A STUDY OF SOME ALKALINE PHOSPHATASES OF *Neurospora crassa*

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ABSTRACT

The activity of the alkaline phosphatase from the mycelium of *Neurospora crassa* was examined in an attempt to learn something of the mechanism of action of the enzyme responsible for that activity.

Product inhibition studies on a relatively impure preparation showed that the catalytic mechanism involved the ordered release of products from the enzyme, the alcoholic or phenolic product being released first, followed by the release of phosphate.

Inorganic phosphate was a linear competitive inhibitor of the hydrolysis of either p-nitrophenyl phosphate or β-glycerophosphate. p-Nitrophenol was a non-linear non-competitive inhibitor of p-nitrophenyl phosphate hydrolysis, whereas the noncompetitive inhibition by glycerol was linear when β-glycerophosphate was the substrate.

It was shown that not one, but two constitutive alkaline phosphatases were present in *Neurospora*. They were separable by DEAE cellulose chromatography and by electrophoresis, and differed in molecular weight, electrophoretic mobility and in their response to some cations. Due to the presence of phosphate in the growth medium neither of the enzyme activities could be attributed to the alkaline phosphatase, repressible by phosphate, known to be present in

Neurospora during phosphate deficiency. Both enzymes required Zn^{++}, which was tightly bound to the protein and could not be replaced by any other ion tested. A second, loosely bound metal ion was also required for maximum activity, Mg^{++} being by far the most effective of the ions tested.

A purification procedure whereby one of the two activities could be obtained free of the other was devised. It was purified approximately 400 fold as compared to the activity in the crude extract and appeared to be about 50% pure enzyme. The effects of pH on the activity of the purified enzyme were studied in detail, using p-nitrophenyl phosphate and glucose 6-phosphate as substrate. In both cases the optimum pH increased with the substrate concentration and was shown to be a linear function of the logarithm of the substrate concentration. Dixon plots for both substrates were prepared and are qualitatively, but not quantitatively, interpreted.

Product inhibition experiments using p-nitrophenyl phosphate and glucose 6-phosphate with the purified enzyme confirmed and extended the results obtained earlier. The non-linear nature of the inhibition due to p-nitrophenol of p-nitrophenyl phosphate hydrolysis was found to be more complex than was evident in the early work. A two-substrate

mechanism, involving hydroxyl ion as the second substrate, is proposed and appears to offer satisfactory explanations of the observed pH effects and substrate inhibition.

A possible explanation for the competitive inhibition by glucose of p-nitrophenyl phosphate hydrolysis and for the apparent lack of inhibition by p-nitrophenol of glucose 6-phosphate hydrolysis is also presented. The proposed model involves the hypothesis that two substrate binding sites exist in the enzyme.

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pK_m *versus* pH and Log V_{max} *versus* pH curves from various sources.

Scales have been changed so that all figures are to the same scale.

A. Redrawn from Motzok (1959)
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B. Redrawn from Morton (1957)
   (a) Milk phosphatase with phenyl phosphate,
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C. Redrawn from Anagnostopoulos and Matsudaira (1958)
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D. Redrawn from Anagnostopoulos and Matsudaira (1958)
   Human placental enzyme in ethanolamine buffer with phenyl phosphate.

E. Redrawn from Lazdunski and Lazdunski (1966)
   E. coli enzyme (ionic strength was 0.4)
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   (b) with p-nitrophenyl phosphate at 45°,
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H. Redrawn from Kadner and Nyc (1969).
   Repressible enzyme from *Neurospora crassa* with p-nitrophenyl phosphate, at 37° in 0.2M buffers.
   (a) in the presence of 1mM EDTA,
   (b) in the absence of EDTA.

INTRODUCTION

Numerous individual phosphomonoesterases exist in all cells. They may be divided into two large groups (specific and non-specific phosphomonoesterases) on the basis of substrate specificity. The non-specific enzymes have been divided into two groups; those with pH optima in the alkaline range, known as alkaline phosphatases and the acid phosphatases whose pH optima lie on the acid side of neutrality.

Since only a few non-specific phosphatases have been obtained in high purity, very little is known of the properties of these enzymes. Thousands of scientific papers have dealt with some aspects of the phosphatases, such as their distribution and varying levels in diseased organs or tissues. Despite the volume of the literature on the subject little is understood of the mechanism of action or physiological function of the non-specific phosphatases. It has even been suggested that the phosphatase activity observed *in vitro* is an artifact.

Due to the ubiquitous presence and extreme importance of phosphorylated compounds in living material it is of major academic and probably practical interest to clarify our understanding of those enzymes potentially responsible for massive non-specific dephosphorylation. In addition to

their hydrolytic activity at least some of these enzymes also exhibit phosphotransferase activity. It is possible that phosphate transfer may be a physiological function, but the high concentrations of acceptor required to produce a measurable reaction rate do not encourage the idea.

The present work is an attempt to extend our knowledge of the properties of the alkaline phosphatases of *Neurospora crassa*. Particular attention has been paid to the kinetics of the enzyme-catalysed reaction and to the effects of pH on catalysis. These appear to be extremely complex and the data obtained raise more questions than they answer. No attempt has been made to investigate the potential phosphotransferase ability of the enzyme.

The format of the thesis is unusual in that discussion of the results is not reserved to the end. It seemed more logical to present results in the order in which they were obtained, dividing the body of the work into three phases related to different degrees of purification of the enzymes. Within the three phases, discussion of each topic investigated appears as a natural consequence of the completion of the presentation of experimental results.

HISTORICAL REVIEW

Over fifty years have elapsed since reports of the occurrence of an enzyme responsible for splitting phosphate esters appeared in the literature (Suzuki *et al*, 1906; McCollum and Hart, 1908). Since that time such enzymes have been shown to act on many substrates and their presence has been observed in a wide variety of organisms.

A. Classification of the phosphatases

Attempts to classify the phosphatases are of two kinds, those based on the types of substrates hydrolysed and those based on such characteristics as the optimum pH for the reaction catalysed. Roche (1950) listed four types of phosphomonoesterases as follows:

Roche's classification of the phosphomonoesterases

Type I	optimum pH 8.6 - 9.4	Activation by magnesium ion, inhibition by -SH. More active on β- than on α-glycerophosphate. Optimal stability at pH 7.5 - 8.5.
Type II	optimum pH 5.0 - 5.5	No activation by magnesium ion. Inhibition by fluoride ion. More active on β- than on α-glycerophosphate. Optimal stability at pH 5.0 - 6.0.
Type III	optimum pH 3.4 - 4.2	Inhibition by magnesium ion. More active on β- than on α-glycerophosphate. Optimal stability at pH 6.5 - 7.5.

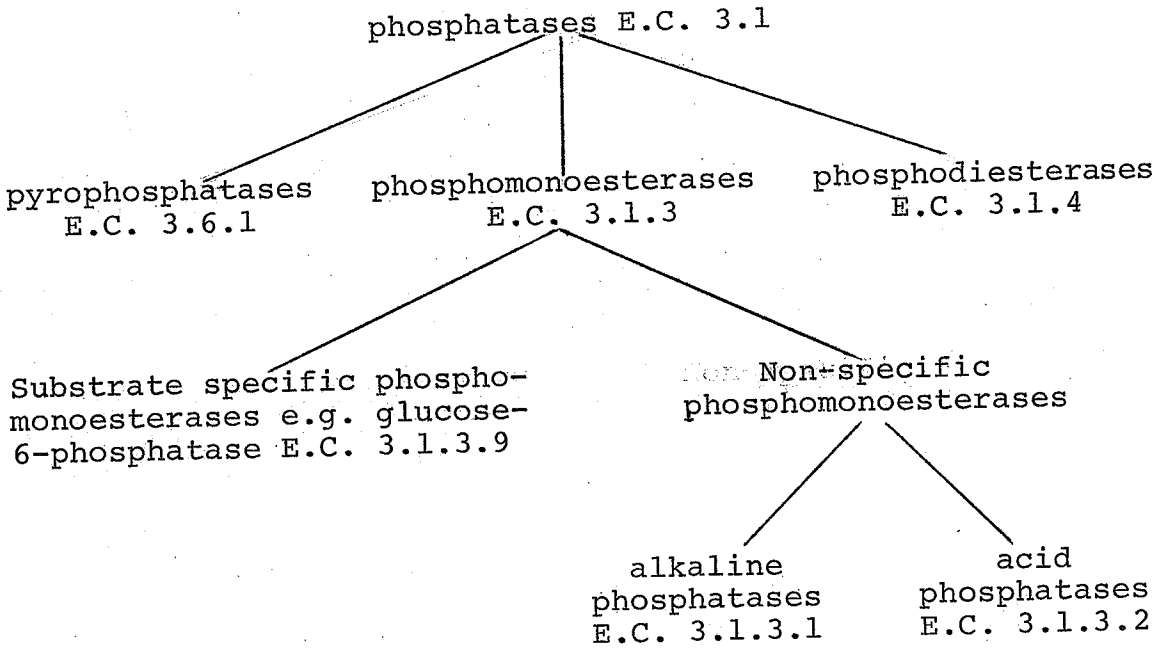
Type IV	optimum pH 5.0 - 6.0	Activation by magnesium ion. More active on α- than on β-glycerophosphate. Optimal stability at pH 6.5 - 7.5.

Type I enzymes are those to which the trivial name of alkaline phosphatase has been applied. In the systematic classification and nomenclature established by the Commission on Enzymes of the International Union of Biochemistry (Florkin and Stotz, 1964) these enzymes are known as orthophosphoric monoester hydrolases and are numbered 3.1.3.1.

Types II, III and IV are commonly referred to as acid phosphatases (orthophosphoric monoester hydrolase E.C. 3.1.3.2).

Schmidt and Laskowski (1961) classified the phosphomonoesterases into two groups based on the degree of specificity they exhibit toward their substrates. There were the substrate-specific phosphatases on the one hand and the non-specific alkaline and acid phosphatases on the other hand. In this way glucose 6-phosphatase (E.C. 3.1.3.9), with a relatively limited ability to hydrolyse phosphomonoesters was considered to be a specific phosphomonoesterase. Information on the substrate specificity of highly purified enzymes, however, is limited and these attempts at classification should be treated as tentative. The following "organizational chart" of the phosphatases illustrates in a simple way the relationships between the various enzymes.

An "organizational chart" of the phosphatases



Many of the non-specific phosphomonoesterases also exhibit the ability to transfer a phosphate group to an alcoholic acceptor (in high concentration) other than water (Anderson and Nordlie, 1967; Wilson *et al*, 1964; Dayan and Wilson, 1964). Such an activity would require these enzymes to be classed as phosphotransferases E.C. 2.7.1, further complicating any attempt at an inclusive scheme of classification.

Only the alkaline phosphatases will be dealt with in detail in the remainder of this review.

B. Distribution of alkaline phosphatase in nature

Alkaline phosphatase has been observed in a list of organisms far too long to enumerate. In man, it has been reported in the blood serum, capillaries, leukocytes, milk, placenta, intestine, liver, urine, bone, teeth and in cell cultures both normal and cancerous. It is practically absent from skeletal muscle and from the heart. Similar lists, which are by no means exhaustive, could be made for other mammals, insects, birds, amphibia and fish. The enzyme has been reported in protozoa, bacteria and fungi. Strangely enough, no reference to the existence of alkaline phosphatase in green plants has been found.

C. Cellular localization and possible function of alkaline phosphatase.

No one has yet been able to assign a specific metabolic role to alkaline phosphatase. Interest in the enzyme was greatly stimulated by the suggestion (Robison and Soames, 1924) that it is involved in the calcification of bone and in tooth formation. The observation that the epiphyses of long bones and the teeth of young animals are rich in alkaline phosphatase, and that the hydrolysis of the calcium salts of phosphorylated hexoses leads to the formation of tricalcium phosphate supported their suggestion

(Stadtman, 1961). The enzyme has been histochemically demonstrated in the chondrocytes of human tibial cartilage (Bona *et al*, 1965).

Anagnostopoulos and Matsudaira (1958) suggested that the enzyme plays a role in the absorption of maternal blood glucose by the human placenta, which has very high alkaline phosphatase activity. The activity was reported to increase during pregnancy and to reach a maximum just before birth. Enzymatic activity in the mammary gland also increases during pregnancy and is maintained at a high level during lactation (Folley and Greenbaum, 1947), again suggesting a possible role for the enzyme in either absorption or secretion. The localization of the enzyme in mouse duodenum has been examined (Hugon and Borgers, 1966a) in considerable detail. Its location in the brush border, in the Golgi zone and in granules below the brush border was demonstrated with the light microscope following specific staining techniques. In the cells of the villi the enzyme occurred on the outer side of the membrane of the microvilli, the inner side and the core of the microvilli being devoid of activity. Small bodies, supposedly pinocytic vesicles derived from the membrane of the microvilli, also contained alkaline phosphatase, located, as one would expect, on the inner side of the membrane. Many Golgi vesicles stained

strongly, enzymatic activity being associated with the membranes leaving the central part of the structure free of stain. The smooth endoplasmic reticulum leading to the Golgi zone was alkaline phosphatase positive, as were various bodies interpreted as lysosomes. The lateral membranes of the villus cells showed no activity. No activity was detected in the migrating leucocytes or in the mucous cells of the villi. In the bottom of the crypts, no alkaline phosphatase was observed in the brush border, the Golgi zone or elsewhere. These workers concluded their discussion with the generalization that alkaline phosphatase activity appeared to be associated with structures all known to play a role in the absorption of fats or glucose.

The change in enzyme content of mouse duodenum as a function of development has been studied (Etzler and Moog, 1968). Enzymatic activity increased sharply before birth and declined in the first week after birth. It then rose 10 to 20-fold to a maximum at 20 days of age. The increase in activity was accompanied by a change in substrate preference as indicated by the ratio of activity with phenyl phosphate to that with β-glycerophosphate. The change appeared to be a result of the appearance of a new isozyme. Column chromatography at this stage separated two components differing in their activity ratio. The

change in isozymal composition paralleled the differentiation of the microvilli into the form characteristic of the mature mouse.

Alkaline phosphatase is distributed throughout the length of the small intestine of the rat, and is most abundant at the anterior end. Triantaphyllopoulous and Tuba (1959) found the distribution of the enzyme among subcellular fractions prepared from rat intestinal homogenates to be as follows: microsomes, about 76%; nuclei, 10%; mitochondria, 8%; supernatant fraction, 9%. Similarly, 97% of the alkaline phosphatase content of rabbit intestine and 83% in the guinea pig were found to be associated with the microsomal fraction (Hers *et al*, 1951). It is possible that the small amount of activity not associated with membrane fragments represented enzyme removed from membranes by the fragmentation process.

In mouse kidney alkaline phosphatase was found (Hugon and Borgers, 1966b) in the brush border, in multivesicular bodies of the tubular cells and in the deep intracytoplasmic extensions of the apical membrane. These workers felt that the light activity they observed along the basal membrane of the cells of the distal and proximal tubules and in the basal membranes of the capillaries of the glomerulus was an artifact due to diffusion.

Histochemical studies (Piatka and Gibley, 1967) of the developing pronephros of the frog suggested two possible roles for alkaline phosphatase. Heavy concentrations of the enzyme were found in the pronephric anlage. By the time the pronephros became functional the cells of the proximal tubule contained a greater quantity of the enzyme than did those of the distal tubule. As cytological differentiation proceeded the enzyme content of the distal and collecting tubules decreased to almost nothing. It was inferred that alkaline phosphatase was involved in morphogenesis and disappeared when the tissues differentiated. In the proximal tubules, which retained activity at the luminal border the enzyme apparently played a role in the reabsorbing and possible secretory functions of the tubules.

The localization of the enzyme in nervous tissue seems to vary. In the nerves of the lateral line of *Saccobrancus fossilis* alkaline phosphatase was demonstrated in the axons, but not in the myelin sheath, whereas in the optic nerve of *Mystus seenghala* the enzyme occurred in the myelin sheath but not in the axons (Tewari and Raybanshi, 1968). The enzyme was shown to be concentrated at the nodes of Ranvier and at the incisures of Schmidt-Lanterman in the sciatic nerve of rats and cats (Pinner *et al*, 1964).

In the cells of the trigeminal ganglion of rats alka-

line phosphatase was shown to be localized at or near the cell membrane (Tewari and Bourne, 1964). The nuclear and cytoplasmic areas of the cells were devoid of activity. The blood vessels and connective tissue of the ganglion provided an identical reaction. Processes emerging from the ganglion cells or those lying between the cells were devoid of activity as were the associated nerves. A close examination of the ganglion cells revealed the absence of enzymatic activity in the main body of the capsular cells. The activity was confined to the interface between the capsular cells and the associated neurons. These authors considered the possibility that the enzyme is involved in trans-membrane transport of metabolic substrates, that it is a structural as well as a functional part of the membrane, and may even be involved in Na^+ and K^+ transport.

Varma and Guraya (1968) compared the location of alkaline phosphatase in the ovaries of fishes, amphibians, reptiles, birds and the white rat. They reported that the theca interna of developing and degenerating follicles of all species was strongly alkaline phosphatase positive, whereas the enzyme was absent from the germinal epithelium, the follicular epithelium and the ooplasm. The sites at which the enzyme was localized were highly vascularized and the authors concluded that the enzyme was involved in the

transfer of nutrient, secretory and waste materials across the cellular membranes of blood vessels and adjacent tissues.

In *Escherichia coli* it was shown (Malamy and Horecker, 1961, 1964) that alkaline phosphatase was located in the space between the cell wall and the cell membrane and was liberated by lysozyme - EDTA conversion of cells into spheroplasts. The spheroplasts themselves were almost devoid of activity, all of which appeared in the medium. Further evidence for the periplasmic location of alkaline phosphatase in *E. coli* was provided by Neu and Heppel (1965) and Brockman and Heppel (1968). Schlesinger and Olsen (1968) have shown in a very elegant experiment that localization is not a property unique to *E. coli* cells and suggest that it is related to the structure of the protein. The *E. coli* structural gene was inserted into *Salmonella typhimurium* strain LT-2 (which according to these authors does not produce alkaline phosphatase) by episomal transfer. The *S. typhimurium* heterogenote produced alkaline phosphatase which appeared to be identical to that produced by *E. coli* and was quantitatively released by spheroplast formation.

The localization of the enzyme in *Bacillus subtilis* was shown to be similar, but there was also an interesting difference. In addition to alkaline phosphatase, ribonu-

clease was also shown (Neu and Heppel, 1964) to be located in the periplasmic space in *E. coli*. By contrast, α-amylase (Nomura *et al*, 1956), two ribonucleases (Nishimura and Nomura, 1959) and proteinase (Matsubara *et al*, 1958) were shown to be secreted to the medium by normal cells of *B. subtilis*. These observations led Cashel and Freese (Cashel and Freese, 1964) to hypothesize that the difference between the exo-enzymes of *B. subtilis* and the peri-emzymes of *E. coli* was due to a difference in the cell walls of the two species rather than a difference in the enzymes themselves. The hypothesis predicted that peri-enzymes in *E. coli* would be exo-enzymes in *B. subtilis* and vice-versa. These workers showed that alkaline phosphatase is, indeed, secreted to the outside by intact cells of *B. subtilis* following derepression by phosphate starvation.

Results obtained by others (Takeda and Tsugita, 1967) partially contradict these findings. Their results indicated that the *B. subtilis* enzyme is not completely soluble. It appeared to be attached to fragments of the cell walls or membranes and sedimented in the centrifuge. The incompatible results may, however, have been due to differences in bacterial strain or growth medium.

Reports of the localization of alkaline phosphatase in fungal cells are relatively rare. Zalokar (1960)

reported its presence in the cytoplasm and nucleoli of *Neurospora crassa*. Eberhart (1961) observed that water extracts of *Neurospora* conidia contained more alkaline phosphatase after acetone treatment than was present without such treatment. He suggested, on this basis, that the enzyme was located either on or within the surface of the conidia.

No reference to the existence of alkaline phosphatase in green plants was found.

If alkaline phosphatase plays an important role in transmembrane transport, and if, indeed, it is not present in green plants one must hypothesize that such plants have a different transport mechanism, or that trans-membrane transport is not of great importance in plants. The second hypothesis is not as unreasonable as it might at first appear. Most, if not all, living cells in a plant are connected by plasmodesmata. These direct cytoplasmic connections reduce, if they do not altogether remove, the need for transmembrane transport within the plant. Only root cells must absorb materials against a concentration gradient, and these materials are all inorganic. Because of its photosynthetic ability, a green plant is not required to absorb organic nutrients from its surroundings. It would be very interesting to examine organisms such as *Euglena* - does

Euglena synthesize alkaline phosphatase when it grows heterotrophically and not when it grows photosynthetically?

It seems, then, to be well established that alkaline phosphatase in animals and in bacteria is membrane associated and is particularly abundant where absorption or secretion are dominant functions. The evidence, however, merely suggests that the enzyme plays a role in these functions. Thus far, there is no evidence that the enzyme is essential for their performance. The fact that *S. typhimurium* grows very well without it is, indeed, contrary evidence. There may, however, be limitations on the sorts of substrates (particularly phosphorylated ones) that *S. typhimurium* can utilize as compared to those suitable for *E. coli*.

D. Mechanism of the hydrolytic reaction catalyzed by alkaline phosphatase.

A good review of this subject, from which most of the material for this section is taken, may be found in the article by Thressa C. Stadtman in "The Enzymes" (Stadtman, 1961).

(i) Type of substrate hydrolysed. All the alkaline phosphatases hydrolyse a wide variety of phosphomonoesters, such as those of primary and secondary aliphatic alcohols,

sugars, cyclic alcohols, phenols and many amines (e.g. creatine phosphate). The alkaline phosphatases of bone, calf intestinal mucosa, milk and *E. coli* hydrolyse phosphomononucleotides. The bone enzyme, for example, hydrolyses 2'-, 3'- and 5'- phosphates of adenosine. There are some exceptions to these generalities. The *E. coli* enzyme, for instance, does not hydrolyse creatine phosphate.

Highly purified preparations of alkaline phosphatase do not attack inorganic pyrophosphate, inorganic polyphosphates or such substances as ADP and ATP.

The phosphate repressible enzyme of *Neurospora crassa*, however, was reported (Nyc *et al*, 1966) to have some inorganic pyrophosphatase activity. The constitutive enzyme (Kuo and Blumenthal, 1961a) on the other hand, did not hydrolyse organic or inorganic pyrophosphate, nor did it hydrolyse phosphoprotein bonds.

In general, the specificity of alkaline phosphatases is very low for the organic moiety of the substrate molecule but is very high for the phosphate portion. Substances such as p-nitrophenyl phosphate are readily attacked, but p-nitrophenyl sulfate is not.

(ii) Bond cleavage. Experiments involving various substrates in the presence of H_2O^{18} have established that alkaline phosphatase catalyzes the rupture of an oxygen-

phosphorus bond according to the equation

$$R\text{-}O\text{-}P + H_2O^{18} \longrightarrow R\text{-}O\text{-}H + HO^{18}\text{-}P$$

In these experiments the expected amount of labelled oxygen was found in the liberated orthophosphate and none was found in the alcohol moiety. Cleavage of the P-O bond also occurs during non-enzymic alkaline hydrolysis, whereas acid-catalysed hydrolysis results in R-O bond cleavage.

E. Genetic control of enzyme synthesis

(i) The *E. coli* enzyme. From the point of view of genetics, the alkaline phosphatase of *E. coli* is undoubtedly better understood than that of any other organism. Horiuchi *et al* (1959) showed that synthesis of the enzyme was repressed by excess phosphate in the medium. The structural gene for the enzyme was reported to be a single cistron (Garen and Garen, 1963a) and the protein itself to consist of two identical subunits (Rothman and Byrne, 1963). Two other genes regulate the rate of synthesis of the enzyme (Garen and Echols, 1962a) and in wild type cells production of the enzyme can vary over a thousandfold range depending on the concentration of phosphate in the medium. In the derepressed state as much as 6% of the total protein is alkaline phosphatase (Schlesinger, 1967; Schlesinger and

Anderson, 1968). Derepression is jointly controlled by both regulator genes (R_1 and R_2) as shown (Echols *et al*, 1961) by the isolation of constitutive mutants carrying mutations in either gene. Induction appeared (Garen and Echols, 1962a) to be under the exclusive control of the R_1 gene because mutations causing a reduction in the rate of enzyme formation in low phosphate medium occur in the R_1 but not in the R_2 gene. (These authors designated as induction the effect of a low concentration of phosphate on enzyme formation. The effect of a high concentration was called repression).

From a study of heterozygous strains (Garen and Echols, 1962a; Garen and Echols, 1962b) it was concluded that repression was mediated by a cytoplasmic repressor, the formation of which depended on both R_1 and R_2. Similarly, it was concluded that induction was mediated by a cytoplasmic inducer specified by the R_1 gene. The situation was summarized (Garen and Echols, 1962a) in the following reaction scheme.

$$\underset{\downarrow}{R_1^+ \text{ gene}} \qquad \underset{\downarrow}{R_2^+ \text{ gene}}$$

$$\text{Inducer} + R_2 \text{ Enzyme} + \text{orthophosphate} \longrightarrow \text{repressor.}$$

If an inducer was involved in alkaline phosphatase synthesis the authors reasoned that mutations should occur in the R_1

gene that block the formation of inducer, thereby producing a phosphatase - negative mutant that maps in the R_1 gene rather than in the structural gene (P) and shows complementation in tests against all P^- mutants. Such mutants were observed.

Genetic evidence that the products of the regulator genes and, therefore, the repressor, were proteins was provided by Garen and Garen (1963b).

Under normal circumstances if phosphate is added to a derepressed culture, production of the enzyme ceases very rapidly. Working with a temperature - sensitive constitutive R_2 mutant (Gallant and Stapleton, 1963) in which repression is temperature-dependent it was shown (Gallant and Stapleton, 1964a) that restoration of repression in excess phosphate (1) is dependent on the presence of a required amino acid; (2) is completely blocked by chloramphenicol; (3) proceeds in the presence of concentrations of 5- fluorouracil which substantially inhibit RNA synthesis but only slightly inhibit protein synthesis; (4) proceeds in the absence of thymine. These properties were interpreted as indicating that the repressor was a protein whose rate of synthesis varied inversely with temperature in the mutant. The apparent requirement for *de novo* synthesis of repressor after a short time of derepressed growth indicated

that the repressor was metabolically unstable.

Similarly, it was shown (Gallant and Stapleton, 1964b) that the inhibition of protein synthesis by either canavanine or chloramphenicol derepressed cultures of the mutant growing in excess phosphate. Relief of inhibition of protein synthesis permitted the reappearance of repression. Repression was restored only slowly at 37° and more rapidly at lower temperatures. It was concluded that these data were consistent with the notion that the repressor was an unstable protein, probably rich in arginine.

The R_2 regulator gene was resolved into two cistrons (R_{2a} and R_{2b}) thereby increasing to three the number of genes involved in the regulation of synthesis of alkaline phosphatase in *E. coli* (Garen and Otsuji, 1964). The protein specified by the R_{2a} gene was purified and some of its physicochemical properties were reported. It was produced under derepressed (low phosphate) conditions and could not, therefore, itself be the repressor though it might be a structural part of the repressor or play a role in its formation. The R_{2a} protein was repressed under the same genetic and environmental conditions that resulted in repression of alkaline phosphatase. Although they shared the same regulatory system, the amount of alkaline phosphatase synthesized under derepressed conditions (6% of total pro-

tein) greatly exceed the amount of R_{2a} protein synthesized (0.25% of total protein) under the same conditions.

(ii) Other bacteria. An alkaline phosphatase activity has been reported (Carrillo-Castenada and Ortega, 1967) in *Salmonella typhimurium* strain LT-2. The enzyme is reported to differ from the *E. coli* enzyme in being: (a) Non-repressible by phosphate, (b) dependent in amount on the nature of the carbon source in the growth medium (low in glucose, high in lactate), and (c) not stable to heating. The report may be contrasted with that quoted earlier (Schlesinger and Olsen, 1968) under heading C above, in which it was stated that *S. typhimurium* strain LT-2 does not produce alkaline phosphatase, but does carry regulator genes for its production which are operative when the structural gene is present on an episome transferred from *E. coli*.

Reports of the occurrence of the enzyme in *Staphylococcus* are also contradictory. Kuo and Blumenthal (1961) indicated the absence of a repressible enzyme in 20 strains of coagulase-positive and coagulase-negative staphylococci. On the other hand Shah and Blobel (1967) report the presence of a repressible alkaline phosphatase in *S. aureus*, in 18 staphylococcal strains of the international phage-typing series and in 6 coagulase-negative cultures.

A phosphate repressible enzyme tightly bound to the cell wall or membrane has been crystallized (Takeda and Tsugita, 1967) from *Bacillus subtilis*. *B. subtilis* 202-7 is also reported (Arima *et al*, 1968) to produce a very active heat stable factor that bursts protoplasts of *B. megaterium* KM. The factor completely inhibited the synthesis of alkaline phosphatase of *B. megaterium* KM as a result of interaction with the cell membrane without inhibiting the activity of the enzyme and without affecting the synthesis of other enzymes tested. The effect of the factor on *B. subtilis*, *B. megaterium* and *E. coli* was not as conspicuous as its effect on *B. megaterium* KM.

Three different types of phosphate repressible enzyme have been reported in *B. anthracis* (Dobozy *et al*,1968).

(iii) Fungi. At least two alkaline phosphatases have been reported in *Neurospora crassa*. One (Nyc *et al*, 1966) was repressible by phosphate and the other (Kuo and Blumenthal, 1961a) was not subject to this form of regulation. In derepressed cultures the former enzyme represented about 1% of the total protein.

Aspergillus nidulans produced two distinct alkaline phosphatases - one being phosphate repressible and the other not (Dorn, 1967; Dorn, 1968). It was reported that the repressible enzyme could be destroyed by a mutation in

any one of 9 or 10 genetically distinct loci whereas no mutations affecting the constitutive enzyme were known.

(iv) Animals. Little is known of the genetic control of the enzyme in animals. It has been reported to be induced by glucocorticoids in human leucocytes *in vivo* (Valentine *et al*, 1954) and during embryonic development in mouse (Moog, 1959), chicken (Moog, 1952; Kato, 1959) and amphibian (Chieffi and Carfagna, 1960) intestinal epithelium. In some human cell cultures enzyme induction by hydrocortisone or prednisolone has been reported (Cox and MacLeod, 1961; Cox and MacLeod, 1962; Griffin and Cox, 1966; Griffin and Ber, 1969). Organic monophosphates have been implicated as inducers of the enzyme in human cell cultures (Maio and DeCarli, 1963) and phosphate has been suggested as an end-product repressor of the rat kidney enzyme (Melani *et al*, 1967).

F. Some properties of highly purified alkaline phosphatases

Alkaline phosphatase from several sources has been obtained in high purity. The purified enzyme from swine kidney, where it constitutes about 0.003% of the tissue, was estimated (Mathies, 1958) to be 80-90% pure. The turnover number was 100,000 moles per 100,000 gms. of protein per minute (Disodium phenyl phosphate, pH 9.7, 25°).

Fully active preparations of the greatest purity contained 0.15 to 0.18 per cent of zinc. Based on the assumption of one zinc atom per protein molecule, the minimum molecular weight was calculated to be 37,000. Diffusion and ultracentrifuge measurements indicated a higher molecular weight, so that it was probable that the enzyme molecule contained several firmly bound zinc atoms.

The human placental alkaline phosphatase has been crystallized (Harkness, 1968a; Harkness, 1968b) and some of its properties have been reported. The molecular weight was approximately 125,000 and the presence of 2 - 3 g. atoms of zinc per mole was indicated. The enzyme was inactivated by EDTA. Its turnover number was 40,500 moles PNPP/mole enzyme/minute. Other workers quoted by Harkness determined that the enzyme was dimeric, the molecular weight of the dimer being 116,000 and that of the monomer 58,000.

The phosphate repressible enzyme from *Aspergillus nidulans* was purified sufficiently (Dorn, 1968) to be homogeneous by chromatography, gel filtration, density gradient centrifugation, starch gel and polyacrylamide electrophoresis. It was stabilized by divalent cations and inactivated by chelating agents. The molecular weight was estimated to be 185,000 by density gradient centrifugation and 150,000 by gel filtration on Sephadex G-150. The authors felt that

the discrepancy in molecular weight determinations was due to a basic difference in the methods and expressed the need for accurate determinations by other methods. It was suggested that the four electrophoretically distinct phosphatases (2 acid phosphatases, plus 2 alkaline phosphatases) of A. *nidulans* represent four heteromultimeric proteins each made up of two or more polypeptide chains, and that some of the polypeptide chains are common to two or more enzyme forms. The enzyme probably contained a carbohydrate moiety and it was suggested that of the 10 genes involved in production of the enzyme some may be concerned with the carbohydrate portion. The enzyme was unusual in having two pH optima, one at pH 8.0 and another above pH 10.0. Its isoelectric point was between 3.6 and 4.0. The amino acid composition of the enzyme was determined.

The constitutive enzyme of A. *nidulans* has been only partially purified and little is known of its properties. Its molecular weight (Dorn, 1967) was given as 150,000. Similarly, little is known of the constitutive enzyme from *Neurospora crassa* (which is the subject of this thesis).

The repressible enzyme of *N. crassa* has been extensively purified (Kadner *et al*, 1968). The native protein had a molecular weight of 154,000 and could be dissociated

into subunits of molecular weight 77,000. Its amino acid composition was reported and this enzyme also was a glycoprotein (11.5% carbohydrate, including glucosamine, mannose, galactose and glucose). The value for $E^{1\%}_{280}$, 1 cm. was 11.3 for the native enzyme at pH 8.3 in 0.01M Tris-HCl. The enzyme activity was stimulated by EDTA (Nyc *et al*, 1966) and was unaffected by Mg^{++}, Zn^{++} or many other metal ions. The enzyme was more stable toward denaturation in the presence of Mg^{++} or Zn^{++}, and these ions facilitated the recovery of activity after exposure to various denaturing agents. The metal requirements of this enzyme remain in doubt (Kadner and Nyc, 1969).

The phosphate repressible enzyme of *Bacillus subtilis* was crystallized and its amino acid composition reported (Takeda and Tsugita, 1967). Activity was destroyed by EDTA and could be recovered by adding zinc to the enzyme. Cobalt, however, was a better activator than zinc. The purified enzyme was homogeneous in the analytical ultracentrifuge. A sample treated with 6M urea showed several faint protein bands in addition to a single main band on polyacrylamide gel electrophoresis.

The *E. coli* enzyme has been the subject of extensive investigation. The enzyme was shown to be a dimeric protein containing two identical subunits (Rothman and Byrne,

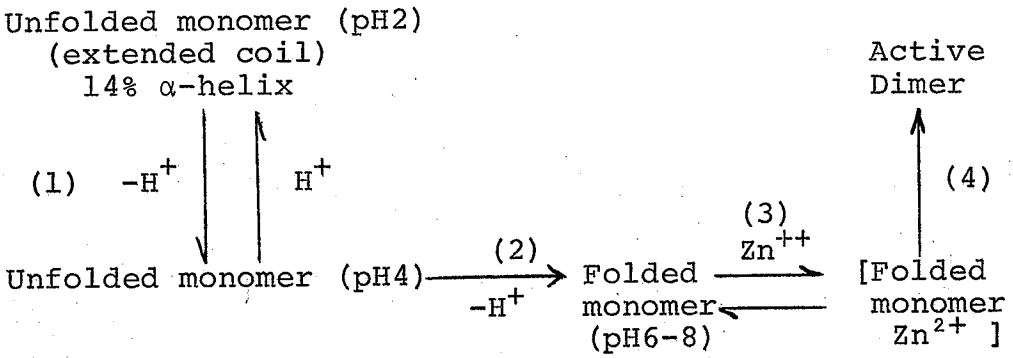
1963). Complementation between structural mutants was demonstrated (Garen and Garen, 1963a) *in vivo* and the properties of the complementing strains were compatible with the suggestion that dimerization occurs randomly. Complementation *in vitro* has also been demonstrated (Schlesinger and Levinthal, 1963). Proteins antigenically related (CRM) to alkaline phosphatase were purified from several phosphatase - negative strains. Each CRM was dissociated into monomers by treatment with thioglycollate in urea (Levinthal, *et al*, 1962). Enzymically active protein was formed by mixing monomers from two different mutants. The active protein was a hybrid composed of one monomer from each mutant. Dimerization was shown to be zinc-activated and proceeded in the pH range 6-9.

Further studies on the reversible denaturation of the *E. coli* enzyme were carried out by Schlesinger and Barrett (1965) and Schlesinger (1965). These authors discussed three different ways in which the enzyme was denatured to yield three different forms of inactive protein. The first form is that produced by reduction in 8M urea. Subunits produced this way contained free sulfhydryl groups. Reactivation involved reoxidation of the sulfhydryl groups. A second type of subunit was produced simply by lowering the pH to values below 3. Disulfide bridges

(intrachain) of the subunits are intact in this form. It had been observed earlier (Garen and Levinthal, 1960) that EDTA reversibly inhibited the enzyme. Plocke *et al* (1962) showed that this effect was due to the removal of two zinc atoms that were essential for activity. The denatured protein (a dimer) was rapidly renatured on addition of zinc. Acidification also released zinc which was essential for reassociation of the acid produced monomers. EDTA inhibited dimerization.

A low activation energy for dimerization was observed which implied that the process would not require special enzyme mediated reactions in the cell. Dimerization resulted in the conversion of a very labile, non-active monomer into an active enzyme that was exceptionally stable. Subunits did not precipitate with antiphosphatase antibody, though there appeared to be some antigenic determinants common to both monomer and active enzyme. Apparently dimerization resulted in large changes in the tertiary structure of the subunits.

The existence of several distinct conformational states of the monomer was shown to depend on the pH of the medium (Reynolds and Schlesinger, 1967). Their results suggested the following protein states and pathway from monomer at pH 2.0 to dimer:



Zinc was found to be necessary only for the dimerization. No postulate was made as to the number of zinc atoms involved or the role of the ion in dimerization. Steps (2) and (4) were not reversible.

The amount of zinc associated with the enzyme varies with the method of purification and past history of the enzyme. Enzyme crystallized from $(NH_4)_2SO_4$ (Malamy and Horecker, 1964) contained 3 g. atoms of zinc per mole based on a molecular weight of 89,000. Enzyme purified in a single step (Simpson *et al*, 1968) contained 4 g. atoms/mole based on the same molecular weight. Treatment of this enzyme with $(NH_4)_2SO_4$ lowered the zinc content to 3 g. atoms/mole or less.

Two of the four zinc atoms were removed rapidly by 8-hydroxyquinoline-5-sulfonic acid (Simpson and Vallee, 1968). Their removal resulted in the loss of activity. The other two zinc atoms were more tightly bound. The existence

of two classes of metal atoms in the enzyme was confirmed by replacement of zinc with cobalt. The first two 2 g. atoms of cobalt bound to the apoenzyme produced no activity. A second 2 g. atoms of cobalt generated activity.

The molecular weight of the pure enzyme is dependent on several factors. Garen and Levinthal (1960) gave the molecular weight of the monomer as 40,000 while Schlesinger and Olsen (1968) gave the value 43,000. Applebury and Coleman (1968) reported that the weight average molecular weight ($\bar{M}w$) of the dimer varied from 80,000 to 100,000 depending on pH and ionic strength. The molecule remained a dimer between pH 8 and 4. Below pH 4 the dimer dissociated and near pH 2 $\bar{M}w$ for the monomer was 40,000. Reassociation of the monomers followed a pathway different from the pathway of dissociation and complete redimerization did not occur until pH 6.0. This hysteresis effect observed in the dependence of $\bar{M}w$ on pH corresponded to the hysteresis observed (Schlesinger and Barrett, 1965) in acid titration of the enzyme. Determinations of $\bar{M}w$ for the apoenzyme as a function of pH showed that the apoenzyme underwent dissociation and reassociation as did the native dimer. On return of the apoenzyme to neutral pH, however, 25% monomer remained. Addition of either zinc or cobalt caused the residual monomers to form dimers.

At neutral pH the native enzyme or the apoenzyme labelled with ^{65}Zn bound between 2 and 3 g. atoms of ^{65}Zn per mole. ^{65}Zn dissociated from the protein between pH 6 and 4 and was half dissociated at pH 5. Reassociation of the metal ion followed the same pH function.

Torriani (1968) and Schlesinger and Olsen (1968) suggested that *in vivo* dimerization takes place in the periplasmic space from a pool of subunits in the cytoplasm.

G. Multiple forms of alkaline phosphatase.

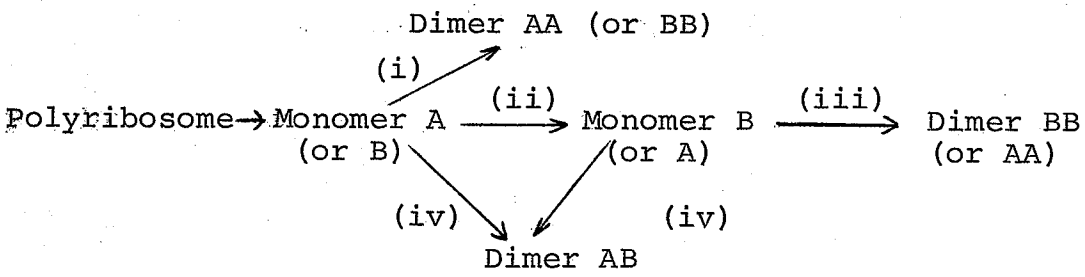
Highly purified preparations of the *E. coli* enzyme which were homogeneous in the analytical ultracentrifuge nevertheless have been shown (Garen and Levinthal, 1960) to consist of several distinct enzymatically active forms when analyzed by starch gel electrophoresis. The isozymes were shown (1) not to be artifacts of isolation, preparation, or analysis, (2) not to be the result of subunit aggregation and (3) to be the product of a single gene (Signer *et al*, 1961).

Lazdunski and Lazdunski (1967) succeeded in separating three isoenzymes of the *E. coli* alkaline phosphatase by DEAE cellulose chromatography. All three isoenzymes were shown to be dimers.

On polyacrylamide gel electrophoresis three separated

bands could also be observed. The slowest (Band I) corresponded to the isoenzyme that eluted earliest from the DEAE cellulose column. Band III, the fastest band on the gel was the isoenzyme that eluted last from the column. Band II was intermediate in both cases.

If pure Band I or pure Band III enzyme was dissociated into monomers and allowed to reassociate no new bands appeared. If an equimolar mixture of Band I and Band III was dissociated and allowed to reassociate all three bands were formed. If Band II was dissociated and reassociated all three bands appeared. These observations were interpreted as follows: Bands I and III were dimers each consisting of identical subunits. They were designated AA (or BB) and BB (or AA). Band III was, then a hybrid dimer AB. Monomers A and B had very similar amino acid compositions. A scheme accounting for the existence of the three isoenzymes *in vivo* was proposed.



The scheme was considered to be in accord with (a) complementation studies which required the free monomer to exist

in the cytoplasm and (b) the final dimerization to the active form taking place in the periplasm where, presumably, the modifying enzyme that catalyzes reaction (ii) is located.

Further work on the isoenzymes has been reported (Schlesinger and Andersen, 1968) in which the existence of three isoenzymes is confirmed. These workers showed that the composition of the electrophoretic pattern varied with the carbon source in the growth medium but offered no explanation of the fact. They provided evidence that upon derepression Band I isoenzyme first appears and undergoes some converson *in vivo* to yield the other forms. The same relationships existed between the three bands when they were dissociated and reassociated either singly or in a mixture as were shown by Lazdunski and Lazdunski, (1967). It was not possible to bring about *in vitro* conversion of Band I to Band III. Fingerprint analysis of tryptic digests of Bands I and III showed one peptide in Band I that was absent from Band III.

Schlesinger and Olsen (1968) reported that the isoenzymes also appeared when the *E. coli* enzyme was synthesized in *S. typhimurium* heterogenotes.

The occurrence of multiple forms of the enzyme seems to be quite widespread. These forms may differ in the

sense that the *E. coli* isoenzymes differ, or they may be completely different enzymes, such as the repressible (apparently not resolvable into isoenzymes) and non-repressible enzymes of *A. nidulans* (Dorn, 1968) or *N. crassa*.

Boyer (1961) reported (on the results of starch gel electrophoresis) the existence of 16 alkaline phosphatase activities in human sera, though not all 16 were found in the serum of any one individual. Some of these forms could also be separated by column chromotography. Placental and intestinal alkaline phosphatases could be completely separated by chromatography. Antibodies produced in rabbits against bone alkaline phosphatase did not cross react with purified intestinal or placental enzymes. Such antibodies did, however, react with and remove from serum one of the bands normally seen on starch gel electrophoresis of serum. Purified bone enzyme moved on starch gel in the same manner as the removed serum band. Placental enzyme was resolved into three bands, but only two were present in any one placenta.

The crystallized ultracentrifugally homogeneous enzyme purified from multiple placentae demonstrated only one band, on polyacrylamide gel. A non-crystallized sample purified by chromatography to the same specific activity as the crystalline material and judged 95% pure was resolved

into three active bands on polyacrylamide gel (Harkness 1968a). Harkness wondered why the crystalline material produced only one band, and reported that other workers had observed the same result with freshly prepared enzyme. On storage, however, additional bands of activity appeared.

H. The effects of pH on the activity of alkaline phosphatase and kinetic studies on its mechanism of action.

In the words of Stadtman (Stadtman, 1961) the number of reports concerning the effect of pH on the activity of various alkaline phosphatases is legion. The more complete earlier studies are those of Morton (1957), Motzok (1959) and Motzok and Branion (1959). These studies have shown that the optimum pH depends on the nature of the substrate and on its concentration, on the source of the enzyme and its concentration, on the nature and concentration of metal activator and on the type of buffer used.

The optimum pH for the enzyme from several sources (rats, goslings, chickens, rabbits, pigeons and pigs) was shown (Motzok and Branion, 1959) to be a linear function of the logarithm of the substrate concentration. The optimum shifted to higher pH values with higher substrate concentrations. The pH activity curves prepared for several concentrations of the same substrate (Motzok, 1959; Moss *et*

al, 1961) were broader and flatter at lower substrate concentrations than at higher ones. An exception to this general pattern was reported (Anagnostopoulos and Matsudaira, 1958) in the behaviour of human placental alkaline phosphatase. The pH optimum for that enzyme was higher (pH 10.2) than for other alkaline phosphatases and did not vary with substrate concentration over the concentration range tested.

These considerations make it very difficult to compare data from different sources. Measurements for comparison would have to be made very carefully under identical conditions.

Several figures demonstrating the relationship of V_{max} and K_m to pH have appeared in the literature. They are presented in Fig. H1 for comparison with each other and with the data in the experimental portion of this thesis.

The results of several workers (Schwartz, 1962; Engstrom, 1962; Pigretti and Milstein, 1965) have shown that hydrolysis by the *E. coli* alkaline phosphatase takes place in two steps.

$$EH + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EHS \xrightarrow{k_3} \underset{P}{EHS'} \xrightarrow{k_4} EH + Q$$

where EHS' is an intermediate phosphoryl-enzyme, P is the alcoholic or phenolic part of the substrate and Q is

phosphate. Wilson *et al* (1964) demonstrated that k_3 is much larger than k_4. The value of k_4 is, of course, the same for all substrates and is a probable explanation of the observation that V_{max} is nearly the same for all substrates (Melani *et al*, 1967).

Lazdunski and Lazdunski (1966) discussed three states of ionization of the enzyme:

$$EH_2 \rightleftharpoons EH + H^+ \qquad pK\ 7.1$$

$$EH \rightleftharpoons E + H^+ \qquad pK\ 8.6$$

The state of ionization of these two groups at the active center affected the apparent affinity for substrate and the rate of breakdown of the enzyme-phosphate complex. The first group, with a pK of 7.1 in the free enzyme and 7.4 in the phosphorylated enzyme had to be in the basic form for the enzyme to be active. The group took part in substrate binding and in the phosphorylation and dephosphorylation steps during catalysis. It had been shown earlier (Schwartz, 1962; Schwartz *et al*, 1963) that the *E. coli* enzyme possessed an essential serine at the active center to which phosphate was bound. These workers discussed similarities in the mechanism of catalysis by the *E. coli* alkaline phosphatase, chymotrypsin and trypsin.

Rzhekhina (1963) showed that the rate of hydrolysis

of sodium phenylphosphate by dog intestinal alkaline phosphatase could be expressed as

$$v = \frac{rC_E.C}{1+aC+bC^{n+1}} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad I$$

where r, a and b are constants, n is the number of molecules of substrate bound to the active complex with formation of an inactive complex ES_{n+1}. C_E is the enzyme concentration and C is the substrate concentration. The values of r, a, b and n varied with pH. Methods for their evaluation were given. These workers analyzed their results starting from a modified Michaelis-Menten equation

$$v = \frac{V_{max}C}{K_s+C+KC^{n+1}} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad II$$

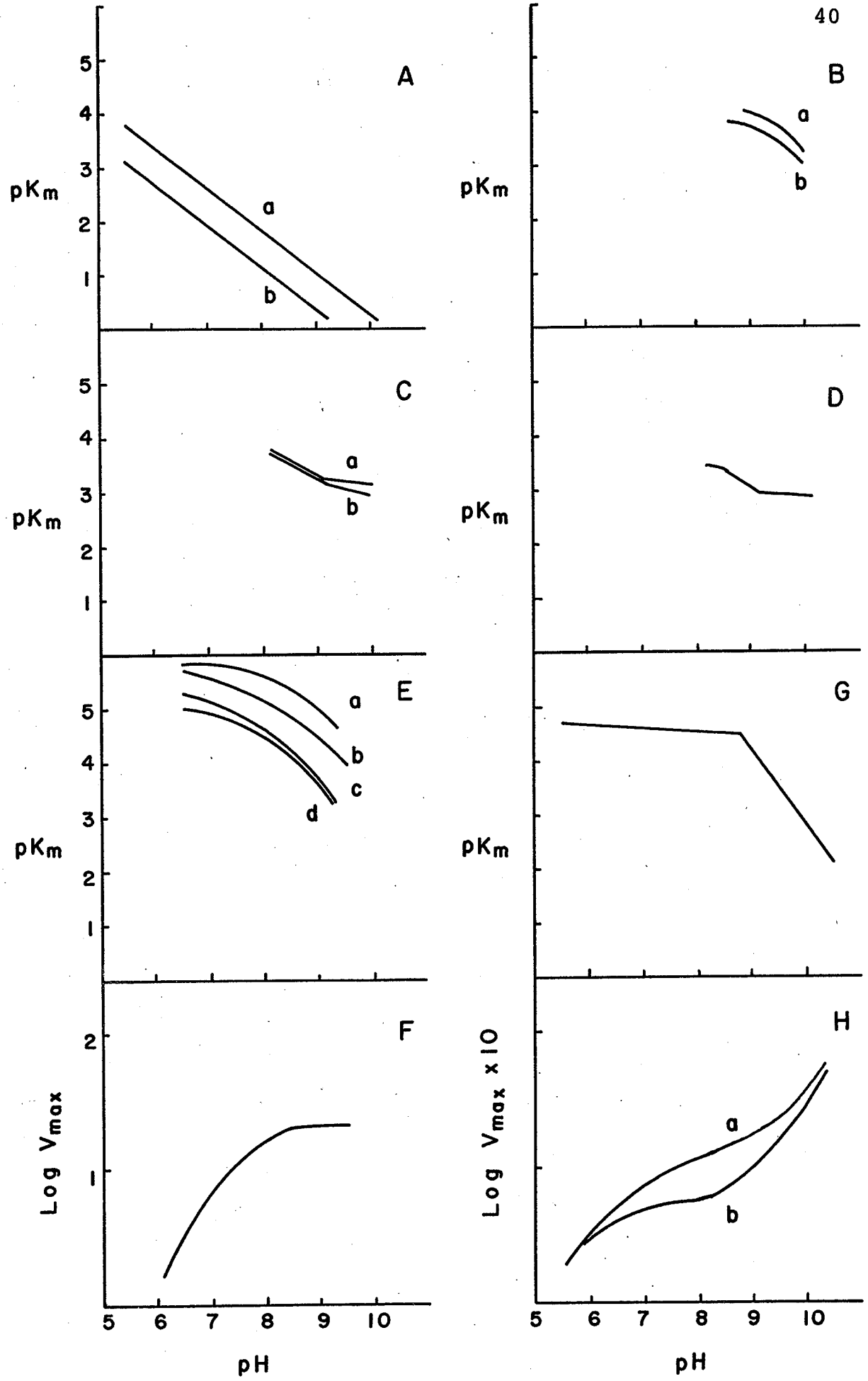
Equation I can be converted to the form of II on division by a. Incorporating the values of constants determined by Rzhekhina (1963) the kinetic equations at three different values of pH (10.0, 9.4, and 8.8) yield values of K_s and V_{max} that increase with pH over the range studied. With a fall in pH, however, K_s fell more rapidly than V_{max}. At small substrate concentrations, when the value of the denominator in equation II is determined largely by the value of K_s the rate of the reaction increased with decrease of pH. At high substrate concentrations, however, the equation

predicted that the rate would increase with pH.

Over the short pH range studied, these results described well the dependence of reaction rate on pH and substrate concentration characteristic of alkaline phosphatases.

Data relating to the alkaline phosphatases have been accumulating in the literature at an accelerating rate. Most of the references used in the compilation of this review are dated in the decade just past. Isolated portions of the total body of knowledge one would wish to have about an enzyme, such as the genetics of the *E. coli* phosphatase, are well developed. On the other hand only the first furrows have been ploughed in the field of kinetics or physiological function of the enzyme. Information relating to the enzyme in the fungi is particularly sparse and to the author's knowledge, product inhibition studies (except phosphate inhibition) have not been performed with the enzyme from any source.

The present study was undertaken with a view to extending our knowledge of the fungal alkaline phosphatases and to add to the general body of knowledge by means of a detailed study of pH effects and product inhibition.


A
B
C
D
E
F
G
H
pKm
Log Vmax
Log Vmax x 10
pH
a
b
c
d
1
2
3
4
5
6
7
8
9
10

MATERIALS AND METHODS

Growth of *Neurospora crassa*

The organism used in this study was *Neurospora crassa* strain 533 (a "wild" type). The fungus was grown in Vogel's medium (Vogel, 1956) containing 2% sucrose as the carbon source. A two liter flask of the medium was inoculated with conidiospores and grown for three days at room temperature on a rotary shaker. The contents of the flask were used to inoculate 10 l of the medium in a glass carboy. Growth with vigorous aeration was allowed to continue for 30 hours, at which time the contents of the carboy were used to inoculate 140 l of the medium in a stainless steel tank. Growth in the tank was allowed to continue for 40 - 50 hours at room temperature. The mycelium was harvested by straining the medium through cheesecloth. The mycelium was wrung dry, weighed, shredded in a Waring blendor, and lyophilized. The lyophilized mycelium was reduced to a powder and stored at -20°.

Chemicals

All organic chemicals used during the investigation were purchased from the Sigma Chemical Co. except the Glucostat reagents used for the determination of glucose, which were purchased from the Worthington Biochemical Cor-

poration. Cleland's reagent (dithiothreitol) was purchased from Calbiochem.

Standard enzyme assay

During purification the enzyme was assayed with p-nitrophenyl phosphate as substrate. The reaction was followed by measuring the increase in optical density at 410 mμ which is the absorption maximum of one of the products, p-nitrophenylate. All measurements were made on the Unicam SP 700 spectrophotometer. Each cuvette, with an optical path length of 1 cm. contained $MgCl_2$, 12 μmoles; p-nitrophenyl phosphate, 30 μmules; Bicine (N,N-bis [2-hydroxyethyl] glycine), 150 μmoles; in a final volume of 3 ml at pH 9.0. One unit of enzyme activity was defined as that amount of enzyme which produced an increase in optical density at 410 mμ of 0.01 optical density units in 1 minute under the above conditions. The reaction rate remained linear for at least 5 minutes.

Protein determination

Protein was determined by the method of Lowry *et al* (1951).

Phosphate determination

Inorganic phosphate was determined by the method of

Peel and Loughman (1959) except that the concentration of sodium acetate buffer was doubled.

Glucose determination

Glucose was determined by the Glucostat method. Glucostat is a prepared reagent for the quantitative colorimetric determination of glucose. It makes use of the coupled enzyme reactions represented in the following scheme:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} H_2O_2 + \text{Gluconic acid}$$

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{peroxidase}} \text{oxidized chromogen} + H_2O$$

The reduced chromogen is colourless; in the oxidized state it is blue. The optical density of the oxidized solution was read in the Klett colorimeter with a red filter. A standard with a known concentration of glucose was run with each series of determinations. The concentration of glucose in an unknown sample was calculated from the formula:

$$\frac{A_u}{A_s} \times C_s = C_u$$

where A = absorbancy (Klett reading)
C = concentration of glucose
u = unknown
s = standard

Hemoglobin determination

Hemoglobin was determined by measuring the optical density at 415 mμ in the SP 700 spectrophotometer.

Alcohol dehydrogenase

Alcohol dehydrogenase was assayed by measuring the increase in optical density at 340 mμ due to the reduction of NAD^+ by the enzyme in the presence of ethanol in phosphate buffer at pH 8.7.

Analytical gel electrophoresis

Analytical electrophoresis on polyacrylamide gel was performed according to the method of Davis (1964).

EXPERIMENTAL RESULTS AND DISCUSSION

Attempts to purify alkaline phosphatase from *Neurospora crassa* mycelium met with a great deal of difficulty. Work on the properties of the enzyme can be divided into three parts, corresponding to different degrees of enzyme purity. Accordingly, results are here presented in three phases.

PHASE I

Enzyme purification

Step I. Crude extract. The lyophilized mycelium (20.0g.) was vigorously stirred in 300 ml of water at 0°-4°C for one hour. The extract was centrifuged at 14,600 x g for 15 minutes. The precipitate was discarded.

Preparation	Volume (ml)	pH	Enzyme	protein	activity
Step I	245	6.6	72,960	2,569	53.5
Step II	258	6.4	77,900	1,569	49.6
Step III	35	7.4	65,200	505	51.2
Step IV	40	-	58,600	386	151.8

Step II. One tenth of a volume of cold 2% protamine sulfate was added with gentle stirring to the supernatant fluid from Step I. After 10 minutes without stirring the solution was centrifuged as before and the precipitate was discarded.

Step III. Enough 0.5M Tris-HCl buffer, pH 7.4 was added to the supernatant fluid from Step II to make the solution 0.05M in Tris-HCl at pH 7.4. Solid $(NH_4)_2SO_4$ was added until the solution was 65% saturated. The precipitate

was collected by centrifugation at 14,600 x g for 15 minutes and dissolved in 0.02M Tris-HCl pH 7.4.

Step IV. The dissolved precipitate was heated to 60° and maintained at that temperature in a water bath for 15 minutes. It was then cooled in an ice-water bath and the heavy precipitate removed by centrifugation at 27,000 x g for 10 minutes. The precipitate was resuspended in 0.02M Tris-HCl pH 7.4 and centrifuged again. The supernatant fluids from both centrifugations, which contained the enzyme, were combined. The enzyme preparation was stored at -20°. The results of a typical purification are presented in Table I:

TABLE I

PURIFICATION OF ALKALINE PHOSPHATASE

Preparation	Volume (ml)	pH	Units of Enzyme	Total protein (mg)	Specific activity units/mg.
Step I	245	6.6	79,960	2,389	33.5
Step II	258	6.4	77,900	1,569	49.6
Step III	35	7.4	65,200	803	81.2
Step IV	40	-	58,600	386	151.8

This enzyme preparation, representing a 5-fold purification, was used in initial experiments to determine some of its properties.

Influence of pH on enzyme activity

The results of experiments on the influence of pH on the activity of the enzyme with p-nitrophenyl phosphate as substrate are presented in Fig. 1. The pH of the reaction mixture was determined immediately after the initial velocity measurement. Maximum activity was obtained at pH 9.4 - 9.5.

Effect of enzyme concentration

The results of experiments on the influence of enzyme concentration are presented in Fig. 2. The initial velocity was proportional to the enzyme concentration.

Effect of Heating on enzyme activity

A sample of the crude extract was rapidly heated to 70° and was maintained at that temperature in a water bath. Samples were removed at various times and assayed for enzyme activity. Similar experiments were performed at holding temperatures of 55°, 60° and 65°. The results are presented in Fig. 3. The enzyme was stable for at least 45 minutes at 50°. Boiling destroyed all enzyme activity.

Influence of magnesium ion

The effects of varying the magnesium ion concentration in the assay mixture are presented in Fig. 4. An absolute requirement for magnesium was not demonstrated,

FIG. 1. The influence of pH on the initial velocity of alkaline phosphatase. (PNPP)

Each cuvette contained in a final volume of 3 ml:-

p-nitrophenyl phosphate, 0.033 mmoles
N,N-bis (2-hydroxyethyl) glycine, 0.138 mmoles at the pH indicated
$MgCl_2$, 3.3 µmoles.
Enzyme, 35 units. Boiled enzyme was used in the control cuvette.

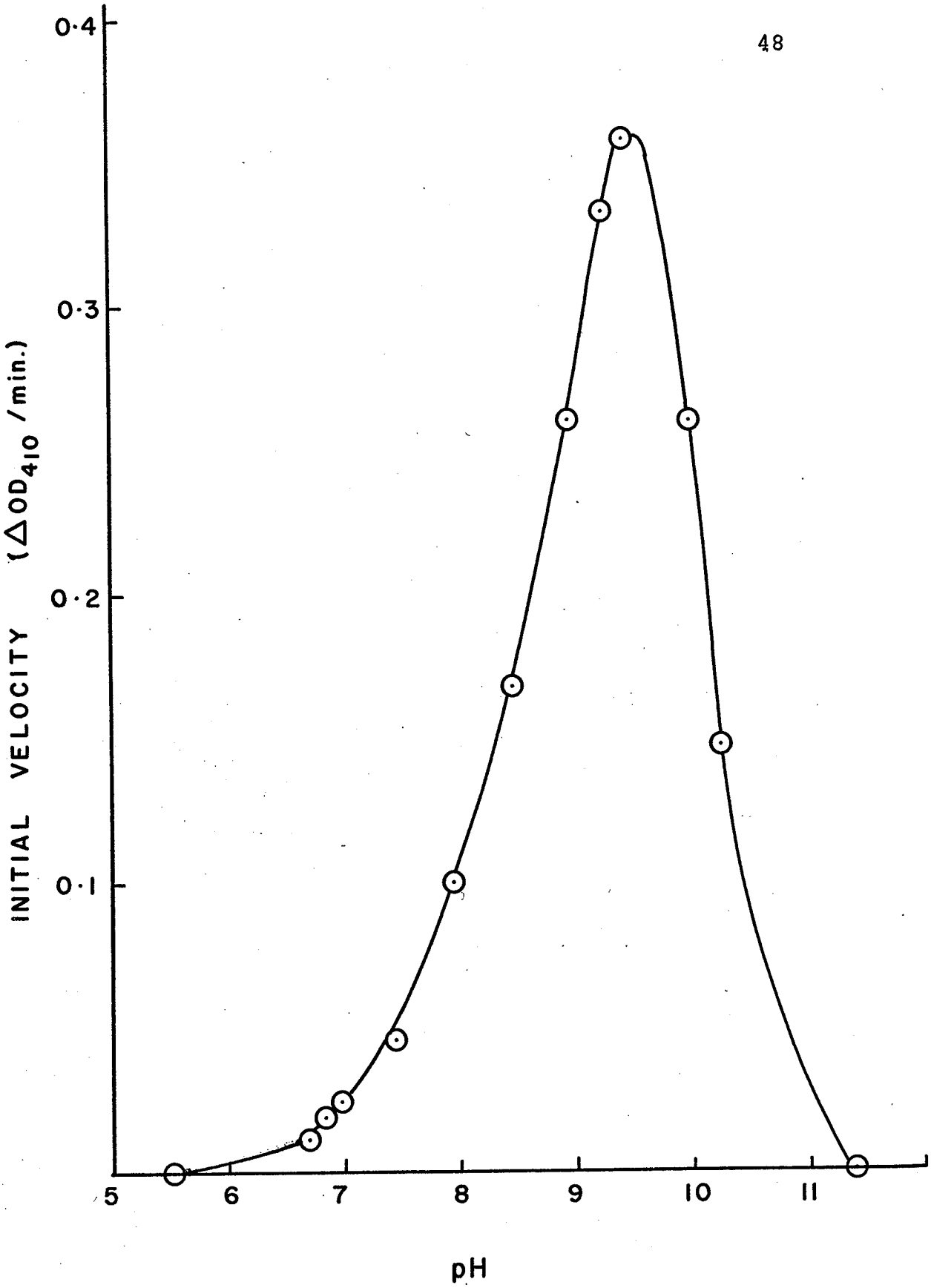

0·4
0·3
0·2
0·1
INITIAL VELOCITY (ΔOD410 /min.)
5
6
7
8
9
10
11
pH

FIG. 2. Effect of enzyme concentration on initial velocity. (PNPP)

The standard assay (See Methods and Materials) was used except that the amount of enzyme was varied.

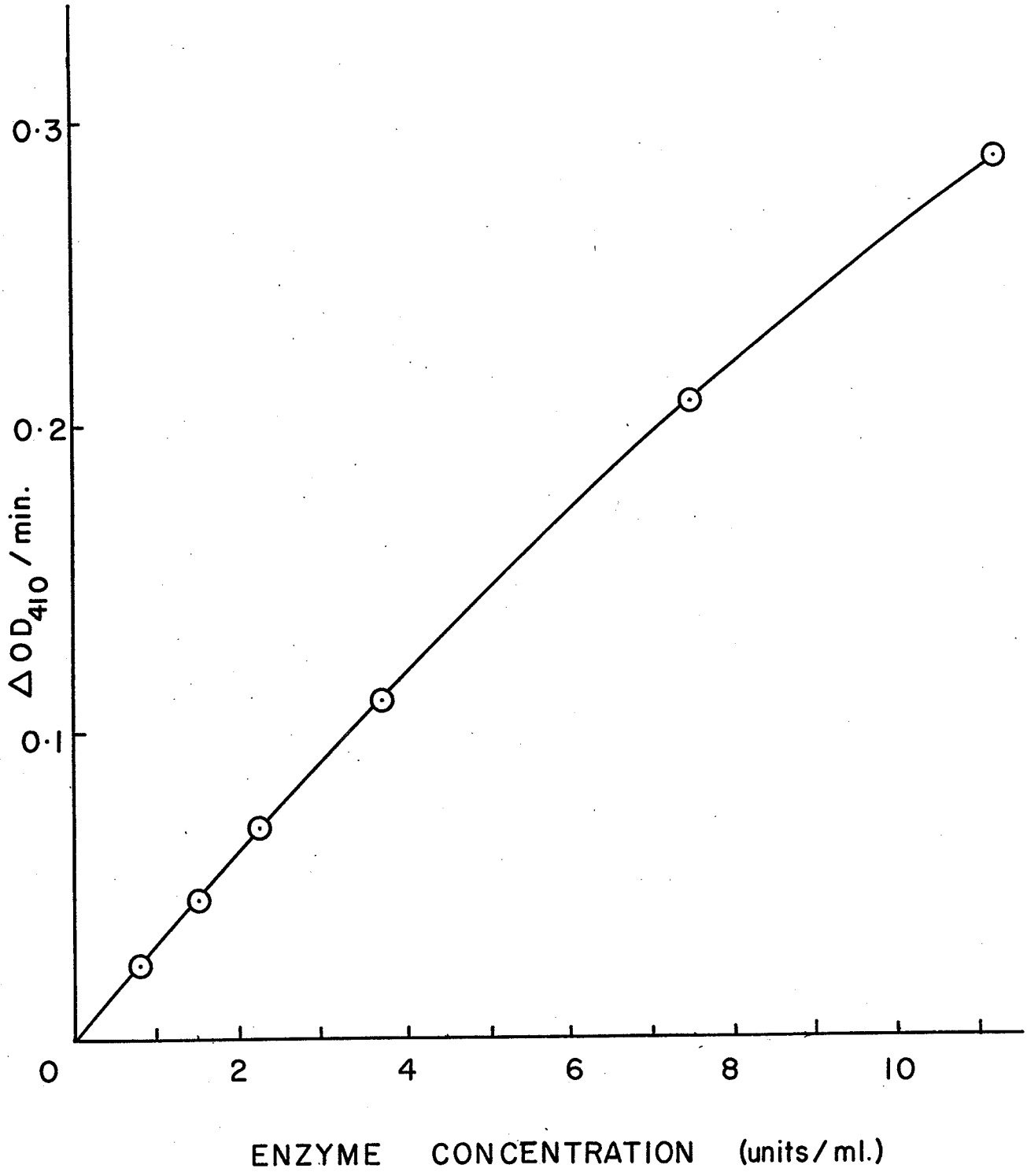

0·3
0·2
0·1
0
2
4
6
8
10
ΔOD410 / min.
ENZYME CONCENTRATION (units / ml.)

FIG. 3. Denaturation of alkaline phosphatase by heat. The enzyme was maintained at the temperature indicated. Aliquots were assayed by the standard assay (See Methods and Materials) at various times.

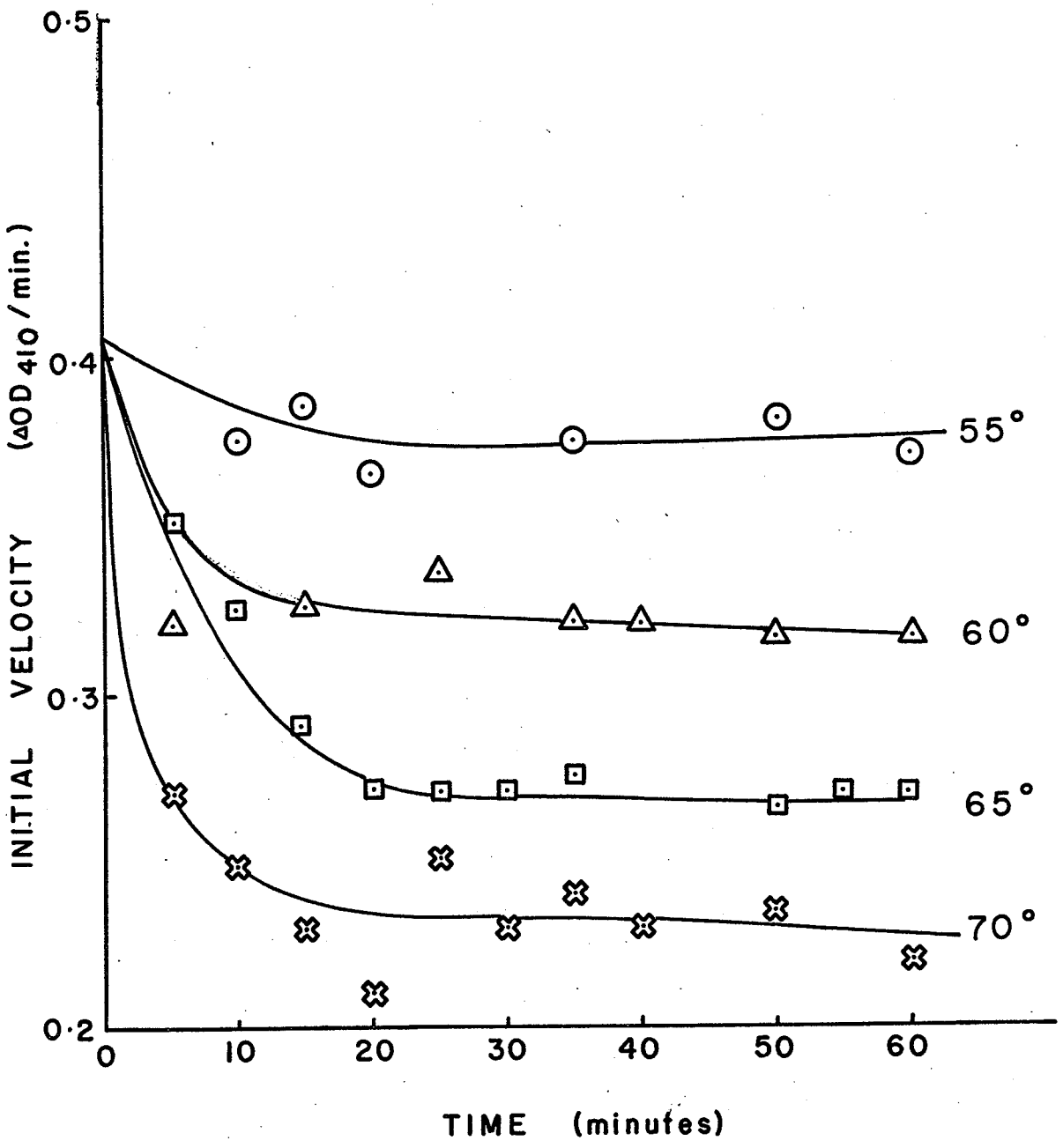
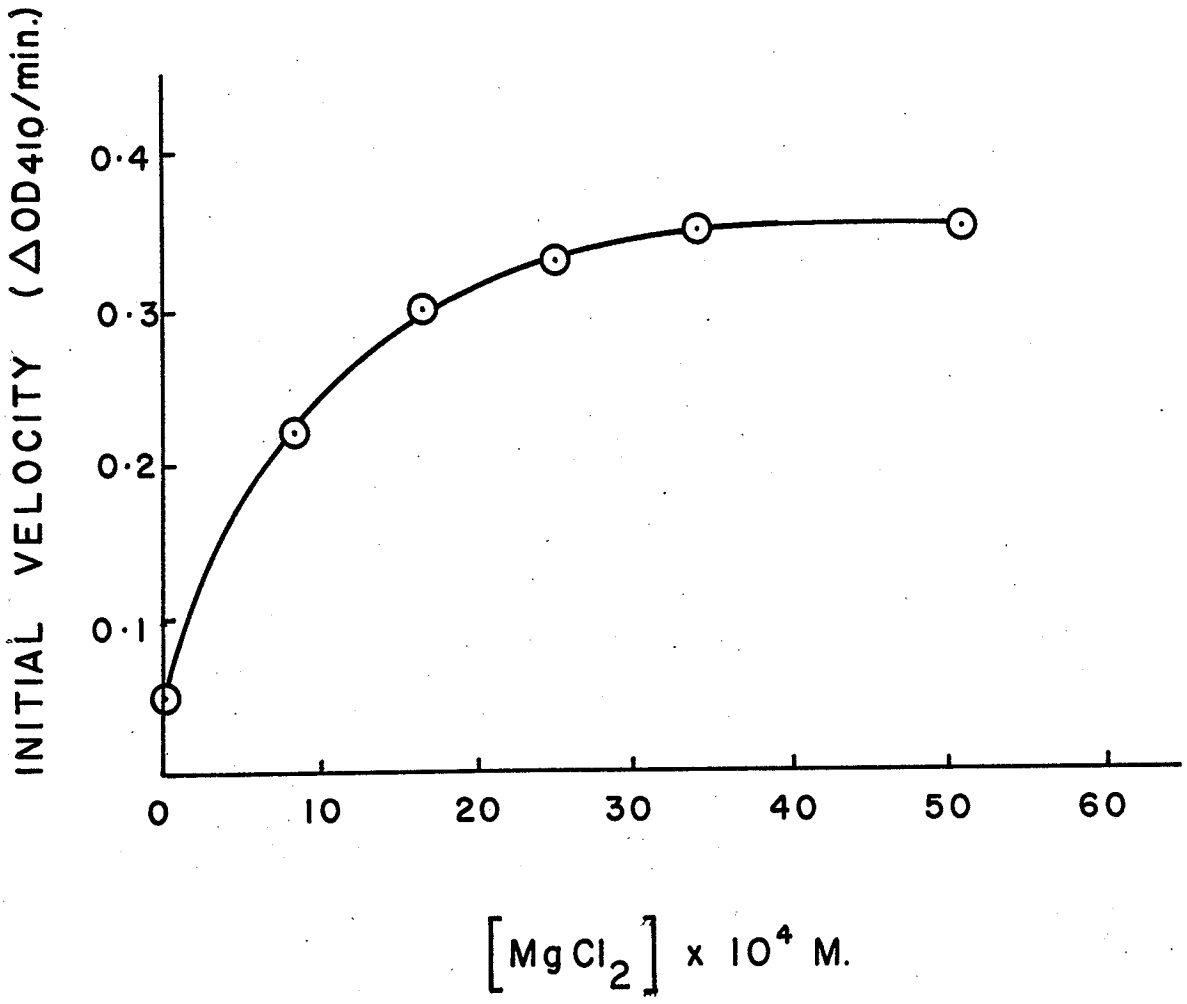

0·5
0·4
0·3
0·2
INITIAL VELOCITY (ΔOD 410 / min.)
0
10
20
30
40
50
60
TIME (minutes)
55°
60°
65°
70°

FIG. 4. Influence of magnesium ion on initial velocity. (PNPP)

The standard assay (See Methods and Materials) was used, except that the concentration of $MgCl_2$ was varied as indicated.


INITIAL VELOCITY (ΔOD410/min.)
0·4
0·3
0·2
0·1
0
10
20
30
40
50
60
[MgCl2] x 10⁴ M.

but its presence greatly enhanced reaction velocity. Maximum activity was obtained at 3.5×10^{-3}M $MgCl_2$ and was unaltered at concentrations up to 5×10^{-3}M.

Influence of substrate concentration

The effect of substrate concentration on the velocity of hydrolysis of p-nitrophenyl phosphate was determined at pH 9.4 and at 25°. Substrate inhibition occurred when the concentration of p-nitrophenyl phosphate exceeded 1.4×10^{-2}M. These results are presented in Fig. 5. The Lineweaver-Burk plots of $\frac{1}{v}$ *versus* $\frac{1}{S}$ for this substrate are presented in Fig. 6. The K_m value obtained was 1.7×10^{-3}M.

Inhibition by products

The initial velocity of hydrolysis of p-nitrophenyl phosphate was measured at various levels of substrate concentration and at three levels of inorganic phosphate concentration. Double reciprocal plots of $\frac{1}{v}$ *versus* $\frac{1}{S}$ were prepared. Phosphate was shown to be a linear competitive inhibitor. The data are presented in Fig. 7 and a replot of the slopes *versus* phosphate concentration is given in Fig. 8. The results of a similar experiment, using p-nitrophenol as inhibitor, are presented in Fig. 9. In this instance the inhibition was shown to be of a non-linear non-competitive type. Slope and intercept replots are

presented in Figs. 10 and 11.

Similarly, with β-glycerophosphate as substrate, glycerol was shown to be a linear non-competitive inhibitor. These results are presented in Figs. 12, 13 and 14. Data derived from these plots are summarized in Table II.

FIG. 5. Effect of substrate concentration on initial velocity. (PNPP)

Each cuvette contained, in a final volume of 3.0 ml, pH 9.4, at 25°:

p-nitrophenyl phosphate as indicated.
N,N-bis (2-hydroxyethyl) glycine, 0.128 mmoles.
$MgCl_2$, 3.3 μmoles.
Enzyme 12 units.

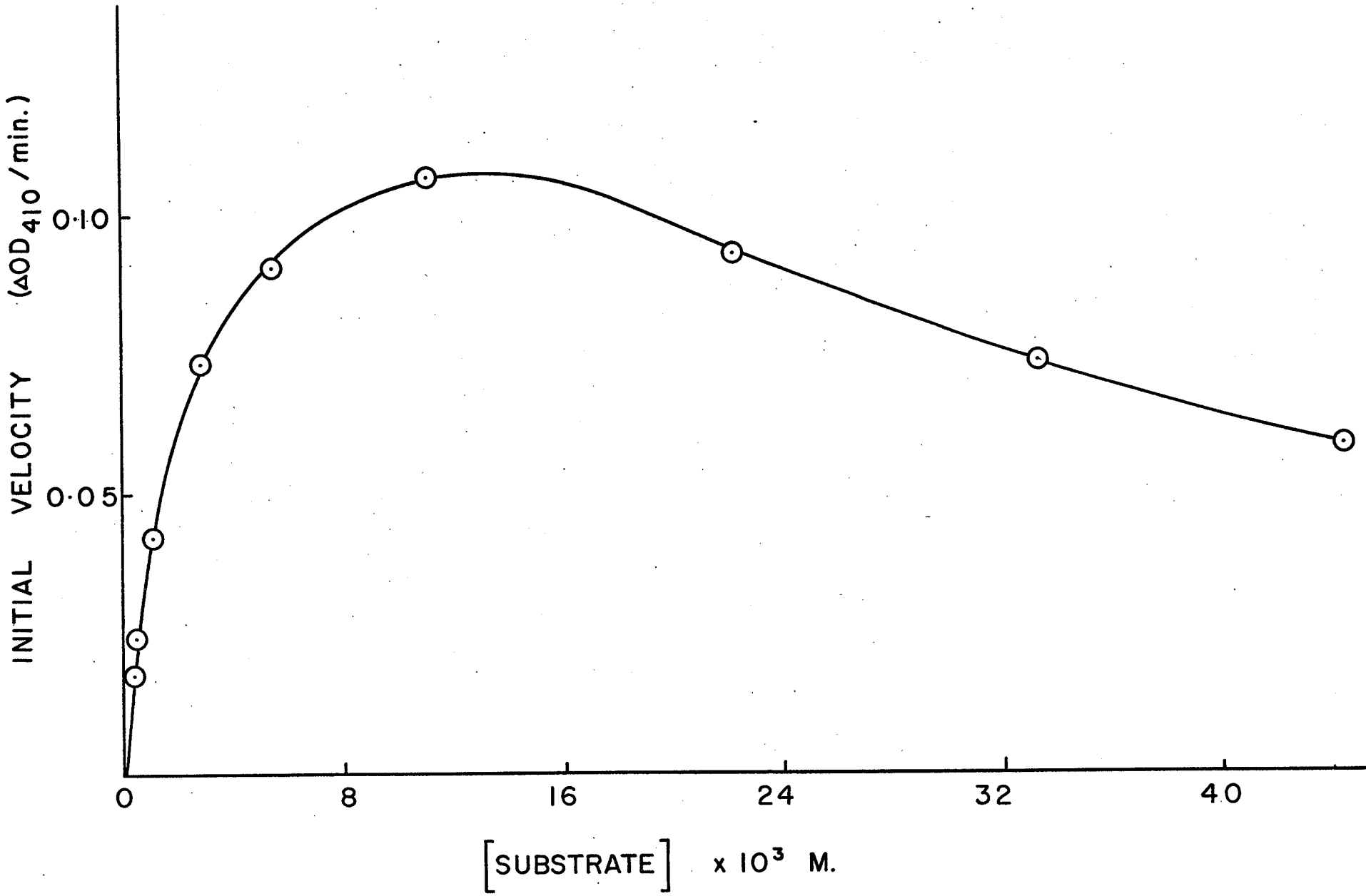

INITIAL VELOCITY (ΔOD410/min.)
0·10
0·05
0
8
16
24
32
40
[SUBSTRATE] x 10³ M.

FIG. 6. Lineweaver-Burk double reciprocal plot and determination of K_m. (PNPP)
The data are the same as those of Fig. 5. Vertical bars indicate the range of values obtained in triplicate determinations.

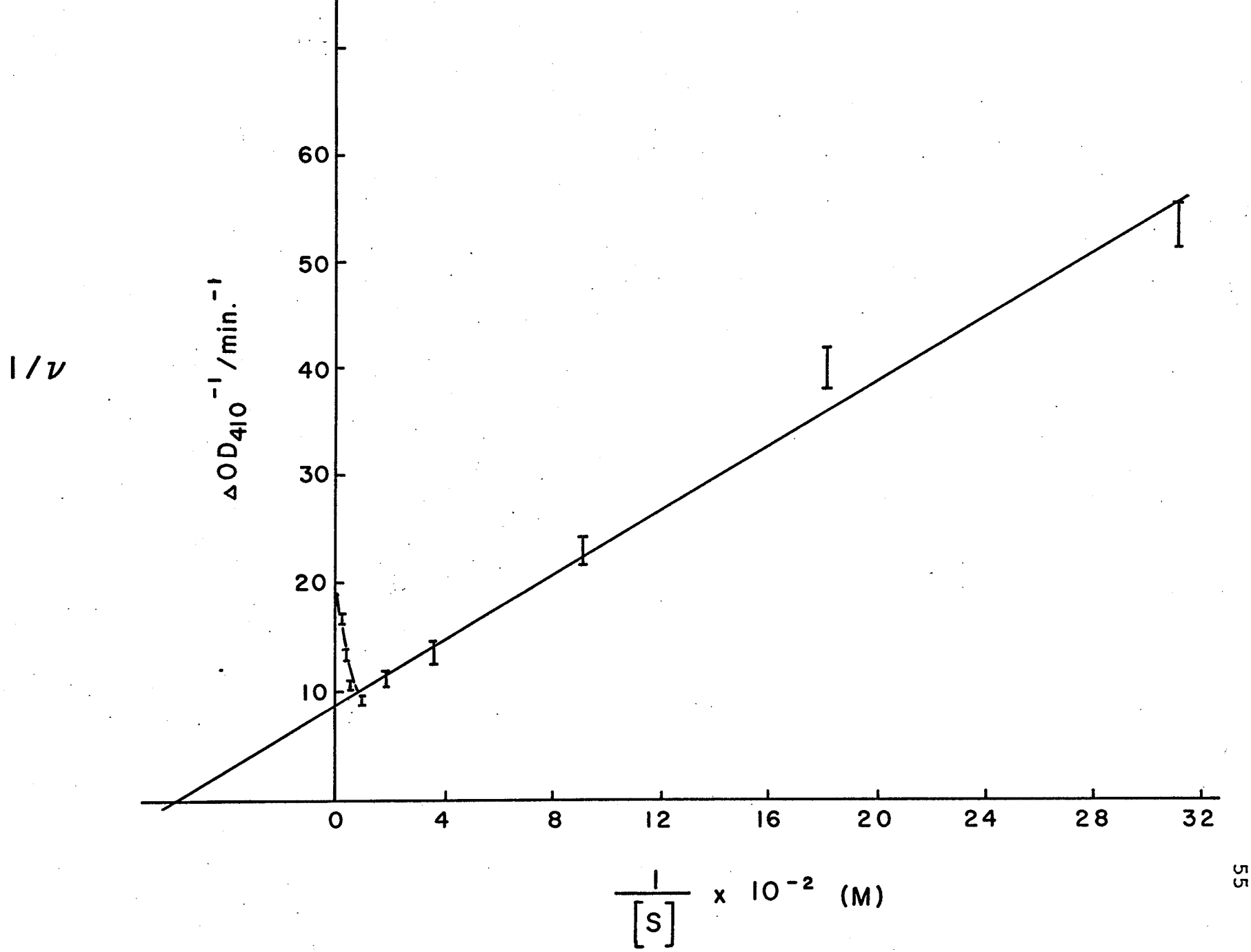

1/v
ΔOD410 -1 /min.-1
60
50
40
30
20
10
0
4
8
12
16
20
24
28
32
1/[S] x 10-2 (M)

FIG. 7. Inhibition by phosphate. (PNPP)

Each cuvette contained, in a final volume of 3.0 ml, pH 9.4, at 25°:

p-nitrophenyl phosphate varied as indicated.
N,N-bis (2-hydroxyethyl) glycine 0.125 mmoles.
$MgCl_2$, 3.3 μmoles.
Na_2HPO_4 at concentration A, B, C or D.
Enzyme, 24 units.

A 3.33×10^{-2}M.
B 1.67×10^{-2}M.
C 8.33×10^{-3}M.
D No inhibitor

The control cuvette contained the same substances except that the enzyme had been boiled.

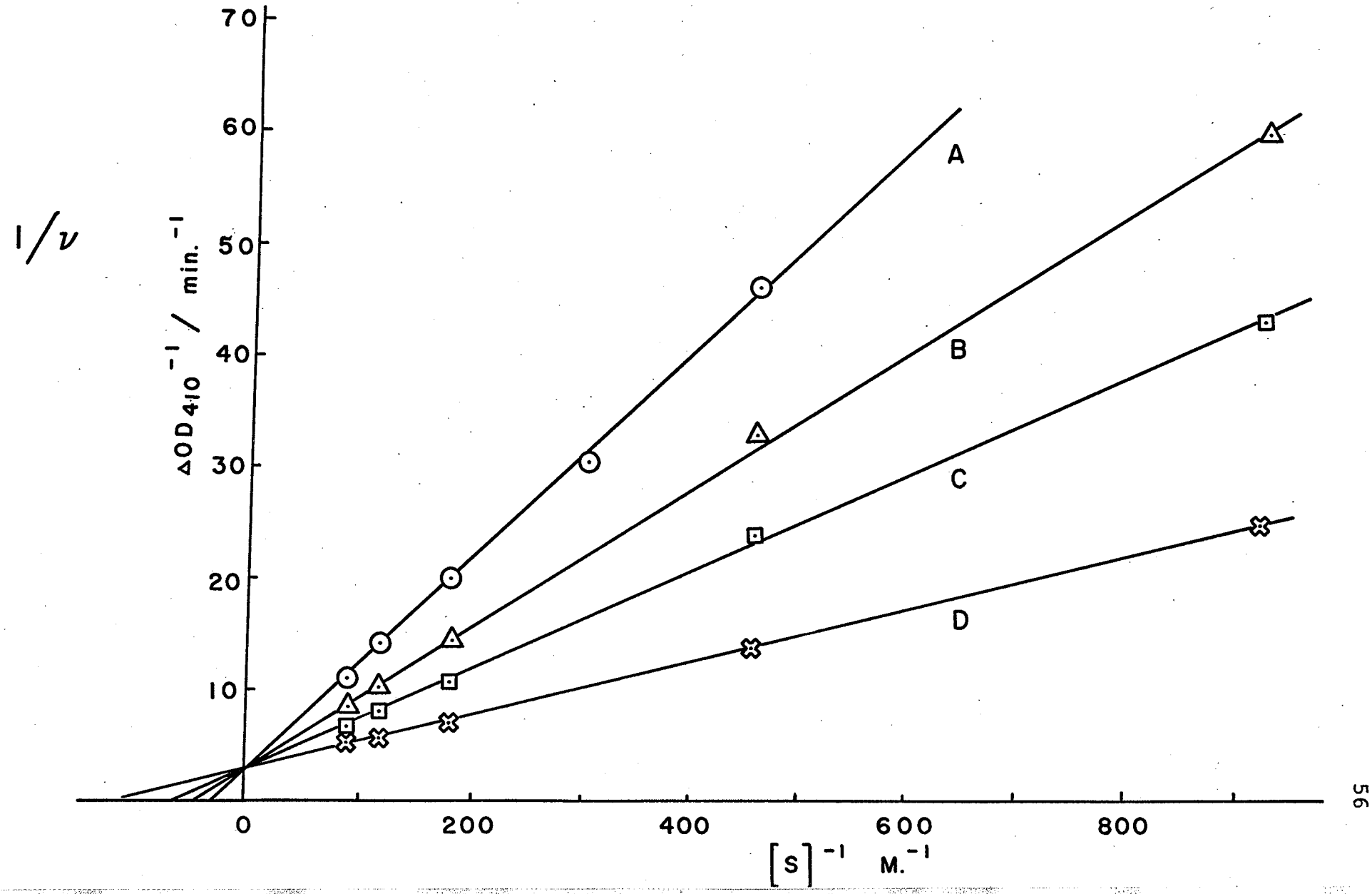

1/v
70
60
50
40
30
20
10
ΔOD410 -1 / min.-1
A
B
C
D
0
200
400
600
800
[S]-1 M.-1

FIG. 8. Replot of slopes of Fig. 7 *versus* inhibitor concentration.

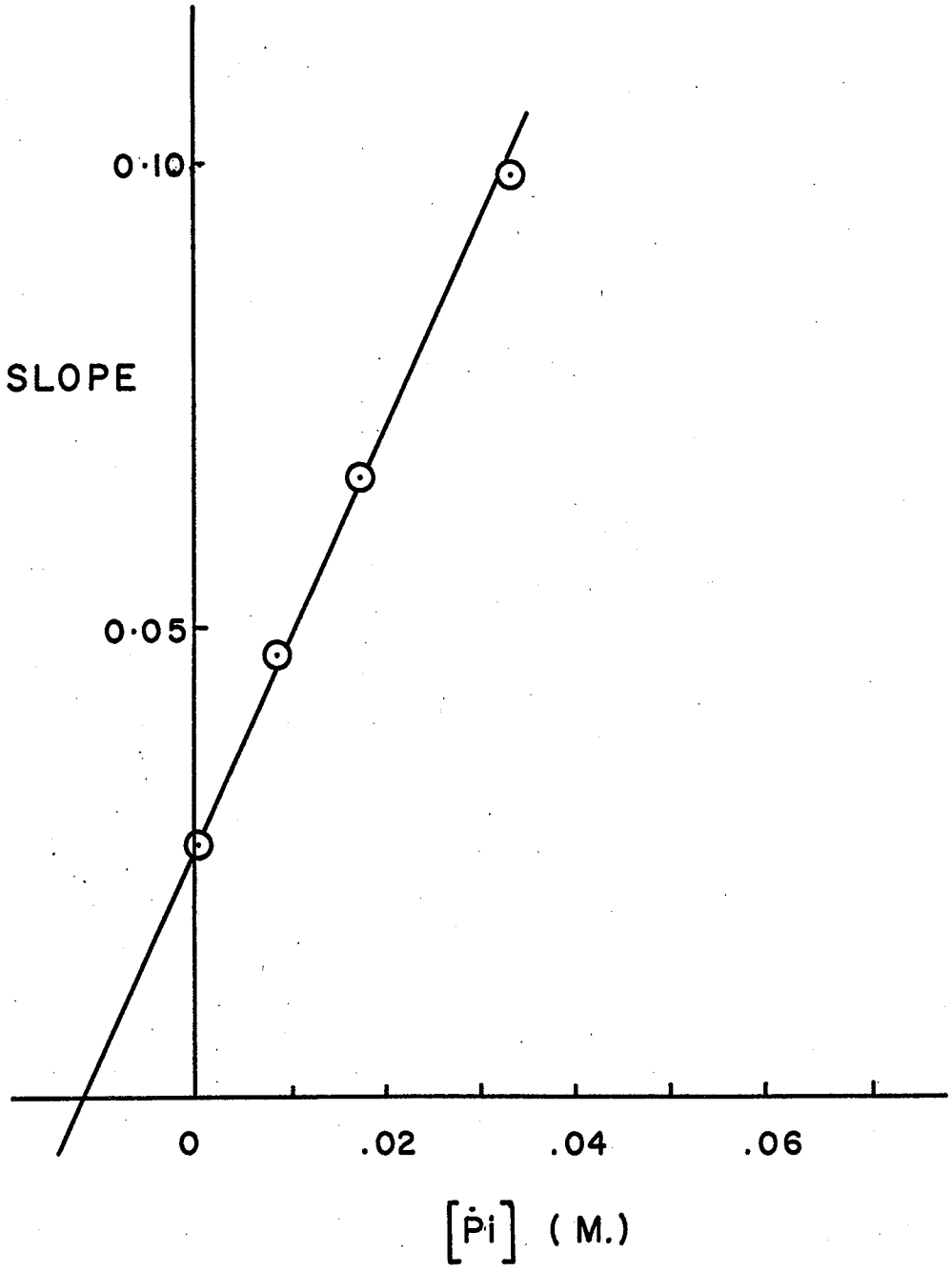

0·10
SLOPE
0·05
0
.02
.04
.06
[Pi] (M.)

FIG. 9. Inhibition by p-nitrophenol. (PNPP)

Each cuvette contained in a final volume of 3.0 ml, pH 9.4, at 25°:

p-nitrophenyl phosphate, varied as indicated.
N,N-bis (2-hydroxyethyl) glycine, 0.125 mmoles.
$MgCl_2$, 3.3 µmoles.
p-nitrophenol at concentration A, B, C or D.

A	1.25×10^{-4}M
B	1.00×10^{-4}M
C	8.33×10^{-5}M
D	No inhibitor

The control cuvette contained the same substances except that the enzyme had been boiled.

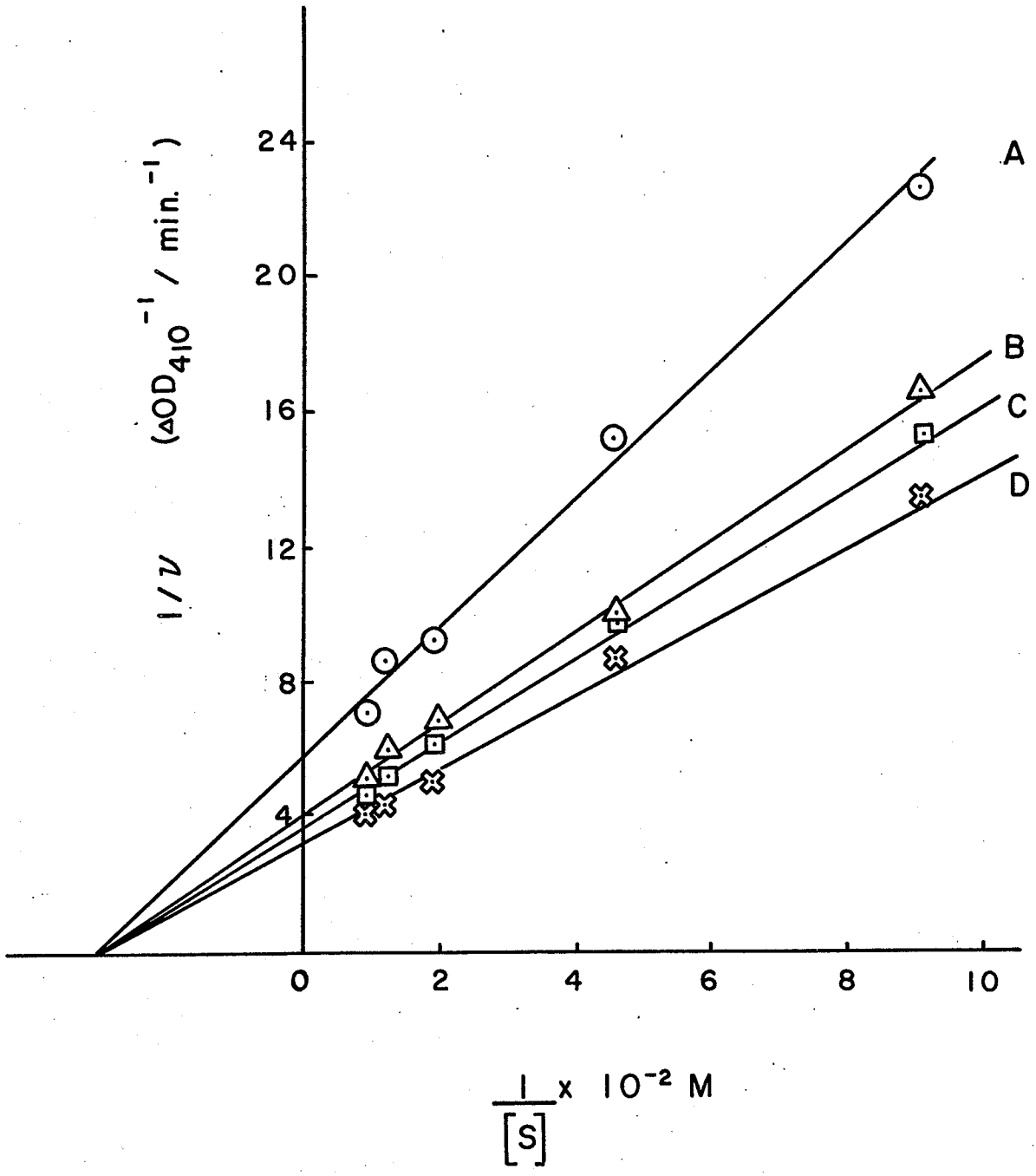

1/v (ΔOD410 -1 / min.-1)
24
20
16
12
8
4
0
2
4
6
8
10
A
B
C
D
1/[S] x 10-2 M

FIG. 10. Replot of slopes from Fig. 9 *versus* inhibitor concentration.

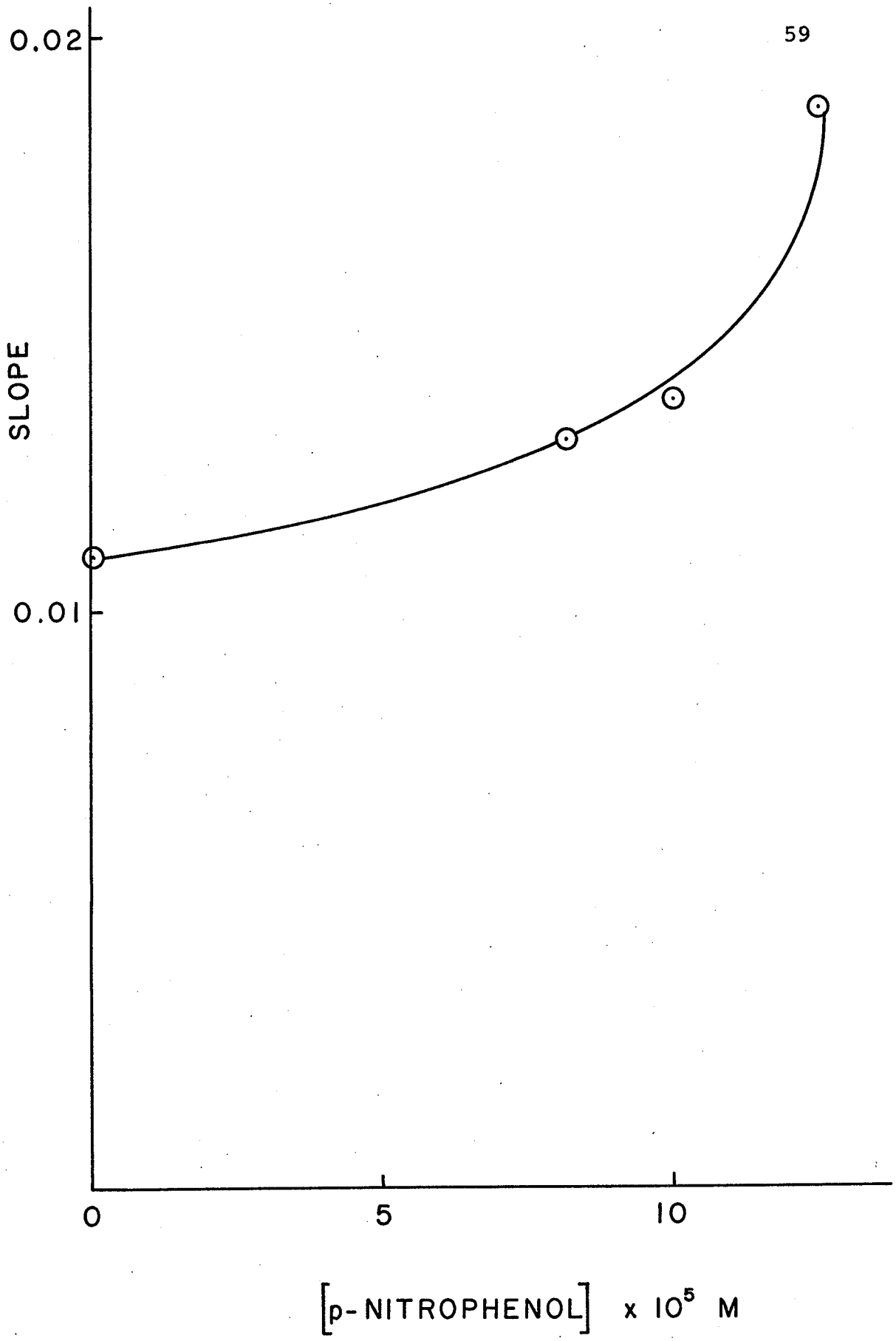

0.02
0.01
SLOPE
0
5
10
[p-NITROPHENOL] x 10⁵ M

FIG. 11. Replot of intercepts from Fig. 9 *versus* inhibitor concentration.

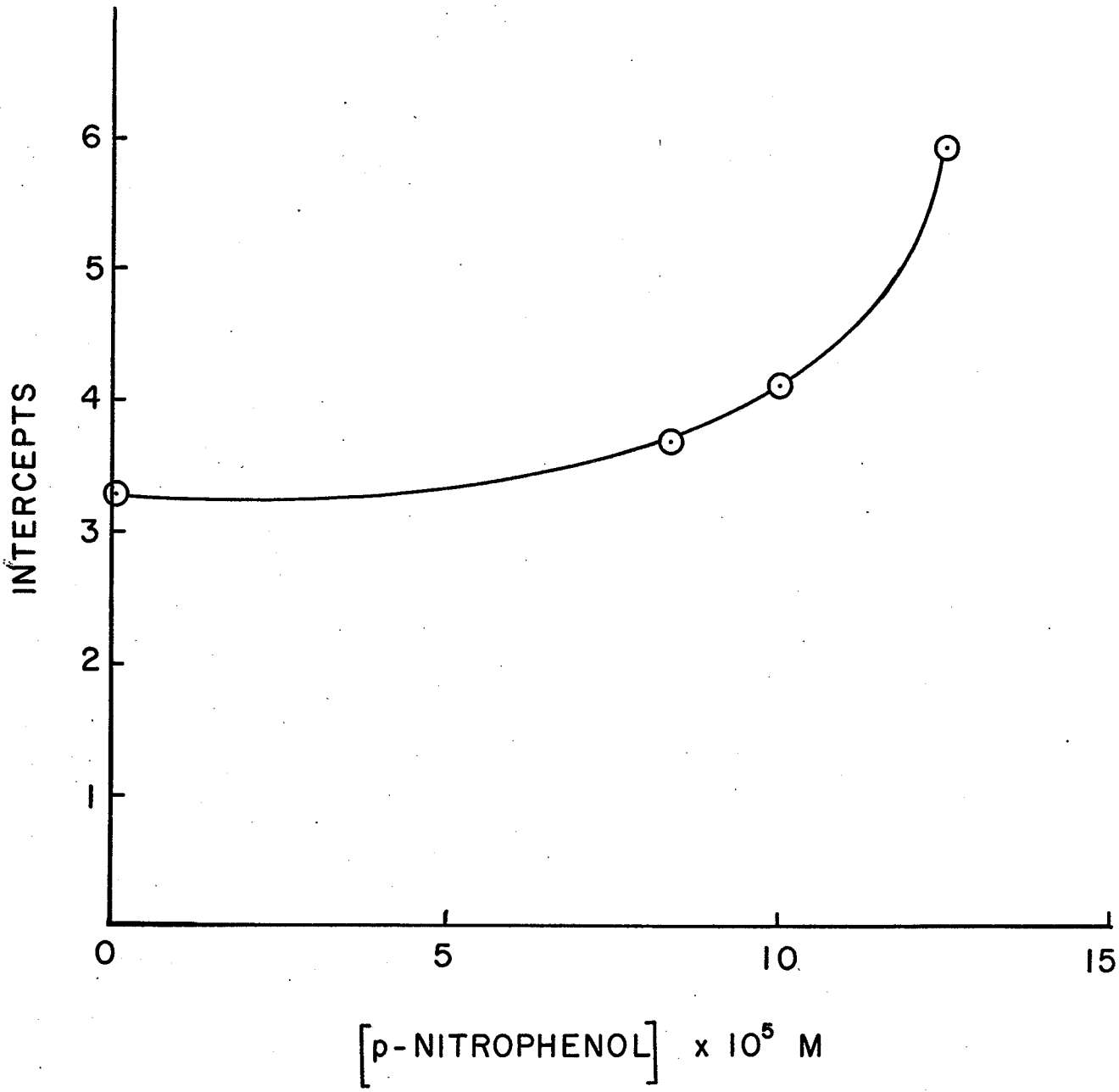

INTERCEPTS
6
5
4
3
2
1
0
5
10
15
[p-NITROPHENOL] x 10⁵ M

FIG. 12. Inhibition by glycerol. (β-glycerophosphate).

Each assay tube contained, in a final volume of 1.0 ml, pH 9.4:

β-glycerophosphate varied as indicated.
N,N-bis (2 hydroxyethyl) glycine, 30 μmoles.
$MgCl_2$, 2 μmoles.
Glycerol at concentration A, B or C.
Enzyme, 0.05 ml. (75 units).

A 1.93 M
B 0.48 M
C No inhibitor.

The reaction was started by adding enzyme and was allowed to run for 30 minutes at 30°. The reaction rate was linear for at least 35 minutes as determined in a preliminary experiment. After 30 minutes 0.1 ml of 10% trichloroacetic acid was added. Protein was removed by centrifugation and the supernatant was assayed for phosphate. A blank tube, which differed from the reaction tubes only in that enzyme was added after trichloroacetic acid at zero time, was run with each determination. The blank values were subtracted from values obtained from the test samples.

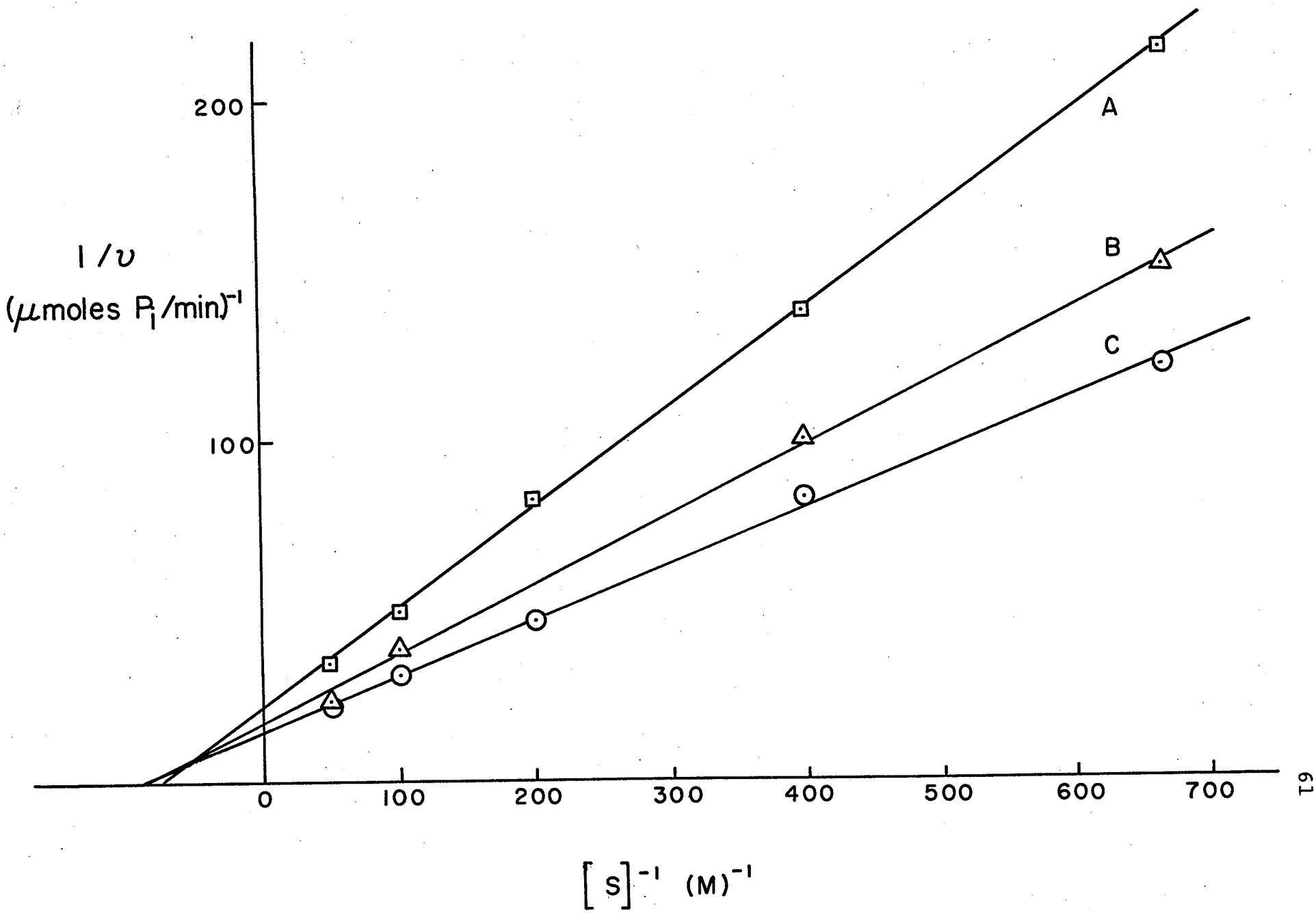

200
100
1/v
(μmoles P_i/min)$^{-1}$
A
B
C
0
100
200
300
400
500
600
700
[S]$^{-1}$ (M)$^{-1}$

FIG. 13. Replot of slopes of Fig. 12 *versus* inhibitor concentration.

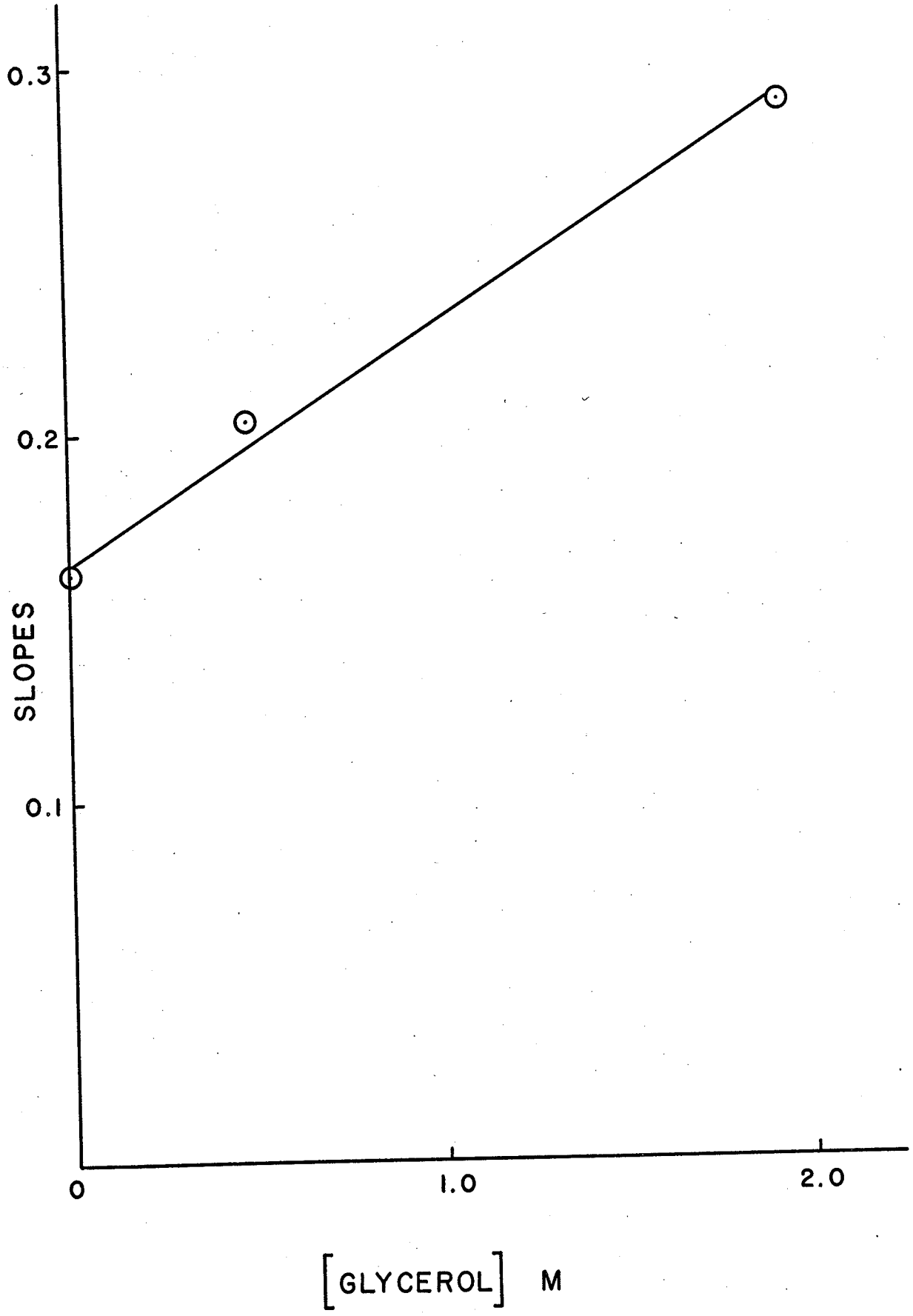

0.3
0.2
0.1
SLOPES
0
1.0
2.0
[GLYCEROL] M

FIG. 14. Replot of intercepts of Fig. 12 *versus* inhibitor concentration.

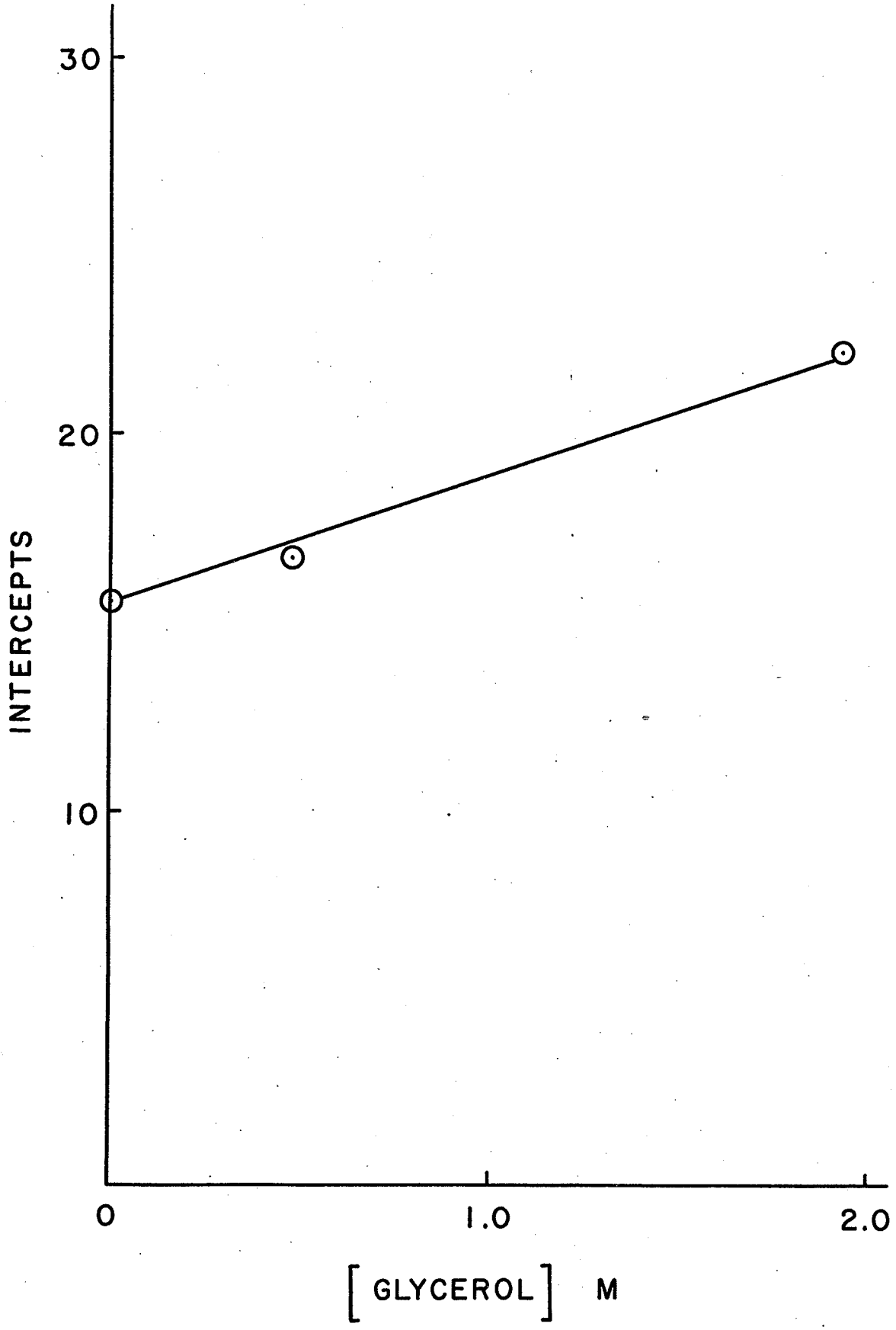

30
20
10
INTERCEPTS
0
1.0
2.0
[GLYCEROL] M

TABLE II

A SUMMARY OF THE DATA DERIVED FROM FIG. 6 AND FROM PRODUCT INHIBITION EXPERIMENTS

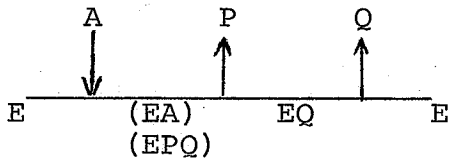
Substrate	Inhibitor	Product determined	†Type of Inhibition	*Michaelis constants (M)	Inhibition constants	
					K_{ip} (M)	K_{ia} (M)
p-nitrophenyl phosphate	none	PNP	-	1.7×10^{-3}	-	-
p-nitrophenyl phosphate	phosphate	"	linear, C	9.3×10^{-3}	-	-
p-nitrophenyl phosphate	PNP	"	non-linear, NC	3.3×10^{-3}	-	-
β-glycero-phosphate	glycerol	phosphate	linear, NC	1.05×10^{-2}	4×10^{-1}	1.7×10^{-2}

† C = competitive, NC = Non-competitive.

* Values obtained for the indicated substrate in the presence of the inhibitor listed.

Discussion of the results obtained in Phase I

The results of product inhibition experiments with p-nitrophenyl phosphate as substrate indicate that the reaction is ordered, with phosphate being the second product released from the enzyme. The simplest such mechanism may be represented as:



MECHANISM I

where A is p-nitrophenyl phosphate, P is p-nitrophenol and Q is phosphate. Mechanism I predicts (a) that P will give linear non-competitive inhibition, and (b) that Q will give linear competitive inhibition. Prediction (b) is confirmed by the results shown in Figs. 7 and 8. However, the inhibition by p-nitrophenol, although it is non-competitive, is certainly not linear (Figs. 9,10 and 11).

The rate equation* for an ordered Uni-Bi reaction is (Cleland, 1963):

$$v = \frac{V_1 V_2 (A - \frac{PQ}{K_{eq}})}{K_a V_2 + V_2 A + \frac{K_q V_1 P}{K_{eq}} \quad \frac{K_p V_1 Q}{K_{eq}} \quad \frac{V_1 PQ}{K_{eq}} \quad \frac{V_2 AP}{K_{ip}}} \quad \cdots\cdots\cdots (1)$$

*The kinetic terminology and symbolism used in this thesis is that of Cleland (1963).

When Q = O

$$v = \frac{V_1 V_2 A}{K_a V_2 + V_2 A + \frac{K_q V_1 P}{K_{eq}} + \frac{V_2 A P}{K_{ip}}} \quad \cdots\cdots\cdots\cdots\cdots\cdots (2)$$

In the event that an inhibitor forms a dead end complex with all enzyme forms, and has an equal affinity for all such forms, all terms in the denominator of eq. (2) must be multiplied by the factor $(1 + \frac{I}{K_i})$ where K_i is the the dissociation constant for each dead end complex containing enzyme. Expressed in the reciprocal form and rearranged, eq. (2) then becomes

$$\frac{1}{v} = \frac{1}{A}\left[(1 + \frac{I}{K_i})(\frac{K_a}{V_1} + \frac{PK_q}{V_2 K_{eq}})\right] + \frac{1}{V_1}\left[(1 + \frac{I}{K_i})(1 + \frac{P}{K_{ip}})\right] \cdots (3)$$

The slope term in eq. (3) may be rewritten, substituting P for I, as

$$\text{Slope} = P^2(\frac{K_q}{V_2 K_{eq} K_i}) + P(\frac{K_q}{V_2 K_{eq}} + \frac{K_a}{K_i V_1}) + (\frac{K_a}{V_1}) \quad \cdots\cdots (4)$$

where all the bracketed terms are constants.

If the slopes are plotted against P one obtains a parabola. The slope of the parabola at any value of P is given by:

$$\frac{d}{dP}(\text{Slope}) = P\left(\frac{2K_q}{V_2K_{eq}K_i}\right) + \left(\frac{K_q}{V_2K_{eq}} + \frac{K_a}{K_iV_1}\right) \quad \cdots\cdots\cdots \quad (5)$$

where, again, the bracketed terms are constants. Thus the slope of the slope replot should be a linear function of P.

Similarly, the intercepts of the double reciprocal plot are a parabolic function of P.

$$\text{Intercept} = P^2\left(\frac{1}{V_1K_{ip}}\right) + P\left(\frac{1}{V_1K_{ip}} + \frac{1}{V_1K_i}\right) + \frac{1}{V_1} \quad \cdots\cdots \quad (6)$$

$$\text{and } \frac{d}{dP}(\text{Intercept}) = P\left(\frac{2}{V_iK_{ip}}\right) + \left(\frac{1}{V_iK_{ip}} + \frac{1}{V_iK_i}\right) \quad \cdots\cdots \quad (7)$$

The slope of the intercept replot should therefore be a linear function of P. A plot of such slope values against P would yield values of K_{ip} and K_i.

Unfortunately, 4 points are hardly sufficient to prove a fit to a second degree equation with accuracy great enough to justify any conclusions drawn from the plots indicated in eq. (5) and eq. (7). It is, however, reasonable to conclude that the inhibition by p-nitrophenol is mixed dead end and product inhibition. There may be two sites available for the binding of p-nitrophenol, one the active site, which when bound produces normal product inhibition. The second site permits the occurrence of dead end inhibition by p-nitrophenol. The structural similarity

of p-nitrophenyl phosphate and p-nitrophenol suggests that the substrate as well as the product could bind to the dead end site. Such an explanation could account for the observed substrate inhibition.

The pattern of inhibition of β-glycerophosphate hydrolysis by glycerol is different from that observed with p-nitrophenol and p-nitrophenyl phosphate. The inhibition in this case is linear non-competitive, indicating that dead end inhibition does not occur.

Working with potato acid phosphatase Hsu *et al*, (1966) have shown that p-nitrophenol is a linear non-competitive inhibitor of p-nitrophenyl phosphate hydrolysis, and that there is an effect on both the maximum velocity and on the apparent K_m. In this case there is no dead end inhibition, nor is there any substrate inhibition. These workers also indicate that alternate product inhibition by p-nitrophenol of β-glycerophosphate hydrolysis is hyperbolic uncompetitive, while the normal product inhibition is linear non-competitive. One would expect from the results presented in this thesis that p-nitrophenol should produce an effect on both the slopes and intercepts of the double reciprocal plots obtained from β-glycerophosphate hydrolysis by the *Neurospora* alkaline phosphatase. One would also expect dead end inhibition to occur. On the other

hand, if glycerol is used to inhibit p-nitrophenyl phosphate hydrolysis one would expect linear inhibition without dead end inhibition.

Results of product inhibition experiments with glycerol as inhibitor and β-glycerophosphate as substrate are consistent with mechanism I, if one assumes that product inhibition by phosphate is competitive. The reciprocal form of the rate equation when Q is zero is then,

$$\frac{1}{v} = \frac{1}{A}\left(\frac{K_a}{V_1} + \frac{K_q P}{V_2 K_{eq}}\right) + \frac{1}{V_1}\left(1 + \frac{P}{K_{ip}}\right) \quad \ldots\ldots\ldots\ldots \quad (8)$$

The equation to the slope replot is

$$\text{Slope, } S = P\left(\frac{K_q}{V_2 K_{eq}}\right) + \frac{K_a}{V_1} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (9)$$

If one substitutes for K_{eq}, from the Haldane relationship,

$$K_{eq} = \frac{V_1 K_{ip} K_q}{V_2 K_{ia}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (10)$$

eq. (9) becomes

$$S = P\left(\frac{K_{ia}}{V_1 K_{ip}}\right) + \frac{K_a}{V_1} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (11)$$

The equation to the intercept replot is

$$\text{Intercept, } I = P\left(\frac{1}{V_1 K_{ip}}\right) + \frac{1}{V_1} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (12)$$

The values of V_1 and K_{ip} may now be readily determined. Substitution of these values into eq. (11) allows the evaluation of K_{ia} and K_a. These values are listed in Table II.

The kinetic results obtained using two different substrates with the same enzyme can evidently differ considerably. The data obtained with one substrate may be no more than an indication of the type of data to be expected with another substrate. Further study, involving a large number of lines in the double reciprocal plots is necessary to evaluate the kinetic constants defined for p-nitrophenol inhibition of p-nitrophenyl phosphate hydrolysis. The work should be extended to other substrates, with particular attention being paid to alternate product inhibition. Present evidence indicates that the mechanism of action of alkaline phosphatase is ordered Uni-Bi, with the alcoholic product being released first and phosphate second. Hsu *et al.* (1966) have demonstrated the same mechanism for potato acid phosphatase. This mechanism, however, includes an isomerization of two enzyme phosphate complexes. Such an isomerization may also form a part of the mechanism of the

alkaline phosphatase of *Neurospora*.

The experiment performed to study the influence of pH on enzyme activity is subject to criticism. No account was taken of the variation in degree of ionization of p-nitrophenol with pH. The pK of the hydroxyl group of p-nitrophenol is 7.15 (Handbook of Chemisty and Physics, p. D-90 College edition. 48th edition. 1967-68. The Chemical Rubber Co., Cleveland, Ohio, U.S.A.) and the ionized form is responsible for the yellow colour of the acid in alkaline solution. At pH 5.6 an aqueous solution is colourless. Below about pH 8.0, therefore, the pH curve bears little relation to the activity of the enzyme.

To obtain valid measurements of enzymatic activity at low pH values the experimental procedure should be altered. Test reactions at all pH values must be adjusted to a constant high pH for the determination of p-nitrophenol.

PHASE II

Further purification of the enzyme

Every attempt to purify the enzyme beyond the degree of purity obtained in Phase I ran into difficulty. Of the methods tried, the following were either completely useless or gave such variable results that they had to be abandoned: acetone fractionation at various temperatures down to -20° and in various buffers, Sephadex column chromatography at various values of pH and ionic strength even in the presence of substrate, selective denaturation with chloroform, ethanol fractionation, and selective denaturation by pH changes. These methods were tried in addition to and in many different combinations with the steps worked out in Phase I. The problem was usually that an unacceptably large loss of activity occurred during one or more steps. Some modifications in buffer systems and the addition of $MgCl_2$ to all solutions were found to be helpful and were adopted. Details of these changes are given in the purification procedure outlined below.

Step I

The lyophilized mycelium (50 g.) was stirred in 750 ml of Tris-HCl buffer (0.02M Tris, 4×10^{-3}M $MgCl_2$, pH 7.5) for 1 hour. The extract was centrifuged at 14,000 x g

for 25 minutes and the precipitate was discarded. The crude extract was prepared at room temperature but was centrifuged at 0-4°

Step II

The supernatant fluid from Step I was rapidly heated to 60° and maintained at that temperature for 12 minutes in a water bath. The heavy precipitate that formed was removed by centrifugation as before.

Step III

The pH of the clear yellow extract was adjusted to 7.5 with 1.0N NaOH and finely ground $(NH_4)_2SO_4$ was added to a final concentration of 40% saturation (0.243 g/ml). After 30 minutes a small precipitate was removed by centrifugation. The supernatant fluid was adjusted to pH 7.5 and brought to 65% saturation by adding $(NH_4)_2SO_4$ (0.165 g/ml). The precipitate after 30 minutes was collected by centrifugation, dissolved in 0.01M Tris-HCl, pH 7.5 (4×10^{-3}M $MgCl_2$) and dialyzed for 3 hours against 1 liter of the same buffer with three changes of external buffer.

Step IV

Medium mesh DEAE cellulose was washed with 1.0 N KOH, followed by water, and was packed into a cylindrical column

(2.5 x 45cm.) The column was equilibrated with 0.01M Tris-HCl, pH 7.5 ($4x10^{-3}$M $MgCl_2$). The dialyzed enzyme was adsorbed to the column and eluted with a linear concentration gradient of KCl (0-0.3M) in the same buffer. Each reservoir contained 400 ml. Fractions of 8.0 ml each were collected and the fractions containing enzyme activity were pooled. The results of a typical purification are presented in Table III.

TABLE III

PURIFICATION OF ALKALINE PHOSPHATASE

Preparation	Enzyme units	Total protein, mg	Specific activity
Step I	607,000	9,720	62
Step II	558,000	4,278	134
Step III	508,000	850	597
Step IV	241,000	174	1,385

The protamine sulfate step used in Phase I was abandoned. It contributed nothing to the final degree of purification obtained in the new procedure. It is evident from Table III that a considerable loss of activity occurred during DEAE cellulose chromatography. Despite many variations in column characteristics, such a loss always occurred.

In no case did the enzyme elute from the column in a symmetrical peak. A shoulder always occurred on the later eluting side of the elution curve. In one instance, as shown in Fig. 15 the activity eluted in two distinct peaks. Advantage was taken of this result to study the peaks individually. It was found that two distinct enzymes were present.

Molecular weight determination

The molecular weight of the enzyme in each peak was determined by the method of Martin and Ames (1961). Samples of the enzyme were layered on to a sucrose density gradient (4% to 20% sucrose) together with a small amount of bovine hemoglobin as a standard. The tubes (0.5 x 2 cm) were centrifuged at 35,000 r.p.m. for 12 hours in the SW 39 swinging bucket rotor in a Spinco model L ultracentrifuge. After the run, 2-drop fractions were collected by making a small hole in the bottom of the tube. The fractions were assayed for enzyme activity and for hemoglobin content. Runs were made with the enzyme from each peak and with a mixture of the two peaks. The results, which are the average of three determinations, are presented in Table IV. The molecular weight of hemoglobin was taken as 66,000; this happened to coincide with the molecular weight of

peak II enzyme. It should be noted that the peak I enzyme was difficult to detect after the run. Presumably it had lost much of its activity during the run.

TABLE IV

MOLECULAR WEIGHT DETERMINATIONS

Molecular weight		
Peak I	Peak II	Mixture of Peak I and Peak II
193,000	66,000	89,300

Polyacrylamide gel electrophoresis

Analytical disc electrophoresis on polyacrylamide gel was performed on dialyzed samples from peak I and peak II, and on a mixture of the two peaks, according to the method of Davis (1964). After electrophoresis, the separating gels were placed in a small test tube containing the same substances as the standard assay. Within 1 to 3 minutes a yellow band appeared in the gel wherever enzyme activity was localized. Color photographs of the developing bands were prepared. The rapidity with which diffusion of the yellow color occurred necessitated the taking of photographs within the 1 to 3 minute period. During this

time the tracking dye (bromo phenol blue) also diffused and its location was marked with ink on the outside of the tubes. Typical color photographs are presented in Fig. 16.

Peak I contained a single band of enzyme activity. Peak II contained two distinct bands of activity, the slower of which is considered to be the same enzyme as that present in Peak I. Analysis of a mixture of the two peaks showed two bands of activity. In all cases where there were two bands of enzyme activity the faster band was weaker in activity than the slower, as judged by the rapidity and intensity of the developing color. Comparison of these gels with gels stained in Coomassie blue (Chrambach *et al*, 1967) demonstrated that the homogeneous enzyme of peak I coincided with the major protein band. The slower enzyme of peak II coincided with a light protein band, the faster with a very light protein band. In the mixture of the two peaks the slower enzyme corresponded with a heavy protein band, the faster one with a light protein band.

Effect of cations on the activity of peak I and peak II enzyme

Several metal ions are known to enhance the activity of alkaline phosphatase, some being loosely bound and perhaps concerned in ion-substrate complexes (Mathies, 1958)

FIG. 15. Chromatography of alkaline phosphatase on DEAE cellulose.

The method is described in the text (see page 73). Fractions were assayed for enzyme activity (---). Absorbance at 280 mμ (——) was used as an indication of protein content. The earlier eluting peak of enzyme activity is designated peak I and the later peak as peak II.

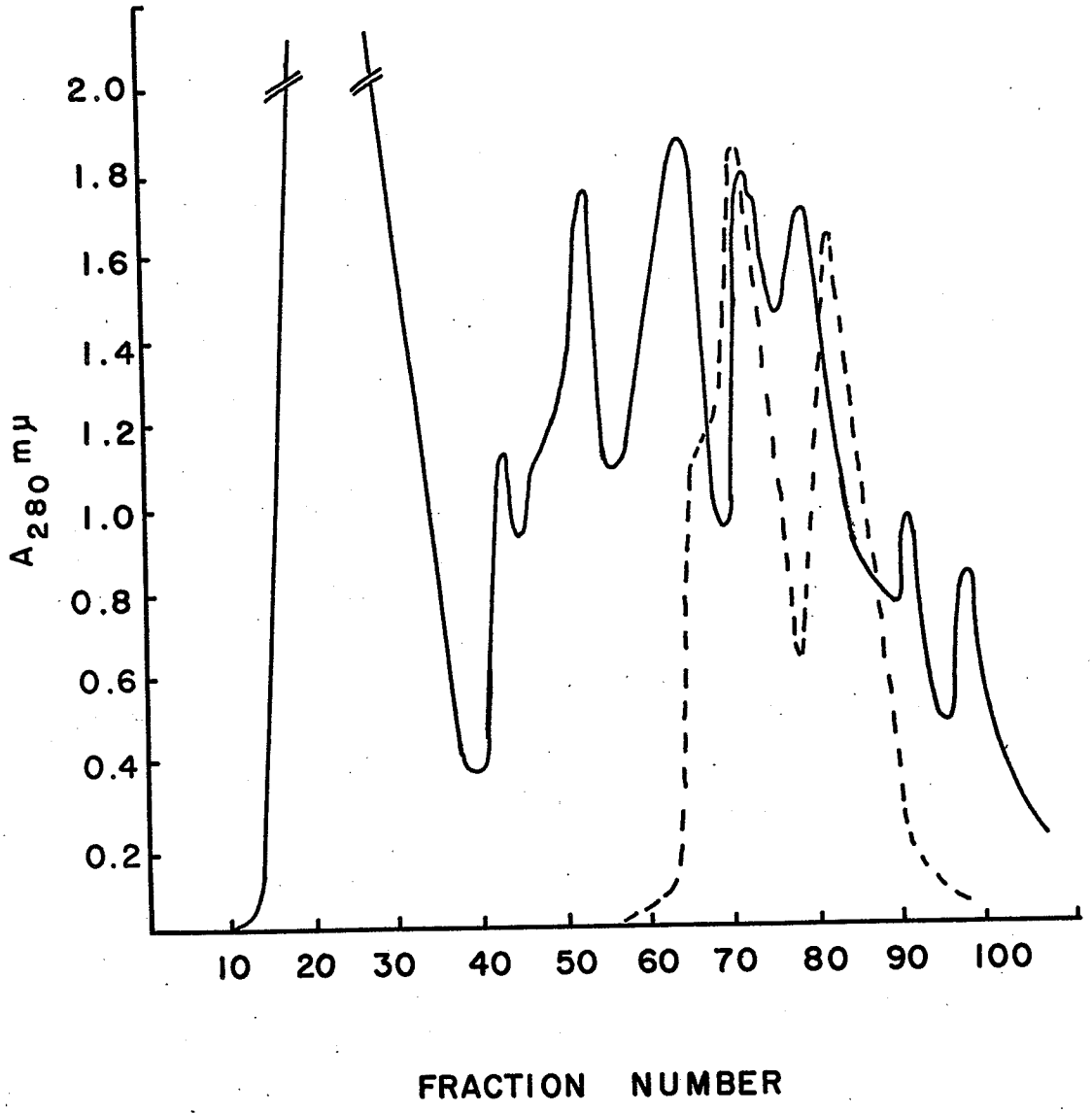

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A_{280} mμ
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

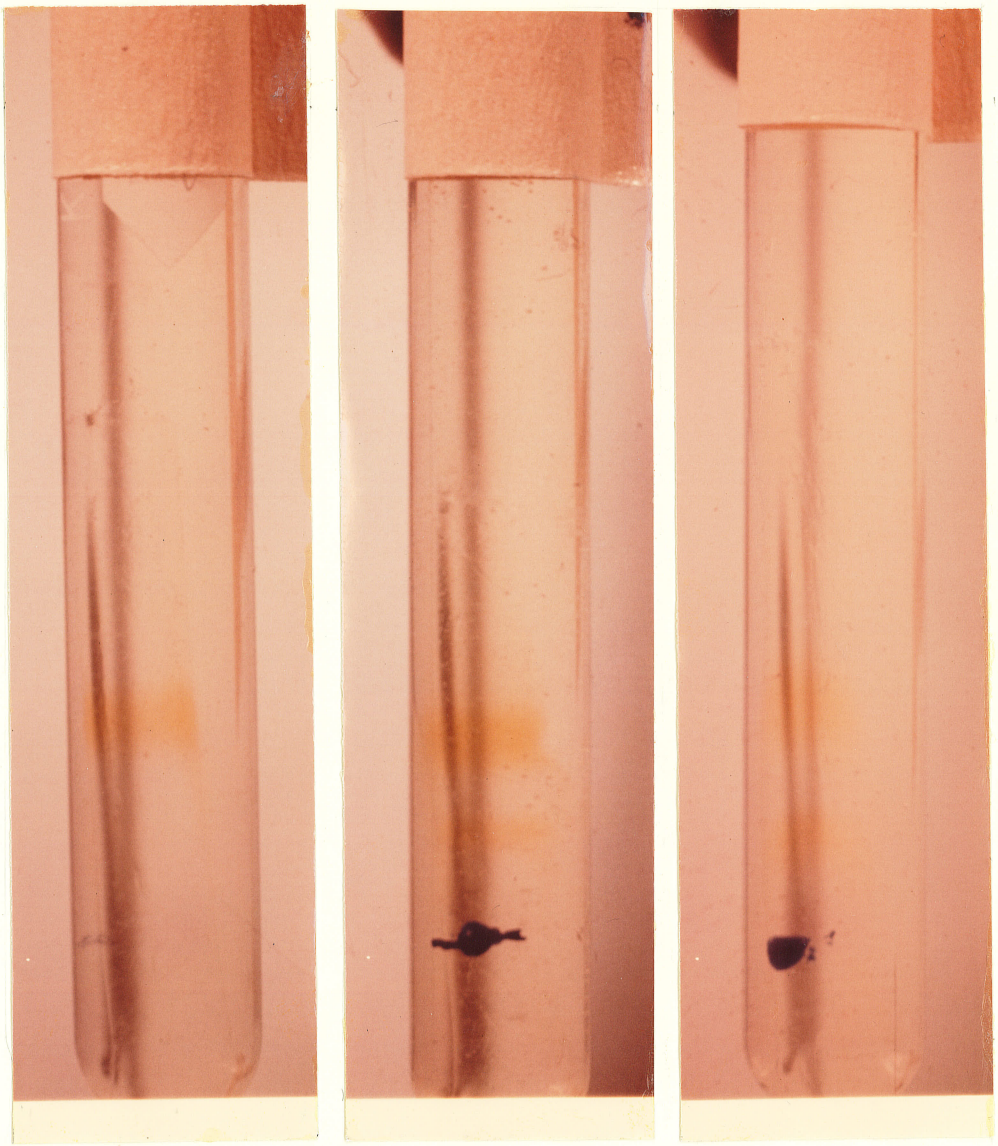
FIG. 16. Electrophoresis of alkaline phosphatase on polyacrylamide gel.

(A) Peak I enzyme showing a single band of activity.

(B) Mixture of peak I and peak II enzymes showing two bands of activity.

(C) Peak II enzyme showing two bands of activity.

Enzyme bands are yellow. The position of the tracking dye was marked with ink on the outside of the tubes. Electrophoretic migration was from the top of the tube to the bottom.



A B C

and others being more tightly bound to the enzyme protein. Experiments were performed to determine the effect of various ions as activators of the enzyme which had been dialyzed under conditions suitable for the removal of loosely bound ions.

Enzyme from peak I, peak II, and a mixture of these was dialyzed against 1000 volumes of glass-distilled water (neutralized with NaOH) for 6 hours, with four changes. Little or no loss of activity (as measured in the standard assay) resulted. With no Mg^{2+} added to the assay, peak I retained 23% and peak II retained 26% of the initial activity.

The assay conditions used to determine the effect of each ion were the same as those of the standard assay except that Mg^{2+} was replaced by varied quantities of the ion under study. The results are presented in Table V and are in good agreement with the results of Kuo and Blumenthal (1961).

The effect of pH on the activity of peak I and peak II enzyme.

The method of determining pH activity profiles used in Phase I, while subject to the criticism discussed there, is valid when used to compare the profiles of different forms of the same enzyme under the same conditions. For

TABLE V

THE EFFECT OF VARIOUS CATIONS
IN THE ASSAY AS ACTIVATORS OR INHIBITORS OF ALKALINE PHOSPHATASE

Enzyme	Compound studied	Optimum concn., M	Activation %	Concn., M	Inhibition, %
Peak I	$ZnCl_2$	1.0×10^{-2}	134	7×10^{-2}	18
Peak II	$ZnCl_2$	1.0×10^{-2}	190	7×10^{-2}	47
Mixture	$ZnCl_2$	1.0×10^{-2}	176	7×10^{-2}	19
Peak I	$MgCl_2$	Not determined	700	-	None
Peak II	$MgCl_2$	Not determined	700	-	None
Mixture	$MgCl_2$	Not determined	700	-	None
Peak I	$MnSO_4$	3×10^{-3}	240	1.5×10^{-1}	Slight
Peak II	$MnSO_4$	3×10^{-3}	19	1.5×10^{-1}	Slight
Mixture	$MnSO_4$	3×10^{-3}	210	1.5×10^{-1}	Slight
Peak I	$CuCl_2$	No effect between 1×10^{-5} M and 4×10^{-3} M			
Peak II	$CuCl_2$				
Mixture	$CuCl_2$				
Peak I	$BeSO_4$	1.0×10^{-7}	5	7.8×10^{-4}	82
Peak II	$BeSO_4$	1.0×10^{-6}	7	7.8×10^{-4}	47
Mixture	$BeSO_4$	1.0×10^{-6}	5	7.8×10^{-4}	61

Note: The compounds under study were added to the assay cuvette in varying amounts and the initial velocity was determined in the spectrophotometer at 410 mμ. The activation or inhibition produced is expressed as a percentage of the activity observed in the absence of added ions. The optimum concentration is that ion concentration which resulted in maximum activation. The concentration resulting in the given percentage inhibition is also quoted.

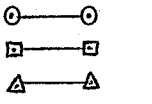
this reason the same method was used to determine whether or not the two peaks of activity differed in their response to hydrogen ion concentration. The results are presented in Fig. 17. It is evident that the pH profiles of the two activities are different. Peak II enzyme has a greater tolerance to a drop in pH than does peak I enzyme. If the pH dropped from 9.15 to 7.5, peak II enzyme retained 63% of its activity whereas peak I enzyme retained only 20%. The behaviour of a mixture of the two is intermediate. The pH optima for the two forms of enzyme under these conditions, however, are essentially the same (pH 8.95 for peak II and pH 9.15 for peak I).

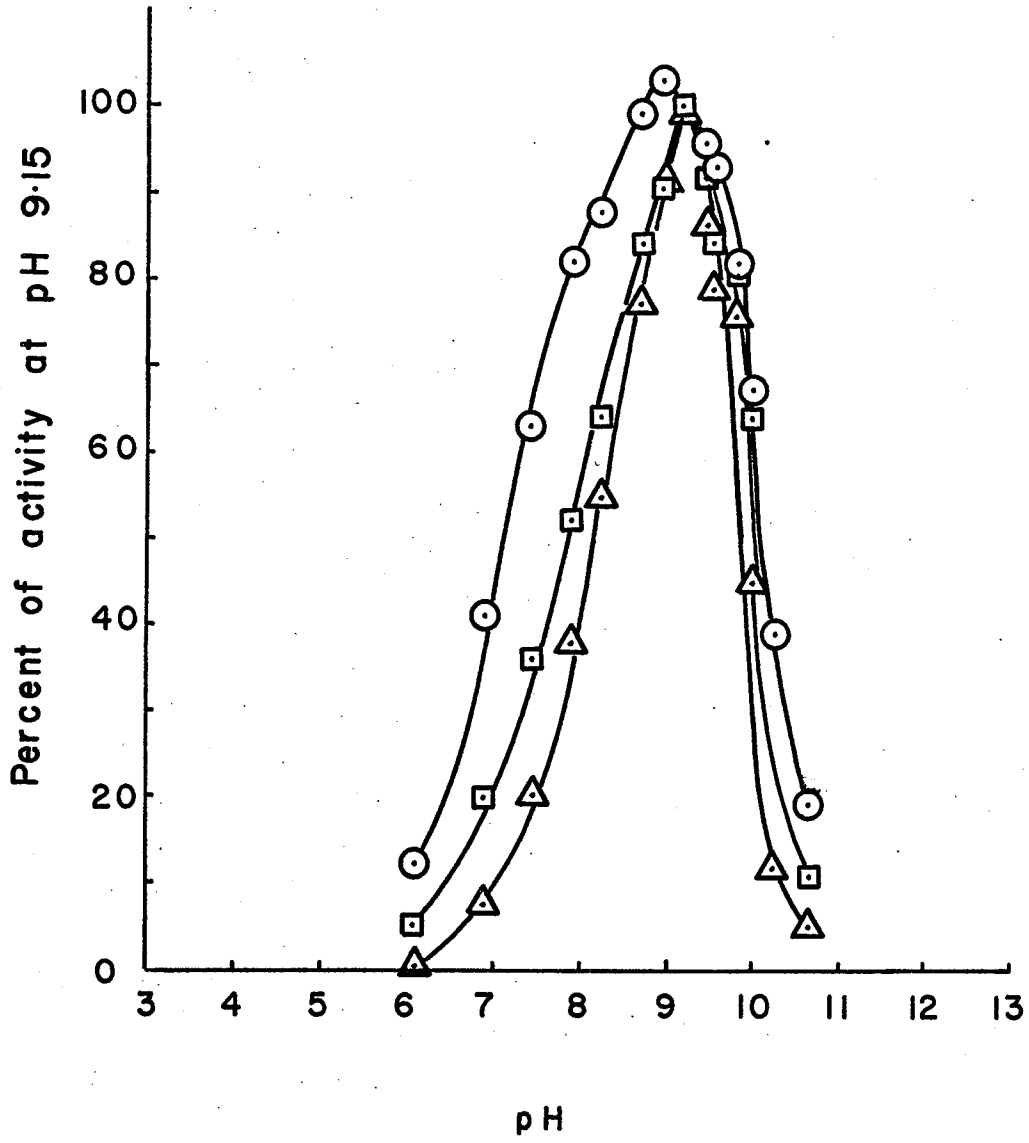
Enzyme stability under various conditions of storage

The loss of enzyme activity during DEAE chromatography and in other attempted purification procedures prompted a search for some way to stabilize it.

The purification procedure used to obtain the enzyme for this experiment differed from that given earlier in Phase II in that the enzyme from step IV was dialyzed for three hours against 0.02M Tris-HCl buffer (pH 8.4, 4×10^{-3}M $MgCl_2$). The dialyzed enzyme was run on to a Sephadex G-150 column previously equilibrated with the same buffer and was eluted from the column with that buffer. The active

FIG. 17. Effect of pH on the activity of the two peaks of enzyme activity obtained by DEAE column chromatography and on a mixture of the two peaks.(PNPP) Conditions for each measurement were the same as those of the standard assay except that the pH was adjusted to various values with HCl or NaOH and was determined immediately after the reaction rate had been measured. Initial velocity values for each curve are plotted as a percentage of the value for the corresponding enzyme obtained at pH 9.15.




Percent of activity at pH 9·15
100
80
60
40
20
0
3
4
5
6
7
8
9
10
11
12
13
pH

fractions (a single peak) were pooled. The preparation contained two enzymatic activities as determined by analytical electrophoresis on polyacrylamide gel. (Note:- this procedure produced little or no increase in specific activity, and resulted in a large loss (60%) of enzyme. It was abandoned as a means of purification.)

The enzyme preparation obtained in the above manner was divided into two portions. One portion was adjusted to pH 7.4 with HCl and the other portion was left at pH 8.4, so that the effects of several treatments could be determined at two pH values. Samples of the enzyme preparations were placed in test tubes, treated in the appropriate manner, assayed immediately in the standard assay and placed in the cold room (0-4°). The activity of each experimental tube was assayed at 5.5 hours, 23 hours, 42.5 hours and 162 hours after treatment. Details of the different treatments and assay results at 5.5 hours and 162 hours are given in Table VI. None of the treatments improved the stability of the enzyme, but it is evident that under dilute conditions the enzyme is more stable at pH 7.4 than at pH8.4.

The dramatic loss of activity in the presence of Cleland's reagent and EDTA was hypothesized to be due to the chelating effect of EDTA. An experiment was performed

to test this hypothesis and to identify the metal ions involved.

TABLE VI

STABILITY OF ALKALINE PHOSPHATASE UNDER VARIOUS CONDITIONS OF STORAGE

Treatment	pH 7.4		pH 8.4	
	% of Initial activity remaining after			
	5.5 hrs.	162 hrs.	5.5 hrs.	162 hrs.
No further treatment	100	93	100	94
Dilute with 4 volumes H_2O.	100	96	100	50
Add equal volume of 40% sucrose	100	80	100	85
Add equal volume of 40% glycerol	100	90	100	86
Make to 1.0×10^{-5}M Cleland's reagent and 1×10^{-3}M EDTA.	62	nil	89	nil

The effect of various metal ions as restorers of activity to alkaline phosphatase dialyzed against EDTA

For this experiment enzyme purified through step IV (a mixture of both enzymes) was dialyzed against 2,000 volumes of 0.02M Tris-HCl, pH 7.4 containing 1.0×10^{-3}M EDTA. Dialysis was continued for six hours with four changes of external buffer. The enzyme was then dialyzed against the same volume of buffer without EDTA for three hours with four changes. At the same time another sample of the enzyme was dialyzed for ten hours against the same buf-

fer without EDTA to serve as a control. After dialysis samples of the EDTA-treated enzyme were incubated with various ions (Co^{2+}, Mn^{2+}, Zn^{2+}, $FeSO_4$) both alone and with Mg^{2+} present at room temperature. At various times samples of the incubating enzyme were assayed (a) with the same ions added to the assay as were present in the incubation and (b) with the addition of Mg^{2+} to the assay for those enzyme samples incubated in its absence. The results are presented in Table VII.

TABLE VII

THE EFFECT OF VARIOUS CATIONS AS ACTIVATORS OF THE MIXED ALKALINE PHOSPHATASES WHEN INCUBATED WITH THE ENZYMES

Incubation tube no.	Ions in incubation†	Ions in assay†	% activity compared to control enzyme* (av.of duplicates)
Control enzyme*	None	None	Trace
1*	None	Mg^{2+}	100
2	None	None	Trace
3	Mg^{2+}	Mg^{2+}	13.2
4	Mg^{2+},Zn^{2+}	Mg^{2+},Zn^{2+}	58.3
5	Zn^{2+}	Zn^{2+}	9.6
6	Zn^{2+}	Mg^{2+},Zn^{2+}	60.0
7	Mg^{2+},Co^{2+}	Mg^{2+},Co^{2+}	13.2
8	Co^{2+}	Co^{2+}	0
9	Co^{2+}	Co^{2+},Mg^{2+}	13.0
10	Mg^{2+},Mn^{2+}	Mg^{2+},Mn^{2+}	12.3
11	Mn^{2+}	Mn^{2+}	Trace
12	Mg^{2+},$FeSO_4$	Mg^{2+},$FeSO_4$	9.6
13	$FeSO_4$	$FeSO_4$	Trace
14	$FeSO_4$	Mg^{2+},$FeSO_4$	9.6
15	None	Zn^{2+},Mg^{2+}	17.9

*Enzyme dialyzed in the absence of EDTA.

†The concentrations of the various ions were $MgCl_2$, 3.92×10^{-3}M; $ZnCl_2$, 1×10^{-3}M; $CoCl_2$, 1×10^{-5}M; $MnCl_2$, 1×10^{-3}M; $FeSO_4$, 1×10^{-3}M.

The activities quoted are the maximum obtained which occurred after incubation periods of 30 to 95 minutes. In all cases incubation was continued for at least 700 minutes but little further activation occurred and the activity decreased in some instances. The activity of the control enzyme in the standard assay remained constant throughout the 95 minute period.

It is evident from Table VII that, of the ions tested, only zinc can significantly restore activity to the enzyme after inactivation by EDTA and that a second ion (Mg^{++} in this case) enhances activity in the assay system.

Discussion of the results obtained in Phase II

The results presented here demonstrate clearly that mycelial extracts of *Neurospora crassa* contain two alkaline phosphatases. The two enzymes differ in molecular weight, in their response to some cationic activators and inhibitors, in their electrophoretic mobility and in the shape of their pH activity curves. The presence of phosphate in the growth medium precludes the possibility that either of these enzymes is the phosphate-repressible enzyme. Furthermore, the repressible enzyme is unaffected by Mg^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ca^{2+}, and Cu^{2+} at concentrations up 1 mM and is stimulated by EDTA (Nyc *et al*, 1966). Both

enzymes dealt with here are inactivated by EDTA and are markedly affected by some of these cations.

In addition to its acid phosphatase, *Neurospora crassa*, therefore, has the ability to produce at least three alkaline phosphatases. What the function of such a multiplicity of similar enzymes could be is at present a matter of conjecture.

Zinc has been shown to be absolutely required by the non-repressible enzymes and is firmly bound to the enzyme protein. It cannot be replaced by any other ion tested. A second metal ion is also needed, Mg^{2+} being by far the best activator tested.

The molecular weight results raise the possibility that the two enzymes represent two different states of aggregation rather than two different proteins. One might expect that a mixture of two enzymes of different molecular weight would be resolved into its components on sucrose density gradient centrifugation. Results obtained, however, show that this is not the case - a single activity of intermediate molecular weight was obtained. It is possible that an activity of M.W. 193,000 was also present but not detected because of the loss of activity of this enzyme during centrifugation. A similar explanation could account for the detection of but one activity in peak II. There

may, therefore, be three active states of the enzyme. The observed molecular weights could then represent dimeric, trimeric and hexameric forms of a subunit of molecular weight near 30,000.

The question then arises as to why three bands of activity were not detected on polyacrylamide gel electrophoresis. The concentration of acrylamide used (7 1/2%) does not yield a gel of sufficiently small pore size to resolve proteins of molecular weight less than 100,000. Therefore, the activities of molecular weight 66,000 and 89,300 would run as a single band.

PHASE III

The demonstration in Phase II that two alkaline phosphatases are present in *Neurospora crassa* made it imperative that they be separated before any further studies be undertaken. The search for a means whereby the separation might be accomplished extended over a period of two years.

In an attempt to prevent the large losses of activity during DEAE cellulose chromatography experienced in Phase II, all buffers used in purification procedures in Phase III were made 1.0×10^{-4}M $ZnCl_2$. Large losses on DEAE columns continued as before. A fortunate accident occurred during one purification. The pooled active fractions from the DEAE column were assayed and showed the usual loss of activity. Since we were willing to try anything once, a dash (there is no other valid description!) of 1.0M $ZnCl_2$ was added. A cloudy white precipitate formed which was removed by centrifugation. The supernatant fluid was assayed but was devoid of activity. The precipitate was extracted with phosphate buffer (pH 6.4, 0.01M phosphate) and centrifuged again. Most of the original activity was recovered with a 4.5 fold purification. Precipitation with $ZnCl_2$ was incorporated into future purification procedures, the first of which is detailed below.

First purification incorporating $ZnCl_2$ precipitation

Crude extract was prepared as detailed in Phase I except that the extracting buffer was 1.0×10^{-4}M with respect to $ZnCl_2$.

Heat step. As detailed in Phase I except that the temperature was 61°.

Ammonium sulfate fractionation. The pH of the extract was adjusted to 7.5 with NaOH and finely ground ammonium sulfate was added (0.277 g/ml, 45% saturation). After 30 minutes in the cold (0-4°) the precipitate was removed by centrifugation. The pH of the supernatant fluid was adjusted as before and the ammonium sulfate concentration was increased to 65% saturation (0.134 g/ml). The precipitate was collected by centrifugation after 1/2 hour and was dissolved in a small volume of 0.01M Tris-HCl pH 7.5 (4×10^{-3}M $MgCl_2$, 1×10^{-4}M $ZnCl_2$). The dissolved precipitate was dialyzed for 2 hours against the same buffer (100 volumes, 4 changes of external buffer) in the cold.

DEAE cellulose column chromatography. A column (13 x 3 1/2 cm) of DEAE cellulose was equilibrated with the same buffer as that used in dialysis. The enzyme was adsorbed to the column and was eluted with a linear

gradient of KCl in the same buffer (0 - 0.5M KCl, 400 ml in each reservoir). Fractions of 9.0 ml each were collected. Fractions containing enzyme eluted as a single peak and were pooled. The enzyme was precipitated by adding $(NH_4)_2 SO_4$ to a final concentration of 80% saturation. The precipitate was collected by centrifuge, dissolved in about 20 ml of 0.025M Tris-HCl pH 7.5 ($4x10^{-3}$M $MgCl_2$, 1.0×10^{-4}M $ZnCl_2$) and dialyzed for 3 hours against the same buffer.

$ZnCl_2$ precipitation. The volume of the dialyzed preparation was adjusted so that the protein concentration was 2.5 mg/ml and 0.5 µ liters of 1.0M $ZnCl_2$ was added per milligram of protein. After mixing well, precipitation was allowed to continue overnight in the refrigerator. The precipitate was collected by centrifuge and homogenized in about 10 ml of 0.01M phosphate buffer pH 6.4 and centrifuged again. The extract contained only about half the expected amount of enzyme. Accordingly, an additional 1.0 µ liter of 1.0M $ZnCl_2$ was added per mg of protein to the supernatant fluid retained from the first centrifugation. After 3 hours a further precipitate was collected and was extracted with the phosphate buffer containing the first half of the recovered

activity. The preparation was then dialyzed against phosphate buffer (0.01M phosphate pH 6.4, 2 1/2 hours). Results of this purification procedure are presented in Table VIII.

TABLE VIII

FIRST PURIFICATION OF ALKALINE PHOSPHATASE INCORPORATING $ZnCl_2$ PRECIPITATION

Preparation	Volume (ml)	Enzyme units	Total protein (mg)	Specific activity (units/mg)
Crude extract	635	425,000	7,593	56
Heat Step	595	312,000	2,618	119
$(NH_4)_2SO_4$ Fractionation	31.8	181,000	716	253
DEAE $(NH_4)_2SO_4$ ppt.	25.0	83,000	135	615
$ZnCl_2$ ppt. (combined)	13.5	88,000	29.7	2,960

Analytical electrophoresis on polyacrylamide gel was performed on samples from each stage of purification by the method described in Phase I. Photographs were not prepared. Instead, small test tubes containing the resolved gels were developed in the same substances as were present in the routine assay. The tubes were laid on their sides on a sheet of squared paper (1 mm squares). The positions of the tracking dye, the top of the gel (origin) and the developing bands of enzyme activity were marked on the paper. From these data rough values for the relative movement (Rm)

were calculated for each enzyme band. The data are presented in Table IX. The Rm values were calculated as follows:

$$Rm = \frac{\text{Distance from origin to enzyme band}}{\text{Distance from origin to tracking dye}}$$

TABLE IX

Rm VALUES FOR ALKALINE PHOSPHATASE ON POLYACRYLAMIDE GEL

Preparation	µg protein Applied to gel	Enzyme units	Rm of enzyme bands	
			slow band	fast band
Crude extract	118	5.6	.60	.80
Crude extract	236	11.2	.64	.82
Heat step	44	5.2	.58	.81
Heat step	88	10.4	.60	.80
$(NH_4)_2SO_4$ Fractionation	112	28.3	.65	.83
$(NH_4)_2SO_4$ Fractionation	56	14.2	.62	.88
After DEAE	54	33.2	.58	-
After DEAE	27	16.6	.62	-
$ZnCl_2$ Precipitate	22	65.2	.62	-
$ZnCl_2$ Precipitate	11	32.6	.61	-

From the data in Table IX it can be seen that the two bands of activity were well separated and that the Rm values were consistent enough to be used as a means of distinguishing between the two enzymes even if they were separately purified.

In this particular purification the more slowly

moving band was obtained free of contamination by the faster moving band. The slower band corresponds to the enzyme designated peak I in Phase II. Purification to a single band of activity could not be routinely obtained by this procedure, however, and the purification procedure eventually had to be modified and extended.

Effect of pH on activity at various substrate concentrations

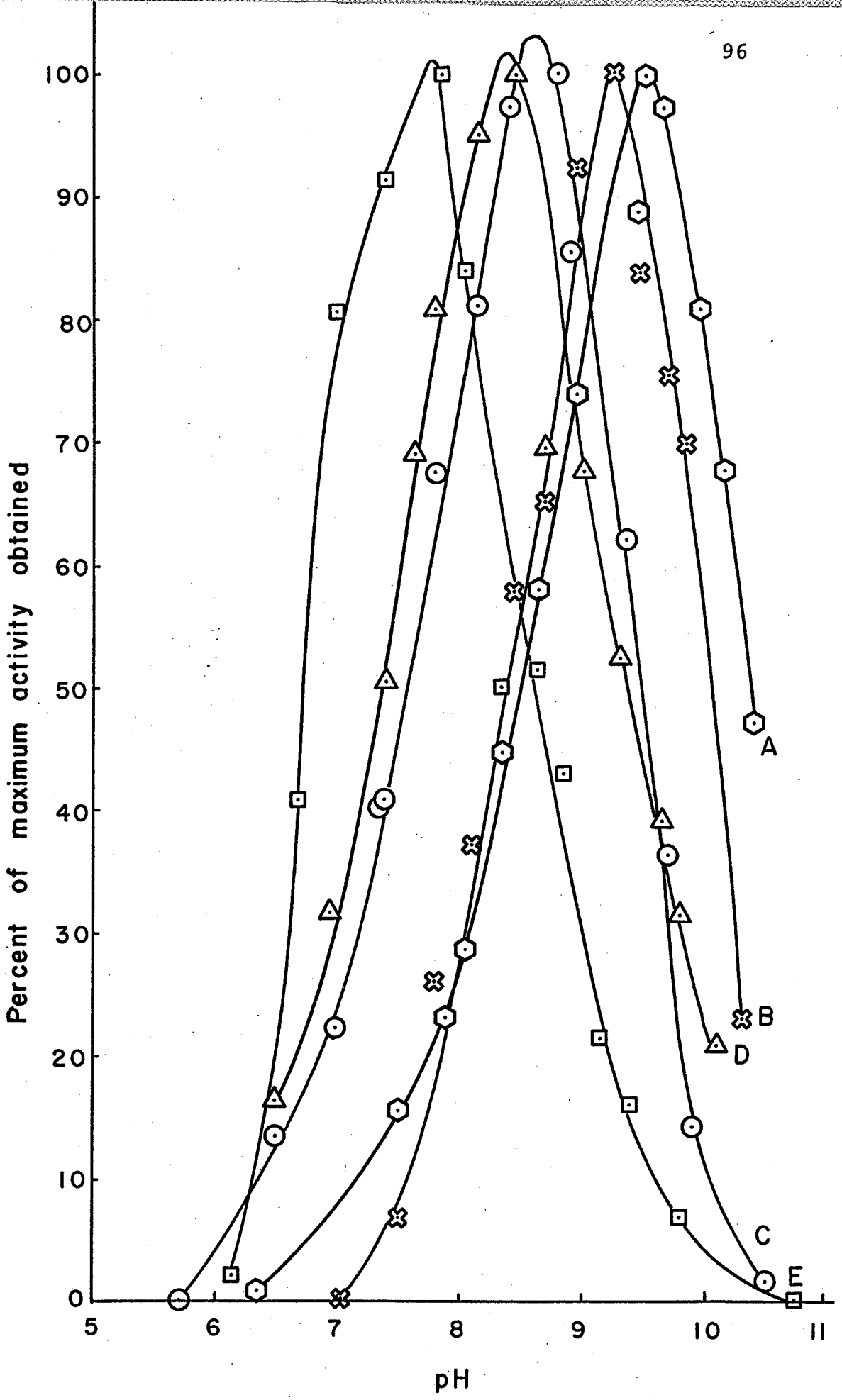
The effect of pH on the activity of the purified (approximately 53-fold) peak I enzyme was studied under conditions not subject to the criticism applied to earlier, similar experiments. The effect was studied at 5 different concentrations of p-nitrophenyl phosphate as substrate.

Method. Enzyme was added to 2 ml of reaction mixture at the appropriate pH. For each pH value five values of substrate concentration were used. Each reaction was run in triplicate. The reaction was allowed to proceed for 3 minutes at 25° and was stopped by the addition of 2 ml of 60% KOH. The volume was adjusted to 5 ml with H_2O and the optical density at 410 mμ was read in the SP700 spectrophotometer against a blank to which no enzyme had been added. Separate blanks were prepared for each substrate concentration. The results are presented in Fig. 18. It is evident that the pH optimum for the reaction approaches neutrality from

FIG. 18. Effect of pH on p-nitrophenyl phosphate hydrolysis at several values of substrate concentration (PNPP).

The method is described in the text. (See p.95) Activities are plotted as percentages of the maximum activities measured for each substrate concentration. Substrate concentrations used were:

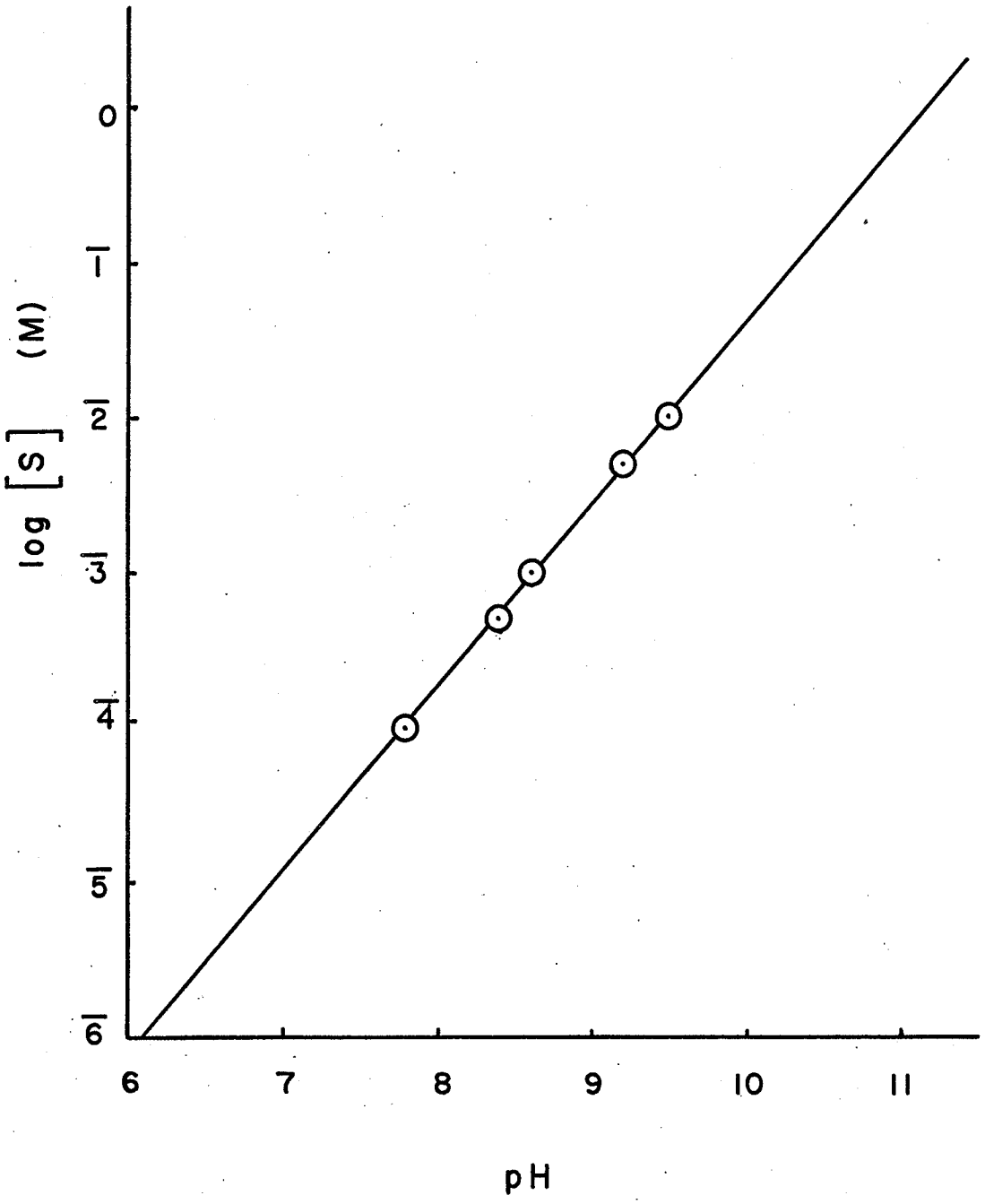
	Maximum activity ΔOD_{410}/reaction/3 mins.
A - 1.0×10^{-2}M PNPP	0.469
B - 5.0×10^{-3}M PNPP	0.290
C - 1.0×10^{-3}M PNPP	0.185
D - 5.0×10^{-4}M PNPP	0.158
E - 1.0×10^{-4}M PNPP	0.093


Percent of maximum activity obtained
100
90
80
70
60
50
40
30
20
10
0
5
6
7
8
9
10
11
pH
A
B
C
D
E

the alkaline side as the substrate concentration is decreased. A graph, plotting log (substrate concentration) *versus* the optimum pH for the reaction at that substrate concentration is presented in Fig. 19. The optimum pH is seen to be a linear function of the logarithm of the substrate concentration.

At this point in the investigation it became evident that only in cases where large losses of enzyme occurred could the enzyme be purified to a single activity. All steps in the purification procedure were re-examined. Since the two bands of activity were readily separable by electrophoresis on polyacrylamide gel at the analytical scale, it seemed reasonable to attempt their separation by this method on a preparative scale. All attempts to do so, using the preparative apparatus designed and sold by Canalco, failed. The two activities separated and could be demonstrated on the gels, but the amount of activity that could be eluted from the gel was so small as to be almost undetectable. Variations in gel porosity and length, buffer systems, electric current, temperature, amount of protein and its method of application to the gel (in a sample gel, in 20% sucrose without a sample gel) all failed to improve the yields. It was concluded that movement through the gel exposed the enzyme to forces (chemical or

FIG. 19. Optimum pH as a function of the logarithm of substrate concentration. (PNPP) Data taken from Fig. 18.


log [S] (M)
0
$\bar{1}$
$\bar{2}$
$\bar{3}$
$\bar{4}$
$\bar{5}$
$\bar{6}$
6
7
8
9
10
11
pH

physical or both) that destroyed its activity, leaving only enough activity to be detected in the highly concentrated migrating bands. The method was abandoned.

Use of DEAE cellulose column chromatography was also abandoned. It was found that use of the batch method on the crude extract gave similar results with a great saving in time and inconvenience.

Further investigation into the conditions required for the precipitation of alkaline phosphatase by $ZnCl_2$ revealed that the only condition of any importance was that the pH of the preparation be alkaline and that it be dialyzed to remove ammonium sulfate. One simply added 1.0M $ZnCl_2$ from a micropipette until a precipitate formed, and then one added a little more. Precipitation was allowed to continue for an hour or overnight. If too much activity remained in the supernatant fluid after centrifugation, it could be precipitated by the addition of more $ZnCl_2$.

At this time several preparations of enzyme resulting from investigations into purification methods were on hand. These were pooled, yielding a total of 235,000 units in a volume of 70 ml. The enzyme was precipitated by adding $(NH_4)_2SO_4$ to a final concentration of 75% saturation. The precipitate was collected by centrifugation, dissolved

in 0.01M Tris-HCl ($4x10^{-3}$M $MgCl_2$, $1.0x10^{-4}$M $ZnCl_2$, pH 7.5) and dialyzed for 3 hours against 1,000 volumes of the same buffer with 4 changes of external buffer. The dialyzed enzyme was centrifuged. The clear supernatant contained 180,000 units in a volume of 35.5 mls.

$ZnCl_2$ (0.25 ml, 1.0M) was added and precipitation continued overnight in the refrigerator. The precipitate was collected by centrifugation and was dissolved in 0.01M phosphate buffer, pH 6.5. A total of 91,000 units was recovered (20,000 units were discarded in the pH 7.5 supernatant) with a specific activity of 2070. The preparation was stored in the refrigerator. Ten days later the specific activity had increased to 2890, and after 23 days it was 3330. The increase was due to an increase in the number of units of activity per mg. of protein. The protein concentration was constant. Some of the enzyme preparation was used to investigate a new purification method. Electrophoresis still appeared to offer the greatest possibilities, but it remained to discover a suitable supporting medium. It was found that electrophoresis in Cellogel blocks gave very satisfactory results.

Electrophoresis in Cellogel blocks

Cellogel is a trade name for pure cellulose acetate in gelatinized microporous form. The distribution of the

pores, which are of macromolecular size, is three dimensional. The blocks used were 3.5 cm. wide, 12 cm. long and 2.5 mm. thick, and were purchased from Consolidated Laboratories (Canada) Ltd., Weston, Ontario.

The blocks were shipped and stored in methanol, which was removed before use by soaking them in Tris-glycine buffer (0.6g Tris, 2.88 g glycine in a volume of 1 liter, pH 8.3) for 3 hours with several changes of buffer. Excess buffer was removed by pressing the blocks between sheets of filter paper. Five blocks were placed in the electrophoresis tray (Colab) in the horizontal position, supported at each end and near the center. Filter paper wicks connected the ends of the Cellogel blocks to the Tris-glycine buffer in the electrode compartments.

A row of pinpricks 2 mm. wide was made across each block 2 cm. from the cathode end. A total of 1.5 ml of enzyme (specific activity 3330, 19,700 units) was applied with a hypodermic syringe to the pinpricked areas and was allowed to sink into the blocks. The current was switched on and was maintained at 20 m.a. for the duration of the run. The voltage was 800 volts. The enzyme migrated from cathode to anode. Migration was monitored by switching off the current for a moment and pressing a narrow strip of filter paper to the block along its length, until the strip

became moist. The strip was then pressed onto the surface of some paper towels wetted with the solution used in the routine assay. Within a minute a yellow band appeared on the filter paper strip. By laying the strip beside the Cellogel block, the position of the enzyme on the block was easily determined.

When the enzyme had migrated to within one or two centimeters of the anode end the block was removed from the tray. The enzyme band, located as described, was cut out of the block with a razor blade.

The section of Cellogel containing the enzyme was broken into small pieces. The fluid was removed from the pieces by pressure in a 10 ml syringe. The pieces were then wetted with about 0.3 ml of phosphate buffer (0.01M, pH 6.4) and again subjected to pressure.

The recovered enzyme solution was assayed. It contained 17,600 units of activity, and the specific activity was 11,700. Analysis on polyacrylamide gel showed the presence of only one band of activity.

The amount of time required to perform the electrophoresis on Cellogel varied. Sometimes the enzyme remained at the origin for an hour and then completed its migration in a half hour. Sometimes migration commenced as soon as the current was switched on and was complete in about

three-quarters of an hour. No reason for the variation was found, and it made no difference to the results.

From this point on all enzyme preparations were stored in the refrigerator after the $ZnCl_2$ precipitation. Cellogel electrophoresis was performed whenever enzyme was required for use.

The following purification procedure represents the sum of all modifications discussed earlier, and was the routine procedure used for the remainder of this investigation.

Purification of alkaline phosphatase to a single band of activity

Step I. Preparation of crude extract: 75.0g of freeze dried mycelium was stirred in 1,000 ml of Tris buffer (pH 7.7, $4x10^{-3}$M $MgCl_2$, $1.0x10^{-4}$M $ZnCl_2$, buffer A) for 1 hour at room temperature. The extract was centrifuged at 14,800xg for 20 minutes and the precipitate was discarded.

Step II. DEAE cellulose batch purification: Coarse mesh DEAE cellulose was equilibrated with buffer A and was sucked dry in a Buchner funnel. To 1,500 ml of the same buffer 360 g of the dried (still moist) DEAE was added. The crude extract was then added and stirred at room temperature for 20 minutes. The suspension was dried in a Buchner funnel, and 3 liters of buffer A was washed through

the DEAE in the funnel. The filtrates were discarded.

The DEAE was then mixed with 2 liters of 0.1M KCl in buffer A. After 10 minutes of stirring the DEAE was again dried on a Buchner funnel, the filtrate was discarded and the precipitate was suspended in 1.5 liters of 0.15M KCl in buffer A to solubilize the enzyme. After 10 minutes of stirring the suspension was filtered through a Buchner funnel. The precipitate was washed with 500 ml of 0.1M KCl in buffer A. The combined filtrates contained the enzyme.

Step III. Heat denaturation of unwanted proteins: The combined filtrates (approximately 2 liters) from step II were heated on an electric stir plate to 60° and maintained at that temperature for 12 minutes. The preparation was then cooled under the tap and centrifuged. The precipitate was discarded.

Step IV. Ammonium sulfate fractionation: Solid $(NH_4)_2SO_4$ was added slowly to the supernatant fluid from Step III to a final concentration of 65% saturation. After 1 to 12 hours the precipitate was collected by centrifugation, and was thoroughly homogenized with 30% (w/v) $(NH_4)_2SO_4$ in buffer A. Following centrifugation the supernatant fluid was discarded, the precipitate was homogenized with 20% (w/v) $(NH_4)_2SO_4$ in buffer A and centrifuged again.

Usually two extractions with 20% $(NH_4)_2SO_4$ were required to solubilize most of the enzyme. The two extracts were combined and the enzyme was precipitated again by the addition of 0.2g $(NH_4)_2SO_4$ per ml. After 1 to 12 hours the precipitate was collected by centrifuge, dissolved in 10-15 ml of Buffer A and dialyzed for 4-5 hours against the same buffer.

Step V. $ZnCl_2$ precipitation: $ZnCl_2$ was added until a white precipitate formed. The amount required varied from 1 to 2 µmoles per mg of protein. After 1 to 12 hours in the refrigerator the precipitate was collected by centrifugation. The supernatant fluid usually contained little or no enzyme. If necessary, $ZnCl_2$ precipitation was repeated. The precipitate was homogenized in 0.01M phosphate buffer pH 6.4 (2 to 5 ml) and the insoluble material was removed by centrifugation. The enzyme solution was stored in the refrigerator.

Step VI. Electrophoresis on Cellogel was performed as described above whenever enzyme was needed for experimental purposes.

The results of a typical purification are presented in Table X. Analytical electrophoresis on polyacrylamide gel was performed as described earlier on samples from each step of purification. Photographs (black and white) of the

bands of enzymatic activity as they developed in a Petri dish were taken through a blue filter. The photographs reproduced in Fig. 20 clearly show the two bands of activity present at all stages of purification, except the last. No trace of a second band of activity could be observed after Cellogel electrophoresis. As judged from the density of the bands on the gel when stained for proteins with Coomassie blue, the final preparation was approximately 50% pure enzyme.

TABLE X

PURIFICATION OF ALKALINE PHOSPHATASE

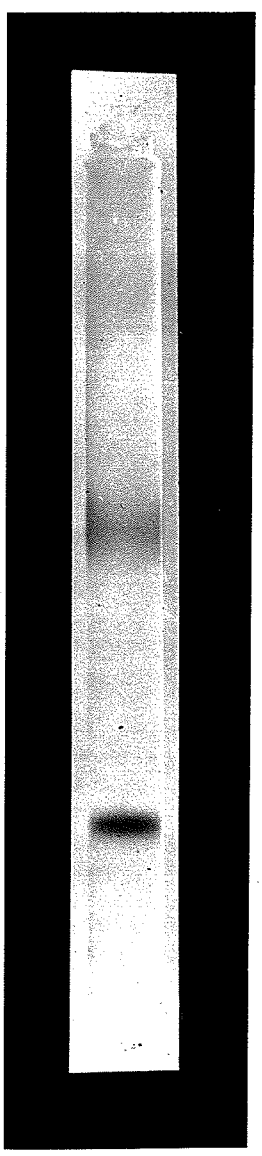
Preparation	Vol. (ml.)	Enzyme units	Total protein (mg)	Specific activity (units/mg. protein)
Step I	900	383,000	11,070	35
Step II	1,925	167,000	1,330	126
Step III	1,915	147,000	955	154
Step IV (dialyzed)	10.5	82,000	80	1,025
Step V	2.3	80,000	23.8	3,361
Step VI	Performed as required. Specific activity ranged between 6,000 and 12,000.			

Enzyme purified in the above manner was used for the remainder of the investigation.

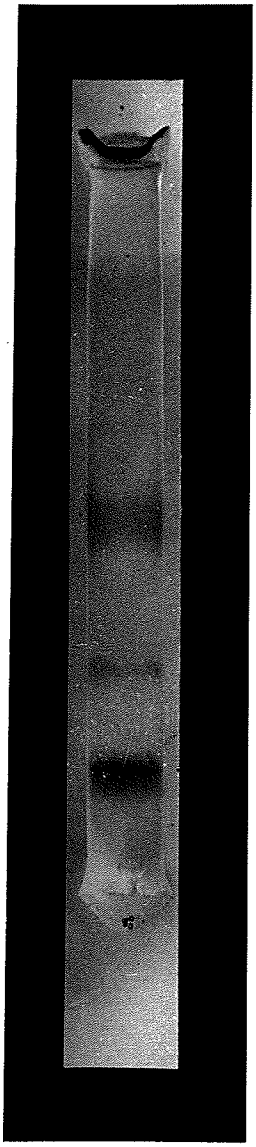
FIG. 20. Polyacrylamide gel electrophoresis of alkaline phosphatase at two stages of purification.

(A) Shows two bands of enzyme activity above the tracking dye. This photograph is typical of photographs taken at all stages of purification except after Cellogel electrophoresis.

(B) Shows one band of enzyme activity above the tracking dye. This photograph is typical of photographs taken after purification by Cellogel electrophoresis.



B



A

Further studies of the effect of pH on alkaline phosphatase with p-nitrophenyl phosphate as substrate.

The effect of pH on the K_m for p-nitrophenyl phosphate at high substrate concentration.

In order to determine the effect of pH on the K_m double reciprocal plots of $\frac{1}{v}$ versus $\frac{1}{S}$ were prepared. Reactions at the appropriate pH and substrate concentration were run in triplicate at 27°. A blank, to which no enzyme was added was run for each substrate concentration. After 4 minutes, the reaction was stopped by the addition of 6.0 ml of 0.1M EDTA in 20% KOH. The optical density of the reacted mixtures at 410 mμ compared to that of the appropriate blank was determined in the SP700 spectrophotometer. The optical density readings were converted to μmoles of p-nitrophenol by use of a standard curve, and velocities (v) were calculated as μmoles of p-nitrophenol produced per reaction per minute. The data are plotted in Fig. 21 and in Fig. 22 in the reciprocal form. Under these conditions the reaction rate remained linear for at least 7 minutes.

These data apparently have no simple explanation and K_m values cannot be derived from the plots but two generalizations are possible:

(1) As the pH falls, the linear portion of the double reciprocal plot moves further into the low substrate con-

FIG. 21. Velocity of p-nitrophenyl phosphate hydrolysis as a function of pH at various substrate concentrations.

Each point was determined in triplicate at 27°. Each reaction vessel contained the following concentrations of substances:

Bicine 3.0×10^{-2}M
$MgCl_2$ 4×10^{-3}M
p-nitrophenyl phosphate - varied as indicated,
alkaline phosphatase - 27 units,

in a final volume of 4.0 ml at the appropriate pH. The method is described in the text. (See page 108)

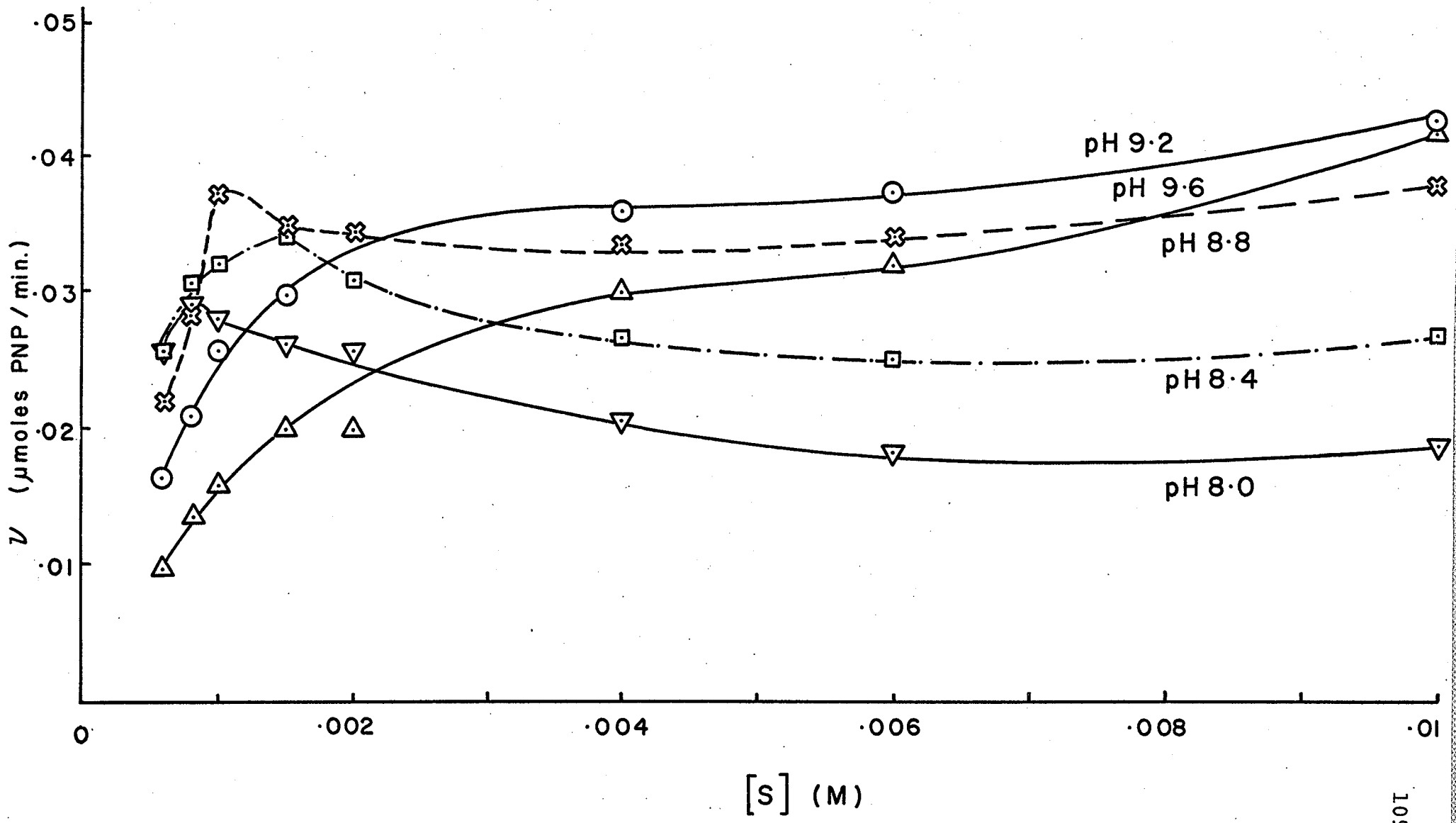
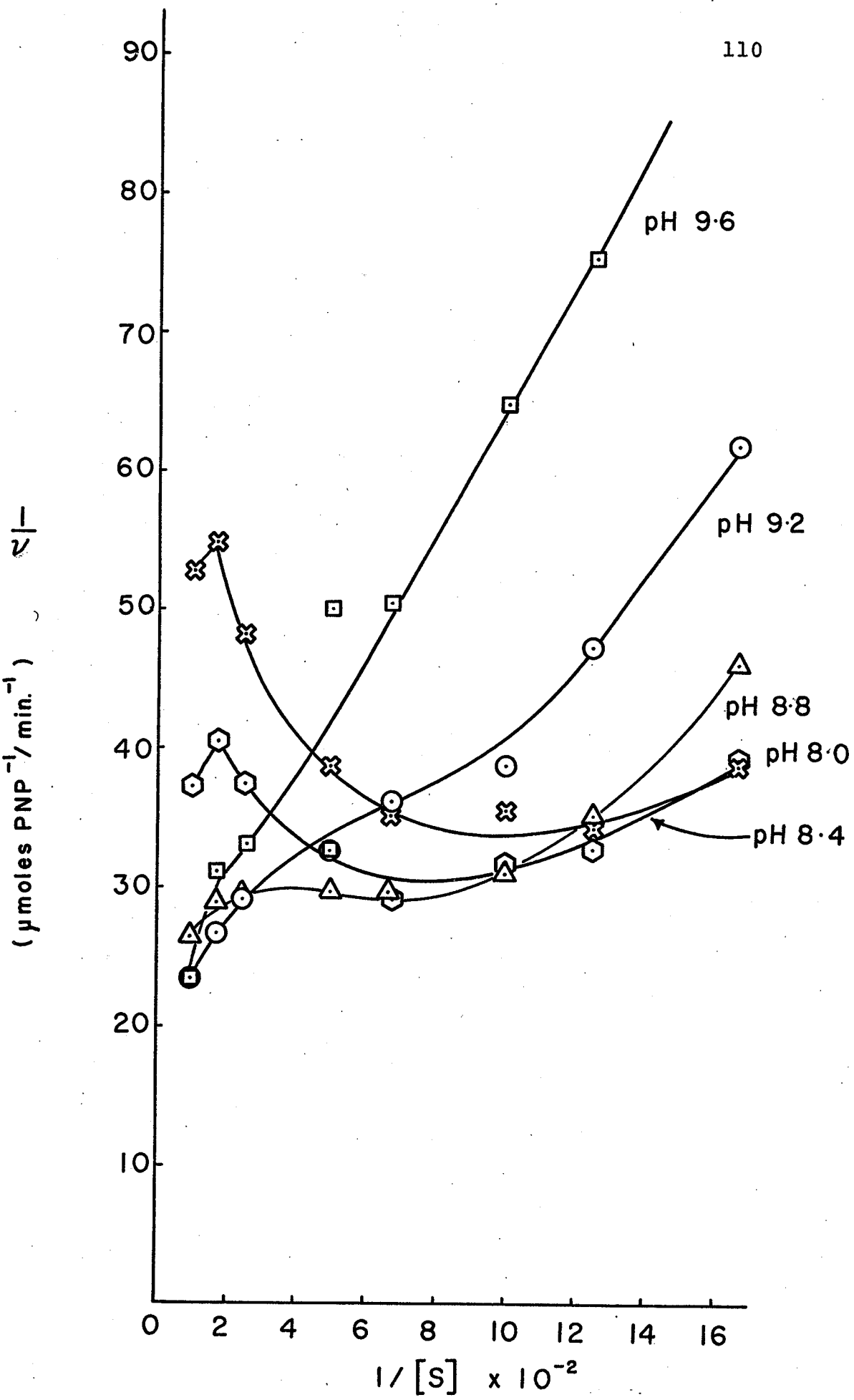

·05
·04
·03
·02
·01
v (μmoles PNP / min.)
pH 9·2
pH 9·6
pH 8·8
pH 8·4
pH 8·0
0
·002
·004
·006
·008
·01
[S] (M)

FIG. 22. Double reciprocal plot of the data presented in Fig. 21.


90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
1/ν
(μmoles PNP⁻¹/min.⁻¹)
1/[S] x 10⁻²
pH 9·6
pH 9·2
pH 8·8
pH 8·0
pH 8·4

centration range.

(2) As the pH falls substrate inhibition occurs at lower substrate concentration.

It seemed that such curves could arise in only two ways (a) by an effect of pH on the substrate or, (b) an effect of pH on the enzyme itself. A titration curve of the substrate is presented in Fig. 23. Over the pH range 7.5 to 9.7 there appears to be little or no change in the ionic species present. A direct effect of pH on p-nitrophenyl phosphate cannot, therefore, account for the peculiarities of these curves.

Samples of the enzyme were exposed to various conditions of pH in $5x10^{-2}$M Bicine (adjusted to pH values between 5.0 and 12.0) for 10 minutes and were then assayed in the standard assay. The results are presented in Fig. 24 and clearly demonstrate that pH values between 6.0 and 11.5 have no permanent effect on the activity of alkaline phosphatase. The unusual effect of pH on activity cannot, therefore, be accounted for in terms of enzyme denaturation.

The effect of pH on the K_m for p-nitrophenyl phosphate at low substrate concentrations.

It appeared from the previous experiment that data obtained at low substrate concentration (on the linear portions of the $\frac{1}{v}$ *versus* $\frac{1}{S}$ plots) might be more easily under-

FIG. 23. Titration curve of p-nitrophenyl phosphate. 15.0 ml of 0.01M p-nitrophenyl phosphate in water, adjusted to pH 12.0 with NaOH was titrated with HCl (1 ml conc. HCl in 250 ml H_2O).

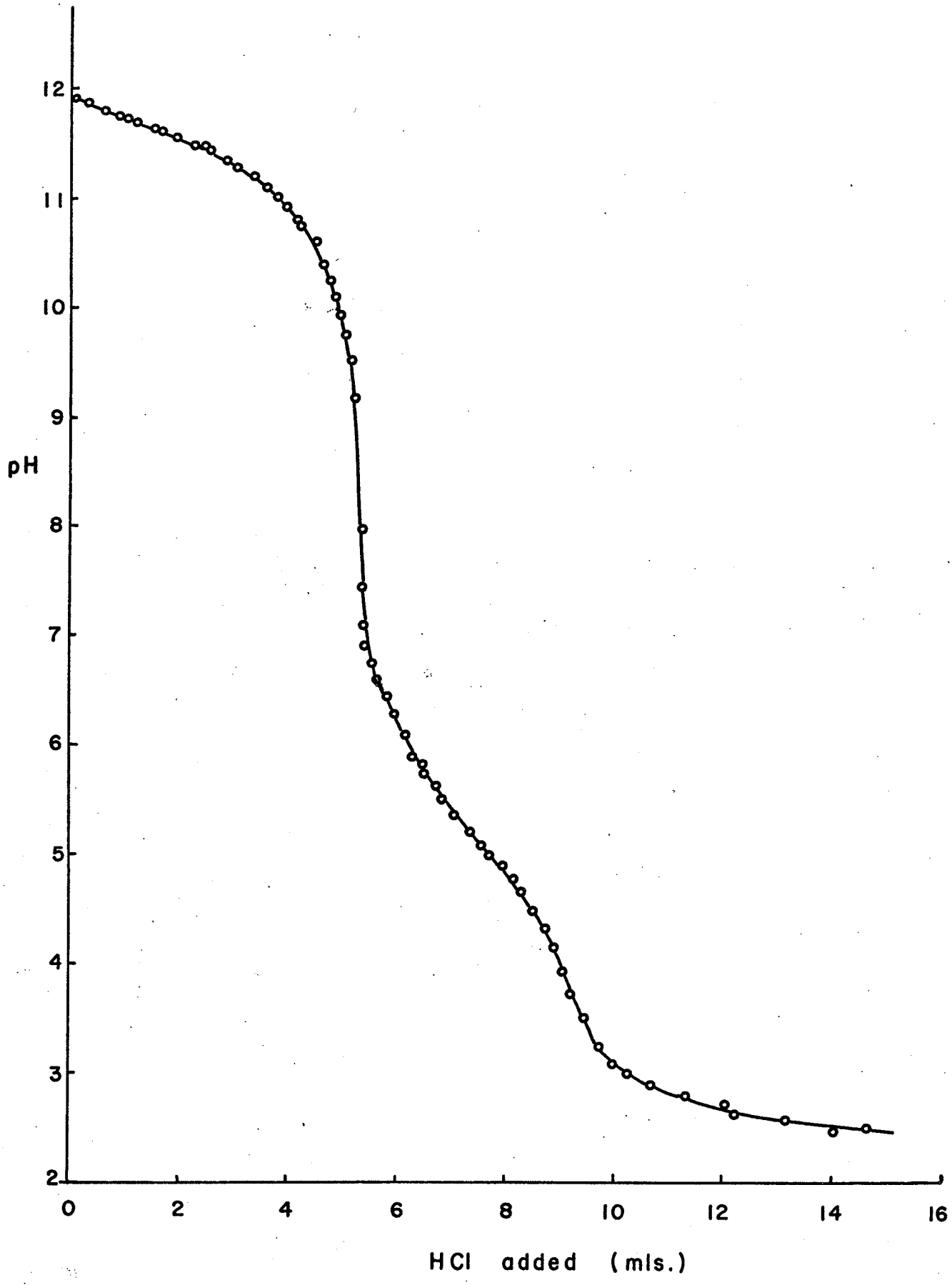
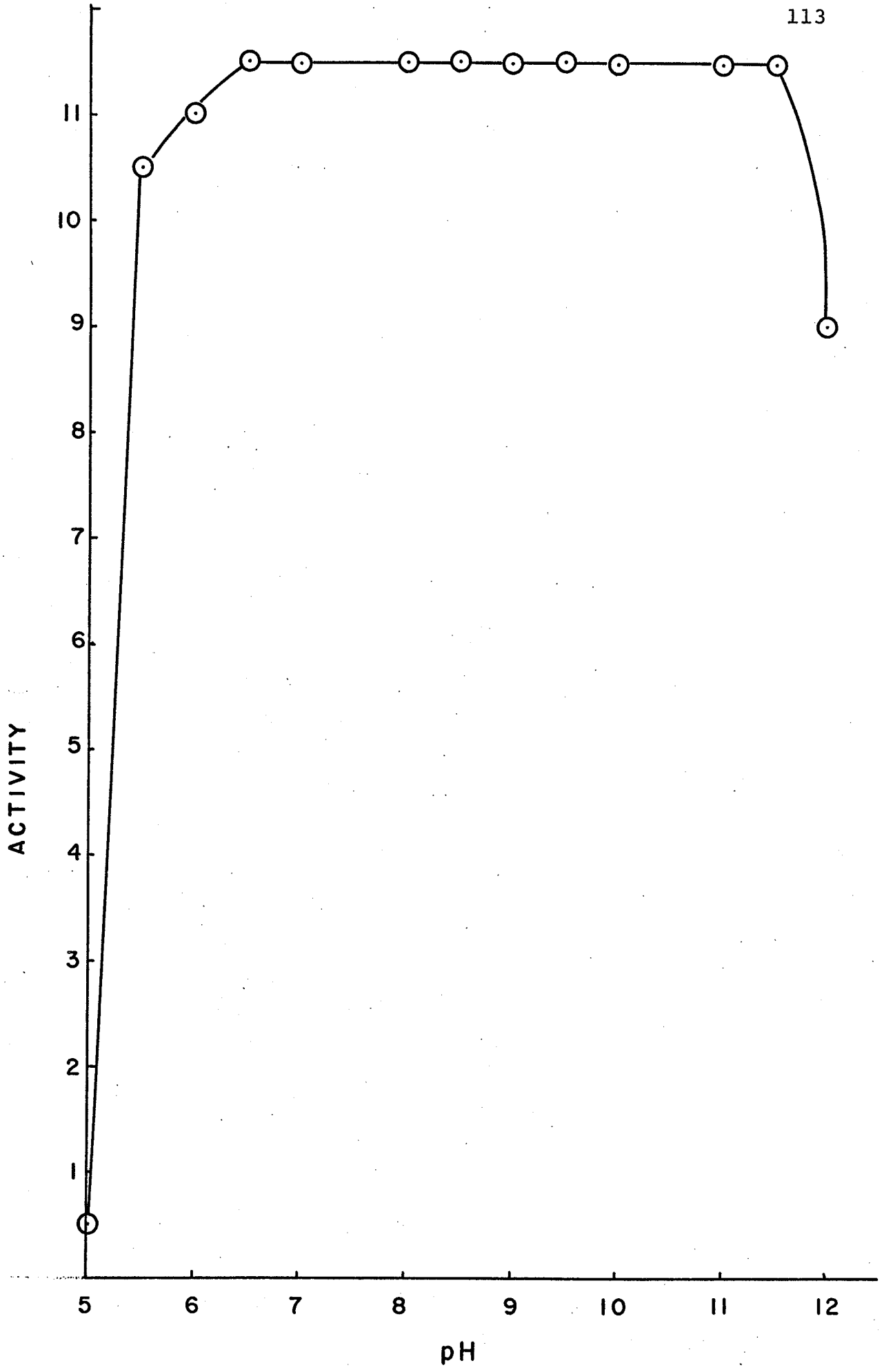

12
11
10
9
pH
8
7
6
5
4
3
2
0
2
4
6
8
10
12
14
16
HCl added (mls.)

FIG. 24. The stability of alkaline phosphatase to a 10 minute exposure to various values of pH. The method is described in the text. (See page 111.)


ACTIVITY
1
2
3
4
5
6
7
8
9
10
11
5
6
7
8
9
10
11
12
pH

stood. Such data were obtained, but a simpler experimental method was employed. Reaction rates were determined in a Gilford model 2400 spectrophotometer at 410 mμ and the initial velocities were recorded as ΔOD_{410}/minute. Optical density readings at the different pH values were converted to values at a constant pH (9.5) by use of a standard curve relating the optical density of a p-nitrophenol solution to pH. Determinations for each point were repeated until random errors were eliminated as judged by the deviation of the points from a straight line.

Data obtained are presented in the form of double reciprocal plots in Figs. 25, 26, 27, 28, 29. A composite of the lines obtained at the different pH values is presented in Fig. 30. Dixon plots (Dixon and Webb, 1964) are presented in Fig. 31. Values of K_m and V_{max} at the various pH values are listed in Table XI.

TABLE XI

VALUES OF K_m AND V_{max} AT VARIOUS VALUES OF pH (PNPP HYDROLYSIS)

pH	K_m (M)	V_{max}
7.0	1.04×10^{-5}	.0287
7.5	6.83×10^{-5}	.0621
8.0	7.78×10^{-5}	.0556
8.4	3.51×10^{-4}	.0926
8.7	1.91×10^{-3}	.345
9.0	1.67×10^{-3}	.244
9.2 9.5	1.81×10^{-3}	.222

The peculiar effects of pH on the activity of alkaline phosphatase with PNPP as substrate raised the question as to whether these effects would be similar if a more "natural" substrate were to be used. The types of experiment performed with PNPP as substrate were repeated using glucose 6-phosphate as substrate.

FIG. 25. Double reciprocal plots at pH 9.5 and 9.2 (PNPP). Each cuvette contained the following substances and concentrations:

Bicine 3×10^{-2}M
$MgCl_2$ 4×10^{-3}M
PNPP varied,
Enzyme 17 units,

in a final volume of 3.0 ml. The reaction was started by the addition of enzyme. The method is given in the text. (See page 111.)

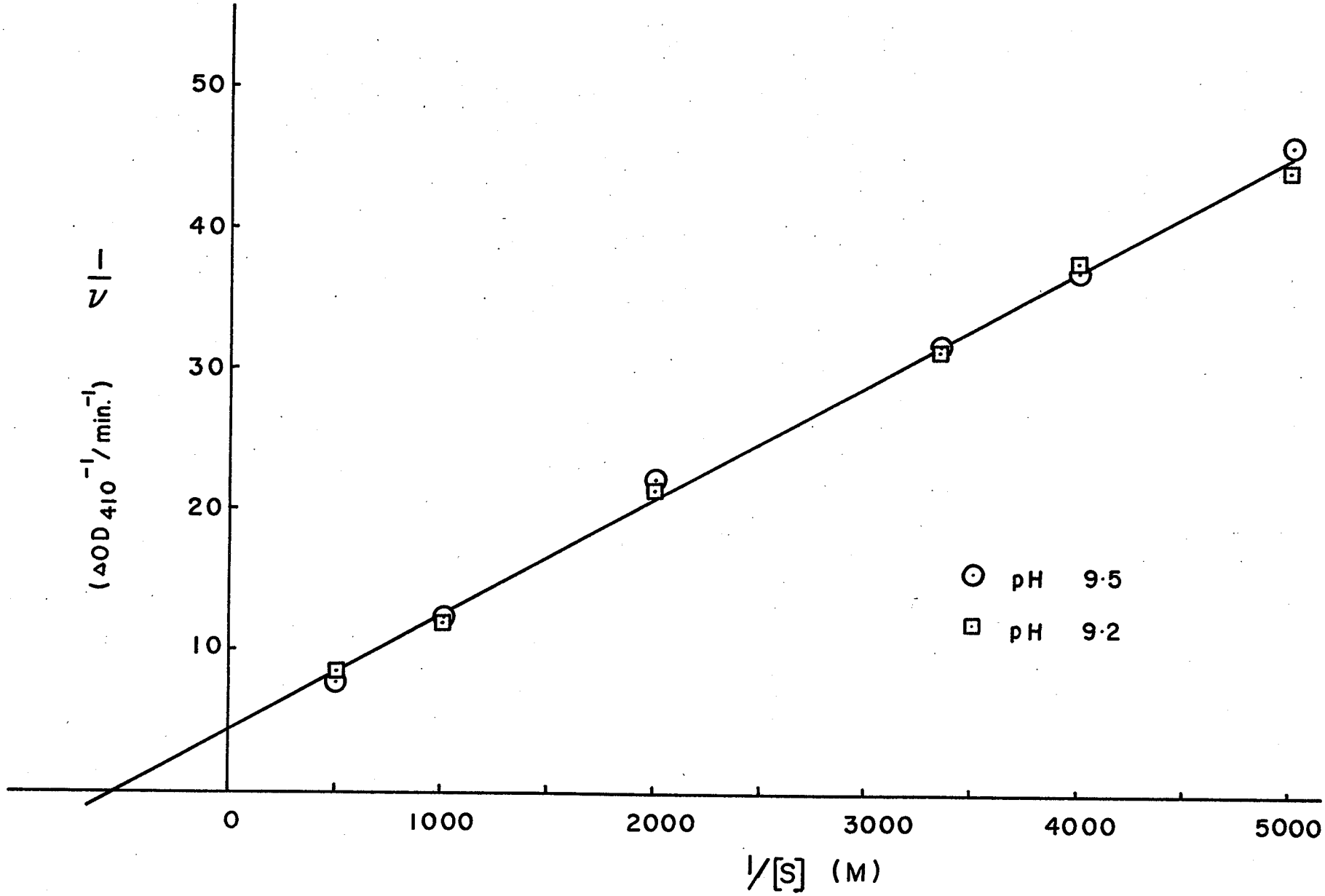

50
40
30
20
10
0
1000
2000
3000
4000
5000
1/v
(ΔOD 410 -1/min.-1)
1/[S] (M)
pH 9·5
pH 9·2

FIG. 26. Double reciprocal plot at pH 9.0 (PNPP).
The method is given in the text (see page 111)
and in Fig. 25.

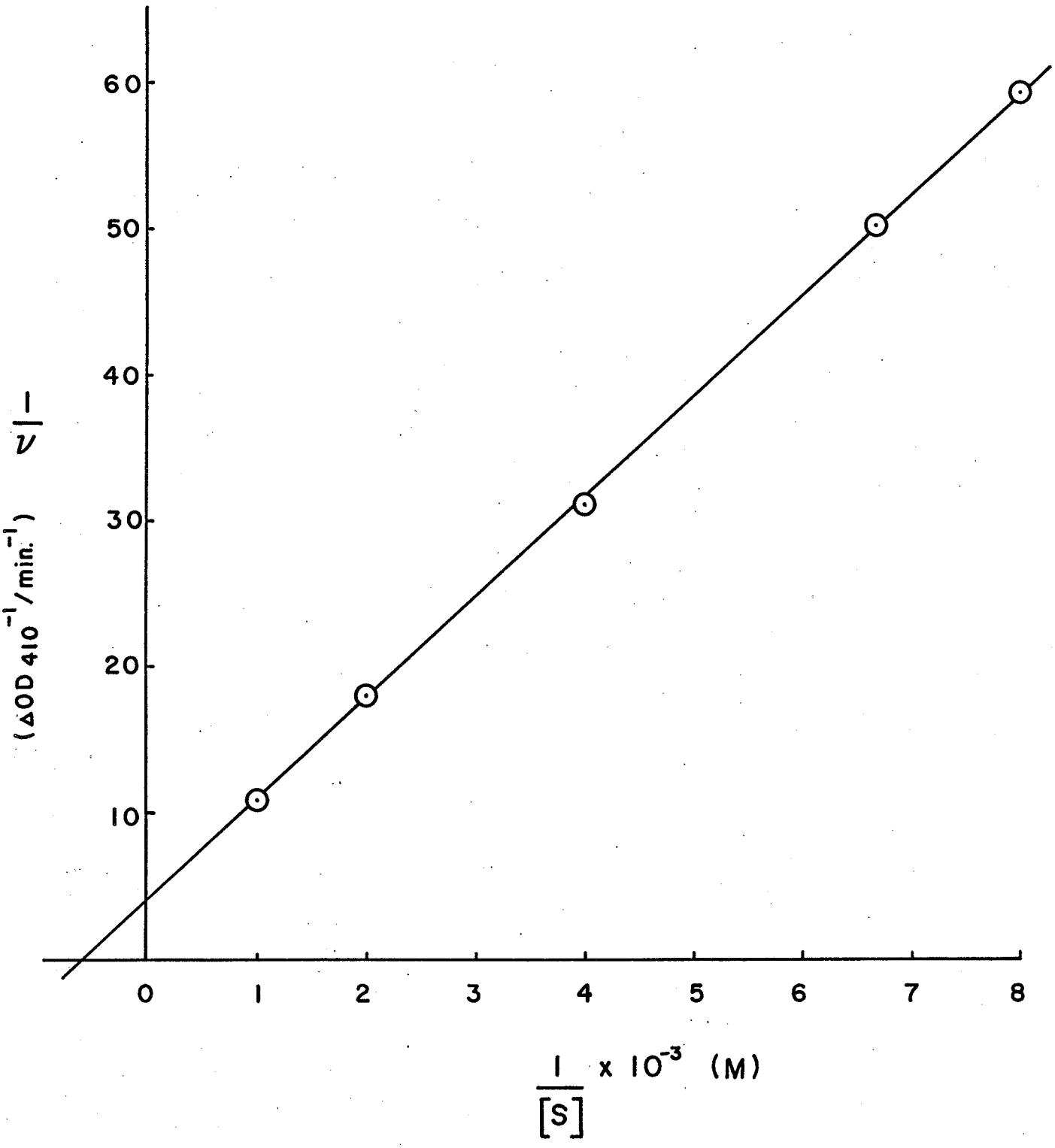

60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
1/v
(ΔOD 410 -1/min.-1)
1/[S] x 10-3 (M)

FIG. 27. Double reciprocal plots at pH 8.7 and 8.4 (PNPP). The method is given in the text (see page 111) and in Fig. 25.

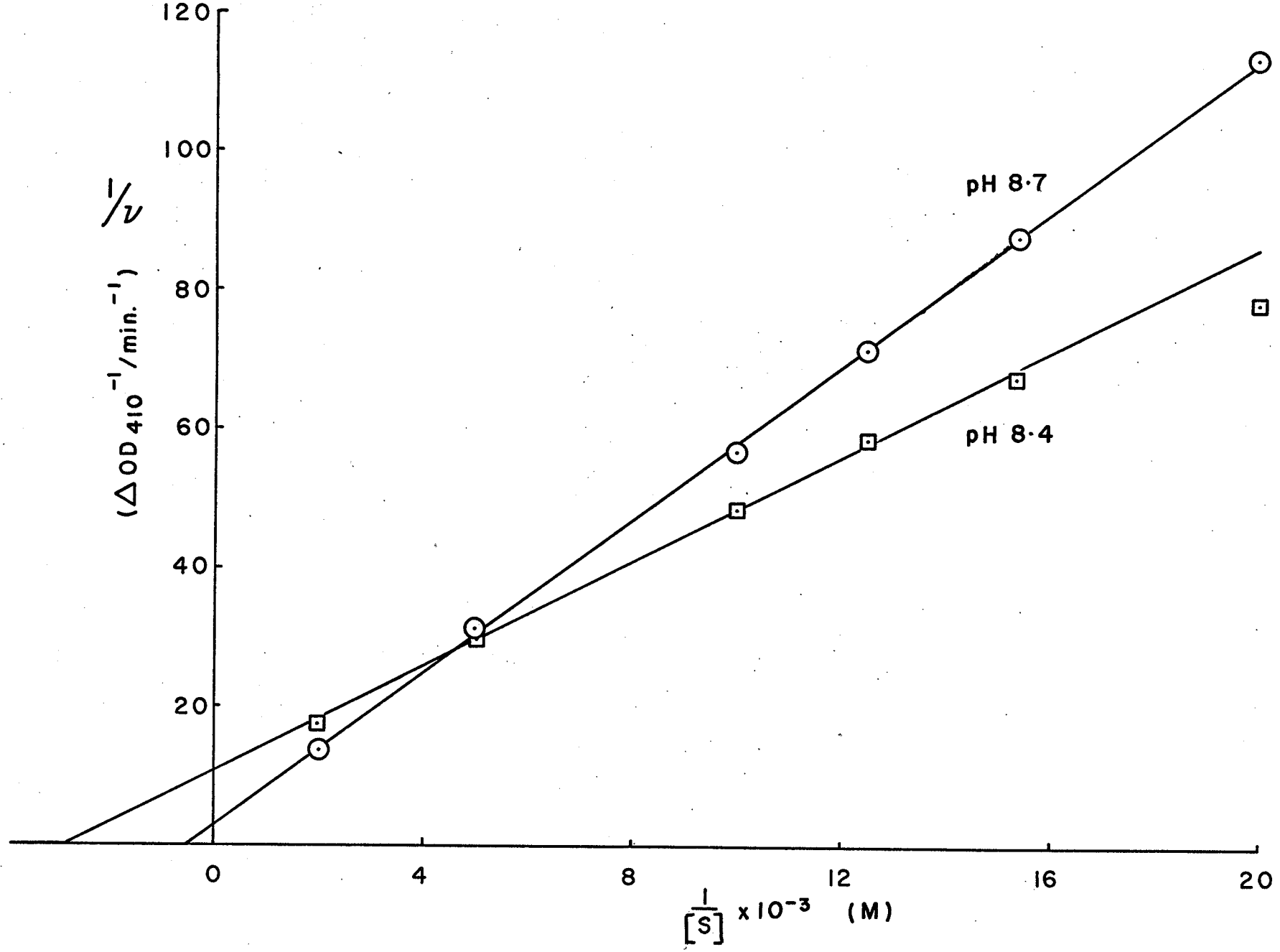

120
100
80
60
40
20
1/v
(ΔOD 410 -1 /min.-1)
pH 8·7
pH 8·4
0
4
8
12
16
20
1/[S] x10-3 (M)

FIG. 28. Double reciprocal plots at pH 8.0 and 7.5 (PNPP). The method is given in the text (see page 111) and in Fig. 25.

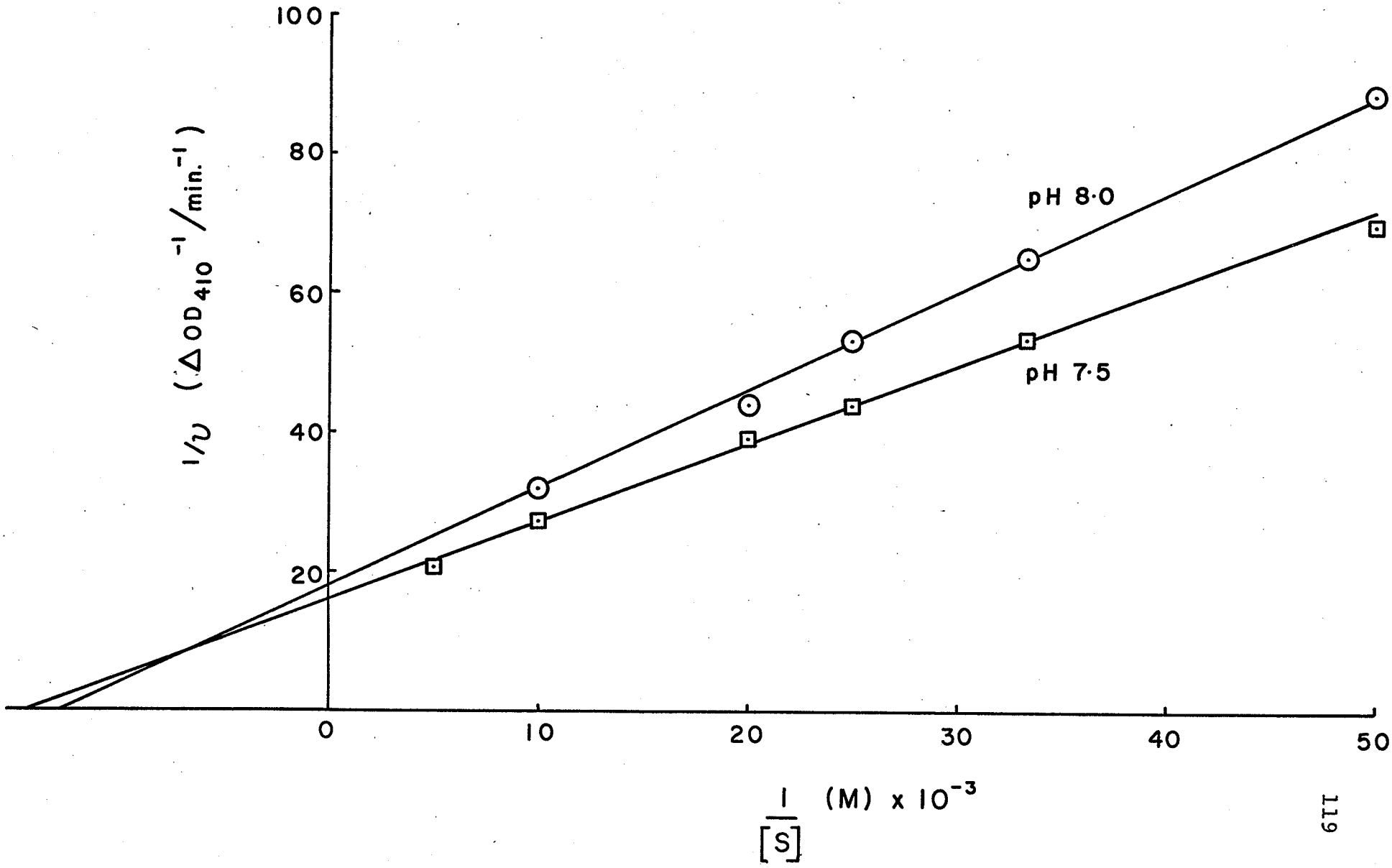

100
80
60
40
20
0
10
20
30
40
50
1/v (ΔOD410 -1 /min.-1)
pH 8·0
pH 7·5
1/[S] (M) x 10-3

FIG. 29. Double reciprocal plot at pH 7.0 (PNPP). The method is given in the text (see page 111) and in Fig. 25.

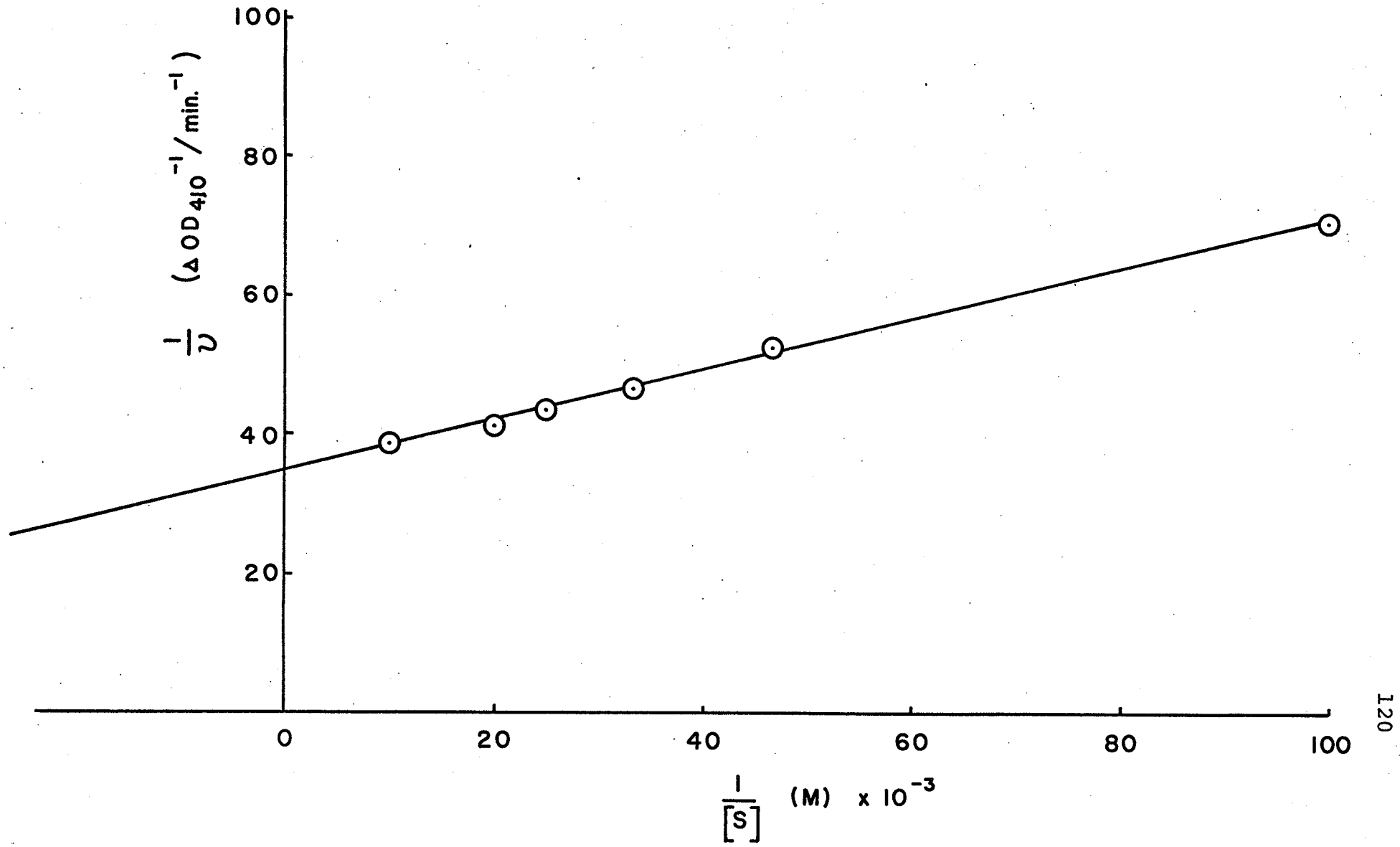

100
80
60
40
20
0
20
40
60
80
100
$\frac{1}{v}$ (ΔOD_{410}^{-1}/min.$^{-1}$)
$\frac{1}{[S]}$ (M) x 10^{-3}

FIG. 30. Double reciprocal plots at various pH values and at low PNPP concentration.
The data of Figs. 25, 26, 27, 28 and 29 are redrawn on a single graph.

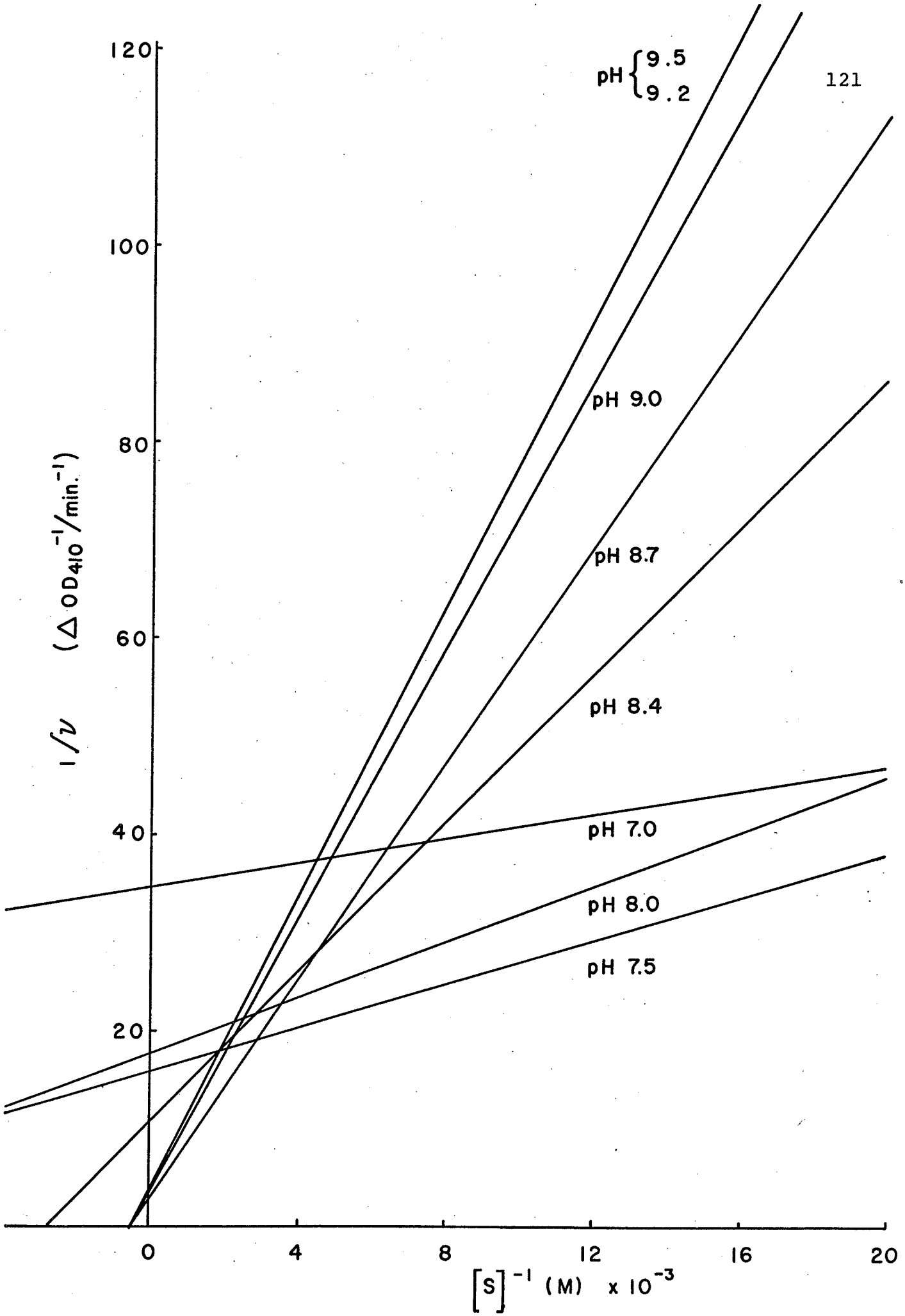
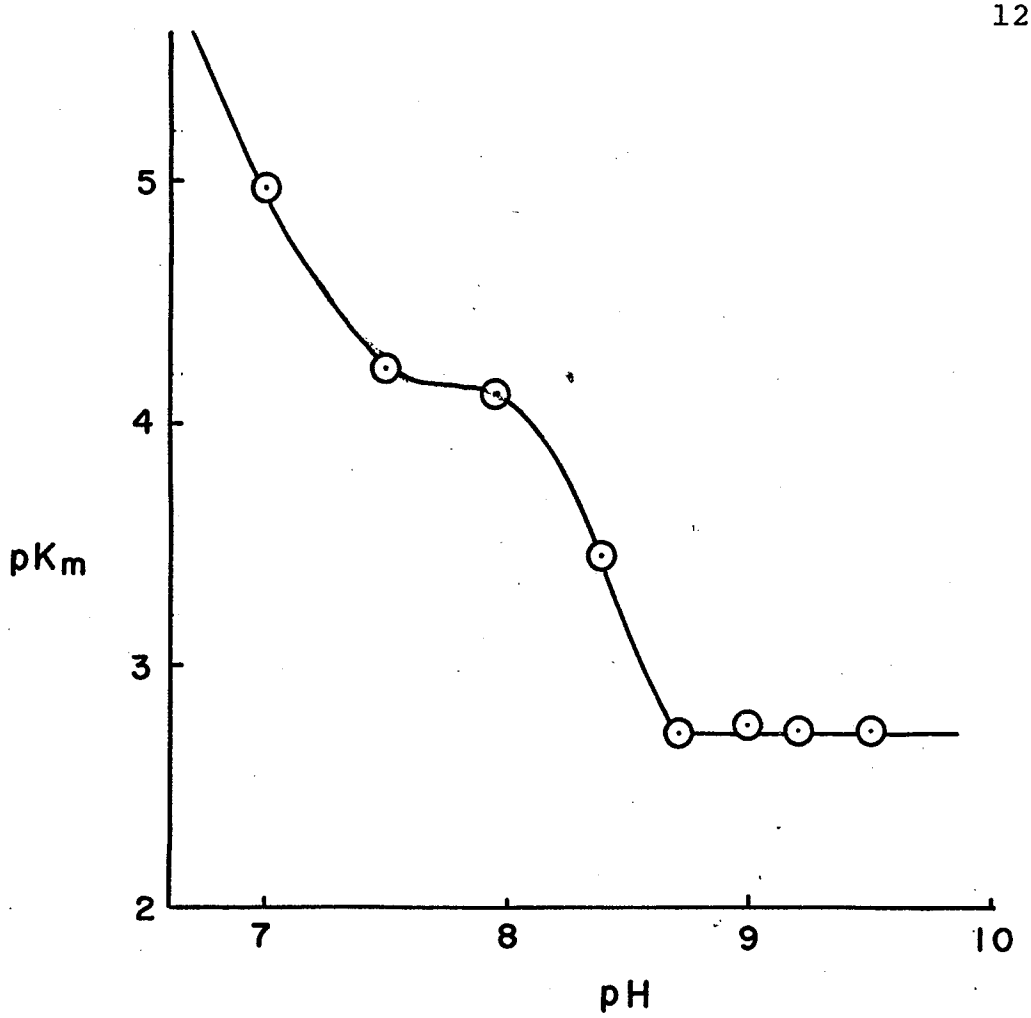
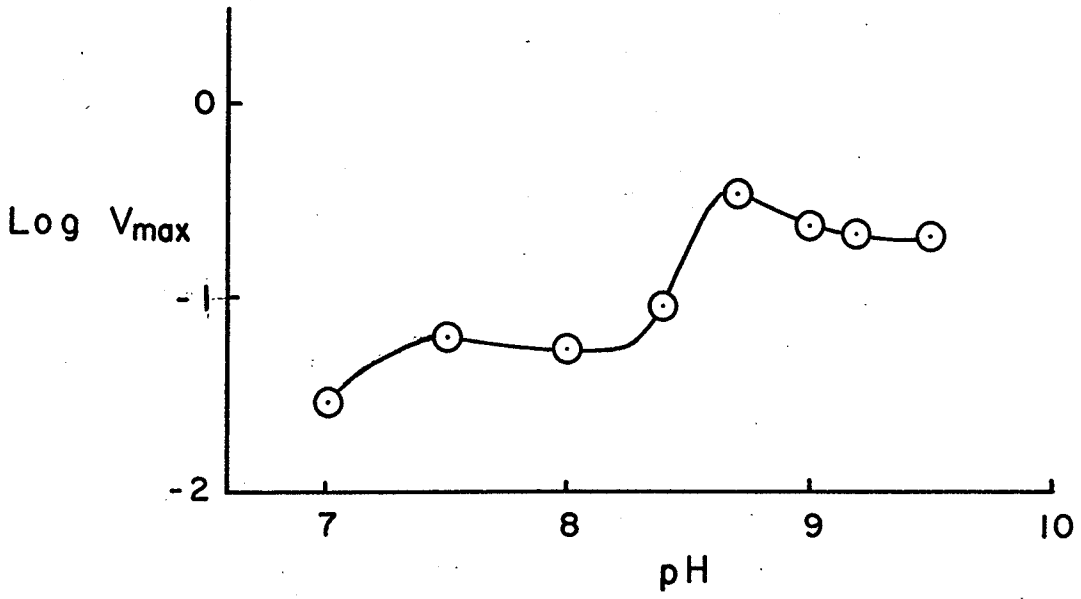

120
100
80
60
40
20
0
4
8
12
16
20
pH {9.5
9.2
pH 9.0
pH 8.7
pH 8.4
pH 7.0
pH 8.0
pH 7.5
1/v (ΔOD410 -1/min.-1)
[S]-1 (M) x 10-3

FIG. 31. Plots of log V_{max} and pK_m *versus* pH prepared by the method of Dixon from the data in Table XI (PNPP).


5
4
3
2
pKm
7
8
9
10
pH


0
-1
-2
Log Vmax
7
8
9
10
pH

Studies of the effect of pH on alkaline phosphatase with glucose 6- phosphate as substrate.

The effect of time on the hydrolysis of glucose 6-phosphate by alkaline phosphatase.

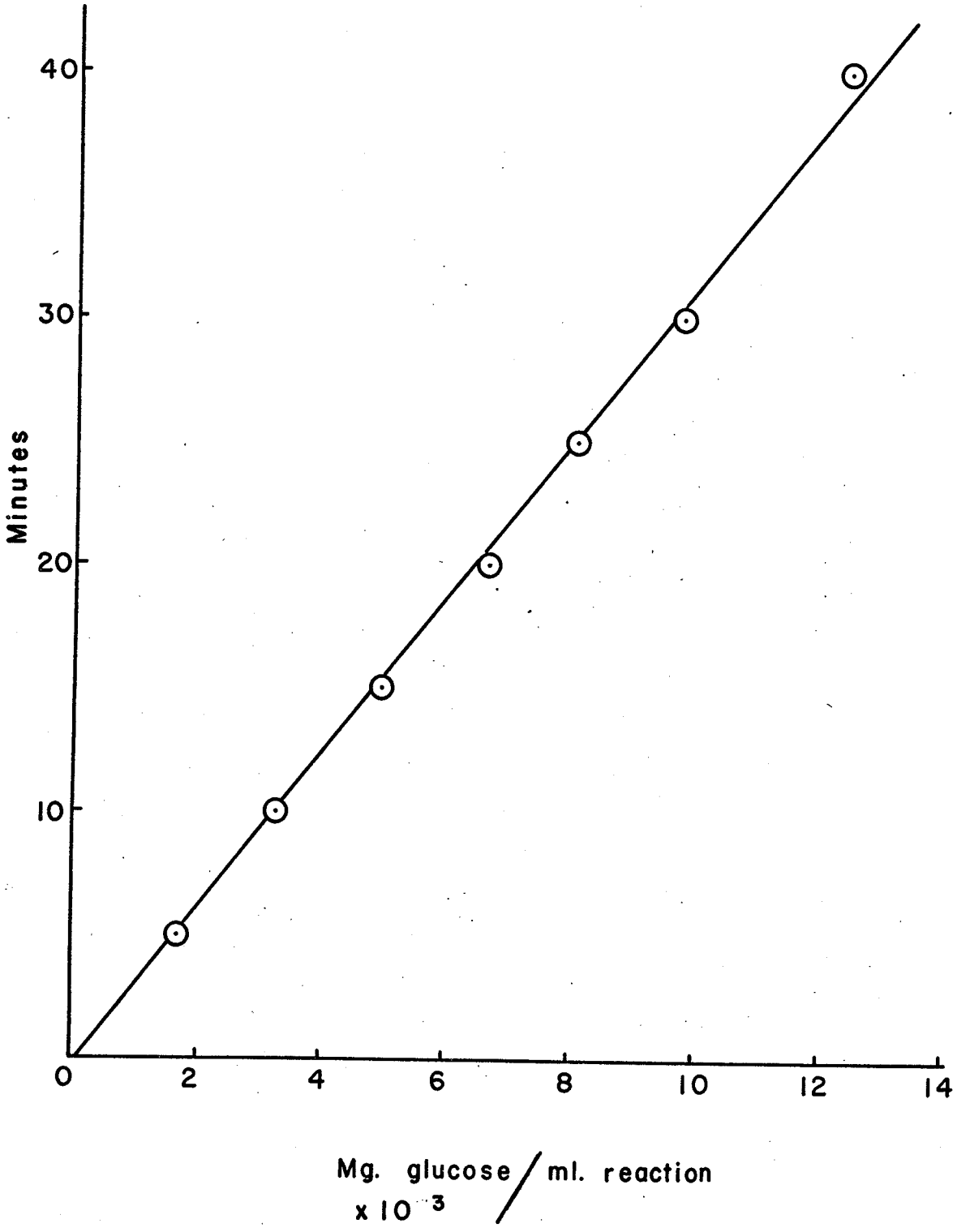
Two reaction vessels containing $3x10^{-2}$M Bicine, $4x10^{-3}$M $MgCl_2$, and $1x10^{-3}$M glucose 6-phosphate at pH 9.0 were brought to 29° in a water bath. The reaction was started by adding 0.4 ml of enzyme (260 units) to one vessel containing 25.0 ml of the reaction mixture. The same volume of H_2O was added to the other vessel, which served as a control. At various times 3.0 ml of the reaction mixture and 3.0 ml of the control were removed and pipetted into test tubes containing 0.2 ml of 10% TCA to stop the reaction and denature the enzyme. After 10 minutes the pH was brought back to near neutrality by the addition of 0.15 ml of 1.0M phosphate buffer pH 7.9. Two ml of the mixture was used for glucose determination by the Glucostat method. The results are presented in Fig. 32. The reaction rate remained linear for at least 40 minutes.

The effect of pH on the hydrolysis of glucose 6-phosphate by alkaline phosphatase.

The rate of the reaction was measured at eight different pH values for each of four different substrate concentrations. 3.0 ml of the reaction mixture ($3x10^{-2}$M Bicine,

FIG. 32. The production of glucose by alkaline phosphatase from glucose 6-phosphate as a function of time.

The method is described in the text. (See page 123.)


40
30
20
10
Minutes
0
2
4
6
8
10
12
14
Mg. glucose / ml. reaction
x 10 -3

$4x10^{-3}$M $MgCl_2$, together with glucose 6-phosphate in the appropriate concentration, at the required pH) was pipetted into each of 3 test tubes. The temperature was brought to 30° in a water bath. Enzyme (47.5 units) was added to two of the three tubes; the third served as a control. The reaction was allowed to proceed for 30 minutes and was stopped by the addition of 0.2 ml of 10% TCA. The pH was then returned to near neutrality by adding 0.15 ml of 1.0 M phosphate buffer pH 7.9. The amount of glucose present in a suitable aliquot was determined by the Glucostat method. Control values were subtracted from test values and the results were expressed as mg. glucose produced per ml of reaction per minute. The data are presented in Fig. 33 from which it is evident that the optimum pH varies with substrate concentration much as it does when PNPP is the substrate. A plot of log (glucose 6-phosphate concentration) versus optimum pH is presented in Fig. 34 and is linear as was the same graph with PNPP as substrate.

FIG. 33. Effect of pH on glucose 6-phosphate hydrolysis by alkaline phosphatase at several values of substrate concentration.

The method is described in the text (see page 123). Activities are plotted as percentages of the maximum activities measured for each substrate concentration.

	G6P concentration	Max. activity (mg. glucose/ml/min.)
A -	1×10^{-2}M	3.6×10^{-3}
B -	5×10^{-3}M	2.89×10^{-3}
C -	1×10^{-3}M	1.52×10^{-3}
D -	5×10^{-4}M	1.47×10^{-3}

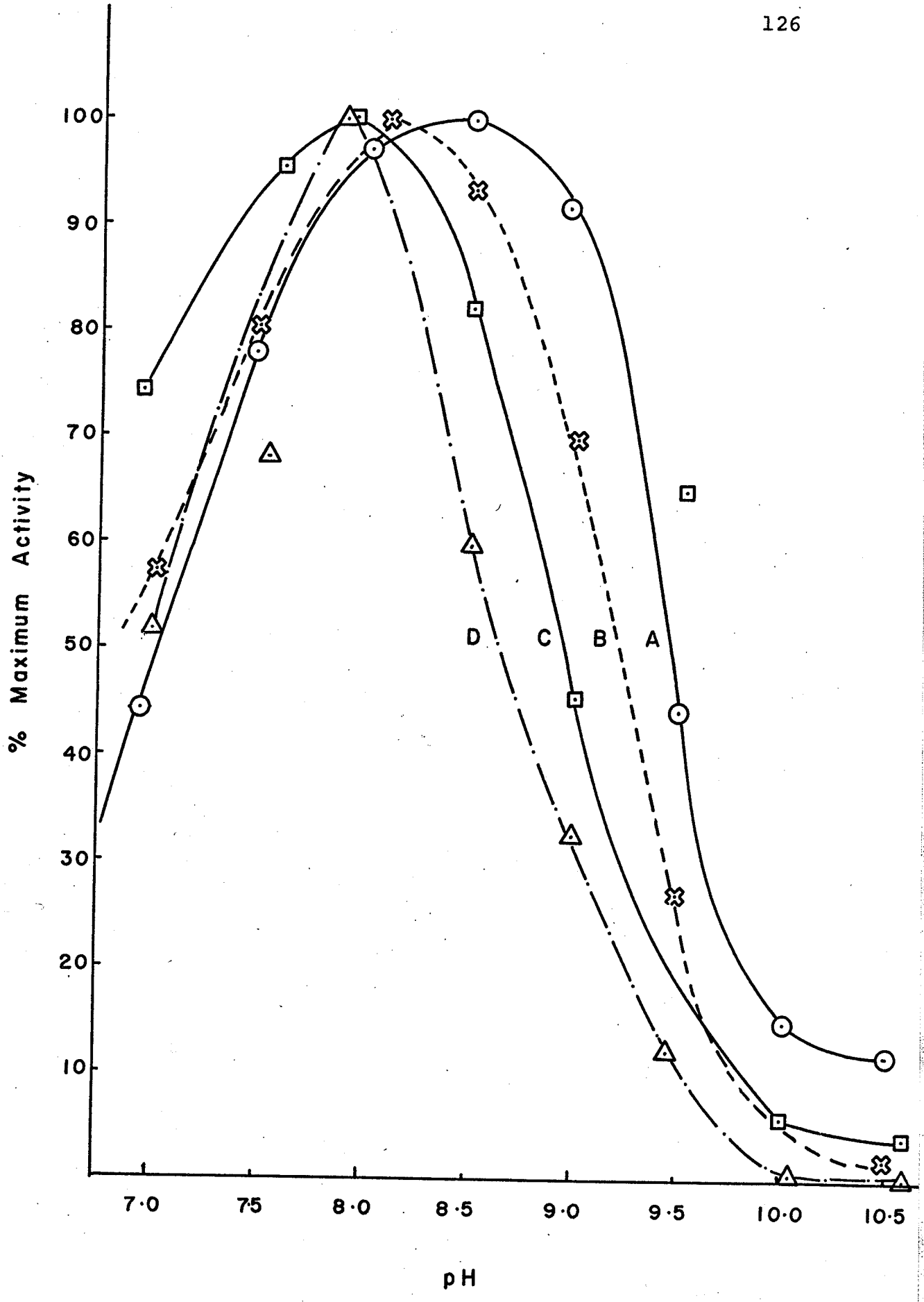
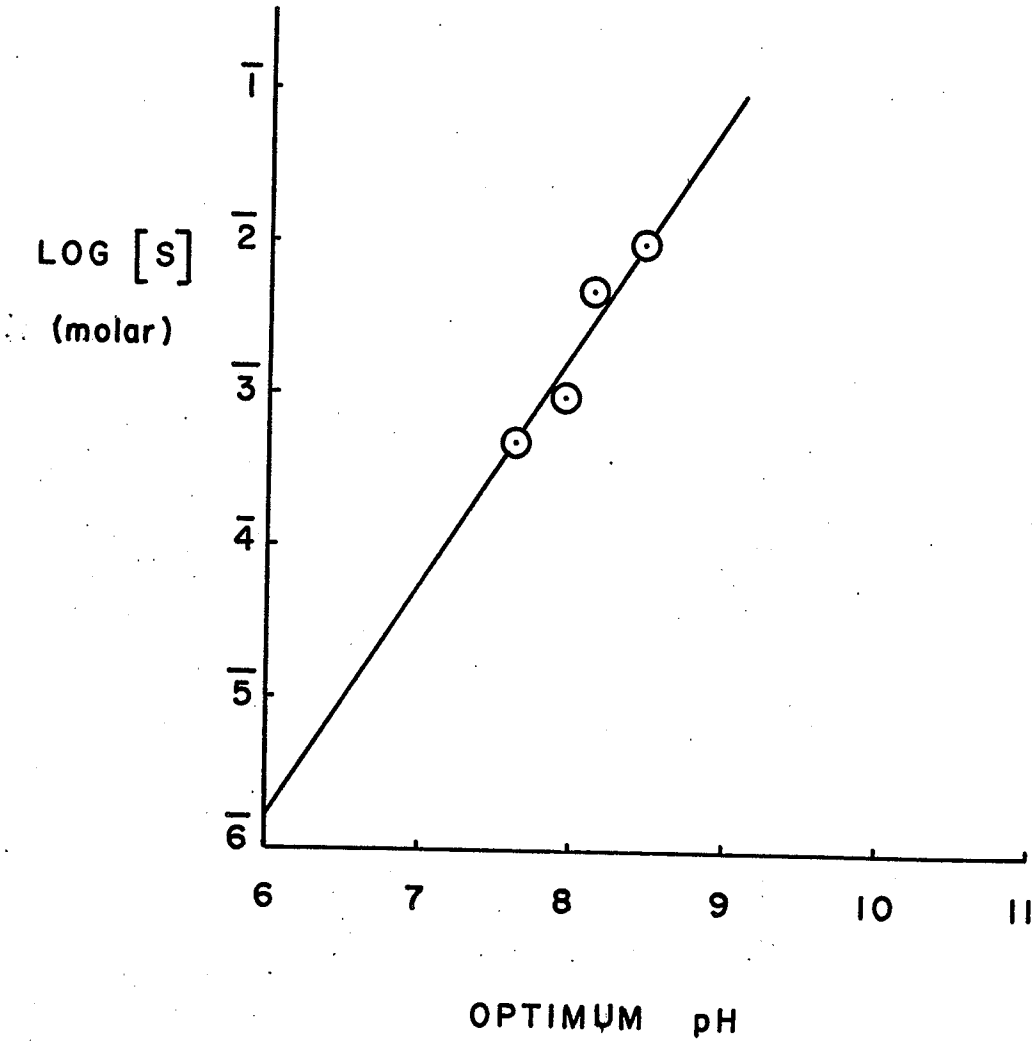

% Maximum Activity
100
90
80
70
60
50
40
30
20
10
A
B
C
D
7·0
7·5
8·0
8·5
9·0
9·5
10·0
10·5
pH

FIG. 34. Optimum pH as a function of the logarithm of glucose 6-phosphate concentration.
Data taken from Fig. 33.


LOG [S]
(molar)
$\bar{1}$
$\bar{2}$
$\bar{3}$
$\bar{4}$
$\bar{5}$
$\bar{6}$
6
7
8
9
10
11
OPTIMUM pH

The effect of pH on the K_m and V_{max} for glucose 6-phosphate hydrolysis.

Double reciprocal plots ($\frac{1}{v}$ versus $\frac{1}{S}$) were prepared at eight different values of pH. All reactions were run in triplicate under the same conditions as those used in the experiment last described except that the concentration of glucose 6-phosphate was varied. The data are plotted in Figs. 35, 36, 37, 38, 39, 40 and 41. The average values of the triplicate determinations were fitted to straight lines by the method of least squares. The individual lines for each pH are plotted in a composite graph in Fig. 42. Data derived from the individual plots are listed in Table XII. Dixon plots of the relevant data in Table XII are presented in Fig. 43.

The shape of the Dixon plots with G6P as substrate differed from the plots obtained with PNPP as substrate. The experiment with G6P was, therefore, repeated, but this time the reaction velocities were calculated from phosphate rather than from glucose determinations. (The pH, therefore, was not returned to neutrality after the TCA addition.) Conditions were indentical except that 60 units of enzyme were used per reaction in the repeated experiment. Only the composite graph, showing all eight double reciprocal plots at once is here presented in Fig. 44. Data derived

FIG. 35. Double reciprocal plots at pH 9.5 and 9.0 (G6P). The method is given in the text (see page 128). The range of values of $\frac{1}{v}$ obtained and the average values are indicated.

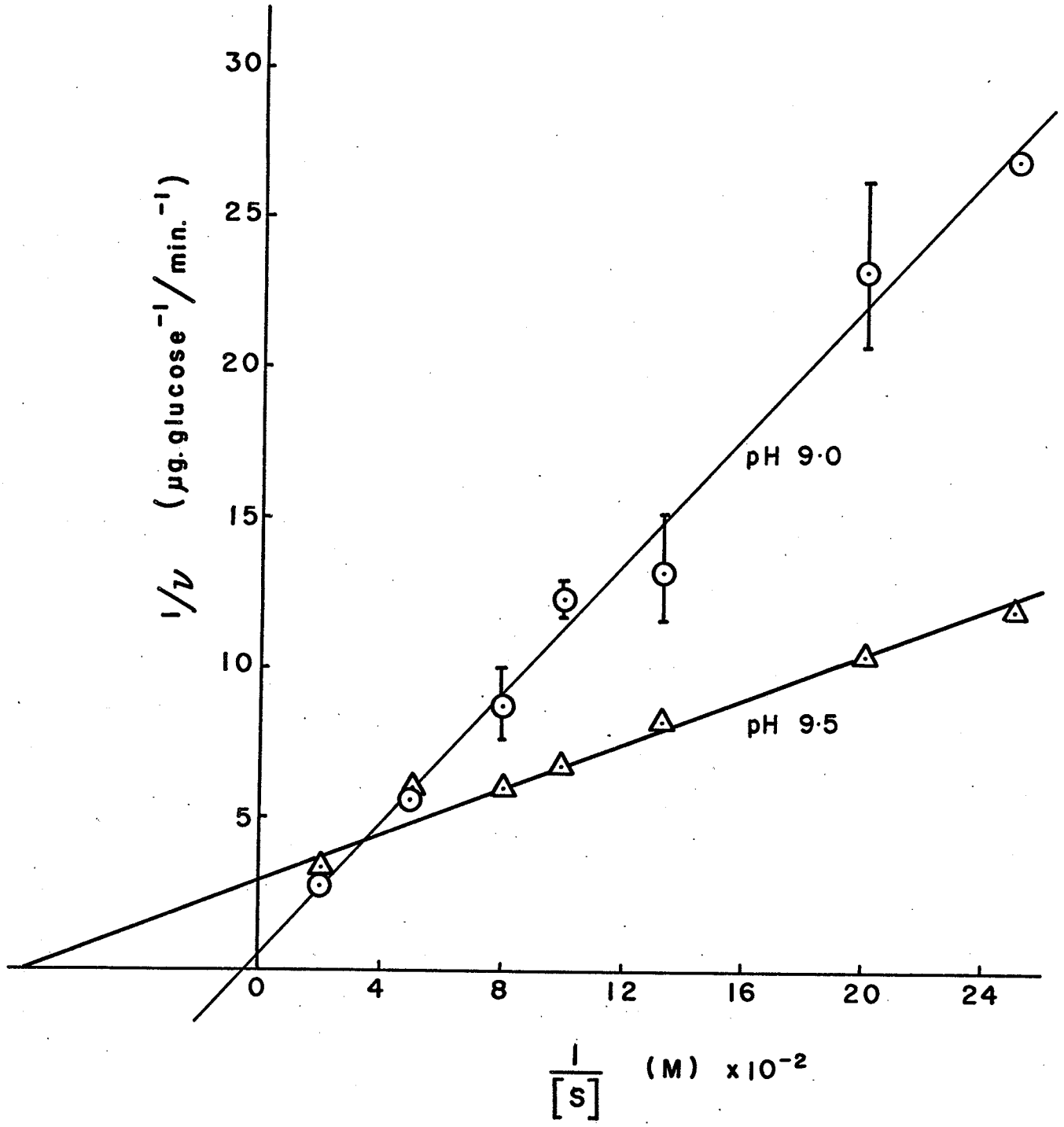

30
25
20
15
10
5
0
4
8
12
16
20
24
1/v (µg.glucose⁻¹/min.⁻¹)
pH 9·0
pH 9·5
1/[S] (M) x10⁻²

FIG. 36. Double reciprocal plot at pH 8.7 (G6P).
The method is given in the text (see page 128).
The range of values of $\frac{1}{v}$ obtained and the
average values are indicated.

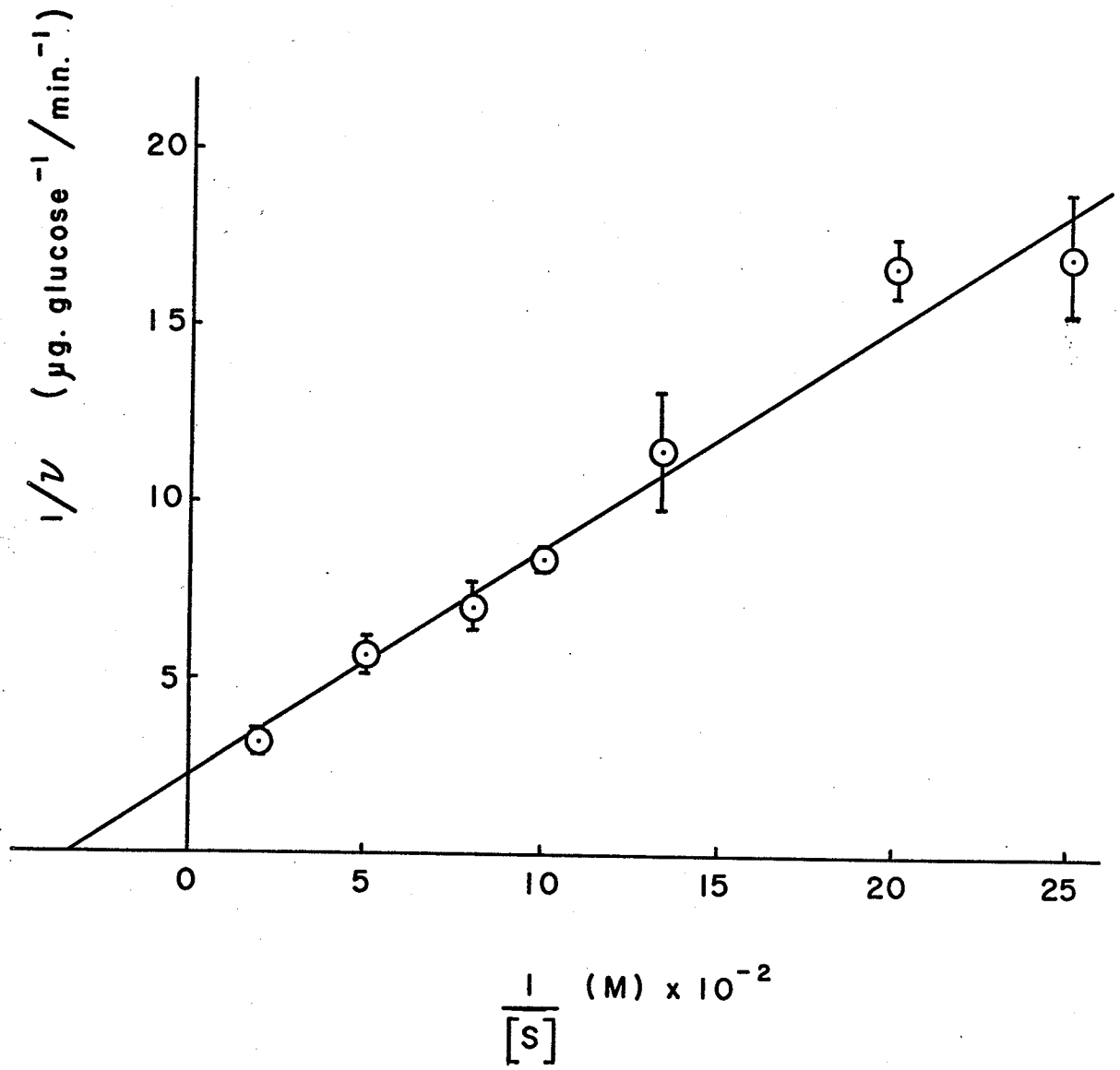

1/v (μg. glucose⁻¹/min.⁻¹)
20
15
10
5
0
5
10
15
20
25
1/[S] (M) x 10⁻²

FIG. 37. Double reciprocal plot at pH 8.4 (G6P).
The method is given in the text (see page 128).
The range of values of $\frac{1}{v}$ and the average
values are indicated.

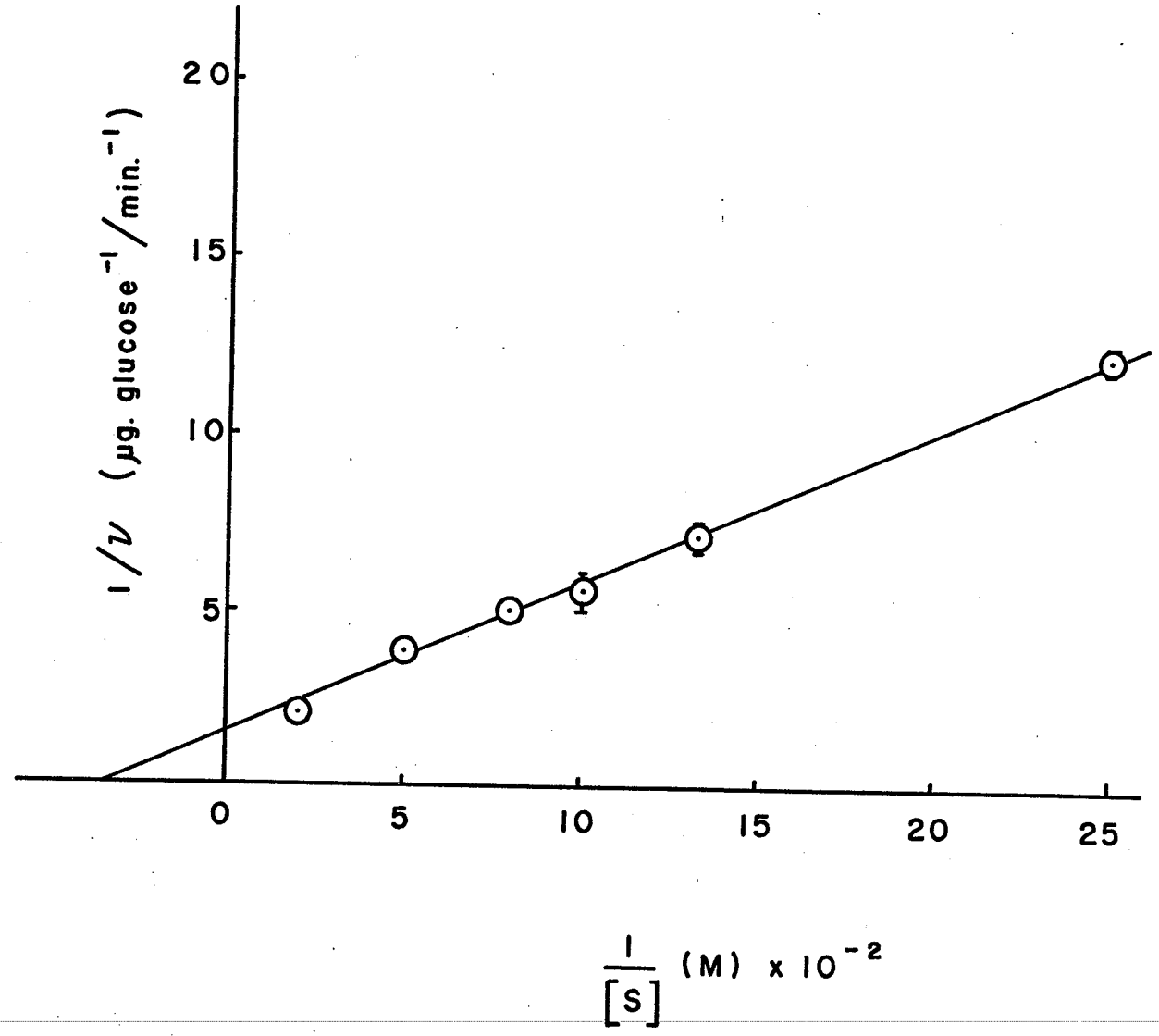

20
15
10
5
0
5
10
15
20
25
1/v (μg. glucose⁻¹/min.⁻¹)
1/[S] (M) x 10⁻²

FIG. 38. Double reciprocal plot at pH 8.1 (G6P).
The method is given in the text (see page 128).
The range of values of $\frac{1}{v}$ and the average values are indicated.

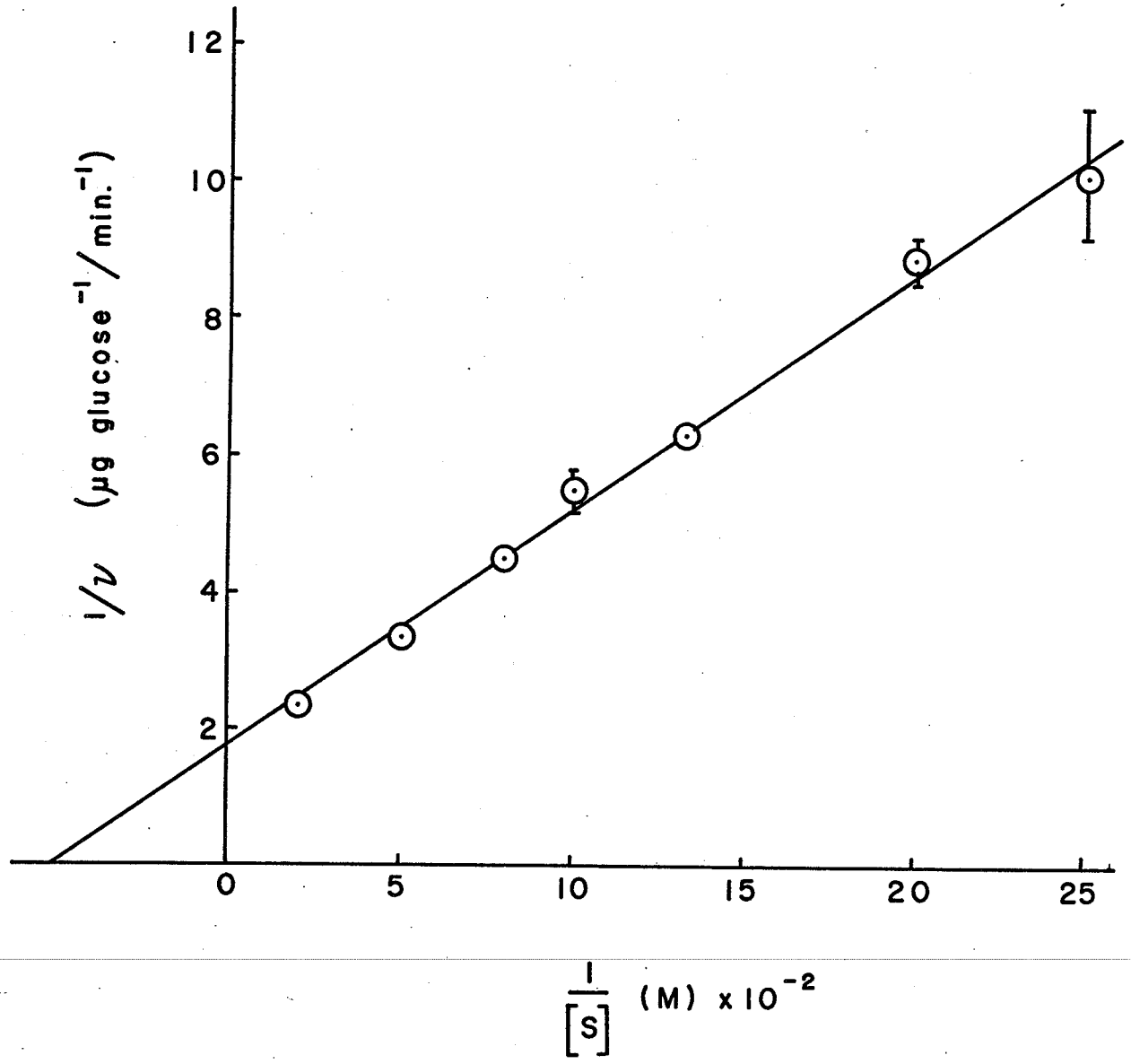

12
10
8
6
4
2
0
5
10
15
20
25
1/v (μg glucose⁻¹/min.⁻¹)
1/[S] (M) x 10⁻²

FIG. 39. Double reciprocal plot at pH 7.8 (G6P).
The method is given in the text (see page 128).
The range of values of $\frac{1}{v}$ obtained and the
average values are indicated.

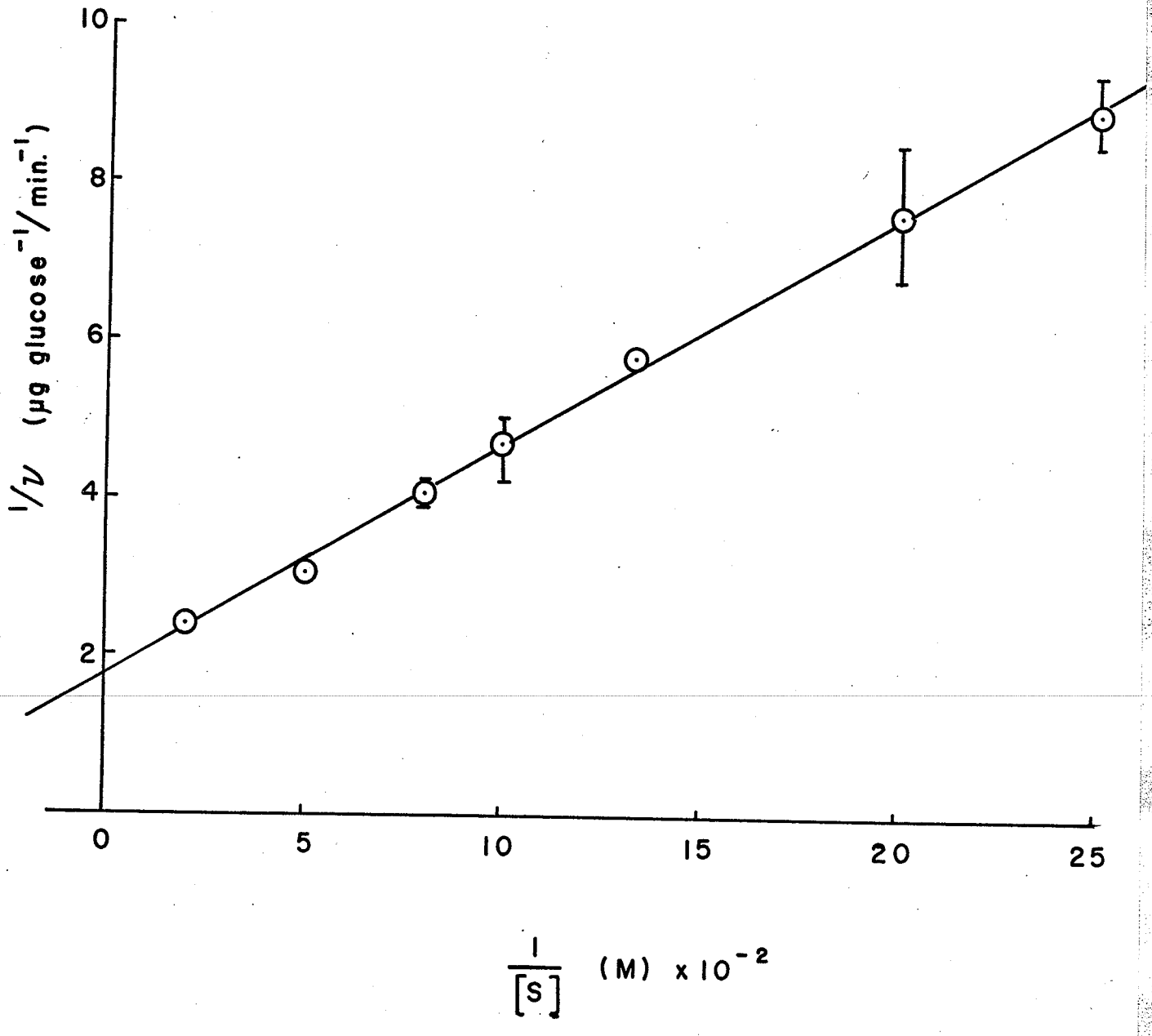

10
8
6
4
2
0
5
10
15
20
25
1/v (µg glucose⁻¹/min.⁻¹)
1/[S] (M) x 10⁻²

FIG. 40. Double reciprocal plot at pH 7.5 (G6P).
The method is given in the text (see page 128).
The range of values of $\frac{1}{v}$ obtained and the
average values are indicated.

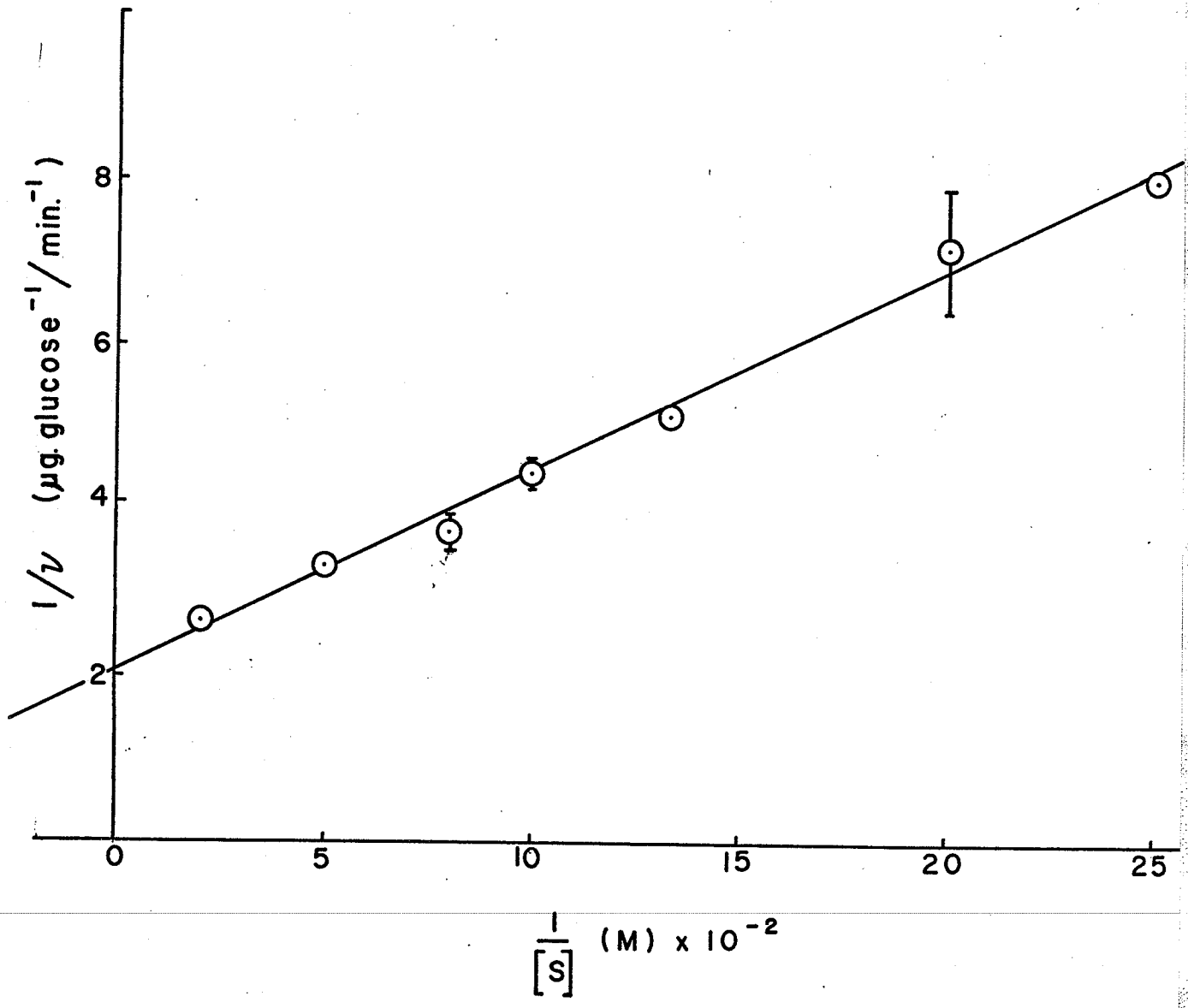

1/v (μg. glucose⁻¹/min.⁻¹)
8
6
4
2
0
5
10
15
20
25
1/[S] (M) x 10⁻²

FIG. 41. Double reciprocal plot at pH 7.1 (G6P).
The method is given in the text (see page 128).
The range of values obtained and the average values are indicated.

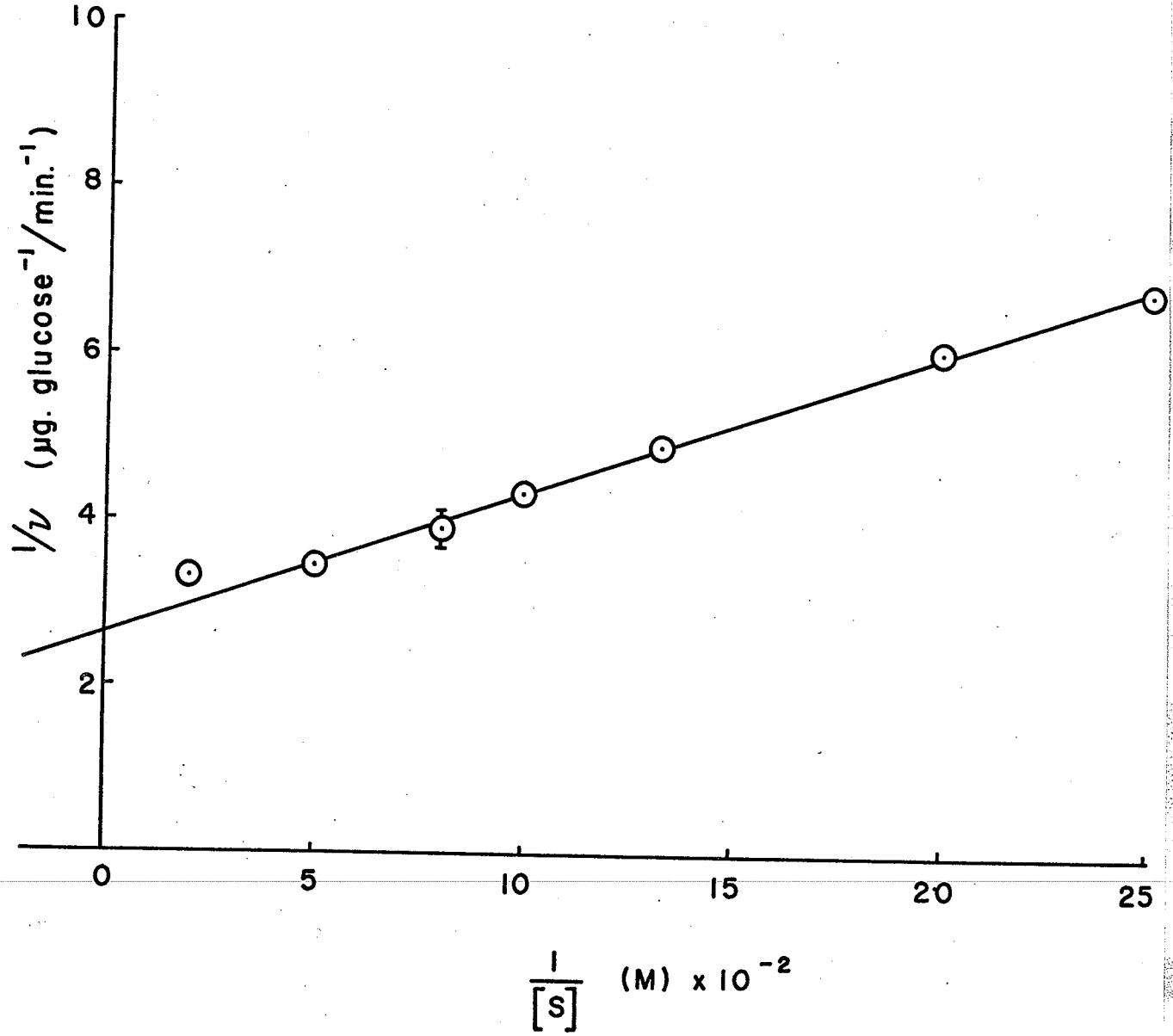

10
8
6
4
2
0
5
10
15
20
25
1/v (µg. glucose⁻¹/min.⁻¹)
1/[S] (M) x 10⁻²

FIG. 42. Double reciprocal plots at various pH values (G6P) (glucose determination).
The data of Figs. 35, 36, 37, 38, 39, 40 and 41 are redrawn on a single graph.

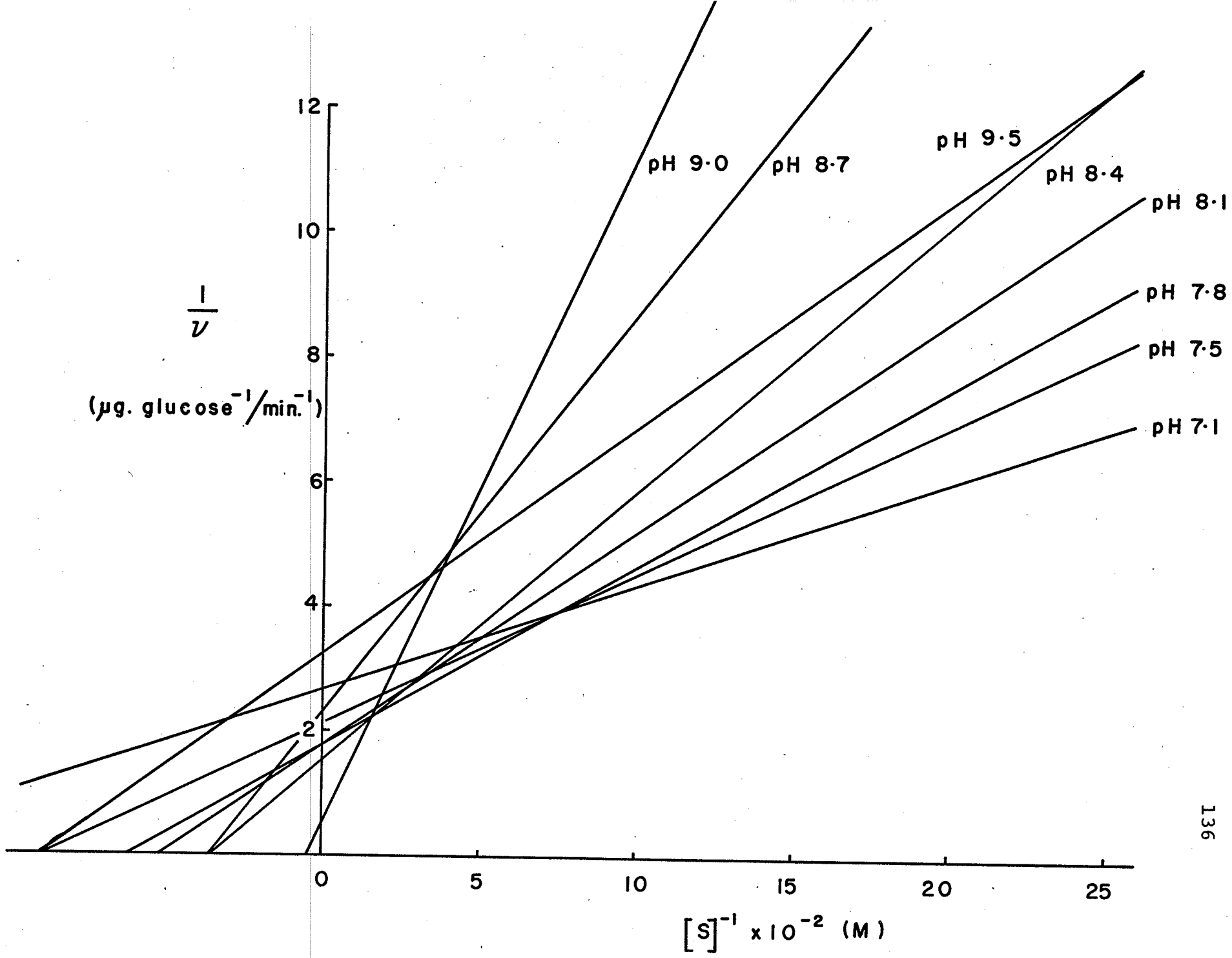

12
10
8
6
4
2
0
5
10
15
20
25
pH 9·0
pH 8·7
pH 9·5
pH 8·4
pH 8·1
pH 7·8
pH 7·5
pH 7·1
1/ν
(μg. glucose⁻¹/min.⁻¹)
[S]⁻¹ x 10⁻² (M)

TABLE XII

VALUES OF K_m AND V_{max} AT VARIOUS VALUES OF pH (G6P HYDROLYSIS).

pH	Glucose determination		Phosphate determination	
	K_m (M)	V_{max}†	K_m (M)	V_{max}*
9.5	1.27×10^{-3}	.340	9.86×10^{-4}	2.02
9.0	2.44×10^{-2}	2.26	1.38×10^{-2}	4.71
8.7	2.85×10^{-3}	.444	2.79×10^{-2}	13.70
8.4	2.96×10^{-3}	.684	4.32×10^{-3}	4.05
8.1	1.94×10^{-3}	.566	3.46×10^{-3}	4.05
7.8	1.66×10^{-3}	.572	1.36×10^{-3}	2.34
7.5	1.18×10^{-3}	.487	8.31×10^{-4}	1.82
7.1	6.33×10^{-4}	.379	8.43×10^{-4}	1.39

†µg glucose/3.2 ml of reaction/minute
*µg phosphate/3.2 ml of reaction/minute

FIG. 43. The effect of pH on K_m and V_{max} for glucose 6-phosphate hydrolysis.

The product determined was glucose. The data are those listed in Table XII.

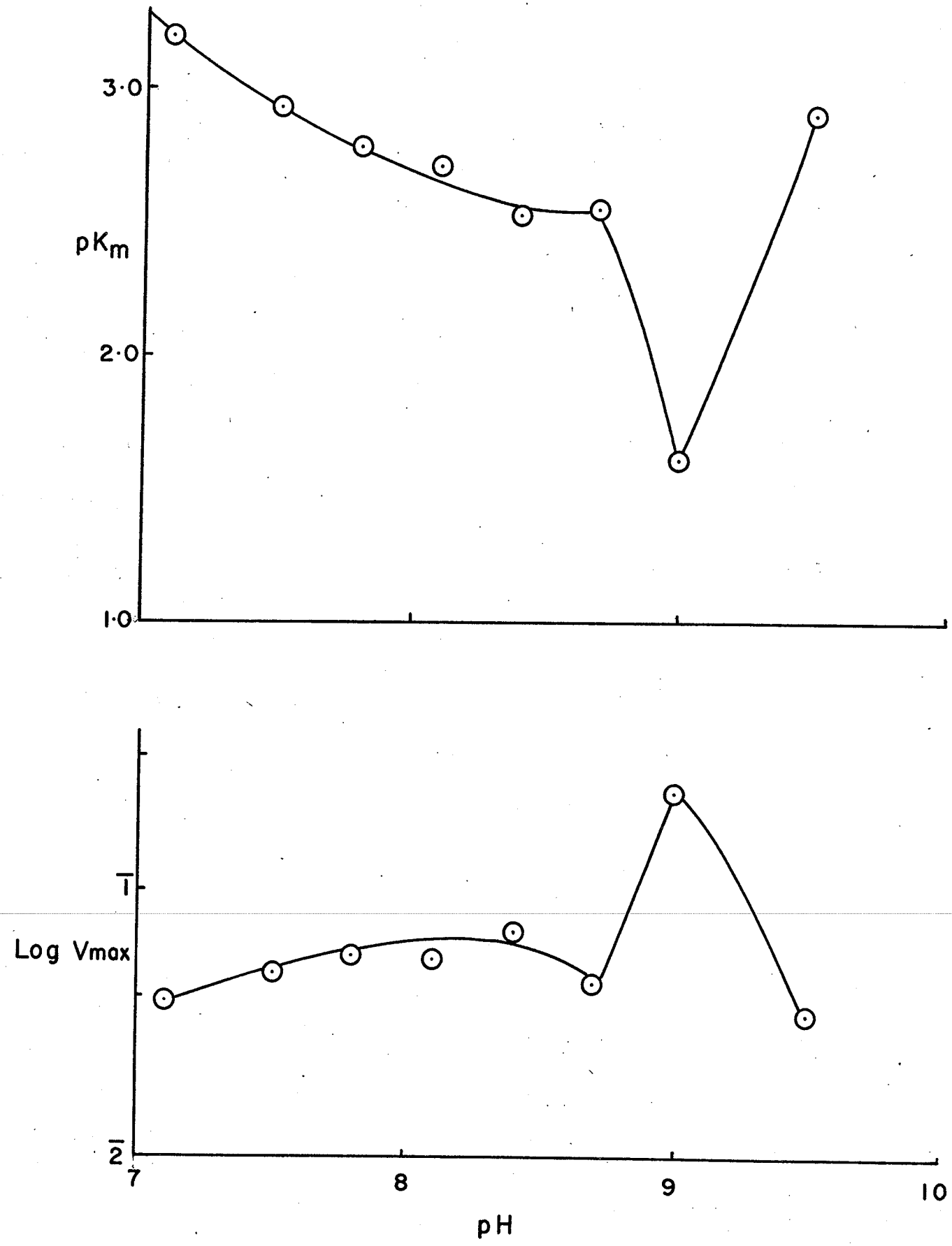
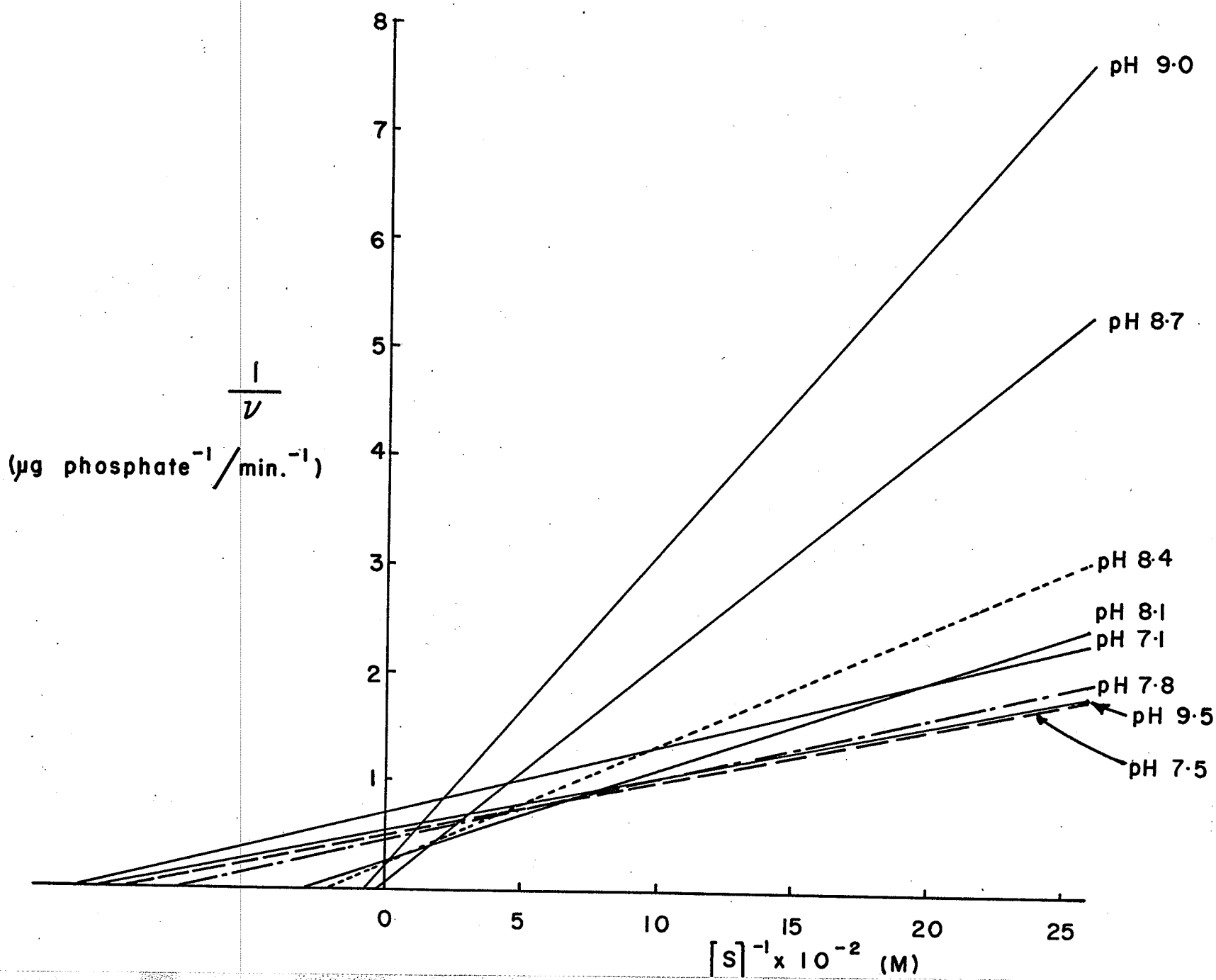

3·0
2·0
1·0
pKm
$\bar{1}$
Log Vmax
$\bar{2}$
7
8
9
10
pH

FIG. 44. Double reciprocal plots at various pH values (G6P).

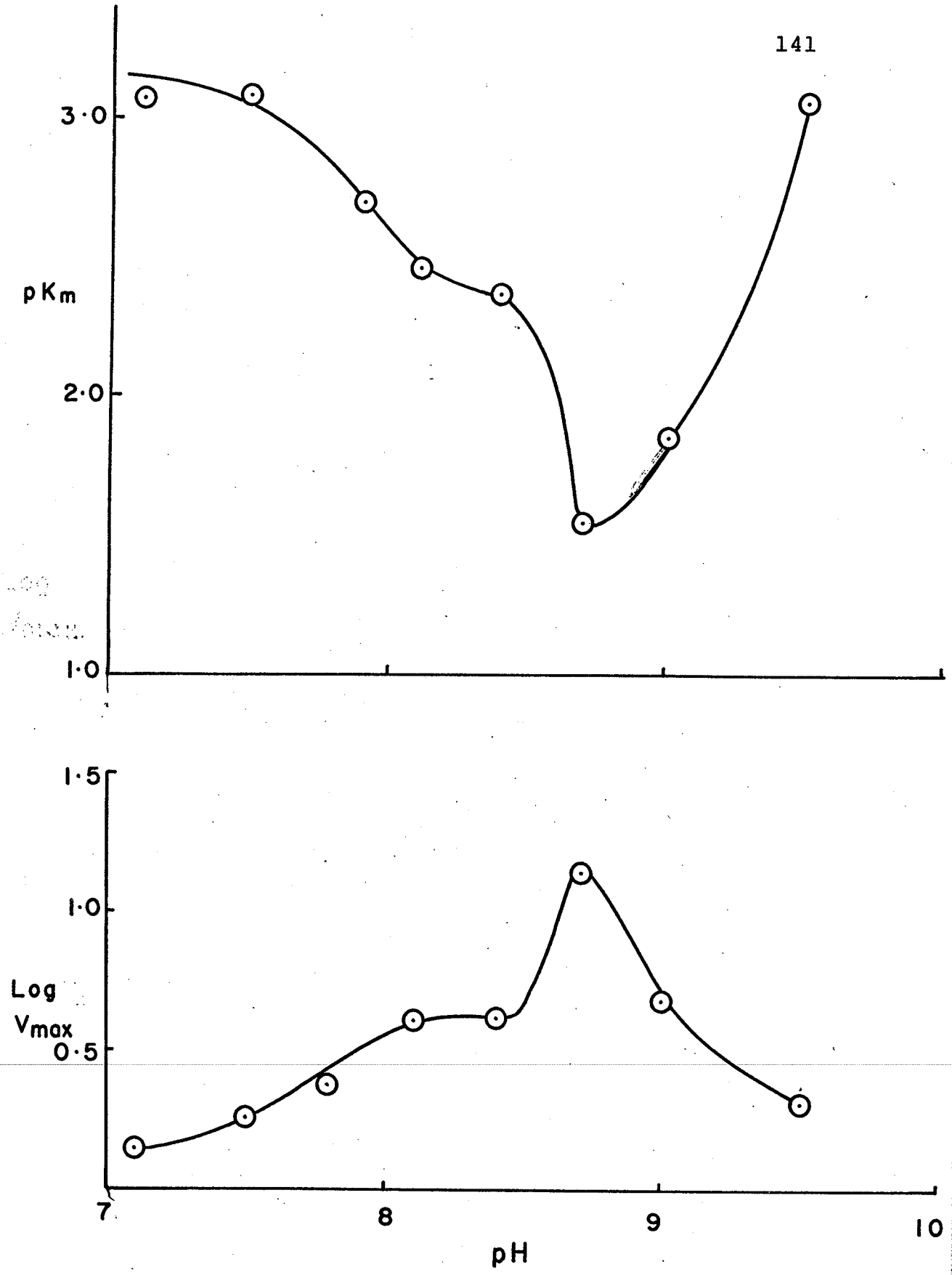
The product determined was phosphate.


8
7
6
5
4
3
2
1
0
5
10
15
20
25
1/v
(µg phosphate⁻¹ / min.⁻¹)
[S]⁻¹ x 10⁻² (M)
pH 9·0
pH 8·7
pH 8·4
pH 8·1
pH 7·1
pH 7·8
pH 9·5
pH 7·5

from the individual plots and used to prepare the Dixon plots of Fig. 45 are listed in Table XII for comparison with the data obtained by glucose determination. Due to the difference in units employed, the V_{max} figures are not comparable in the absolute sense (nor need they be, for the purposes of this experiment). The K_m values, however, which are independent of the velocity units agree very closely, and the Dixon plots are evidently of the same general form.

FIG. 45. The effect of pH on K_m and V_{max} for glucose 6-phosphate hydrolysis.

The product determined was phosphate. The data are those of Table XII.


3·0
pKm
2·0
1·0
1·5
1·0
Log
Vmax
0·5
7
8
9
10
pH

Discussion of the effects of pH on the activity of alkaline phosphatase

It appears that the effects of pH on the activity of alkaline phosphatase are extremely complex. The change in pH optimum with change in substrate concentration has been observed in many instances (Motzok, 1959; Moss *et al*, 1961; Harkness, 1968b) involving a great diversity of organisms, of tissues within any one organism, and of substrates. In all cases the optimum pH increased with increase in substrate concentration, and the phenomenon is now a well established characteristic of alkaline phosphatases. The linear relationship between optimum pH and the logarithm of substrate concentration as shown for the *Neurospora* enzyme in Figs. 19 and 34 is also well established for the enzyme from other sources (Motzok, 1959). The relationship may be used as an argument that alkaline phosphatase has a very real activity in the cell where substrate concentrations are low and the pH is probably far from the "optimum" usually measured *in vitro*. Motzok (1959) and Motzok and Branion (1959) felt that competitive inhibition by hydroxyl ions accounted for their findings, but no one has yet proposed a comprehensive solution to the problem. The same authors have studied the effect of pH on the K_m and on V_{max} for disodium phenyl phosphate hydrolysis by fowl plasma phosphatase. Their double reciprocal plots curve upward as

they approach the $\frac{1}{v}$ axis, as do those in Fig. 22, suggesting the onset of substrate inhibition. The amount of substrate inhibition is less at higher pH than it is at low pH; that is, the higher the pH the more nearly linear is the Lineweaver-Burk plot. By the unusual method of measuring velocities at the optimum pH for each substrate concentration (i.e. pH is also a variable) Motzok produced a Lineweaver-Burk plot that angled downwards as it approached the $\frac{1}{v}$ axis. The curved portion was short and represented a transition from one straight line (at dilute substrate concentrations) to another of different slope (at higher substrate concentrations). Such a resolution of the data into two portions permitted the determination of two different K_m values, one applicable to dilute substrate and one applicable to more concentrated substrate solutions. No attempt has been made to obtain such data in this investigation.

According to Dixon and Webb (1964) if a graph of $- \log K_m$ (pK_m) is plotted against pH, the results can be interpreted by a few rules, as follows:

(a) The graph will consist of straight-line sections (if the pK values are sufficiently separated) joined by short curved parts.

(b) The straight portions have integral slopes, i.e.

zero or one-unit or two-unit slopes, positive or negative.

(c) Each bend indicates the pK of an ionizing group in one of the components and the straight portions when produced intersect at a pH corresponding to the pK.

(d) At each pK a change of one unit in the slope occurs.

(e) Each pK of a group situated in the ES complex produces an upward bend, i.e. an increase of positive slope with increase of pH, or, in other words a bend with the concave side upwards. Each pK of a group situated in either the free enzyme or the free substrate produces a downward bend.

(f) The curvature at the bends is such that the graph misses the intersection of the neighbouring straight parts by a vertical distance of 0.3 units (= log 2); if two pKs occur together the distance is equal to log 3.

(g) The slope of any straight-line section is numerically equal to the change of charge occurring in that pH range when the complex dissociates into free enzyme and substrate.

One cannot distinguish in this way between ionizations of the free enzyme and those of the free substrate, but those of the free substrate can usually be determined by other means. The rules are independent of assumptions

as to which forms of the components react. It is not necessary to be able to write the equations for a given system at all in order to interpret the pK_m *versus* pH curves by means of the rules.

A wave may be produced in the graph when an ionizing group is not actually involved in the link between enzyme and substrate, but when the effect of the combination is to produce a change in its pK. When the vertical and horizontal scales are the same the difference in level due to the wave is equal to the change in the pK on combination. When the combination is actually with the ionizing group itself, its ionization is usually entirely suppressed and an angle in the graph (half a wave) is produced.

The effects on K_m of the ionizations of all groups which ionize equally strongly in the free enzyme and in the enzyme substrate complex cancel out, though they may affect V_{max} and appear in the log V_{max} curve. This means that only those groups which are intimately associated with the active center are revealed by the graphs. Ionizing groups in the protein which are remote from the active center produce no disturbing effect. The rules are valid for K_m under both Michaelis-Menten and Briggs-Haldane conditions.

A few pK_m *versus* pH curves for alkaline phosphatase appear in the literature. One, given by Dixon and Webb

(1964) from the data of Morton (1957) covers the pH range from about 8.6 to 10. It consists of a concave downwards curve (two straight lines joined by a short curved portion) and yields an ionization constant for the free enzyme (pK_e) at pH 9.3. Lazdunski and Lazdunski (1966) have prepared similar curves for alkaline phosphatase from *E. coli* with PNPP, glucose 1-phosphate and β-glycerophosphate as substrates. They found it necessary to consider three different ionization states for the active center. The pKs of the ionizations

$$EH_2 \rightleftharpoons EH + H^+ \text{ and } EH \rightleftharpoons E + H^+$$

were found to be 7.1 and 8.6 respectively. The substrate bound to EH only. Inorganic phosphate also bound to the EH form. The pK_m *versus* pH curves for the three substrates are similar in shape, concave downward curves without waves or irregularities. Although these authors give the pK for p-nitrophenol as 7.15 at 25° they do not indicate that they made corrections to the velocity measurements necessitated by making these measurements over a wide pH range. Such corrections have no effect on the K_m, but they do affect V_{max}, and should be taken into account in graphing any function of V_{max}. On a log scale, however, the necessary corrections are small and have their greatest effect at one

end of the pH range.

Melani *et al* (1967) working with rat kidney phosphatase present a pK_m *versus* pH curve over the pH range of 8.5 to 10.3, with PNPP as substrate. An angle appears in the graph, indicating a pK of 8.9 for a group involved in enzyme-substrate complex formation. The discontinuity at pH 8.9 is close to the value of 9.2 observed by Dabich and Neuhaus (1966) working with alkaline phosphatase from synovial fluid. The results presented in Figs. 31, 43 and 45 show that the pK_m *versus* pH curves for PNPP and glucose 6-phosphate are not identical. The work presented in Phase I indicates that there are differences in the kinetics of the reaction when different substrates are used. These observations are in opposition to the statement by Lazdunski and Lazdunski (1966) that the catalytic mechanism is identical for the three substrates they studied. The statement may indeed be true for the *E. coli* enzyme but is certainly not true for the *Neurospora* alkaline phosphatase.

Kadner and Nyc (1969) have prepared Dixon plots for the repressible enzyme in *Neurospora crassa* (see Historical section) in the presence and absence of EDTA and with PNPP as substrate. Their log V_{max} curve had a plateau in the pH 7-8 range, as is shown for the non-repressible enzyme in Fig. 31. The presence of EDTA tended to eliminate the

plateau, changing the curve to one of nearly constant slope. The pK_m curve had one bend rather than the three bends apparent in Fig. 31.

No comprehensive interpretation of the Dixon plots presented here has yet been possible. Some general observations, however, can be made. It appears useful to consider the data of Fig. 31 as being composed of two parts, separated by a vertical line through pH 8.0. Approaching pH 8.0 from the acid side both K_m and V_{max} increase rapidly as the pH approaches 7.3 (approx.) which is a pK for the ES complex. On the alkaline side of the pK both K_m and V_{max} remain at a constant maximum value as the pH increases to 8.0.

A pK for the free enzyme occurs at approximately 8.2. As the group involved becomes more ionized with increase in pH the affinity of the enzyme for PNPP decreases (K_m increases). On the alkaline side of the pK both K_m and V_{max} rapidly increase. Again the increase is stopped by the occurrence of a *second* pK of the ES complex, at about 8.6, and thereafter both reaction parameters remain constant.

It is now possible to gain a better understanding of the shift in pH optimum that occurs with changes in substrate (PNPP) concentration. At any pH three factors work together to influence the actual velocity of the

reaction:- (a) the affinity ($\frac{1}{K_m}$) of the enzyme for its substrate, (b) inhibition by excess substrate and (c) the rate of breakdown (V_{max}) of the ES complex to yield products. The algebraic sum of these three contributions represents the actual velocity. The optimum pH is simply the pH at which the actual velocity is greatest for any given substrate concentration. Put another way, at any pH there is only one substrate concentration for which the algebraic sum of the three factors is maximum.

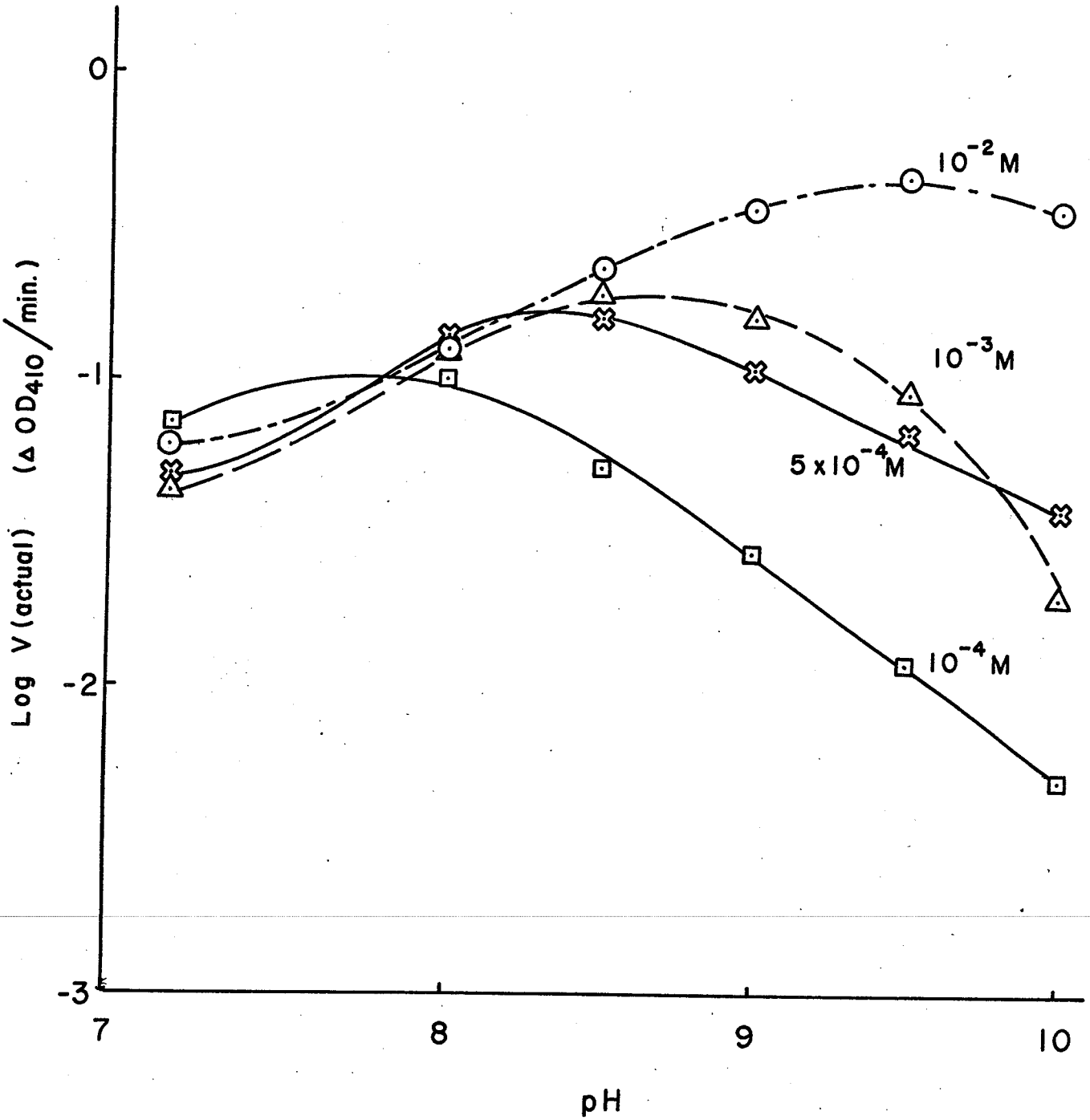
At low pH substrate inhibition is very marked (see Fig. 22). Release of inhibition by a lowering of the substrate concentration increases the actual velocity. Because the K_m is also low the enzyme remains saturated and there is no loss of velocity from this factor. At low pH, then, one expects optimum substrate concentrations to be low. The actual velocity will also be low because V_{max} is low.

At high pH substrate inhibition occurs only at the upper end of the whole range of substrate concentrations tested. The enzyme can, therefore, successfully deal with large substrate concentrations. Indeed, high concentrations become necessary since the K_m increases 200 fold in the pH range 7-8.7. The actual velocity will also be high since V_{max} increases with pH.

The actual velocity for any given substrate range may either increase or decrease with increase in pH, depending on the range of pH involved. One would predict, from the pK_m *versus* pH, and log V_{max} *versus* pH curves shown in Fig. 31 that (1) actual velocities for all substrate concentrations would increase with pH from pH 7.0 to about 7.5., (2) in the pH range occupied by the horizontal portions of the curves actual velocities would be independent of substrate concentration, (3) actual velocities for low substrate concentrations would decrease, and actual velocities for high substrate concentrations would increase with increase of pH beyond about 8.0 and (4) in the pH range occupied by the horizontal portions of the curves (above pH 9.0) the actual velocity should be a function of the degree of saturation of the enzyme by substrate. It would, therefore, decrease rapidly with increase of pH for low concentrations and decrease more slowly for higher concentrations of substrate.

To check these predictions some of the data obtained in the preparation of the pH/activity curves of Fig. 18 have been replotted. Fig. 46 plots the logarithm (to fit the data range on the page) of experimental values of velocity at four substrate concentrations, as a function of pH. Each of the 4 predictions seems to be correct.

FIG. 46. Variation of reaction velocities at four PNPP concentrations as a function of pH.
Data are the absolute experimental values of the velocities (v) plotted in a different way in Fig. 18.


0
-1
-2
-3
Log V (actual) (Δ OD410 / min.)
10^-2 M
10^-3 M
5 x 10^-4 M
10^-4 M
7
8
9
10
pH

The Dixon plots for glucose 6-phosphate (Figs. 43 and 45) are different from those for PNPP, but there are also some similarities. Fig. 43 shows two pKs for the ES complex at about 7.9 and 9.0, and one for the free enzyme at about 8.7 (pK_e). Fig. 45 yields values for the corresponding pKs of about 7.9, 8.8 and about 8.5. For convenience all pK values are listed in Table XIII.

TABLE XIII

VALUES FOR pKs TAKEN FROM FIGS. 31, 43 AND 45

	PNPP as substrate (Fig. 31)	Glucose 6-P as substrate	
		glucose determination (Fig. 43)	Pi determination (Fig. 45)
pK_{es}	7.3, 8.2	7.9, 9.0	7.9, 8.8
pK_e	8.2	8.7	8.5

The value of pK_e should be independent of the method of determination. Values reported here are well within the limits of error expected from the methods used. There is of course, no reason why pK values for different substrate-enzyme complexes should be the same.

The shape of the curves for glucose 6-phosphate (especially Fig. 45) below pH 8.7 are very similar to the curves for PNPP. Above pH 8.7, however, where the K_m and V_{max} for PNPP remain constant, the K_m and V_{max} for glucose

6-phosphate both decrease dramatically. With these differences in mind, the effect of pH on glucose 6-phosphate hydrolysis is qualitatively the same as its effect on PNPP hydrolysis. A precise mathematical formulation of the pH effects has not been found.

The ability of various substances to act as substrates for alkaline phosphatase.

Eighteen different compounds were tested as substrates for the purified enzyme. The results are listed in Table XIV. The enzyme hydrolyzes a wide variety of monophosphorylated substances, but has no effect on inorganic pyrophosphate, ADP or ATP. No activity was observed with p-nitrophenylsulfate. These results are typical of alkaline phosphatases from all sources. No valid comparison can be made between the tabled rate of reaction for one substrate with the rate for another. While the chemical environment was constant for all substrates the kinetic parameters (e.g. K_m) for each may be very different.

All assays were conducted in $3x10^{-2}$M Bicine, $4x10^{-3}$M $MgCl_2$ at pH 9.0 in a final volume of 3 ml. The assay using p-nitrophenyl phosphate was performed spectrophotometrically at room temperature. Reactions with all other substances were run at 28°. Phosphate determinations

were made at 10, 20 and 30 minutes (on samples of the reaction) to ensure that the rate remained constant with time.

TABLE XIV

HYDROLYSIS OF VARIOUS SUBSTANCES BY ALKALINE PHOSPHATASE

Substrate	Concentration (M)	Reaction rate	
β-Glycerophosphate	0.03	0.56	µg Pi/ml/min.
α-Glycerophosphate	0.03	0.85	"
Acetyl phosphate	0.005	Trace	"
Phosphoenol pyruvate	0.03	1.31	"
Glucose 1-phosphate	0.03	1.20	"
Glucosamine 6-phosphate	0.03	0.35	"
Glucose 6-phosphate	0.03	0.92	"
O-phospho D,L-serine	0.03	0.93	"
Inorganic pyrophosphate (tetra-sodium salt)	0.015	nil	"
6 phosphogluconate	0.015	0.66	"
NADP	0.03	0.86	"
AMP	0.03	0.33	"
ADP	0.03	Trace	"
ATP	0.03	Trace	"
D,L O-phosphothreonine	0.03	0.55	"
D galactose 6-phosphate	0.03	2.20	"
p-nitrophenyl phosphate	0.03	1.40	"
p-nitrophenyl sulfate	0.02	nil	ΔOD_{410}/min.

Molecular weight determinations

Data relating to the molecular weight of alkaline phosphatase obtained in Phase II required re-examination. Accordingly, the molecular weight was investigated under

various conditions in an attempt to clarify the earlier data.

The molecular weight of the purified enzyme was determined by the method of Martin and Ames (1961) on a 4% - 20% sucrose density gradient in $5x10^{-3}$M Bicine at pH 7.5 and 9.0. The enzyme was dialyzed for 3 hours against the appropriate buffer, mixed with bovine hemoglobin and yeast alcohol dehydrogenase as standards and was layered onto the gradient in cellulose nitrate centrifuge tubes. The tubes were spun at 50,000 rpm. for 5 hours in the SW 50.1 swinging bucket rotor using a Beckman model L2-65B ultracentrifuge. After the run equal fractions were collected through a small hole in the bottom of each tube. The fractions were assayed for alkaline phosphatase, alcohol dehydrogenase and hemoglobin. A typical distribution of the three substances over the fractions of one gradient is presented in Fig. 47. Altogether 6 gradients were prepared, run, and analyzed.

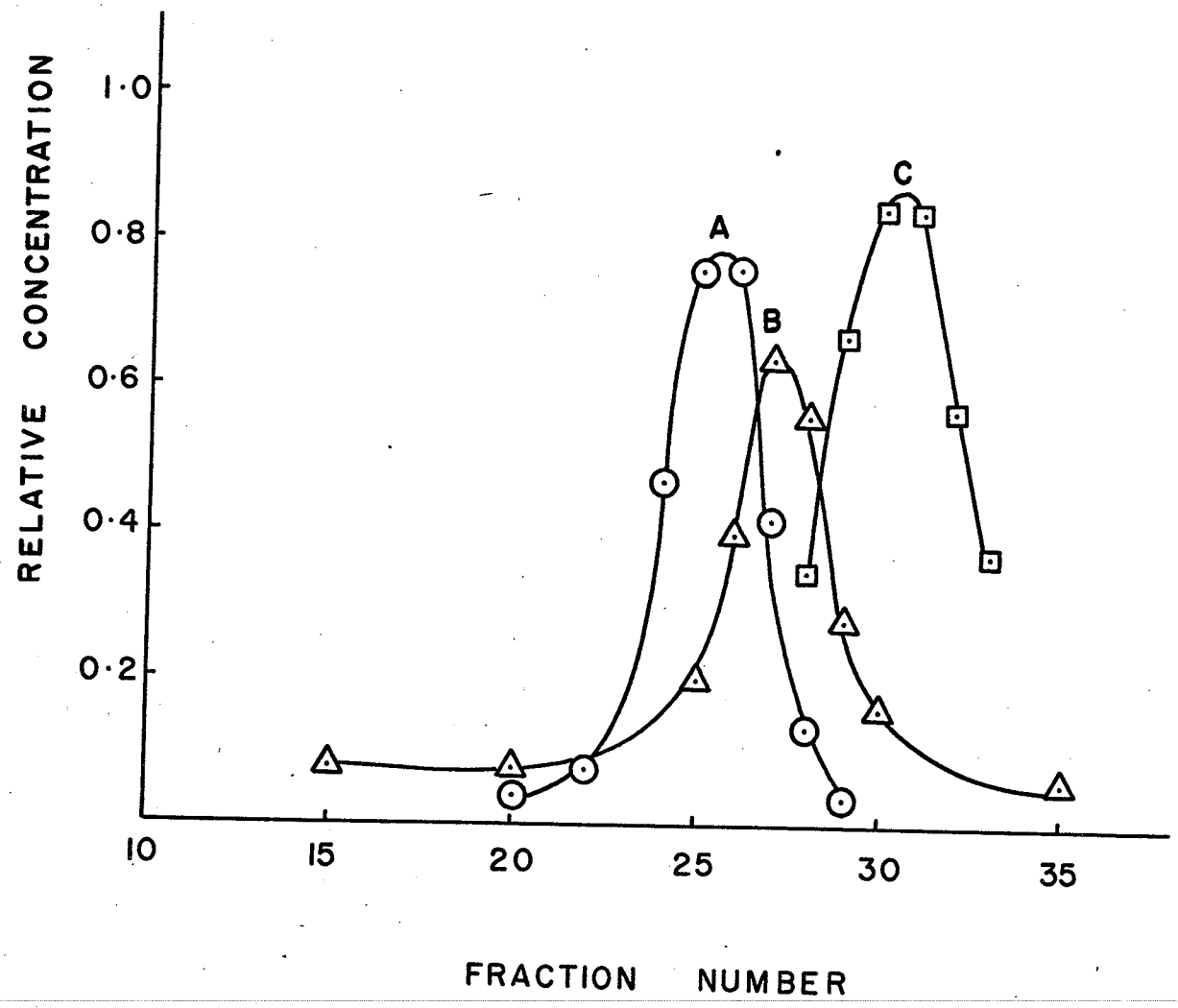
These yielded twelve estimates of the molecular weight, which are listed in Table XV. An additional eight gradients at pH 7.5 were run in an attempt to determine the molecular weights of both enzymes in the crude extract. Only one of the enzymes could be detected, however, and the failure to detect the other was not explained, but was

FIG. 47. The concentrations of purified alkaline phosphatase, alcohol dehydrogenase and bovine hemoglobin as distributed throughout a sucrose density gradient.

(Gradient #1 of Table XV).

A alcohol dehydrogenase
B alkaline phosphatase
C hemoglobin.

The total number of fractions was 38.


RELATIVE CONCENTRATION
1·0
0·8
0·6
0·4
0·2
A
B
C
10
15
20
25
30
35
FRACTION NUMBER

probably due to low activity. The average molecular weight of the detected activity was 107,000 which identified it as being the same as the purified enzyme. Taking these data in combination with the results in Table XV the average value obtained for the molecular weight of the purified enzyme was 112,000. It is possible that the highly purified enzyme is a tetramer of the subunit of molecular weight near 30,000 proposed in the discussion of Phase II.

TABLE XV

DETERMINATION OF THE MOLECULAR WEIGHT OF PURIFIED ALKALINE PHOSPHATASE

Gradient #	pH	Molecular weight	
		Using Hemoglobin MW = 68,000	Using alcohol dehydrogenase MW = 145,000
1	7.5	118,200	117,700
2	7.5	98,540*	110,400
3	7.5	113,800	111,800
4	9.0	117,300	114,800
5	9.0	145,000*	106,800
6	9.0	114,900	106,500
Average MW		116,000	111,300
Overall Average		113,200	
Range		106,500 -	118,200

*Anomalous values omitted in calculating the averages.

Product inhibition studies

The kinetic studies performed in Phase I indicated a need for more detailed data relating to product inhibition of alkaline phosphatase. Such studies were performed with both p-nitrophenyl phosphate and glucose 6-phosphate.

Inhibition of p-nitrophenyl phosphate hydrolysis by p-nitrophenol

Early experiments (see Fig. 9) indicated that PNP inhibition was non-linear, and there was the possibility that the slope and intercept replots (Figs. 10 and 11) were parabolic. To investigate these details it was necessary to prepare Lineweaver-Burk plots at more concentrations of PNP than had been done earlier. The results are presented in Fig. 48 and the replots of slopes and intercepts are presented in Fig. 49.

Inhibition of p-nitrophenyl phosphate hydrolysis by phosphate

The experiment performed in Phase I was repeated with the pure enzyme. The results are presented in Fig. 50 and a replot of the slopes is presented in Fig. 51.

Inhibition of p-nitrophenyl phosphate hydrolysis by glucose

Initial velocities of p-nitrophenol production in the presence of glucose were determined in the SP 700 spectrophotometer by measuring the increase in optical density at

FIG. 48. Inhibition of p-nitrophenyl phosphate hydrolysis by p-nitrophenol.

Assays were performed in the SP 700 spectrophotometer. Velocity was measured as ΔOD_{410}/min. Each cuvette contained the following concentration of substances in a final volume of 3.0 ml, at pH 9.5.

Bicine 5×10^{-2}M
$MgCl_2$ 4×10^{-3}M
Enzyme 20 units,
PNPP varied as indicated.

The various PNP concentrations were:-

A - 1.3×10^{-4}M
B - 1.248×10^{-4}M
C - 1.201×10^{-4}M
D - 1.149×10^{-4}M
E - 1.103×10^{-4}M
F - 1.004×10^{-4}M
G - 9.0×10^{-5}M
H - 7.02×10^{-5}M
I - None

Blanks containing the same amounts of p-nitrophenol were present in the reference cuvettes for each line.

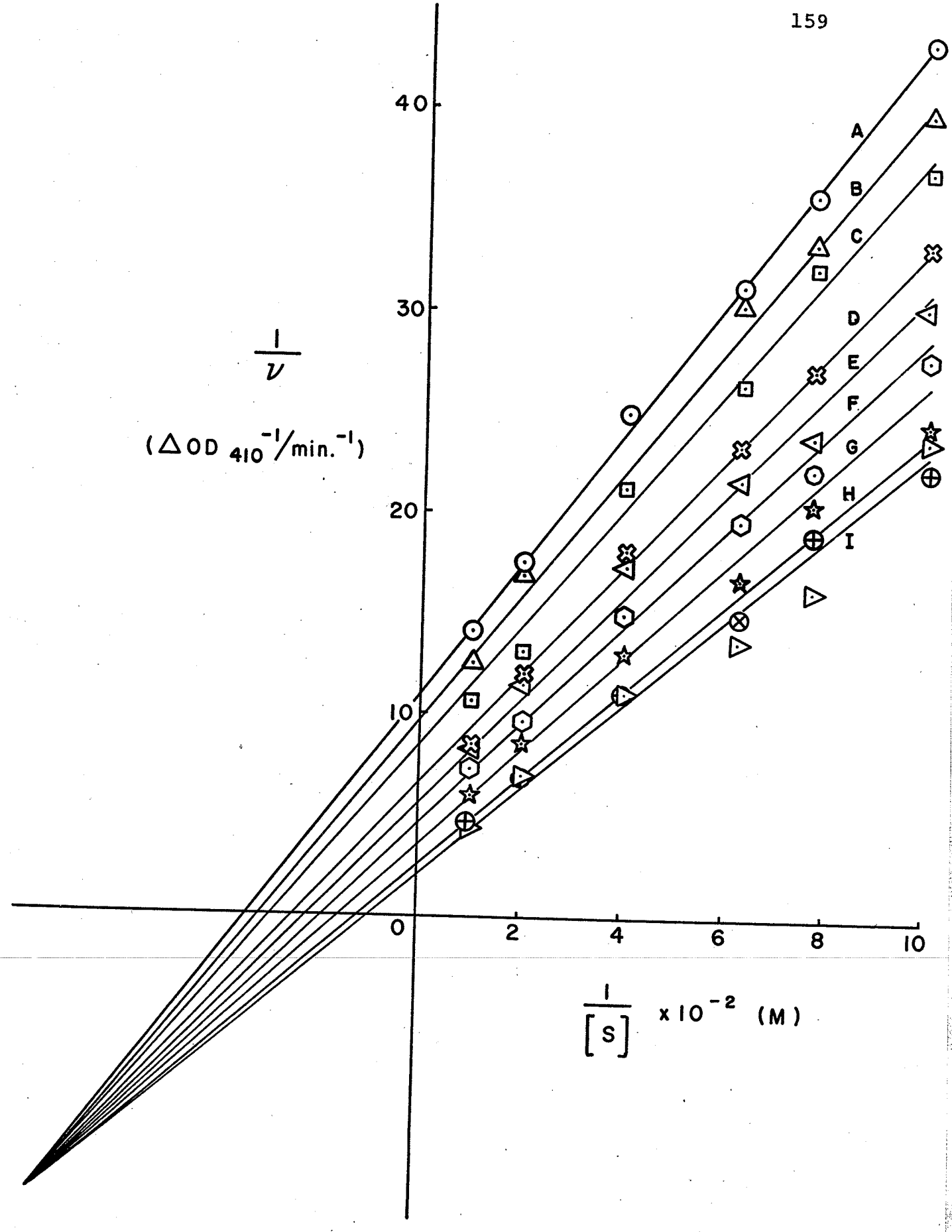
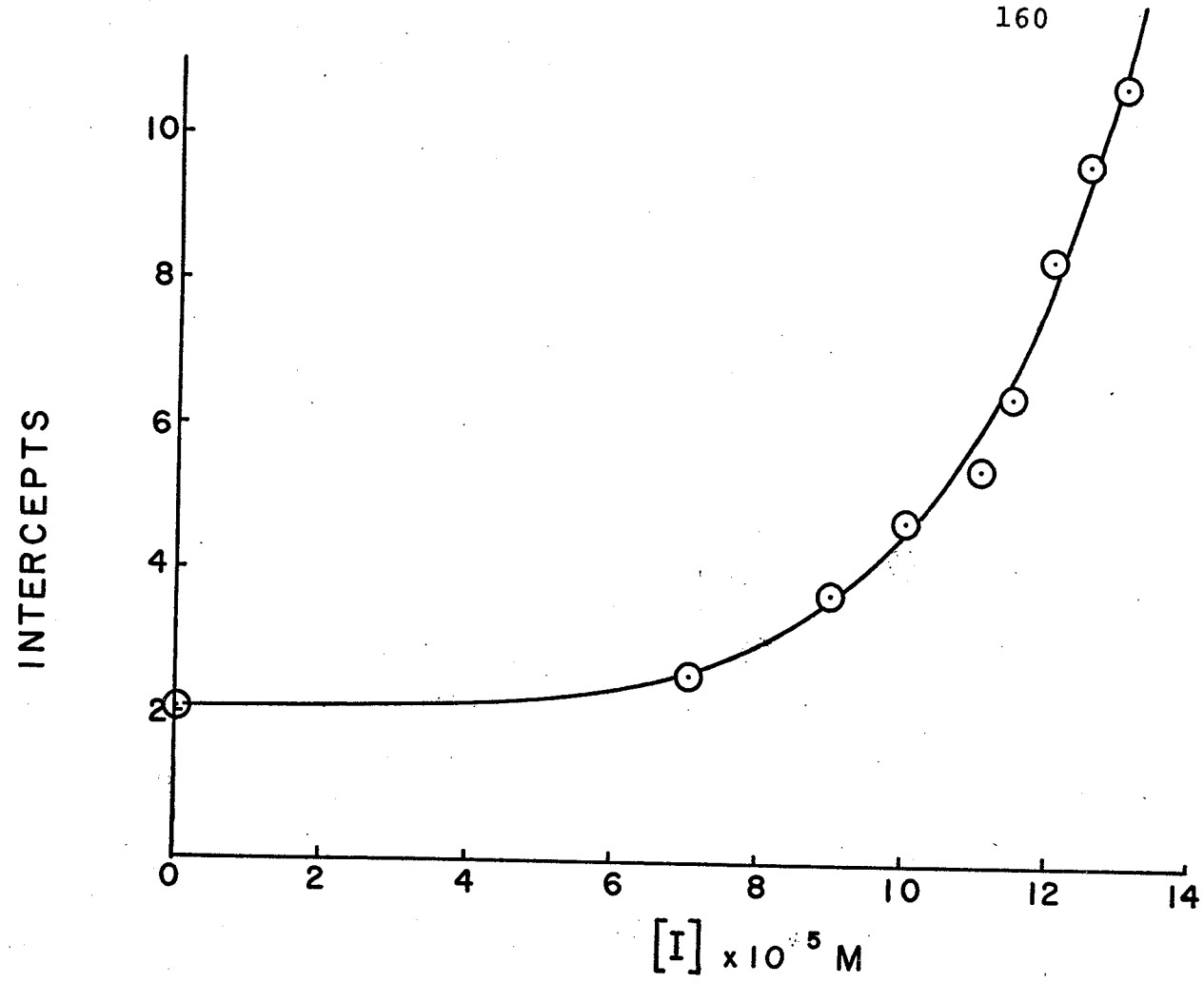

40
30
20
10
0
2
4
6
8
10
A
B
C
D
E
F
G
H
I
$\frac{1}{v}$
$(\Delta OD_{410}^{-1}/min.^{-1})$
$\frac{1}{[S]} \times 10^{-2}$ (M)

FIG. 49. Replots of the slopes and intercepts of Fig. 48 as a function of p-nitrophenol concentration.


INTERCEPTS
10
8
6
4
2
0
2
4
6
8
10
12
14
[I] x 10 5 M

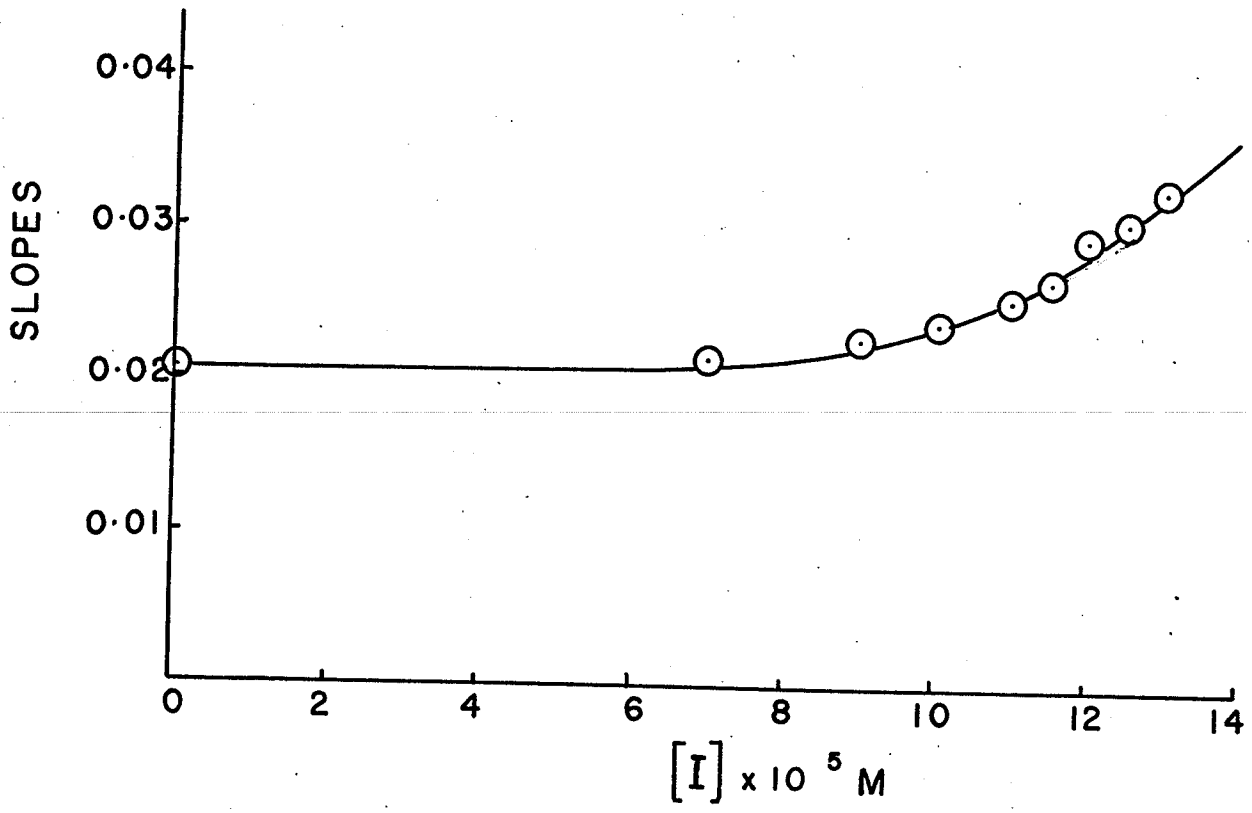

SLOPES
0·04
0·03
0·02
0·01
0
2
4
6
8
10
12
14
[I] x 10 5 M

FIG. 50. Inhibition of p-nitrophenyl phosphate hydrolysis by phosphate.

The conditions for each assay were the same as those used in the p-nitrophenol inhibition experiment except that the concentration of $MgCl_2$ had to be reduced to 1.2×10^{-3}M to prevent the precipitation of magnesium phosphate. The phosphate concentrations were:

A No inhibitor
B 8.33×10^{-3}M
C 1.667×10^{-2}M
D 3.33×10^{-2}M.

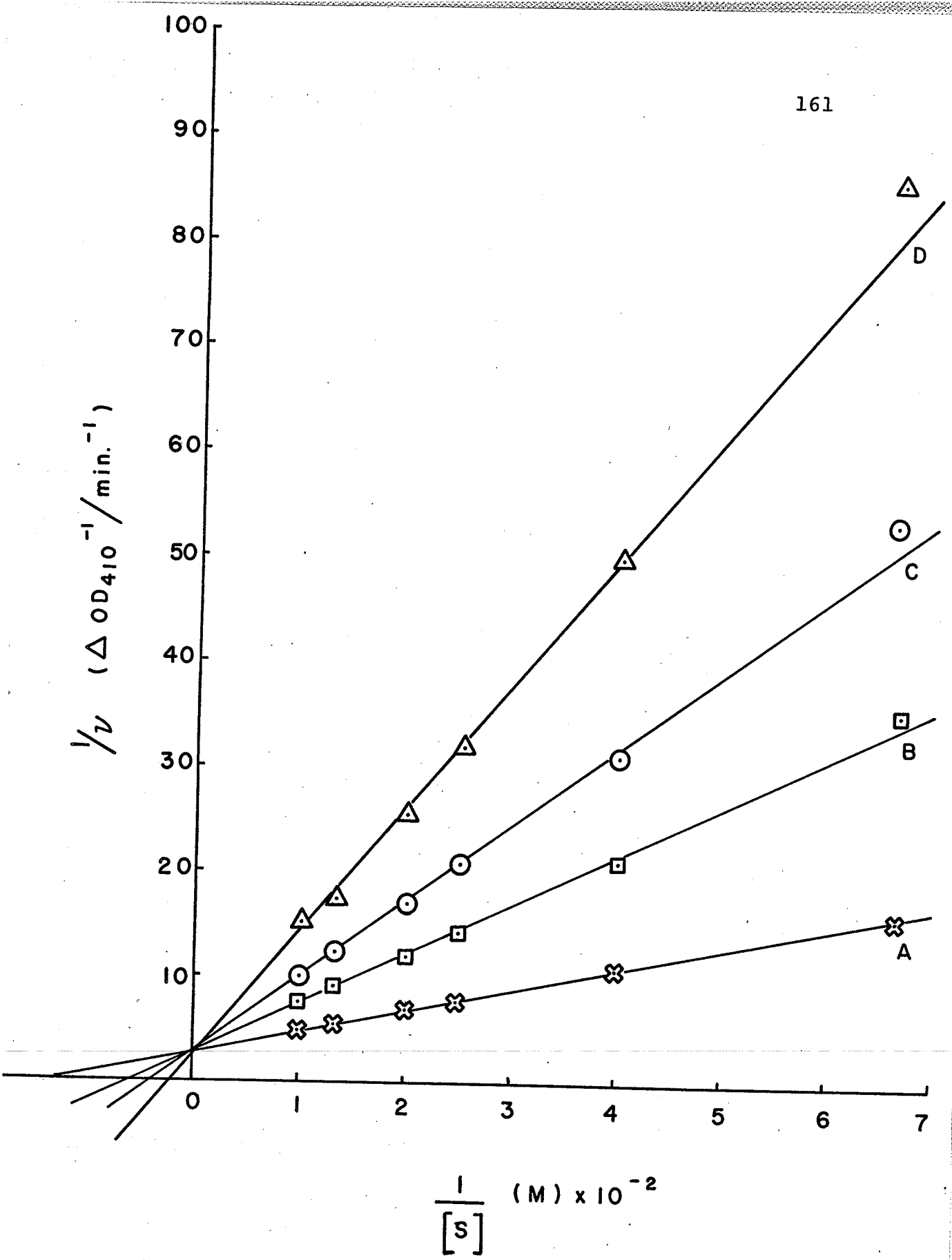
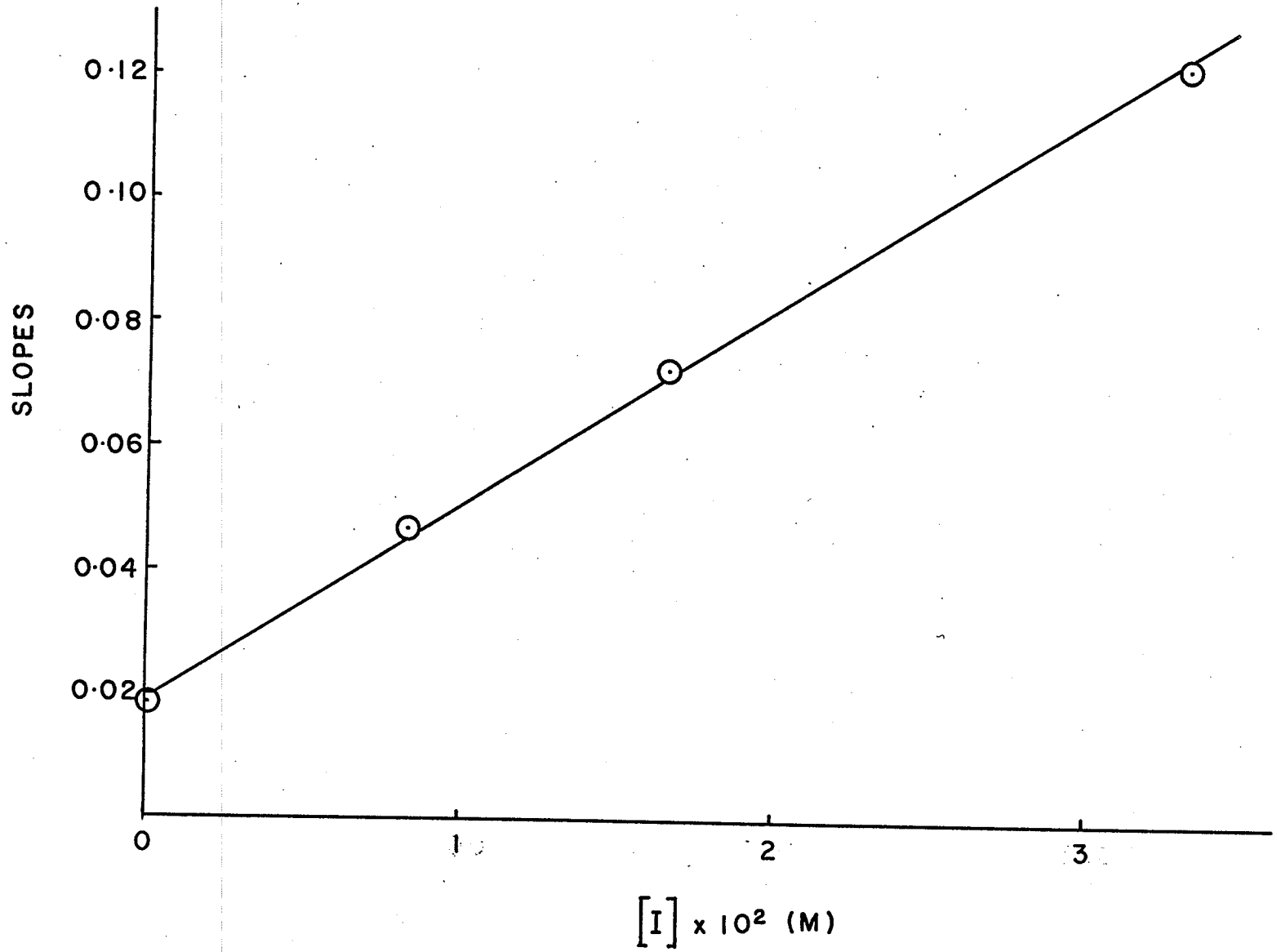

100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
1/v (Δ OD410 -1/min.-1)
1/[S] (M) x 10-2
A
B
C
D

FIG. 51. The slopes of Fig. 50 plotted as a function of inhibitor (Pi) concentration.


SLOPES
0·12
0·10
0·08
0·06
0·04
0·02
0
1
2
3
[I] x 10² (M)

410 mμ. The data are plotted in the double reciprocal form in Fig. 52. A replot of the slopes is presented in Fig. 53. The reciprocal experiment, p-nitrophenol inhibition of glucose 6-phosphate hydrolysis, was impractical. Concentrations of p-nitrophenol up to $3x10^{-3}$M failed to produce any inhibition.

Inhibition of glucose 6-phosphate hydrolysis by phosphate

The rate of production of glucose from glucose 6-phosphate was measured at four different concentrations of inorganic phosphate. Each reaction was run in duplicate, together with a control containing no enzyme for 30 minutes at 30°. The reaction was stopped by the addition of 0.2 ml of 10% TCA. The pH was then returned to near neutrality by the addition of 0.175 ml of 0.05 N NaOH. Aliquots from each reaction and from the control were analyzed for glucose by the Glucostat method. The results are presented in Fig. 54. A replot of the slopes of Fig. 54 is presented in Fig. 55.

Inhibition of glucose 6-phosphate hydrolysis by glucose

The rate of production of phosphate from glucose 6-phosphate was measured at four different concentrations of glucose. Each reaction was run in duplicate together with a control containing no enzyme, for 30 minutes at 30°. The

FIG. 52. Inhibition of p-nitrophenyl phosphate hydrolysis by glucose.

Velocities, expressed as ΔOD_{410}/min. were determined in the SP 700 spectrophotometer. Each cuvette contained the following concentrations of substances:

Bicine 5×10^{-2}M
$MgCl_2$ 4×10^{-3}M
p-nitrophenyl phosphate varied as indicated,
Enzyme 20 units in a final volume of 3.0 ml
at pH 9.5.

The concentrations of glucose were:

A 0.5 M
B 0.3 M
C 0.1 M
D no inhibitor.

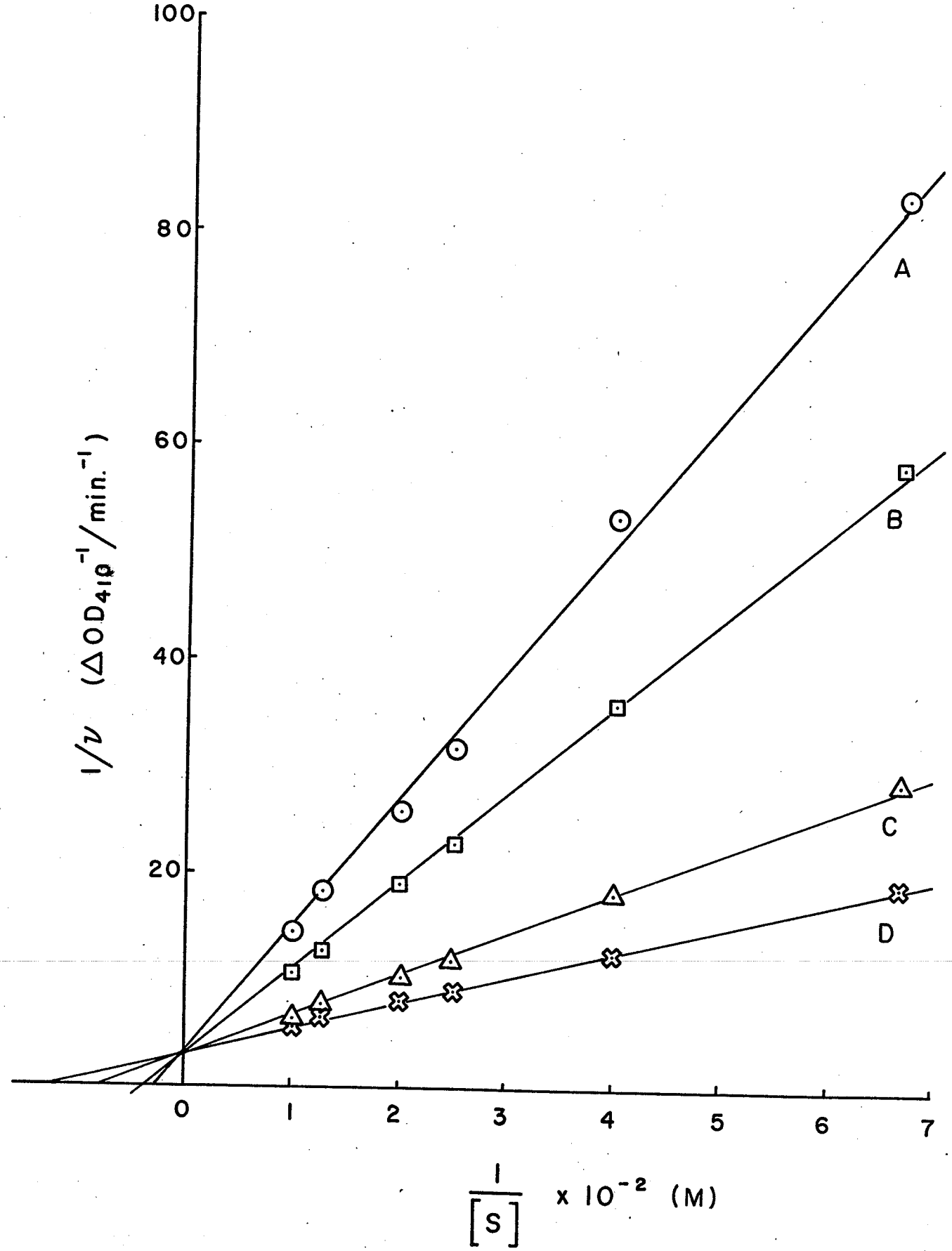

100
80
60
40
20
0
1
2
3
4
5
6
7
A
B
C
D
1/ν (ΔOD410^-1/min.^-1)
1/[S] x 10^-2 (M)

FIG. 53. The slopes of Fig. 52 as a function of glucose concentration.

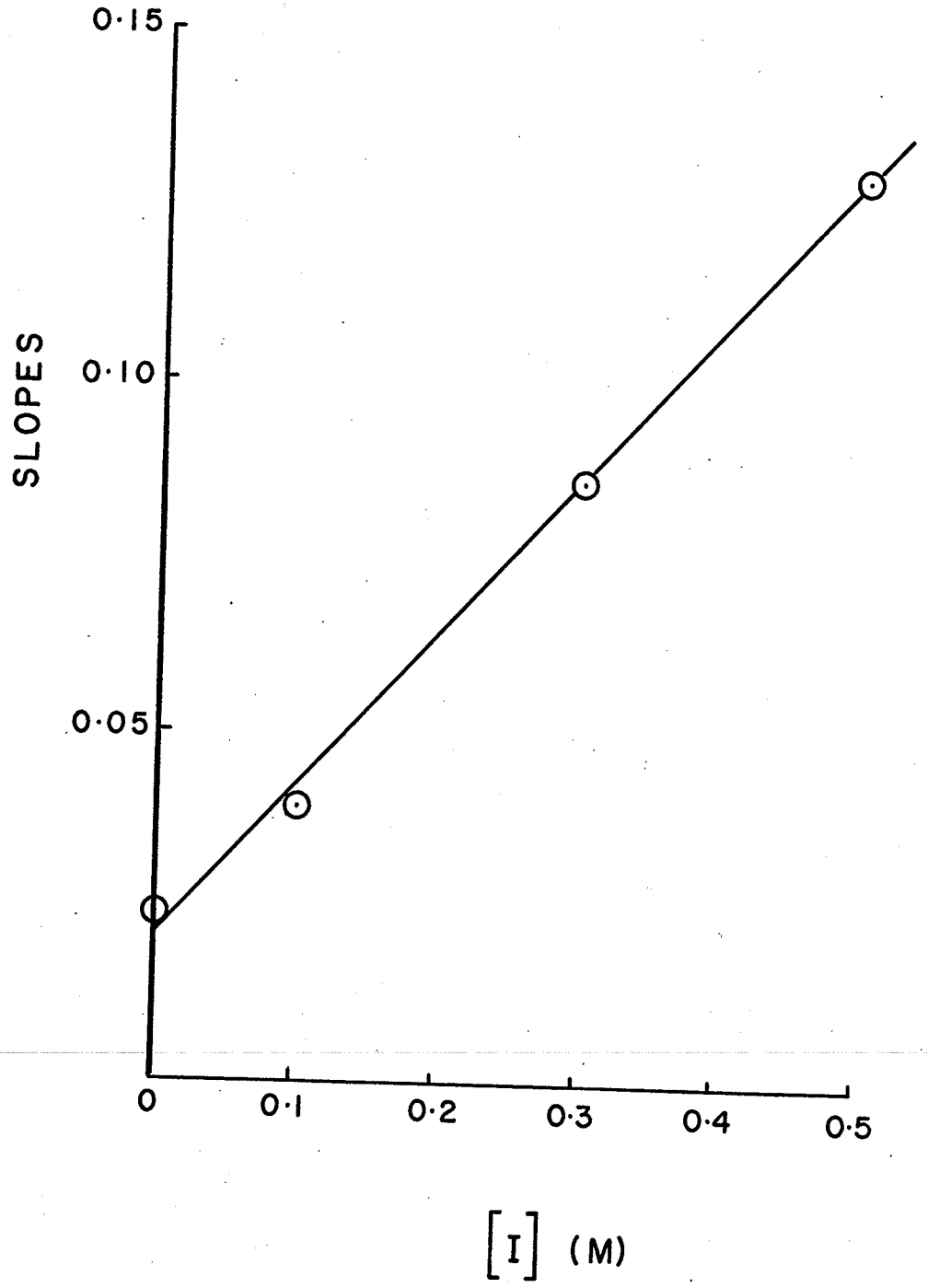

0·15
0·10
0·05
SLOPES
0
0·1
0·2
0·3
0·4
0·5
[I] (M)

FIG. 54. Inhibition of glucose 6-phosphate hydrolysis by phosphate.

Each reaction vessel contained in a final volume of 3.0 ml the following concentrations of substances, at pH 8.0:

Bicine 3×10^{-2}M
$MgCl_2$ 4×10^{-3}M
Glucose 6-phosphate varied as indicated,
Enzyme 50 units.

Inorganic phosphate

A - 2×10^{-3}M
B - 8×10^{-4}M
C - 3×10^{-4}M
D - None

The reaction was started by the addition of enzyme, and ran for 30 minutes at 30°C.

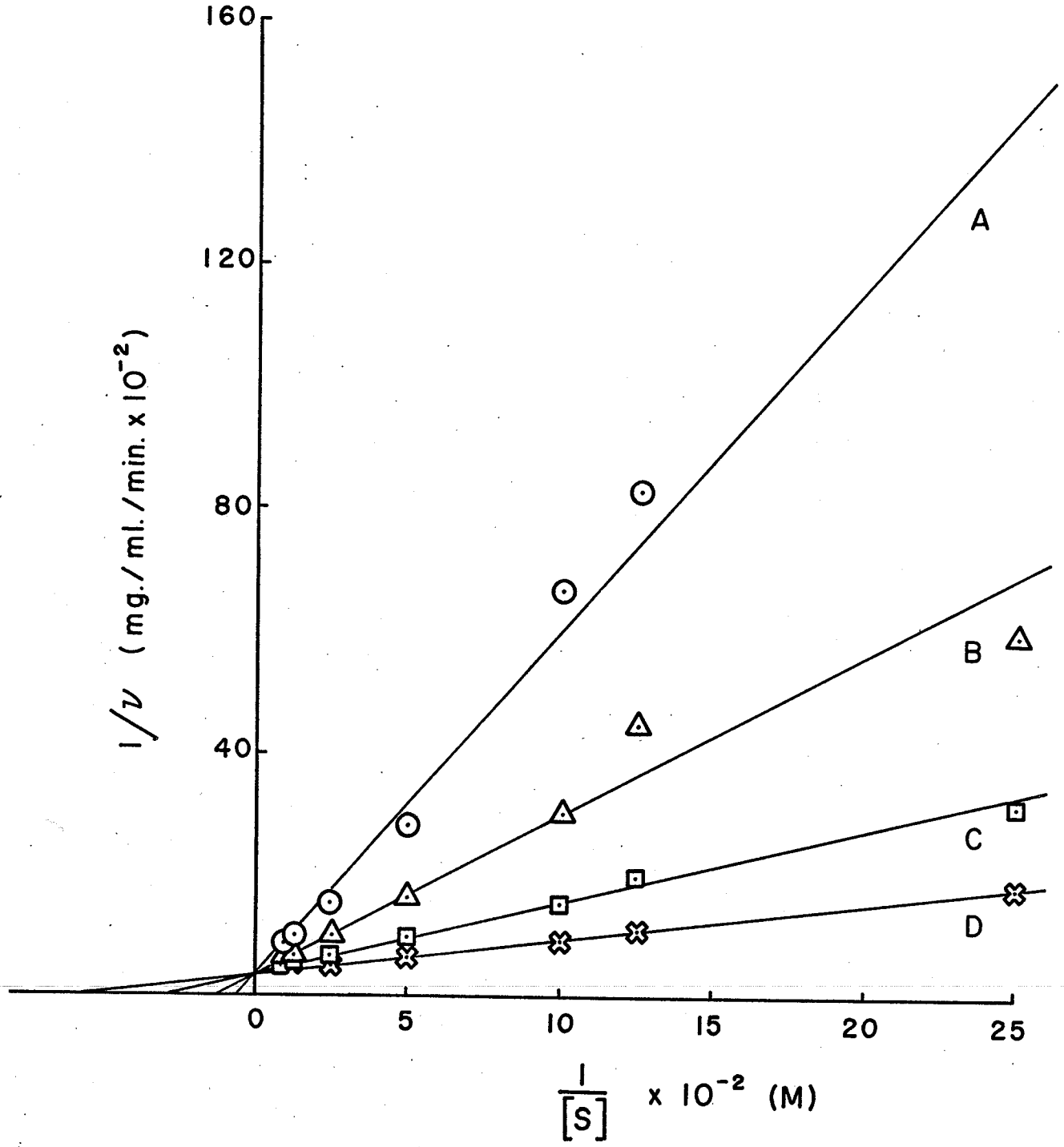
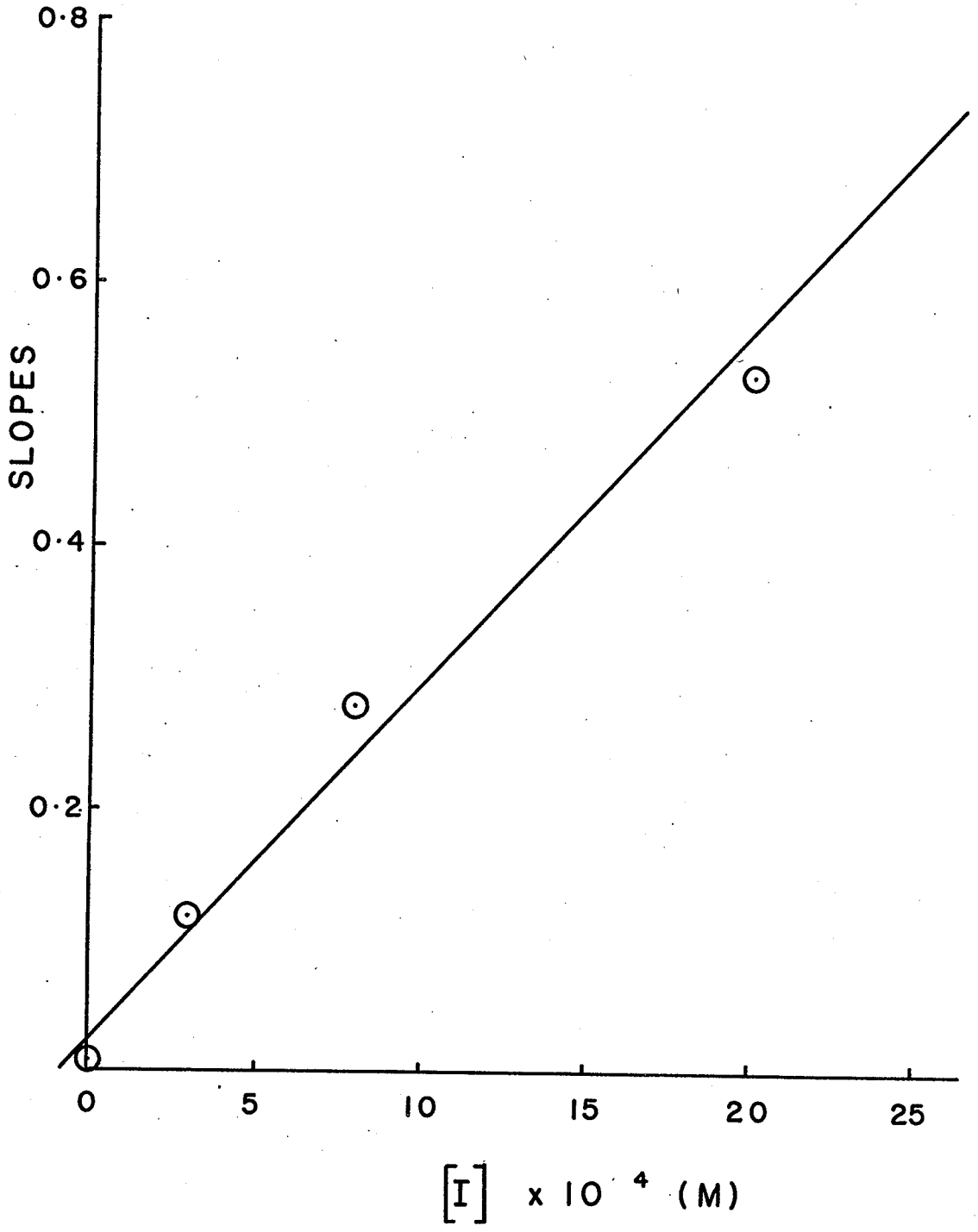

160
120
80
40
1/v (mg./ml./min. x 10^-2)
A
B
C
D
0
5
10
15
20
25
1/[S] x 10^-2 (M)

FIG. 55. Replot of slopes of Fig. 54 as a function of inhibitor (phosphate) concentration.


0·8
0·6
0·4
0·2
SLOPES
0
5
10
15
20
25
[I] x 10 4 (M)

reaction was stopped by the addition of 0.3 ml of 10% TCA and a suitable aliquot was assayed for phosphate. The results are presented in Fig. 56 and replots of the slopes and intercepts are given in Fig. 57.

A summary of the types of inhibition given by the various products of the three reactions studied is presented in Table XVI. The non-linear inhibition of PNPP hydrolysis by PNP observed in Phase I (Figs. 9, 10 and 11) was confirmed. The linear inhibition of glucose 6-phosphate hydrolysis by glucose, taken in conjunction with the linear nature of glycerol inhibition of β-glycerophosphate hydrolysis confirms the suspicion voiced earlier that alkaline phosphatase treats PNPP in a way that is different from the way it treats the other substrates.

TABLE XVI

VARIOUS PRODUCTS AS INHIBITORS OF REACTIONS CATALYZED BY ALKALINE PHOSPHATASE

Substrate	Inhibitory product	Type of inhibition
PNPP	phosphate	linear, competitive
	PNP	non-linear, non-competitive
	glucose	linear, competitive
β-glycero-phosphate	phosphate	linear, competitive
	glycerol	linear, non-competitive
Glucose 6-P	phosphate	linear, competitive
	glucose	linear, non-competitive
	PNP	no inhibition up to 3×10^{-3} M PNP

FIG. 56. Inhibition by glucose of glucose 6-phosphate hydrolysis.

Each reaction vessel contained, in a final volume 3.1 ml, the following concentrations of substances at pH 8.0:

Bicine 3×10^{-2} M
$MgCl_2$ 4×10^{-3} M
glucose 6-phosphate varied as indicated
Enzyme 50 units

Glucose A - 1.45 M
B - 1.25 M
C - 0.75 M
D - None

The reaction was started by the addition of enzyme and ran for 30 minutes at 30°.

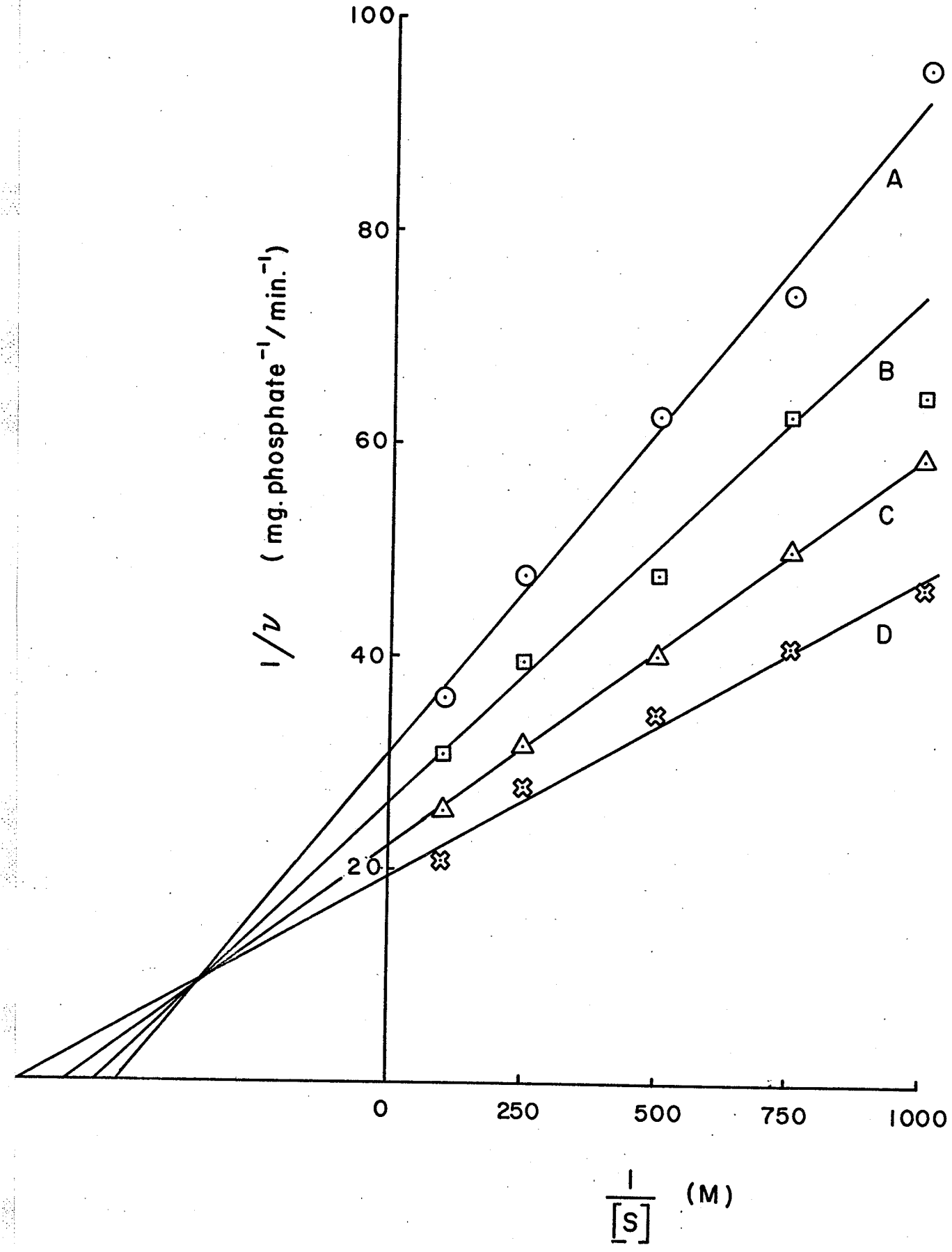
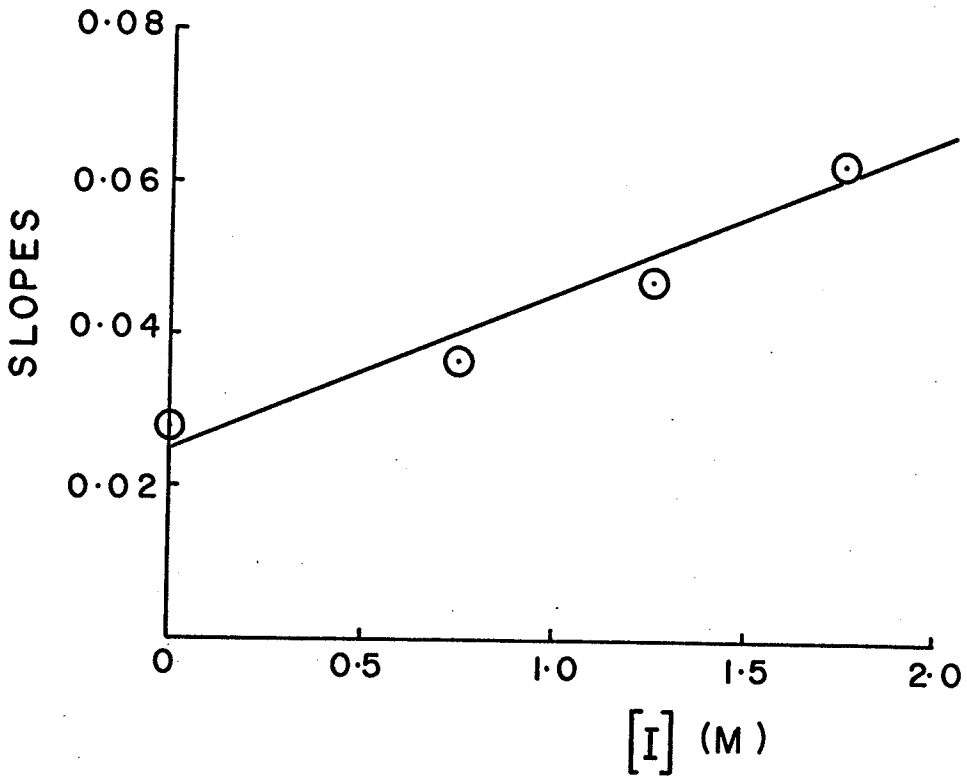
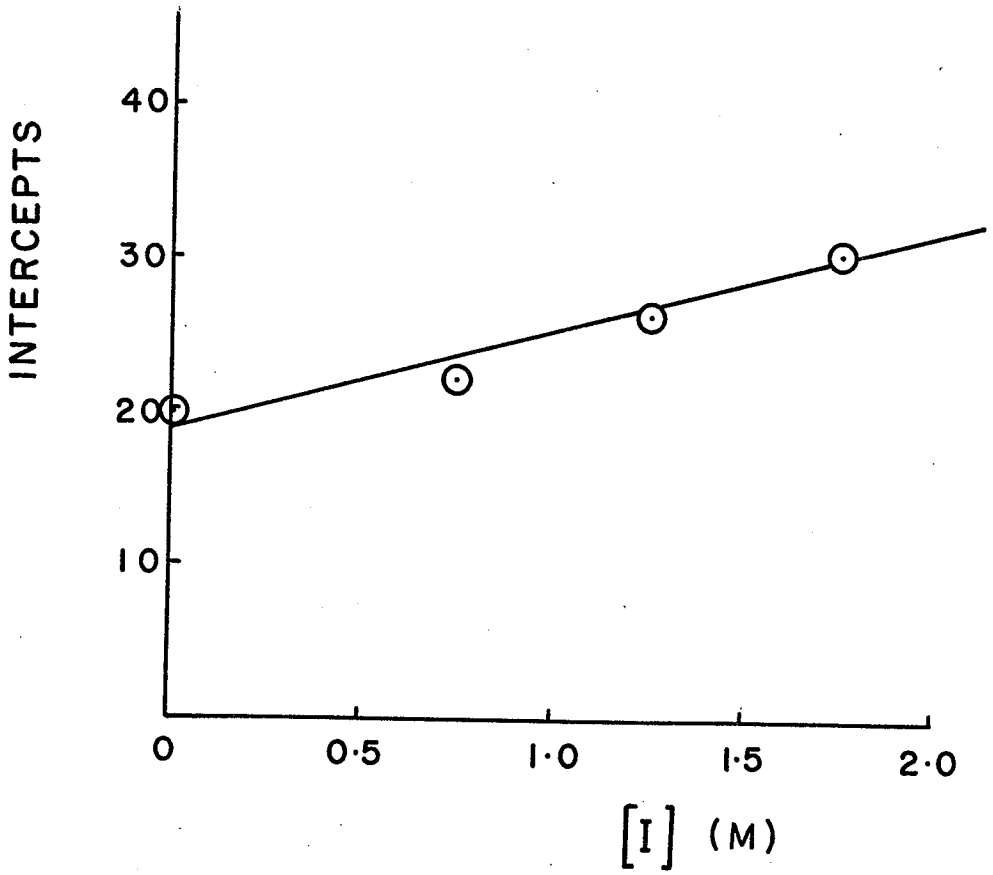

100
80
60
40
20
0
250
500
750
1000
1/v (mg. phosphate⁻¹/min.⁻¹)
1/[S] (M)
A
B
C
D

FIG. 57. The slopes and intercepts of Fig. 56 as a function of inhibitor (glucose) concentration.


0·08
0·06
0·04
0·02
SLOPES
0
0·5
1·0
1·5
2·0
[I] (M)

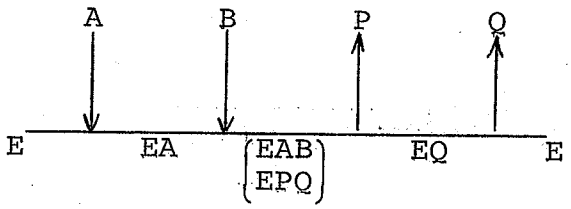

40
30
20
10
INTERCEPTS
0
0·5
1·0
1·5
2·0
[I] (M)

Discussion of the mechanism of the reaction catalyzed by alkaline phosphate

The ordered Uni-Bi mechanism discussed in Phase I is the simplest mechanism that can account for the product inhibition patterns obtained with glucose 6-P and β-glycerophosphate as substrates. It is, however, too simple to account for the non-linear PNP inhibition of PNPP hydrolysis. The complexities introduced by hypothesizing mixed dead-end and product inhibition led, in Phase I, to the prediction of parabolic replots of the slopes and intercepts. Whatever the nature of the curves of Fig. 49 may be they are not parabolic. If they are hyperbolic (which looks reasonable) one must face the fact that there is no reasonable way to get hyperbolic product inhibition in an ordered Uni-Bi mechanism. It is also possible that these curves are not of the second degree at all, but are of higher or of mixed degree.

It is, of course, only a convenient simplification to regard any hydrolytic reaction as being Uni-Bi. There is always at least one other substrate, water. Its concentration, however, is rarely variable and it is usually ignored in kinetic studies. The product inhibition patterns predicted by an ordered Bi Bi mechanism, which can

be represented as:



may be summarized as follows:

Inhibitory product	Types of product inhibition expected in ordered Bi Bi			
	A varies:		B varies:	
	Saturated with B	Unsaturated with B	Saturated with A	Unsaturated with A
P	Uncompetitive	Non-competitive	Non-competitive	Non-competitive
Q	Competitive	Competitive	No inhibition	Non-competitive

In applying this mechanism to PNPP hydrolysis, we find (i) if A is PNPP and B is water (which is certainly present in high enough concentration to be saturating) then one of the products should give uncompetitive inhibition; but neither of them does: and (ii) if B is PNPP and A is water one of the products should give no inhibition; but they both inhibit. It can be concluded that the experimental data for alkaline phosphatase are not consistent with an ordered Bi Bi mechanism where water is considered to be the (saturating)

second substrate.

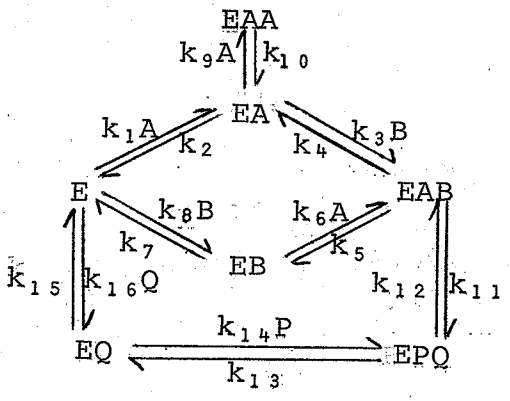
However, it is entirely possible that water itself is not a substrate in hydrolytic reactions - the true substrates may be OH^- or H^+ or both depending on the pH of the reaction medium, and on the nature of the products. Consider the hydrolysis of PNPP and glucose 6-phosphate. At moderately alkaline pH the alcoholic product of one reaction, p-nitrophenol, pK 7.15, is ionized while the alcoholic product of the other, glucose, is not. As a result of the rupture of the P-O bond in the substrate a H^+ must be transferred to the nascent glucose molecule but not to the p-nitrophenylate ion. In both cases, however, a hydroxyl ion must be transferred to the phosphate group. The state of ionization of this hydroxyl and of the remainder of the phosphate group itself introduces further complications.

It may, therefore, be profitable to consider PNPP hydrolysis as a Bi Bi reaction with OH^- as the second substrate. If the release of products is ordered (P= p-nitrophenol, Q= phosphate), and if B is OH^-, the observed inhibition pattern (as far as it goes) is the expected one. It is probably not possible to determine experimentally the nature of the product inhibition when OH^- is saturating. The required high pH would destroy the enzyme. Experiments to determine inhibition patterns with OH^- as the variable

substrate have not been performed.

It was, however, possible to prepare Lineweaver-Burk plots treating OH^- as the variable substrate and PNPP as the changing fixed substrate. The plots were prepared from data taken from Fig. 18 and are presented in Fig. 58. It is evident that at low pH the velocity decreases as the PNPP concentration increases. This may be interpreted as being due to the onset of substrate (PNPP) inhibition, in full agreement with the data of Fig. 22. It is also evident that OH^- is itself an inhibitor when PNPP is present in low concentration. The lower the PNPP concentration the less OH^- is required to inhibit its hydrolysis. At high PNPP concentrations OH^- becomes an activator rather than an inhibitor.

Mechanism II attempts to incorporate these observations and the observations of the effects of pH on K_m and V_{max} (Fig. 31) into a unified scheme.



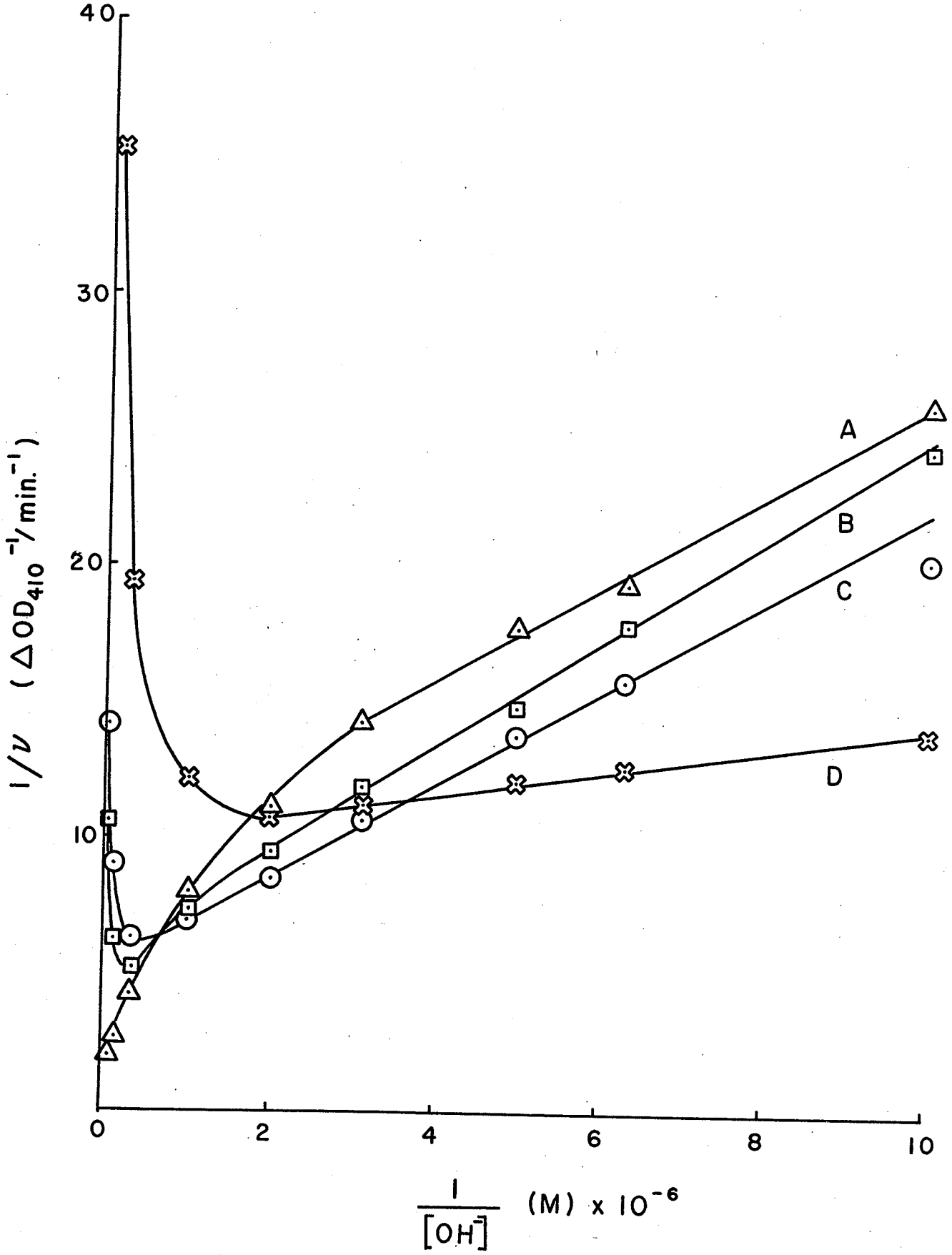
A = PNPP
B = OH^-
P = PNP
Q = Phosphate

Mechanism II

FIG. 58. Lineweaver-Burk plots treating OH^- as a second substrate in PNPP hydrolysis.

Data were taken from Fig. 18 and converted to actual velocities before plotting.

A - 10^{-2}M PNPP
B - 10^{-3}M PNPP
C - 5.0×10^{-4}M PNPP
D - 1.0×10^{-4}M PNPP


40
30
20
10
0
2
4
6
8
10
A
B
C
D
1/ν (ΔOD410 -1/min.-1)
1/[OH-] (M) x 10-6

Mechanism II is a Bi Bi mechanism with random addition of substrates, ordered product release and dead-end substrate inhibition. At low concentration of OH^-, when the affinity of the enzyme for PNPP is greatest (Fig. 31) the upper of the two alternative pathways ($E \rightleftharpoons EA \rightleftharpoons EAB$) is dominant. An increase in the PNPP concentration will result in more of the enzyme being tied up in the dead-end complex EAA. The velocity of the reaction will fall. This is substrate inhibition by PNPP.

An increase in the OH^- concentration (still at low PNPP concentration) should also result in inhibition due to the increase of apparent K_m that occurs with increase of pH. The increased OH^- concentration will increase the concentration of EB at the expense of EA. Presumably the EB form of the enzyme is the form that has a lower affinity for PNPP at high pH.

At high concentrations of both A and B, the lower pathway ($E \rightleftharpoons EB \rightleftharpoons EAB$) dominates. It is necessary to increase the concentration of A enormously to observe substrate inhibition. V_{max} by this pathway is greater than by the upper path, that is, $k_8 k_6 > k_1 k_3$. The activation by hydroxyl ion that occurs at high PNPP concentration is then due to several factors (a) the relief of substrate inhibition by conversion of E to EB, (b) the switch to the

alternate pathway with higher V_{max}, and (c) saturation with PNPP.

The initial velocity equation in the absence of products, derived by steady state treatment of mechanism II* is given in equation 12. The Ks are various combinations of rate constants.

$$\frac{v}{Et} = \frac{K_1AB+K_2A^2B+K_3AB^2}{K_7A+K_8A^2+K_9A^3+K_{11}A^2B+K_{15}AB+K_{16}AB^2+K_{24}B+K_{28}B^2+K} \quad \ldots (12)$$

In reciprocal form and rearranged this becomes:

$$Et\left(\frac{1}{v}\right) = \frac{(K_{28}B^2+K_{24}B+K)\left(\frac{1}{A}\right)^3+(K_{16}B^2+K_{15}B+K_7)\left(\frac{1}{A}\right)^2+(K_8+K_{11}B)\frac{1}{A}+K_9}{(K_3B^2+K_1B)\left(\frac{1}{A}\right)^2+(K_2B)\left(\frac{1}{A}\right)} \quad \ldots (13)$$

This is a 3/2 function which cannot be graphed without knowledge of the constants. At low concentrations of A, however, the equation simplifies to

$$\frac{Et}{v} = \frac{(K_{28}B^2+K_{24}B+K)}{(K_3B^2+K_1B)}\left(\frac{1}{A}\right) + \frac{K_{16}B^2+K_{15}B+K_7}{K_3B^2+K_1B} \quad \ldots\ldots\ldots\ldots (14)$$

and reciprocal plots ($\frac{1}{v}$ *versus* $\frac{1}{A}$) at low A concentration are linear at any given concentration of B. If B is also very

---

* See appendix I for the complete rate equation and definition of all constants.

small Eq. 14 becomes.

$$\frac{Et}{v} = \frac{K_{24}B+K}{K_1B}\left(\frac{1}{A}\right) + \frac{K_{15}B+K_7}{K_1B} \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (15)$$

which may be rearranged and written as:-

$$\frac{Et}{v} = \frac{K_{24}}{K_1}\left(\frac{1}{A}\right) + \frac{K}{K_1}\left(\frac{1}{A}\right)\left(\frac{1}{B}\right) + \frac{K_7}{K_1}\left(\frac{1}{B}\right) + \frac{K_{15}}{K_1} \quad \cdots\cdots\cdots \quad (16)$$

which is identical in form to the initial rate equation for an ordered Bi Bi mechanism and represents the effective limitation of the reaction to the upper of the two alternate pathways. As A is made larger the contributions of all the terms in equation 13 must be taken into account. Without a knowledge of the constants one cannot do this. However, if A is very large

$$\frac{Et}{v} = \frac{K_8+K_{11}B}{(K_2B)} + \frac{K_9A}{K_2B} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (17)$$

and it is evident that the velocity of the reaction decreases with an increase in A, thus accounting for the substrate (PNPP) inhibition that occurs at low pH. At high pH, that is, when B is large, equation (13) becomes

$$\frac{Et}{v} = \frac{(K_{28}B^2+K_{24}B)\left(\frac{1}{A}\right)^3+(K_{16}B^2+K_{15}B)\left(\frac{1}{A}\right)^2+(K_{11}B)\left(\frac{1}{A}\right)}{(K_3B^2+K_1B)\left(\frac{1}{A}\right)^2+K_2B\left(\frac{1}{A}\right)}$$

$$= \frac{(K_{28}B+K_{24})(\frac{1}{A})^2 + (K_{16}B+K_{15})\frac{1}{A} + K_{11}}{(K_3B+K_1)(\frac{1}{A}) + K_2} \quad \cdots\cdots \quad (18)$$

The double reciprocal plot of $\frac{1}{v}$ *versus* $\frac{1}{A}$ will be a 2/1 function. The curve for pH 9.6 in Fig. 22 can be interpreted as being of this form. At low concentrations of A equation (18) becomes

$$\frac{Et}{v} = \frac{(K_{28}B+K_{24})\frac{1}{A} + K_{16}B + K_{15}}{K_3B + K_1} \quad \cdots\cdots\cdots\cdots \quad (19)$$

Under these conditions $\frac{1}{v}$ *versus* $\frac{1}{A}$ plots will be linear. This expectation is realized in all the double reciprocal plots presented here. If the concentration of A is very low the term in $\frac{1}{A}$ of equation (19) becomes dominant and we have

$$\frac{Et}{v} = \frac{(K_{28}B+K_{24})\frac{1}{A}}{K_3B+K_1} \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (20)$$

A plot of $\frac{1}{v}$ *versus* $\frac{1}{B}$ will, then, be hyperbolic and B is expected to be inhibitory. This expectation is realized in the data of Fig. 58.

Equation (18) may be written as

$$\frac{Et}{v} = \frac{\frac{1}{B}(\frac{K_{24}}{A^2} + \frac{K_{15}}{A} + K_{11}) + \frac{K_{28}}{A^2} + \frac{K_{16}}{A}}{\frac{1}{B}(K_2+\frac{K_1}{A}) + K_3} \quad \cdots\cdots\cdots \quad (21)$$

If A is very large, we may be justified in simplifying to

$$\frac{Et}{v} = \frac{\frac{1}{B}(K_{11})}{\frac{1}{B}(K_2) + K_3}$$

$$= \frac{K_{11}}{K_2 + K_3 B} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (22)$$

An increase in B will then result in a decrease of $\frac{1}{v}$: i.e. B is an activator at high A and B concentrations. Both the inhibition by B at low A concentration and the activation by B at high A concentration demonstrated in Fig. 58 can, therefore, be explained by mechanism II. In the presence of the product P, the rate equation becomes (Q=0),

$$\frac{v}{Et} = \frac{K_1AB + K_2A^2B + K_3AB^2}{\begin{aligned} &K_7A + K_8A^2 + K_9A^3 + K_{10}A^3P + K_{11}A^2B + K_{12}A^2BP + K_{13}A^2P \\ &+K_{15}AB + K_{16}AB^2 + K_{17}AB^2P + K_{18}ABP + K_{21}AP + K_{24}B \\ &+K_{25}BP + K_{28}B^2 + K_{29}B^2P + K_{30}P + K. \end{aligned}}$$

If this equation is expressed in the reciprocal form and rearranged the slopes and intercepts of the double reciprocal plots at various levels of P are seen to be linear functions of P. Experimental data, however, require non-linear functions. Such plots will be non-linear if P as well as A can bind to EA to yield a dead-end complex EAP.

The scheme outlined here appears to explain the experimental data for PNPP as substrate quite well. A great

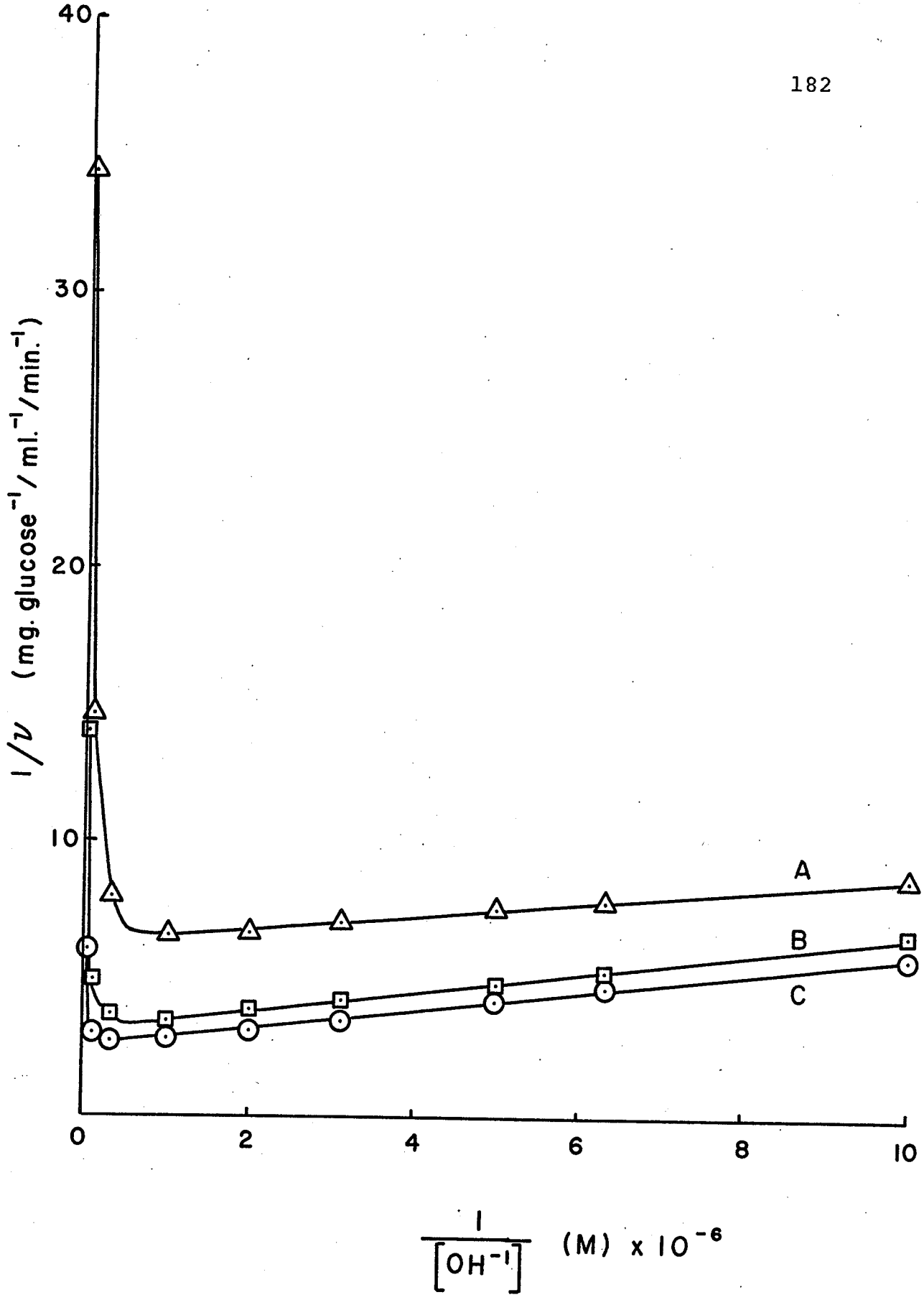
deal more work is required to evaluate the constants in the rate equation. In particular, kinetic studies at several values of pH are required.

The same scheme can explain the experimental data obtained with glucose 6-phosphate as substrate. In this case substrate inhibition by G6P was not observed, though it may occur at concentrations higher than those tested. It is, therefore, not necessary to postulate the occurrence of an EAA complex in the reaction mechanism. Since inhibition by glucose is linear, there is no need to postulate an EAP complex either. Double reciprocal plots, treating hydroxyl ion as variable substrate, prepared from the data of Fig. 33 are presented in Fig. 59. They are linear at low hydroxyl ion concentration. Substrate inhibition by hydroxyl ion occurs at high pH and is more pronounced with lower than with higher G6P concentrations. The necessity to transfer a H^+ to the nascent glucose molecule during G6P hydrolysis need not complicate the situation. The addition may simply occur in the medium with no involvement of the enzyme at all.

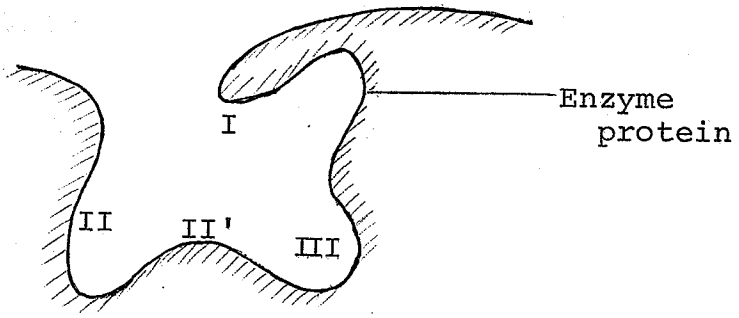
Two data from the product inhibition experiments remain to be explained. These are (1) the linear competitive inhibition by glucose of PNPP hydrolysis and (2) the apparent lack of inhibition by p-nitrophenol of G6P hydroly-

FIG. 59. Lineweaver-Burk plots treating OH^- as a second substrate in glucose 6-phosphate hydrolysis. Data were taken from Fig. 33 and converted to actual velocities before plotting:

A - 1.0×10^{-3}M G6P
B - 5.0×10^{-3}M G6P
C - 1.0×10^{-2}M G6P


40
30
20
10
0
2
4
6
8
10
1/v (mg. glucose⁻¹ / ml.⁻¹ / min.⁻¹)
A
B
C
1 / [OH⁻¹] (M) x 10⁻⁶

sis. These observations are most easily explained by assuming that the active site for PNPP is not the same as that for G6P. If we assume a minimum of 3 points of attachment of the substrate to the enzyme protein, the situation can be visualized in the following diagram.



In the diagram point I represents an attachment site for the phosphate group of any substrate. Points II and II' are the other attachment points for p-nitrophenyl phosphate. The active site for PNPP then consists of points I, II and II'. Similarly points I, II' and III together constitute the active site for glucose 6-P (or any other substrate that behaves like G6P).

Point II is relatively remote from the G6P site, and the binding of PNP to point II does not affect activity at the G6P site. On the other hand, the binding of glucose to point II' hinders the attachment of PNPP to its site and would thereby bring about competitive inhibition of PNPP hydrolysis.

The existence of two substrate sites in alkaline phosphatase is also required by the fact that the enzyme generally seems to possess phosphotransferase activity. (See Historical section.) It is not unreasonable to expect that these sites should interact during the hydrolysis of a single substrate, either in the presence or absence of products.

The two-substrate model for alkaline phosphatase developed here for hydroxyl ion as the second substrate should also apply to phosphotransferase reactions. Here the phosphate may be considered as being transferred to some substrate other than hydroxyl ion. As mentioned in the Historical section of this thesis very high concentrations of substrates such as glucose or Tris are necessary to permit the detection of transphosphorylation. This may be because the reactions have always been run at high pH when strong competition for the phosphate is provided by hydroxyl ion. It would be of great interest to study transphosphorylation at low pH values.

In conclusion, one can fairly state that the kinetics of alkaline phosphatase and the effects of pH on its activity are extremely complex. Much more work is needed before anything like a full understanding of the catalytic reaction is possible. The data presented in this thesis, and the tentative explanation offered, however, provide a reasonable working hypothesis for future research.

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*Not read in the original.

APPENDIX

The complete rate equation for Mechanism II, assuming steady state conditions is as follows:

$$\frac{v}{Et} = \frac{K_1AB + K_2A^2B + K_3AB^2 - K_4BQP - K_5AQP - K_6QP}{\begin{aligned} &K_7A + K_8A^2 + K_9A^3 + K_{10}A^3P + K_{11}A^2B + K_{12}A^2BP \\ &+K_{13}A^2P + K_{14}A^2QP + K_{15}AB + K_{16}AB^2 + K_{17}AB^2P \\ &+K_{18}ABP + K_{19}ABQ + K_{20}ABQP + K_{21}AP + K_{22}APQ \\ &+K_{23}AQ + K_{24}B + K_{25}BP + K_{26}BQ + K_{27}BQP \\ &+K_{28}B^2 + K_{29}B^2P + K_{30}P + K_{31}Q + K_{32}QP + K. \end{aligned}}$$

K, K_1, K_2 *etc.* are combinations of rate constants, as listed below and Et is the total enzyme concentration.

$$K_1 = k_{13}k_{10}k_{15}k_7k_1k_3k_{11} + k_{13}k_{10}k_2k_{15}k_8k_6k_{11}$$

$$K_2 = k_{13}k_{15}k_1k_{10}k_3k_6k_{11}$$

$$K_3 = k_{13}k_{10}k_3k_{11}k_{15}k_8k_6$$

$$K_4 = k_{14}k_{10}k_3k_5k_7k_{16}k_{12}$$

$$K_5 = k_{14}k_{10}k_{12}k_6k_4k_2k_{16}$$

$$K_6 = k_{14}k_{12}k_4k_{10}k_2k_7k_{16} + k_{14}k_{10}k_2k_{16}k_{12}k_5k_7$$

$$\begin{aligned} K_7 = {} & k_6k_{11}k_{13}k_{15}k_{10}k_2 + k_{10}k_6k_4k_2k_{13}k_{15} \\ & +k_{10}k_{12}k_6k_4k_2k_{15} + k_{10}k_{11}k_{13}k_{15}k_1k_7 \\ & +k_{10}k_{15}k_7k_1k_{12}k_4 + k_{10}k_5k_7k_1k_{13}k_{15} \\ & +k_{10}k_{12}k_5k_7k_{15}k_1 + k_{10}k_{13}k_{15}k_7k_1k_4 \end{aligned}$$

$$\begin{aligned} K_8 = {} & k_{10}k_6k_{11}k_{13}k_{15}k_1 + k_{10}k_{13}k_{15}k_1k_6k_4 \\ & +k_{10}k_5k_1k_{12}k_6k_4 + k_{11}k_{13}k_{15}k_7k_1k_9 \\ & +k_{15}k_7k_1k_{12}k_4k_9 + k_{13}k_{15}k_5k_7k_1k_9 \\ & +k_{12}k_5k_7k_{15}k_1k_9 + k_{13}k_{15}k_7k_1k_4k_9 \end{aligned}$$

$$\begin{aligned} K_9 = {} & k_6k_{11}k_{13}k_{15}k_1k_9 + k_{13}k_{15}k_1k_6k_4k_9 \\ & +k_{15}k_1k_{12}k_6k_4k_9 \end{aligned}$$

$$K_{10} = k_{14}k_{12}k_6k_4k_1k_9$$

$$K_{11} = k_{10}k_{13}k_{15}k_1k_6k_3 + k_{10}k_{15}k_1k_3k_6k_{12} + k_{13}k_{15}k_8k_6k_4k_9 + k_{15}k_8k_6k_{12}k_4k_9 + k_{10}k_1k_3k_6k_{11}k_{13} + k_{15}k_1k_{10}k_3k_6k_{11}$$

$$K_{12} = k_{10}k_1k_3k_6k_{14}k_{12} + k_{14}k_{12}k_8k_6k_4k_9 + k_{10}k_1k_3k_6k_{11}k_{14}$$

$$K_{13} = k_{14}k_{12}k_5k_7k_1k_9 + k_{14}k_{12}k_4k_7k_1k_9 + k_{10}k_1k_{14}k_{12}k_6k_4$$

$$K_{14} = k_{16}k_{14}k_{12}k_6k_4k_9$$

$$K_{15} = k_{10}k_3k_6k_{11}k_{13}k_{15} + k_{10}k_{15}k_8k_6k_{12}k_4 + k_{10}k_2k_8k_6k_{13}k_{15} + k_{10}k_2k_8k_6k_{15}k_{12} + k_{10}k_3k_{13}k_{15}k_7k_1 + k_{10}k_3k_1k_7k_{15}k_{12} + k_{10}k_3k_5k_{13}k_{15}k_1 + k_{10}k_{15}k_1k_3k_5k_{12} + k_{10}k_2k_8k_6k_{11}k_{13} + k_{10}k_{15}k_7k_1k_3k_{11} + k_{10}k_2k_{15}k_8k_6k_{11} + k_{10}k_{13}k_{15}k_8k_6k_4 + k_{10}k_7k_1k_3k_{11}k_{13}$$

$$K_{16} = k_{10}k_3k_{13}k_{15}k_8k_6 + k_{10}k_{15}k_8k_6k_3k_{12} + k_{10}k_3k_8k_6k_{11}k_{13} + k_{10}k_3k_{11}k_{15}k_8k_6$$

$$K_{17} = k_{10}k_3k_8k_6k_{14}k_{12} + k_{10}k_3k_8k_6k_{11}k_{14}$$

$$K_{18} = k_{10}k_2k_8k_6k_{14}k_{12} + k_{10}k_7k_1k_{14}k_{12}k_3 + k_{10}k_1k_3k_5k_{14}k_{12} + k_{10}k_2k_8k_6k_{11}k_{14} + k_{10}k_7k_1k_3k_{11}k_{14} + k_{10}k_{14}k_{12}k_8k_6k_4$$

$$K_{19} = k_{10}k_3k_6k_{11}k_{13}k_{16}$$

$$K_{20} = k_{10}k_3k_6k_{16}k_{14}k_{12} + k_{10}k_3k_6k_{11}k_{16}k_{14}$$

$$K_{21} = k_{10}k_2k_4k_6k_{14}k_{12} + k_{10}k_{14}k_{12}k_5k_7k_1 + k_{10}k_{14}k_{12}k_4k_7k_1$$

$$K_{22} = k_{10}k_{16}k_{14}k_{12}k_6k_4 + k_{10}k_2k_{16}k_{14}k_{12}k_6 + k_7k_{16}k_{14}k_{12}k_4k_9 + k_{10}k_2k_{16}k_{14}k_6k_{11} + k_{10}k_6k_4k_2k_{16}k_{14}$$

$$K_{23} = k_{10}k_2k_{16}k_6k_{11}k_{13} + k_{10}k_6k_4k_2k_{16}k_{13} + k_{10}k_{12}k_6k_4k_2k_{16}$$

$$K_{24} = k_{10}k_3k_5k_7k_{13}k_{15} + k_{10}k_3k_{11}k_{15}k_7k_{13} + k_{10}k_2k_8k_{11}k_{13}k_{15} + k_{10}k_2k_{15}k_8k_{12}k_4 + k_{10}k_2k_8k_5k_{12}k_{15} + k_{10}k_4k_2k_8k_{13}k_{15} + k_{10}k_3k_{12}k_5k_7k_{15} + k_{10}k_2k_5k_8k_{13}k_{15}$$

$$K_{25} = k_{10}k_3k_5k_7k_{14}k_{12} + k_{10}k_2k_8k_5k_{12}k_{14} + k_{10}k_2k_8k_{14}k_{12}k_4$$

$$K_{26} = k_{10}k_3k_5k_7k_{16}k_{12} + k_{10}k_3k_5k_7k_{16}k_{13} + k_{10}k_3k_{11}k_{13}k_7k_{16}$$

$$K_{27} = k_{10}k_3k_7k_{16}k_{14}k_{12} + k_{10}k_{16}k_{14}k_{12}k_3k_5 + k_{10}k_3k_{11}k_7k_{16}k_{14} + k_{10}k_3k_5k_7k_{16}k_{14}$$

$$K_{28} = k_{10}k_3k_5k_{13}k_{15}k_8 + k_{10}k_3k_5k_{12}k_{15}k_8 + k_{10}k_3k_{11}k_{13}k_{15}k_8$$

$$K_{29} = k_{10}k_3k_5k_8k_{14}k_{12}$$

$$K_{30} = k_{10}k_2k_{14}k_{12}k_5k_7 + k_{10}k_{14}k_{12}k_4k_2k_7$$

$$K_{31} = k_{11}k_{13}k_{10}k_2k_{16}k_7 + k_{12}k_4k_{10}k_2k_7k_{16} + k_{10}k_5k_7k_2k_{16}k_{13} + k_{10}k_2k_{16}k_{12}k_5k_7 + k_{10}k_4k_2k_7k_{16}k_{13}$$

$$K_{32} = k_{10}k_2k_7k_{16}k_{14}k_{12} + k_{10}k_2k_{16}k_{14}k_{12}k_5 + k_{10}k_2k_7k_{16}k_{14}k_{11} + k_{10}k_2k_5k_7k_{16}k_{14} + k_{10}k_7k_{16}k_4k_{12}k_4 + k_{10}k_7k_1k_3k_{11}k_{14}$$

$$K = k_{10}k_4k_{12}k_2k_7k_{15} + k_{10}k_2k_{12}k_5k_7k_{15} + k_{10}k_4k_2k_7k_{13}k_{15} + k_{10}k_2k_5k_7k_{13}k_{15} + k_{10}k_2k_7k_{15}k_{13}k_{11}$$