hydroxymercuribenzoate, and N-ethylmaleimide have all been found to inhibit intestinal phosphatases (226,302,351-2). But it should be pointed out that relatively high concentrations of these reagents were required for marked inactivation. It therefore still remains doubtful whether intestinal phosphatases contain essential thiol groups. In fact it has not even been determined whether these enzymes possess any free thiol groups. Since Schlessinger and Barrett (353) have shown that the E.coli enzyme lacks free sulfhydryl groups, the basic group of this enzyme cannot be the S-H group of cysteine. This conclusion is further corroborated by results of solvent studies mentioned earlier (311) which indicate that the basic group in the E.coli enzyme is cationic, not neutral. By carrying out similar solvent studies with the intestinal phosphatases it should be possible to eliminate either the  $\alpha$ -amino or the sulfhydryl group from serious consideration as the essential basic group involved in the enzyme catalyzed reaction.

#### Identification of the Acidic Group

The acidic group, shown to be involved in substrate binding, has a  $pK$  of 8.6 in chicken intestinal phosphatase;

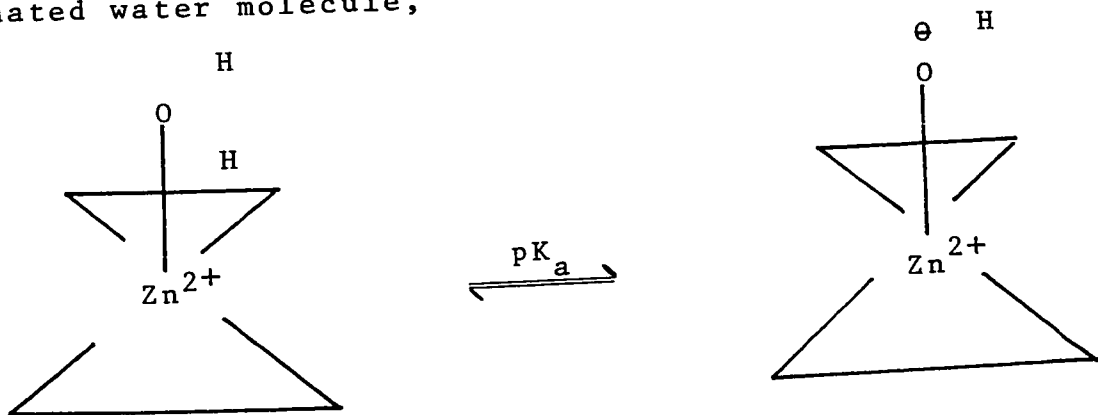
similar pKs have been reported for the E.coli enzyme (225,310-11). The identity of this group in either has not yet been established. The terminal  $\alpha$ -amino group, the  $\epsilon$ -amino group of lysine, the phenolic hydroxyl group of tyrosine, and the sulfhydryl group of cysteine are all possibilities worth considering.

By using arguments similar to those used in discussing the identity of the basic group it is possible to show that the acid group of the E.coli enzyme is unlikely to be one of the above four amino acid residues (311). However, in the case of the intestinal phosphatases all of the above amino acid residues, with the possible exception of the  $\epsilon$ -amino group of lysine, must be considered as likely possibilities until more evidence - particularly the results of detailed solvent and chemical modification studies - becomes available. The chemical modifications of the intestinal enzyme carried out so far have shown that the enzyme is irreversibly inactivated when reacted with amino (226,350-1), thiol (226,350-1), and phenolic hydroxyl (354-6) group reagents. Only one of these groups, at most, can be identified with the acidic group involved in substrate binding; the other groups may not play a direct role in binding and catalysis, but may, instead, be involved in maintaining the conformation integrity of the enzyme.

It is now well-established that the E.coli and intestinal phosphatases are zinc metalloenzymes (357-69).

Gottesman et al. (141) have recently carried out a kinetic study with the alkaline phosphatase of E.coli in which the Zn(II) of the enzyme was replaced by Co(II). They found that the pH profile of  $K_m$  for this enzyme was similar to that obtained with native Zn(II) enzyme: above pH 7  $K_m$  increased with pH. However, the  $pK_a$  was shifted upward by one unit to 9.6. These results and the results of phosphate binding studies to the native and metal free enzyme, and the spectral studies of the Co(II) enzyme suggest that the metal atom plays an important role in substrate binding.

As Lazdunski and Lazdunski have pointed out (151) the magnitude of  $pK_a$  observed for the E.coli enzyme suggests that it may correspond to the ionization of a Zn(II) coordinated water molecule,



which is known to have a  $pK$  of about 9 at  $25^{\circ}C$  (370-71). Since chicken intestinal phosphatase has exactly the same pH dependence on the acid side ( $pK_a = 8.6$ ), an attractive possibility is that in this enzyme too the acid group

involved in substrate binding is a water molecule coordinated to the metal atom at the active site.

CHAPTER IV

THE INHIBITION OF INTESTINAL ALKALINE  
PHOSPHATASE BY L-PHENYLALANINE

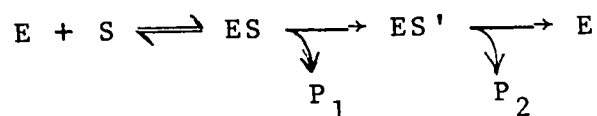
INTRODUCTION

Relatively few detailed kinetic studies have been made of the effects of inhibitors on reactions catalyzed by alkaline phosphatase. Various types of inhibition, however, have been observed with both the mammalian and the E. coli enzymes (22,23). Substances such as phosphate, thiophosphate, and arsenate, which have inhibitor dissociation constants (i.e.,  $K_i$  values) comparable to the  $K_m$  values for good substrates, are potent competitive inhibitors (18,24,225-8); the phosphonates are also competitive inhibitors, but they have much weaker affinities for the enzyme (139,225). Many amino acids, for example, cysteine and histidine, have been found to be weak non-competitive inhibitors (95,226,372,373).

So far the only well-characterized uncompetitive inhibitor of alkaline phosphatase is L-phenylalanine. Preliminary studies by Fishman, Green and Inglis (95) have established that the inhibition by L-phenylalanine is organ-specific: it specifically inhibits purified rat intestinal phosphatase but has little or no effect on the enzyme from several other rat tissues. The stereospecific nature of the inhibition was at the same time also demonstrated: D-phenyl-

alanine was shown to have no inhibitory effect (95). It was later shown that phenylalanine had a similar effect on human tissue phosphatases: of the enzymes from bone, intestine, kidney, liver, lung, and spleen only the intestinal one was appreciably inhibited and only the L-isomer was effective (96). This led subsequently to the development of a convenient method of identifying and estimating alkaline phosphatase of intestinal origin in the presence of the liver and bone enzymes (374). Further studies showed that human placental phosphatase was similarly affected by L-phenylalanine (374-6). The inhibition of both the rat intestinal and the human placental phosphatase, it was later established (374-5), was of an uncompetitive nature. More recently (377) it was shown that L-tryptophan is another organ-specific uncompetitive inhibitor of human placental alkaline phosphatase.

Sufficient data do not at present exist to unequivocally establish the mechanism of inhibition of alkaline phosphatase by L-phenylalanine. Two different hypotheses are found in the literature (374-9). Fishman and Ghosh have, on the one hand, suggested (375,377-9) that an inactive ESI complex is formed by the stereospecific combination of L-phenylalanine with the Michaelis complex, ES, while Fernley and Walker have, instead, proposed (376) that combination with L-phenylalanine has little effect on the reactivity of ES but prevents the breakdown of the phosphoryl-enzyme intermediate, ES', formed later in the hydrolysis sequence:



where E is the free enzyme, S the substrate, ES the Michaelis complex, P<sub>1</sub> the first product of the reaction, ROH, and P<sub>2</sub> the second product, inorganic phosphate.

### Linear Inhibition Patterns

The relation between the initial rate of an enzyme-catalyzed reaction, v, and the initial substrate concentration, S, is usually of the form

$$v = \frac{VS}{K_m + S} \quad (1)$$

where V is the maximum rate and K<sub>m</sub>, the Michaelis constant, is the substrate concentration for which v = V/2. Taking the reciprocal of Eq. (1) gives

$$\frac{1}{v} = \frac{K_m}{V} \left(\frac{1}{S}\right) + \frac{1}{V} \quad (2)$$

In the presence of a reversible inhibitor the rate equation becomes

$$v' = \frac{V'S}{K' + S} \quad (3)$$

so that

$$\frac{1}{v'} = \frac{K'}{V'} \left(\frac{1}{S}\right) + \frac{1}{V'} \quad (4)$$

Reciprocal plots of initial rate versus substrate concentration should therefore be straight lines of the form

$$y = (\text{slope})\left(\frac{1}{S}\right) + (\text{intercept}) \quad (5)$$

both in the presence and absence of inhibitor.

To establish the mode of action of an inhibitor it is usually necessary to plot  $1/v$  against  $1/S$  at several different inhibitor concentrations and determine (1) whether the inhibitor changes the slope, vertical intercept, or both, and (2) whether the slope (and/or intercept) is a linear function of inhibitor concentration. Linear, hyperbolic, and parabolic relationships have been observed; however, if the inhibitor combines with only one form of the enzyme, for example ES, the result will invariably be linear inhibition (380).

The classification of reversible linear inhibitors into three types - competitive, non-competitive, and uncompetitive - is based on their effect on the slope and intercept of Eq. (4). In the presence of a competitive inhibitor

$$\frac{1}{v'} = \left[1 + \frac{I}{K_i}\right] \frac{K_m}{S} + \frac{1}{V} \quad (6)$$

An inhibitor of this type therefore only affects the slope term of Eq. (4) and since

$$K' = K_m \left[1 + \frac{I}{K_i}\right] \quad (7)$$

and

$$v' = v \quad (8)$$

the inhibition will be eliminated at high substrate concentrations. A non-competitive inhibitor affects both the vertical intercept and the slope of Eq. (4). In the presence of this type of inhibitor the rate equation becomes

$$\frac{1}{v'} = \left(1 + \frac{I}{K_i \text{ slope}}\right) \frac{K_m}{V} \left(\frac{1}{S}\right) + \left(1 + \frac{I}{K_i \text{ intercept}}\right) \frac{1}{V} \quad (9)$$

$K_i \text{ intercept}$  may be greater than, equal to, or less than  $K_i \text{ slope}$ . When  $K_i \text{ slope} = K_i \text{ intercept} = K_i$ ,

$$K' = K_m \quad (10)$$

and

$$v' = v / \left(1 + \frac{I}{K_i}\right) \quad (11)$$

The maximum velocity in the presence of a non-competitive inhibitor is therefore lower than in the absence of inhibitor. Since the substrate and inhibitor do not compete for the same binding site on the enzyme, or in some cases for the same form of the enzyme, inhibition can not be eliminated by adding more substrate. In the presence of an uncompetitive inhibitor the rate equation becomes

$$\frac{1}{v'} = \frac{K_m}{V} \left(\frac{1}{S}\right) + \left(1 + \frac{I}{K_i}\right) \frac{1}{V} \quad (12)$$

i.e., only the vertical intercept term of Eq. (4) is affected by this type of inhibition. Since

$$V' = V / (1 + \frac{I}{K_i}) \quad (13)$$

and

$$K' = K_m (1 + \frac{I}{K_i}) \quad (14)$$

$K'$  and  $V'$  will always be smaller than  $K_m$  and  $V$ , but the ratio  $K'/V'$  will be a constant and equal to  $K_m/V$  (i.e.,  $K'$  and  $V'$  are changed by the same extent and in the same direction).

A series of reciprocal plots obtained at different concentrations of the uncompetitive inhibitor will therefore be parallel: each line will have the same slope but an intercept differing from that in the absence of inhibitor by a factor of  $(1 + I/K_i)$ .

## EXPERIMENTAL

The substrate employed was p-nitrophenyl phosphate since it could conveniently be worked with at  $(S) < \tilde{K}_m$  as well as at higher concentrations. The time course of the reaction was followed continuously with a spectrophotometer so that true initial rates could be measured; this is especially important in view of inhibition by the product inorganic phosphate (18,225-8).

The choice of buffer is also a matter of importance. Because the object of the present work was to characterize the inhibition of alkaline phosphatase by L-phenylalanine, the use of other substances which either inhibit or activate the enzyme had to be avoided. It was shown in Chapter II that certain amines with suitably located hydroxyl groups, such as Tris and ethanolamine, enhance the reaction; these compounds could therefore not be employed as buffers. Substances such as borate and carbonate which inhibit the enzyme (316-8) were also unsuitable. It was found that there was no activation or inhibition with barbital (5,5'-diethyl barbituric acid); this substance was therefore used as a buffer in the present study. All the reactions were carried out at 25°C so that the reported thermal instability of the buffer (375) presented no problem and at pH 9.0 at which 0.010M barbital was found to give adequate buffering, especially for the measurement of initial rates.

To avoid the variety of complications arising from the presence of  $Mg^{2+}$  (317,319-23), this ion was excluded from all the reaction mixtures. Ion strength complications (152, 252) were avoided by working at a constant ionic strength of 1.0M, the reaction mixtures being so adjusted by the addition of sodium chloride.

### Materials

The enzyme used was a highly purified preparation of calf intestinal alkaline phosphatase (Code No. P4502) obtained from the Sigma Chemical Company as an ammonium sulfate suspension (5 mg protein/ml) stabilized with  $10^{-3}M$   $MgCl_2$  and  $10^{-4}M$   $ZnCl_2$ . Before use the preparation was dialyzed at  $2^{\circ}C$  against four changes of 0.010M Tris-HCl buffer, pH 8.75, and stored as a 1 mg/ml solution in the above buffer at  $4^{\circ}C$ . A fresh 'working' stock solution of enzyme was prepared immediately before each series of runs by further diluting an aliquot of the 1 mg/ml solution with 0.010M Tris-HCl buffer, pH 8.0 at  $25^{\circ}C$ : 25  $\mu$ l of this 'working' stock solution added to 1.5 ml of the reaction mixture gave an enzyme concentration of about 100  $\mu$ g/ml. The protein concentration of the original stock solution was determined spectrophotometrically at 278 m $\mu$  (specific absorptivity =  $0.72 \text{ ml cm}^{-1} \text{ g}^{-1}$ ).

The p-nitrophenyl phosphate used as substrate was a Calbiochem Grade A reagent (Code No. 4876) obtained in the form of the sodium salt. It was stored in a vacuum desiccator

in the dark at 0°C. The concentration of substrate was found to be most reliably determined from the amount of p-nitrophenol produced on complete hydrolysis, following a modification of the method used by Chanley and Feageson (263); the p-nitrophenol concentration was measured spectrophotometrically at 400 mμ. The unhydrolyzed substrate was found to be essentially free from p-nitrophenol (<0.05%).

Barbital (Code No. B-0375), Tris (Code No. T1503), D- and L-phenylalanine (Code No. P1751 and P2126, respectively) were obtained from the Sigma Chemical Co.; all these materials were of high purity and were used without further purification. The HCl, NaOH and NaCl used to adjust the pH and ionic strength were analytical grade reagents (purity > 99.9%) obtained from Fisher Scientific Co. and were also used without further purification.

All solutions were prepared in doubly-distilled deionized water.

#### Kinetic Procedure

The kinetic procedure and the apparatus used to obtain the initial steady-state rates of hydrolysis of p-nitrophenyl phosphate were that described in Chapter III, PP.210-216.

#### Stopped-Flow Spectrophotometer

In some cases, initial steady-state rates were also determined with a Durrum-Gibson stopped-flow spectrophotometer

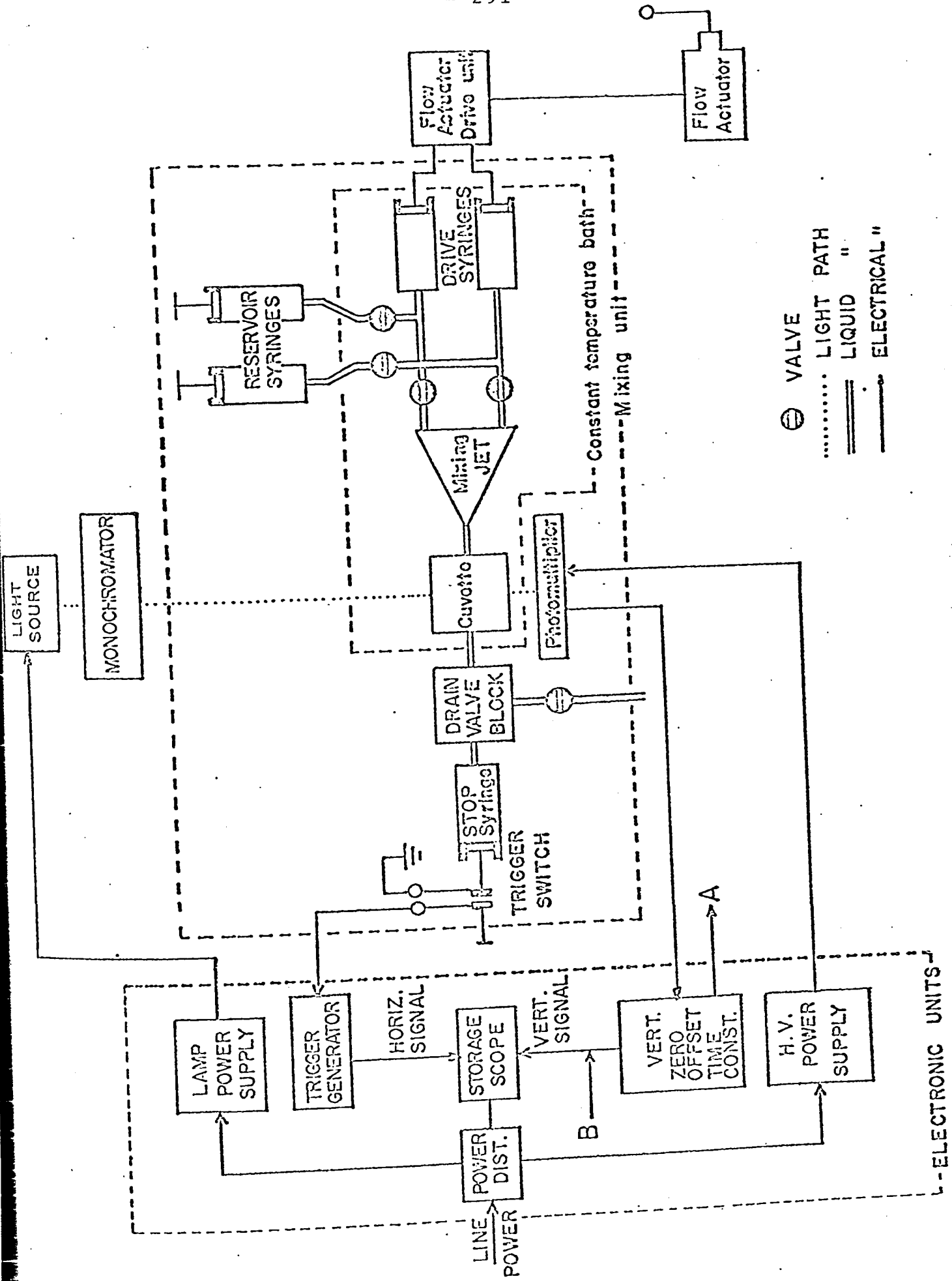
purchased from the Durrum Instrument Corporation, Palo Alto, California. A schematic diagram of the system is shown in Figure 50. Details of the stopped-flow technique are given in the Appendix.

Equal volumes of substrate and enzyme in appropriate buffer solutions are hydraulically forced through the mixing jet into the cuvette and thence into the stop syringe. Just before its plunger hits a mechanical stop, it makes contact with a trigger switch which actuates the horizontal time-base sweep for the oscilloscope display. Since the flow of sample is stopped immediately after mixing, the reaction in the cuvette takes place with a minimum of interference caused by turbulence or flow artifacts. The monochromatic light obtained from the monochromator passes through the mixed solution in the cuvette and the resultant variation in light intensity is sensed by the photomultiplier tube. The photomultiplier output, proportional to the instantaneous light intensity transmitted by the reacting solution, is applied to the vertical axis of the oscilloscope. The display on the storage oscilloscope, which indicates percentage transmittance versus time, started just before the reaction began and ended according to a preselected time-base setting.

All measurements were made at 400 m $\mu$ . The reaction system was maintained at  $25.0 \pm 0.1^{\circ}\text{C}$  by means of a thermostatted water bath.

Figure 50

Functional block diagram of the Gibson-Durrum stopped-flow spectrophotometer system.



## ANALYSIS OF RESULTS

### Determination of V' and K'

Values of V' and K' were obtained by statistically fitting the results to Eq. (3), use being made of the method of Bliss and James (274-5) or of a modified version of the computer program of Cleland (273). The calculations were done on an IBM 360/65 digital computer.

### Determination of K<sub>i</sub>

Upon rearranging, Eqs. (13) and (14) become

$$\frac{1}{V'} = \frac{1}{V} + \frac{1}{VK_i} \quad (I) \quad (15)$$

and

$$\frac{1}{K'} = \frac{1}{K_m} + \frac{1}{K_m K_i} \quad (I) \quad (16)$$

For the case of uncompetitive inhibition, a plot of  $1/V'$  against I will therefore be a straight line with a slope equal to  $1/VK_i$  and a Y-intercept of  $1/V$ ; the inhibitor dissociation constant  $K_i$  will be given by the relation

$$K_i = \frac{(\text{intercept})}{(\text{slope})} \quad (17)$$

Alternatively, if  $1/K'$  is plotted against I,  $K_i$  can be calculated from the Y-intercept and the slope of the straight line obtained, again using Eq. (17).

## RESULTS

The effect of substrate concentration on the activity of calf intestinal phosphatase was studied at three different concentrations of L-phenylalanine in order to determine the dependence of the Michaelis parameters on the concentration of this inhibitor. All the reactions were carried out at pH 9.0,  $I = 1.0$  and  $T = 25.0^{\circ}\text{C}$  and with the same concentration of enzyme ( $0.10 \mu\text{g/ml}$ ). The concentration of substrate, p-nitrophenyl phosphate, was varied from 4.0 to 120  $\mu\text{M}$  and the L-phenylalanine concentrations were 4.96, 9.92 and 14.9 mM.

The double reciprocal plots of velocity versus substrate concentration for the three different L-phenylalanine concentrations studied are shown in Figure 51. The data obtained at a given concentration of L-phenylalanine accurately obey the modified Michaelis-Menten equation, Eq. (3); the slope of each line is therefore equal to  $K'/V'$  and its Y-intercept is  $1/V'$ . The straight lines obtained at the various L-phenylalanine concentrations are parallel: they have different Y-intercepts but identical slopes. This can be seen more clearly from Table 19 which lists the results obtained by statistically fitting the data at each L-phenylalanine concentration to Eq. (3) in the manner described previously. The standard errors reported (calculated as the square roots of the internal variances) show that the kinetic parameters listed

Figure 51

Double reciprocal plots of velocity versus substrate concentration at three different concentrations of L-phenylalanine ( $\Delta$ , 4.96 mM; 0, 9.92 mM; 0, 14.9 mM) for the hydrolysis of p-nitrophenyl phosphate catalyzed by calf intestinal alkaline phosphatase at pH 9.0,  $I = 1.0$  and  $T = 25.0^{\circ}\text{C}$ . The velocity of the reaction is expressed as  $\mu\text{moles of p-nitrophenol released/min/mg enzyme}$ .

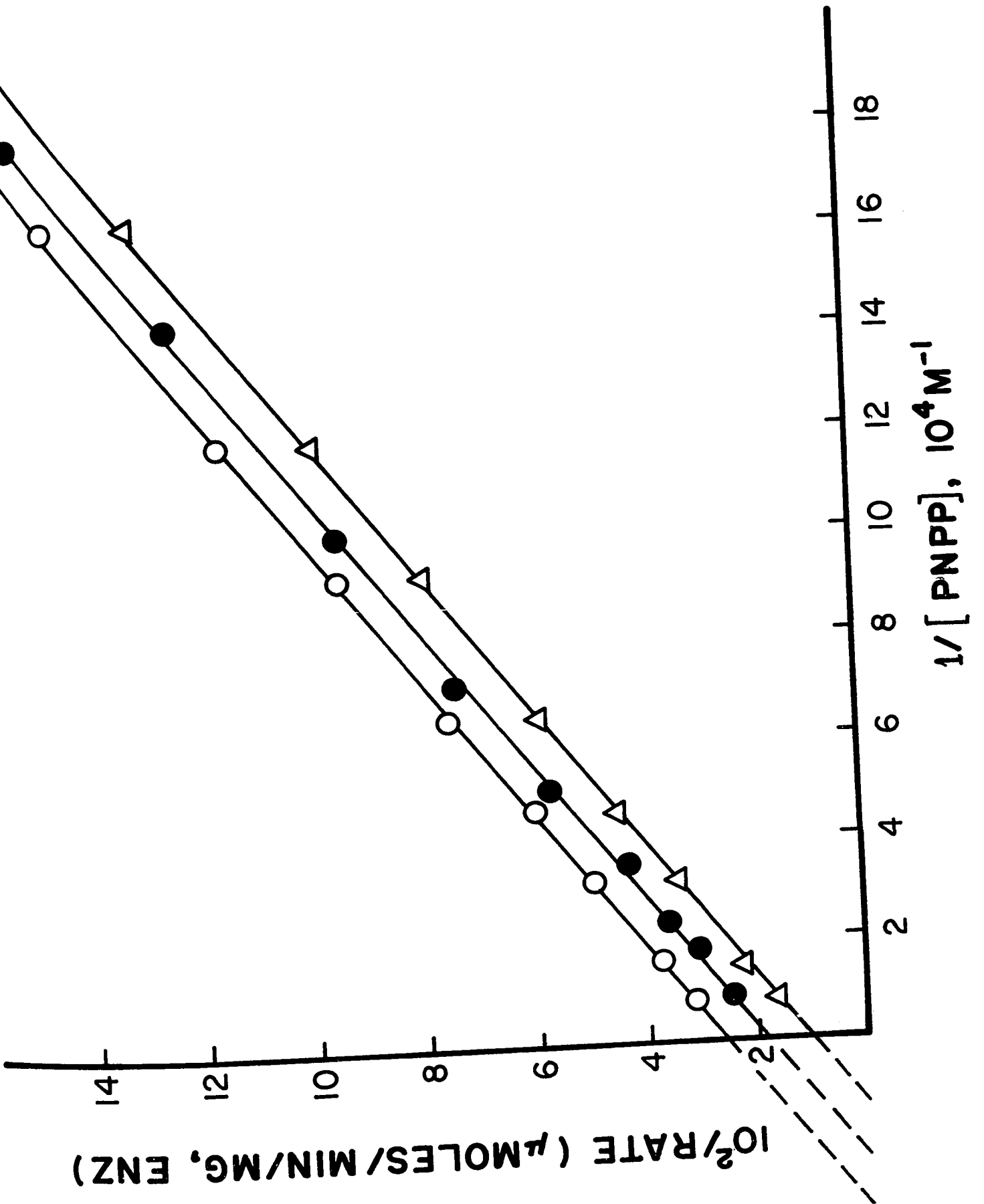


TABLE 19  
 Kinetic Parameters for the Alkaline Phosphatase-Catalyzed Hydrolysis  
 of p-Nitrophenyl Phosphate at pH 9.0, I = 1.0 M and T = 25.0°C

(L-Phenylalanine) mM	V' moles/min/mg Enz	K' moles/liter	V'/K' liters/min/mg Enz
4.96	95.2 ± 1.4	72.3 ± 2.3	0.760 ± 0.13
9.92	55.8 ± 0.99	42.6 ± 1.6	0.763 ± 0.16
14.9	39.6 ± 0.63	30.0 ± 1.0	0.758 ± 0.14

in Table 19 can be considered reliable to better than  $\pm 4\%$ . It is also seen from the last column of the table that the ratio  $K'/V'$  is essentially independent of L-phenylalanine concentration; the relatively small variation in  $K'/V'$  which is observed is well within the 2% experimental error.

The reciprocals of both  $V'$  and  $K'$  are linear functions of L-phenylalanine concentration, their dependencies on (I) being given by Eqs. (15) and (16), respectively. The  $K_i$  values for L-phenylalanine, calculated from the plots of  $1/V'$  and  $1/K'$  against (I) shown in Figures 52 and 53 in the manner described previously, are 1.99 and 1.91 mM, respectively, the average value being  $1.95 \pm .04$  mM.

Figure 52

Variation of  $1/V'$  with L-phenylalanine concentration for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate in 0.010M barbital buffer, pH 9.0, I = 1.0M and T = 25.0°C. The units of  $V'$  are absorbance units/minute and can be converted to  $\mu\text{moles}/\text{min}/\text{mg}$  enzyme by multiplying by  $5.35 \times 10^2$ .

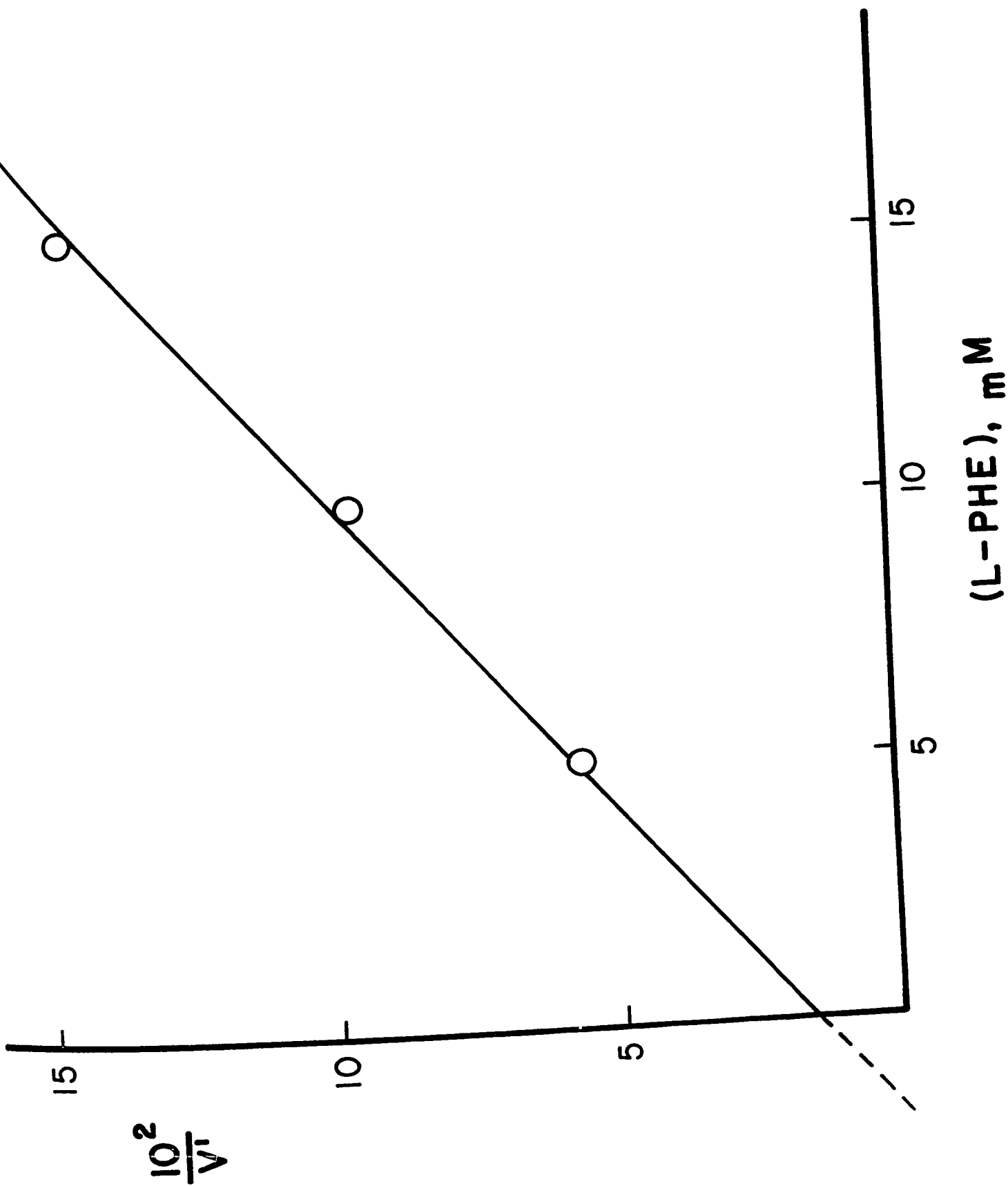
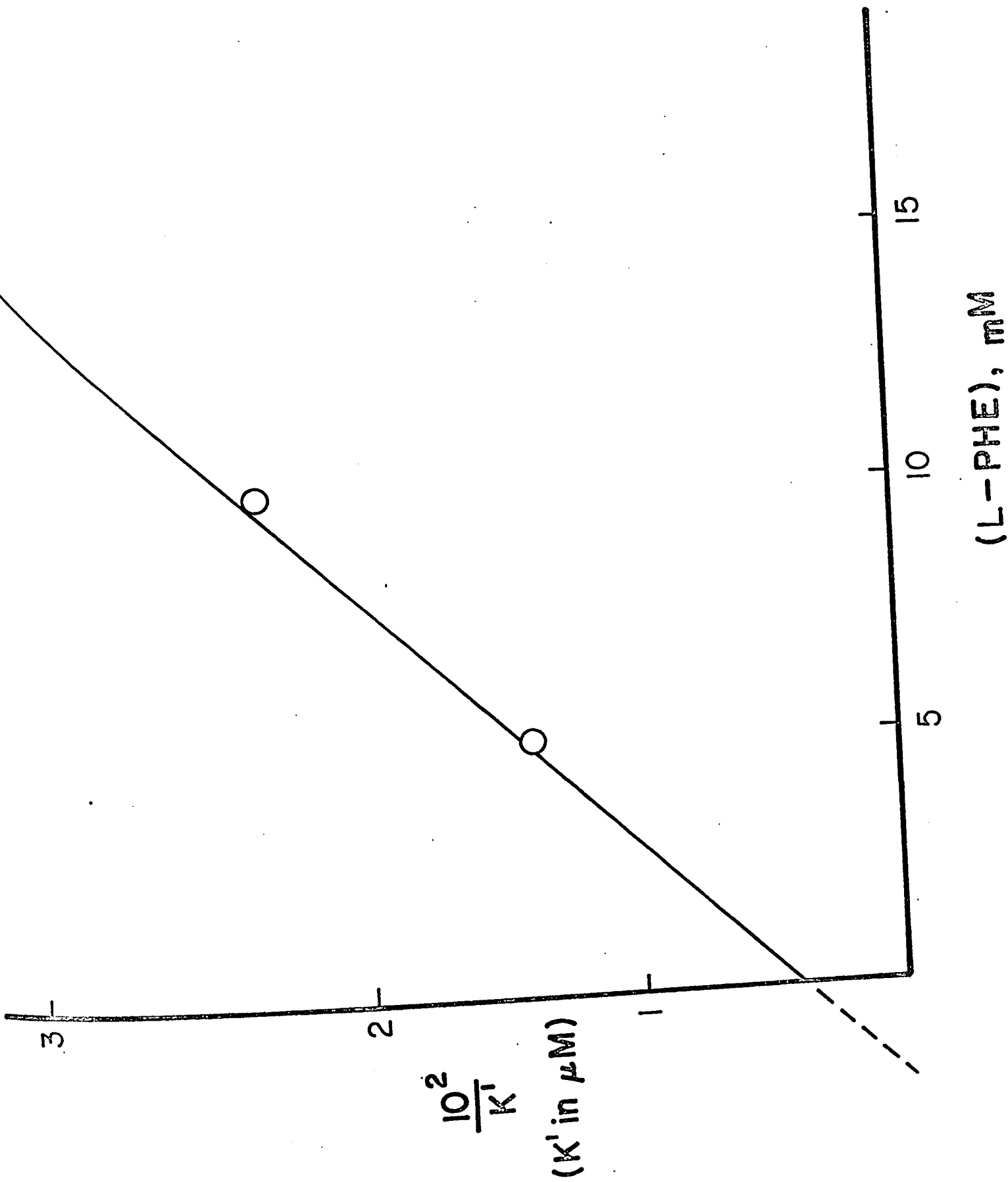


Figure 53

Variation of  $1/K'$  with L-phenylalanine concentration for the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate in 0.010M barbital buffer, pH 9.0, at  $I = 1.0M$  and  $T = 25.0^{\circ}C$ .



## DISCUSSION

The series of parallel straight lines obtained by plotting  $1/v$  against  $1/S$  at various concentrations of L-phenylalanine and the linear dependence of  $1/V'$  and  $1/K'$  on the concentration of L-phenylalanine together represent strong evidence that the inhibition of calf intestinal phosphatase by this inhibitor is uncompetitive. The results of the present study are, in this respect, similar to those obtained earlier with rat intestinal and human placental alkaline phosphatase using other substrates such as phenyl phosphate,  $\beta$ -glycerophosphate and 4-methylumbelliferyl phosphate (374-6,379), although the existence of linear relationships between the reciprocals of  $V'$  and  $K'$  and the L-phenylalanine concentration - a necessary condition for uncompetitive inhibition - was not demonstrated in these previous studies. The present results cannot, however, be easily reconciled with the report by Keiding (381) that L-phenylalanine did not inhibit intestinal alkaline phosphatase when p-nitrophenyl phosphate was used, but did inhibit with phenyl phosphate as substrate. It should be noted that the present study was carried out with the highly purified enzyme freed of extraneous activating metal ions, such as  $Mg^{2+}$ , which could conceivably mask the inhibition of L-phenylalanine. Ghosh and Fishman (379) have previously demonstrated that the inhibition of rat intestinal alkaline phosphatase by

L-phenylalanine is non-allosteric (382). This conclusion was based mainly on (1) the hyperbolic curves relating the percentage inhibition to the inhibitor concentration (2) the relative insensitivity of the inhibition to heat-denaturation urea treatment (3) the relatively low entropy change of the inhibition reaction and (4) a value close to unity for  $n$ , the number of L-phenylalanine molecules that combine with one molecule of enzyme. A similar conclusion has recently been arrived at for the inhibition of the human placental enzyme by L-tryptophan (377). Although the hypothesis that the inhibition of the calf intestinal enzyme by L-phenylalanine is similarly non-allosteric is obviously the most attractive one, a definite conclusion will have to await the availability of more experimental data. In the following sections, however, it will be tentatively assumed that the L-phenylalanine inhibits rat and calf intestinal and human placental alkaline phosphatase by the same mechanism.

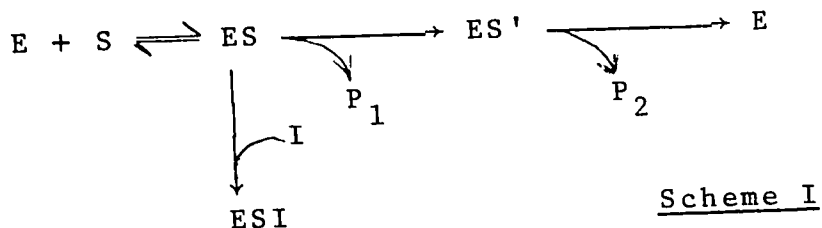
### Mechanisms of Inhibition

Two mechanisms proposed for the inhibition of alkaline phosphatase by L-phenylalanine have already been discussed; the following is a brief summary of these two mechanisms.

#### I. Mechanism of Ghosh and Fishman (379)

The inhibitor I, according to this mechanism, combines only with the Michaelis complex to form a weakly

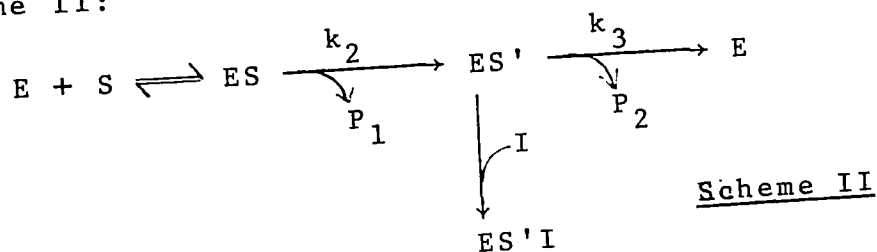
dissociable enzyme-substrate-inhibitor complex, ESI, as shown in the reaction sequence of Scheme I:



where E is the free enzyme, S the substrate, ES the enzyme-substrate complex, ES' the phosphoryl enzyme intermediate, P<sub>1</sub> the first product of the reaction, ROH and P<sub>2</sub> the second product, inorganic phosphate.

### II. Mechanism of Fernley and Walker (376)

Fernley and Walker have instead proposed that the inhibitor has little effect on the reactivity of the ES complex but prevents the breakdown of the ES' intermediate, as shown in Scheme II:

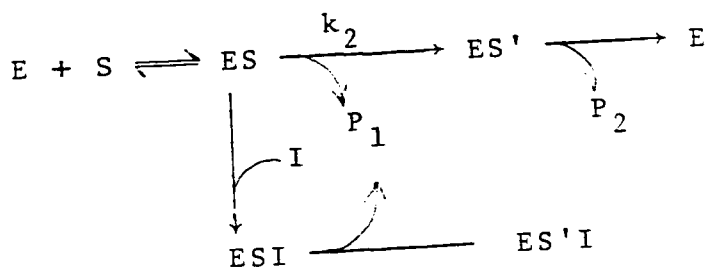


### III. Mechanism of Lin, Sie and Fishman

Both of the above mechanisms have several serious shortcomings. For example, since the inhibitor in Scheme II reacts only with the phosphoryl-enzyme intermediate, Fernley and Walker have predicted (376) that the inhibition will be

independent of the nature of the substrate used. Watanabe and Fishman (384), however, have reported differences in the percentage of inhibition of intestinal alkaline phosphatase by L-phenylalanine when five different phosphate monoesters were compared as substrates. Furthermore, as Lin et al. have pointed out (377), since at  $\text{pH} > 8$   $k_3 \gg k_2$ , it is unlikely that the inhibitor can effectively combine by a random collision with  $\text{ES}'$  before it is hydrolyzed; it is more likely that the inhibitor is combined with the enzyme before the catalytic step. Scheme I, on the other hand, cannot account for stopped-flow data of Fernley and Walker (376) which strongly suggest that  $\text{P}_1$  is split off in the presence of the inhibitor to form a stable  $\text{ES}'\text{I}$  complex.

Lin et al (377) have recently proposed a mechanism which does not suffer from the shortcomings of Schemes I and II. According to this mechanism the inhibitor combines with the Michaelis complex to form  $\text{ESI}$ , which is converted to the more stable  $\text{ES}'\text{I}$  complex by the release of  $\text{P}_1$ . Schematically,

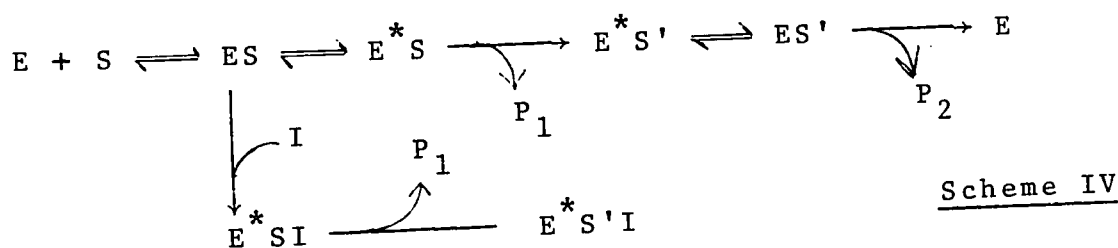


Scheme III

IV. Modification of Mechanism III

Ghosh and Fishman (378) and, more recently, Lin et al. (377) have pointed out that since the optimum temperature for maximum velocity increases in the presence of L-phenylalanine while the extent of L-phenylalanine inhibition decreases with increasing temperature, the inhibition may involve a homosteric transition (383) - a conformational change in the enzyme as a result of the binding of a specific modifier to the active site.

One mechanism which involves such a homosteric transition and yet is consistent with the known data immediately suggests itself by analogy to the scheme proposed in Chapter II to explain the effects of added nucleophiles on the enzyme-catalyzed reaction; this mechanism is shown in Scheme IV.



where ES and E<sup>\*</sup>S are two conformers of the enzyme-substrate complex, and E<sup>\*</sup>S' and ES' two forms of the phosphorylated enzyme. According to this scheme the inhibitor combines with the ES form of the enzyme-substrate complex to give E<sup>\*</sup>SI, a conformational change having occurred in the enzyme as a result of this interaction. The E<sup>\*</sup>SI complex is then converted to the more stable E<sup>\*</sup>S'I complex by the release of P<sub>1</sub>.

APPENDIX TO CHAPTER IV

The Digiscan D-28 Digital Read-Out for the Stopped-Flow Spectrophotometer

The Digiscan D-28 unit was specifically designed by Instronics Techno-Products Ltd., Stittsville, Ontario to digitilize the analog signal received from the photomultiplier tube of the stopped-flow spectrophotometer. The information digitilized consists of a rapidly rising and exponentially decaying d.c. voltage. The system is triggered by the rising voltage and samples the signal at preset time intervals ranging from 2.5 msec to 5 sec, twenty samples being taken per run. The input sensitivity can be varied from 50 mv to 1 v.

The system samples information from one channel only, converts and stores this information and prints the data out after a suitable time interval. An intermediate memory is required to buffer between the input sampling speed and the printer output speed. The unit consists of five basic blocks as follows:

Analog Section

- 1) Input amplifier and offset control
- 2) Analog to digital converter

Digital Section

- 3) Memory

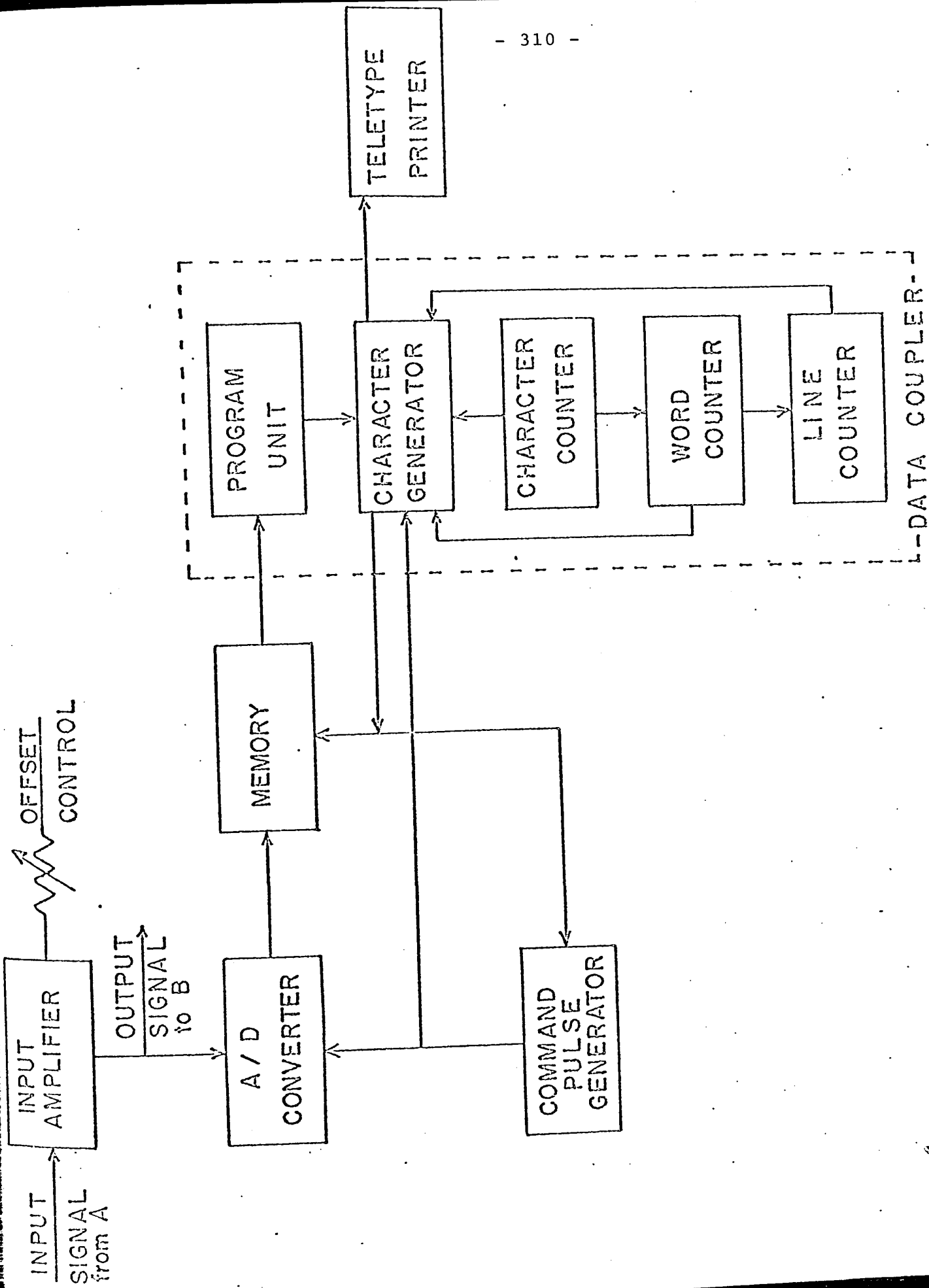
- 4) Data coupler
- 5) Teletype printer

A manually-operated selector switch permits the printing of an identifying number from 0-99 before the start of each run.

The storage oscilloscope of the stopped-flow spectrophotometer system can be operated in series with the digital read-out if desired. The negative voltage at the output of the time-constant coupling circuit of the oscilloscope is amplified and compensated by the operational amplifiers of the digital read-out. The signal is then applied to the analog to digital converter and/or to the positive input of the differential amplifier of the storage oscilloscope. These positions are marked A and B respectively in Figures 50 and 54.

Figure 54

Block diagram of the digital read-out for  
the stopped-flow spectrophotometer.



THE REDUCTION OF 2,6-DICHLOROPHENOL  
INDOLPHENOL BY SODIUM ASCORBATE

Introduction

The performance of the stopped-flow spectrophotometer with the Digiscan D-28 digital read-out attachment was evaluated by measuring the pseudo-first order rate constant for the reduction of  $10^{-5}$  M 2,6-dichlorophenol indolphenol by an excess of sodium ascorbate. Two different concentrations of ascorbate were used: 0.10 and 0.020 M; twenty runs were carried out at each concentration so that a meaningful statistical analysis could readily be performed.

The data for each individual run, which were stored in the digital converter, were analyzed by the standard Guggenheim technique (385); the actual calculations were performed on an IBM 360/65 digital computer.

Kinetic Procedure

Preceding a typical series of runs, the oscilloscope was calibrated for 100% transmittance with 0.1 M phosphate buffer, pH 7.50, in both drive syringes. The calibration was repeated for different settings of amplifier gain and zero offset to give a satisfactory record of the reaction to be studied. For this purpose the zero-offset circuit of the Durrum-Gibson spectrophotometer had been modified to include a calibrated ten-turn potentiometer. The buffer

solution in the drive syringes were then replaced by the two reactants. For each run approximately 0.15 ml of each reactant was injected into the cuvette. The change in absorbance was recorded at 620 m $\mu$  (slit width = 1 mm). At least 5 minutes of thermostating was allowed each time after the drive syringes had been refilled with reactants.

### Results

The oscilloscope traces of two typical experimental runs are shown in Figures 55 and 56. Guggenheim plots for these two runs obtained using the data stored in the digital converter are shown in Figure 57. It should be noted that triggering occurred  $t_i$  seconds before the actual start of the reaction; the true time of a voltage reading from the start of the reaction is always  $(t-t_i)$ , where  $t$  is the time recorded.

The pseudo first-order rate constants calculated for the two different concentrations of ascorbate and the standard errors associated with them are listed in Table 20. The ratio  $k_I/k_{II} = 33.56/6.67 = 5.03$  rather than the expected value of 5.00.

Figure 55

Oscilloscope trace for the reaction of  $10^{-5}$  M  
2,6-dichlorophenol indolphenol with 0.10 M  
sodium ascorbate at pH 7.50, I = 0.1, T = 25.0°C.  
Vertical scale: 50-100% transmittance, horizon-  
tal scale: 10 msec/DIV.; time constant: 0.1  
msec.

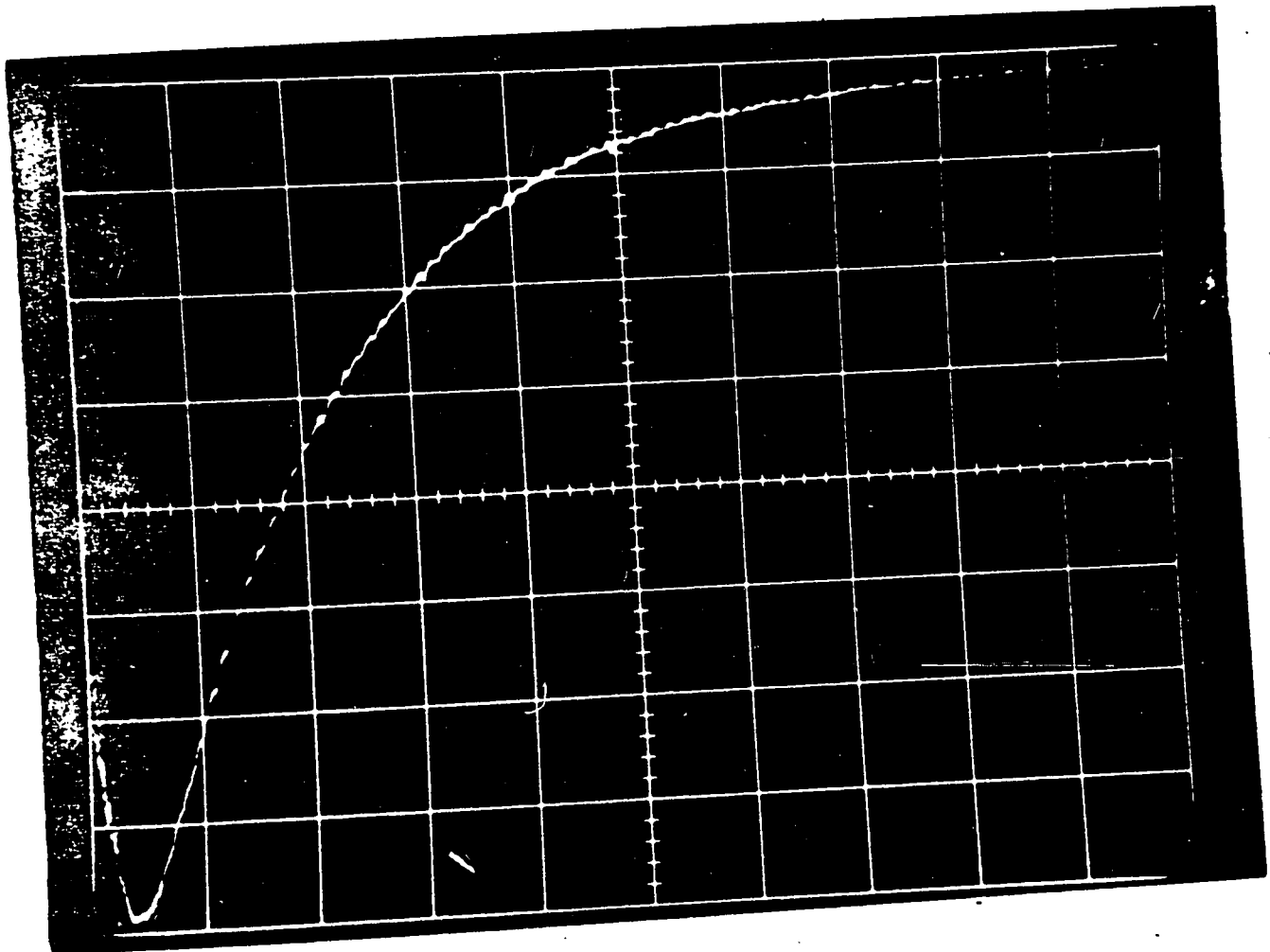


Figure 56

Oscilloscope trace for the reaction of  $10^{-5}$  M  
2,6-dichlorophenol indolphenol with 0.020 M  
sodium ascorbate at pH 7.50, I = 0.1, T = 25.0°C.  
Vertical scale: 50-100% transmittance; horizontal  
scale: 50 msec/DIV; time constant: 0.1 msec.

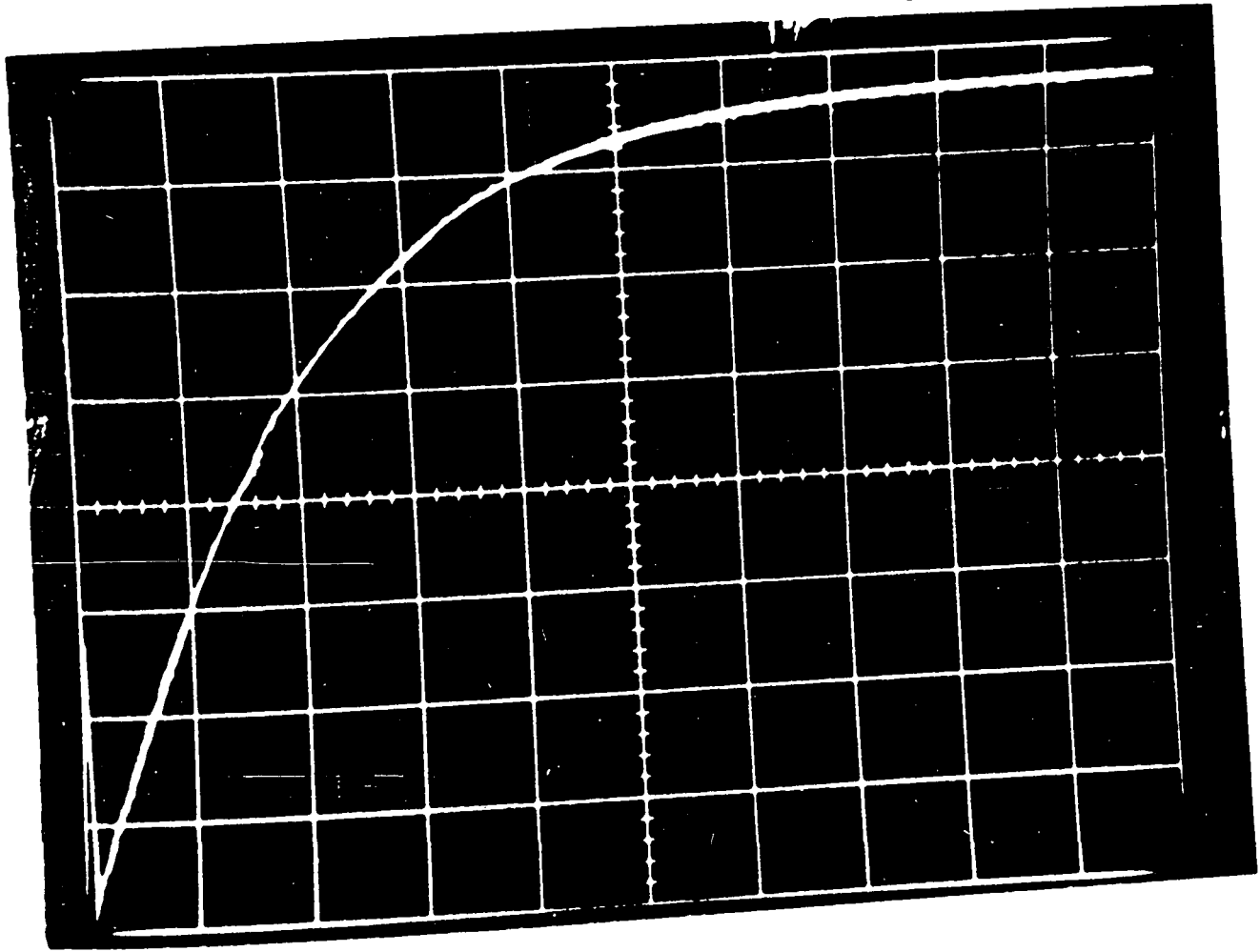


Figure 57

Guggenheim plots to determine the pseudo first-order rate constant for the reaction of  $10^{-5}$  M 2,6-dichlorophenol indolphenol with an excess of sodium ascorbate at pH 7.50,  $I = 0.1$  and  $T = 25.0^{\circ}\text{C}$ . RUN 35: 0.1 M ascorbate, RUN 57: 0.02 M ascorbate.

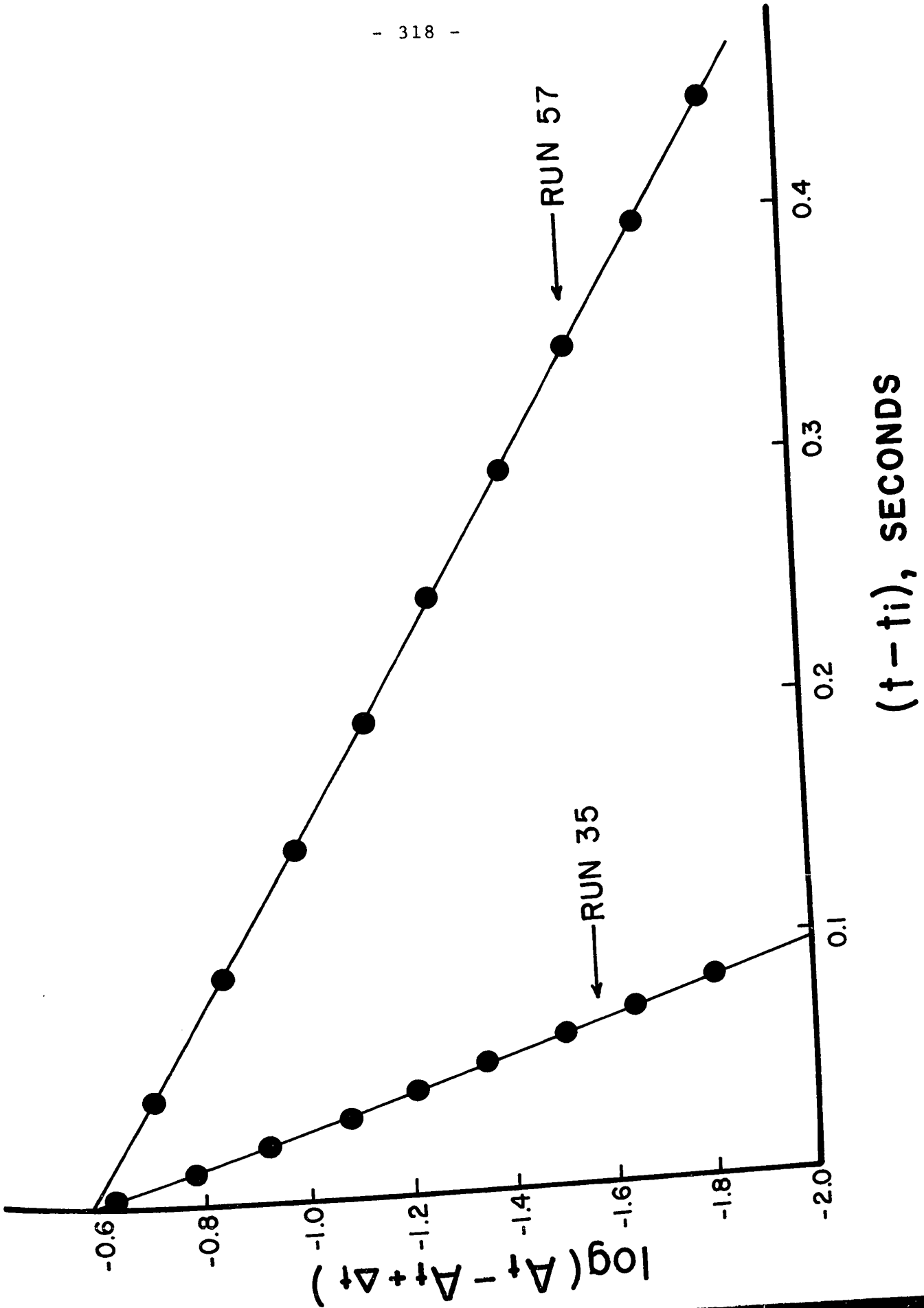


TABLE 20

Pseudo First-Order Rate Constants for the Reduction of  
 2,6-Dichlorophenol Indolphenol by an Excess of Sodium Ascorbate at pH 7.50,  
 I = 0.10 M and T = 25.0°C

Run Series	(Ascorbate) M	Rate Constant Sec <sup>-1</sup>	Standard Error	Percentage Error
I*	1.0	33.56	±0.51	1.5
II*	0.02	6.67	±0.078	1.2

\*Twenty runs per series

CLAIMS TO ORIGINAL RESEARCH

1. The hydrolyses of phenyl phosphate and p-nitrophenyl phosphate catalyzed by chicken intestinal alkaline phosphatase were studied in the presence of Tris and ethanolamine. The variation of the Michaelis parameters for the rates of formation of the three products of the reaction was determined.
2. Equations were derived for the rates of formation of the three products, and the Michaelis constant, as a function of added nucleophile concentration for a number of mechanisms.
3. A novel mechanism was proposed to explain the variation of the Michaelis parameters with added nucleophile concentration observed with chicken intestinal alkaline phosphatase and with the E.coli enzyme.
4. The pH dependencies of the Michaelis parameters for the hydrolysis of p-nitrophenyl phosphate catalyzed by chicken intestinal alkaline phosphatase were determined.
5. The pH dependence observed was explained in terms of the general mechanism for the pH dependence of enzyme-catalyzed reaction involving two intermediates.
6. An attempt was made on the basis of the observed pH dependence and other available data to identify the nature of the ionizable groups at the active site of chicken intestinal alkaline phosphatase.

7. The Michaelis parameters for the hydrolysis of p-nitrophenyl phosphate catalyzed by calf intestinal alkaline phosphatase were determined at different concentrations of L-phenylalanine, and the nature of the inhibition was characterized.
8. A new mechanism consistent with all the data available was proposed to account for the inhibition of alkaline phosphatase by L-phenylalanine.

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