PHOSPHOGLYCOLATE PHOSPHATASE: PURIFICATION AND PROPERTIES

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PURIFICATION AND PROPERTIES

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JOHN T. CHRISTELLER

has been accepted towards fulfillment
of the requirements for

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R. L. Anderson

Major professor

Date August 8, 1974

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ABSTRACT

PHOSPHOGLYCOLATE PHOSPHATASE:
PURIFICATION AND PROPERTIES

By

John T. Christeller

Phosphoglycolate phosphatase (E.C. 3.1.3.18), a specific chloroplastic phosphatase, plays a role in glycolate biosynthesis and photorespiration. The properties of the purified enzyme from tobacco and spinach leaves were studied.

P-glycolate phosphatase was purified 1500 fold from field grown tobacco leaves by acid and acetone fractionation, DEAE-cellulose and molecular sieve chromatography, and preparative polyacrylamide gel electrophoresis. Preparations with specific activity greater than 350 were judged 90-95% homogeneous by rechromatography on DEAE-cellulose, polyacrylamide gels, and by isoelectric focussing. The highest specific activity obtained was 468 μmole phosphate released per minute per milligram protein. The native protein has a molecular weight of 80,500 daltons by Ferguson Plot analysis and 86,300 daltons by sedimentation velocity on sucrose density gradients. SDS-polyacrylamide gels gave a molecular weight of 20,700 daltons, indicating that P-glycolate phosphatase is a tetramer with identical or near identical subunits. The enzyme, freshly purified or in crude homogenates had a pI of 3.8-3.9 pH units by isoelectric focussing.

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John T. Christeller

P-glycolate phosphatase requires divalent cation for activity. Activity- pH curves identified two active site residues with pK's at pH 5.7 and pH 7.5 in the presence of manganese or cobalt, and pH 5.7 and pH 9.1 in the presence of magnesium. Saturation velocity kinetics enabled the identification of two distinct divalent cation binding sites. The first, non specific, site has a $K_{0.5}$ approximately 2-7 x 10^{-5} M, depending on the cation and the pH. The second site, which is specific for magnesium, binds this cation with negative allosteric cooperative kinetics; the affinity appears to vary from about 4 x 10^{-6} M for the $K_{0.5}$ of the first magnesium bound to about 5 x 10^{-4} M for the fourth at pH 8.1. The negative cooperativity is greatest at high pH, suggesting that the conformational changes involved are responsible for the repression of the active site ionization in the presence of magnesium.

Because the pH range of activity is very broad both the phosphate monoanion and dianion of P-glycolate must be bound as the substrate. The concentration of these two species at the apparent Km (P-glycolate) is independent of magnesium concentration. The P-glycolate-magnesium complex is kinetically inactive. The Km (P-glycolate) is 5-6 x 10^{-5} M at pH 6.3 and does not vary appreciably with pH.

Enzymatic hydrolysis proceeds through O-P bond cleavage as determined in (^{18}O)-H_2O and analysis of the trimethylsilyl derivatives of the reaction products. No phosphate, hydroxyl, or carboxyl exchange occurred. End product inhibition was consistent with an ordered release of products, first the alcoholic product, then

phosphate. Analysis of the data indicated that the phosphate-enzyme complex was very unstable; and this was confirmed by use of alternative phosphomonoester substrates. Maximum velocity with these substrates was found to be proportional to the pKa of the corresponding alcoholic product, indicating the rate limiting step in the reaction was protonation of the bridge oxygen. The use of substrate analogs further suggested that enzymatic specificity resides in exacting steric conditions required for binding, and that large alkyl groups were excluded on this basis. P-glycolate phosphatase was found to catalyse transphosphorylation to a wide range of acceptors, and was inhibited at the active site by diisopropylfluorophosphate. This data, with the inhibition studies above, indicates the reaction sequence proceeds via a phosphoenzyme intermediate. P-glycolate phosphatase belongs to the acid phosphatase type on the basis of pH optimum, diisopropylfluorophosphate inhibition kinetics, and S-(carboxymethyl)-phosphorothioate inhibition. The enzyme intermediate is therefore probably a phosphohistidine protein.

N-ethylmaleimide slowly inactivated the enzyme, but the inactivation rate was greatly increased by P-glycolate but not by magnesium. This syncatalytic modification was probably due to an additional sulphydryl becoming exposed on binding the substrate. Glycolate, but not phosphate, would also elicit this conformational change on binding, which is consistent with the failure to observe enzyme mediated H_2O-phosphate oxygen exchange. This conformational change, necessary to induce the transition state complex and phosphoenzyme formation, may account for the broad phosphate acceptor specificity.

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Ribose-5-phosphate is a competitive inhibitor at physiological concentrations, while numerous other chloroplastic metabolites were noninhibitory. Ribose-5-phosphate also inhibited ribulose-1,5-diphosphate oxygenase, which catalyses the formation of P-glycolate *in vivo*.

P-glycolate phosphatase from spinach leaves has a molecular weight of 93,000 daltons and, unlike the tobacco enzyme, is extremely unstable after DEAE-cellulose chromatography and is inactivated by lipase (E.C. 3.1.1.3). The data suggests that the spinach enzyme may contain a lipid component.

PHOSPHOGLYCOLATE PHOSPHATASE:

PURIFICATION AND PROPERTIES

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John T. Christeller

A DISSERTATION

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1974

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I am grateful to Professor N. E. Tolbert for both financial assistance and patience, advice, and encouragement during the span of my research in his laboratory.

Thanks are also due to Professor C. H. Suelter for his willingness to discuss many of my kinetic experiments. His comments proved invaluable. However all errors in interpretation are mine solely.

The mass spectormetry was performed in a most enjoyable collaboration with Dr. R. H. Gerster with the excellent technical assistance of Mr. Jack Herten.

Finally, I wish to thank the many friends, members and supporters of the Michigan State Rugby Club, and especially my wife, Donna, who made the time spent in East Lansing rich, rewarding, and unforgetable.

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phatase

Polyglycolate

LIST OF ABBREVIATIONS

bicine	N,N - bis(2 - hydroxyethyl) glycine
bis	N,N' - methylenebisacrylamide
DEAE	diethylaminoethyl
DFP	diisopropylfluorophosphate
EDTA	ethylenediamine tetraacetic acid
FDP	fructose-1,6-diphosphate
FMN	flavin monomucleotide
GLC	gas-liquid chromatography
HEPES	N-2-hydroxyethylpiperzine-N'-2-ethanesulphonic acid
MES	2(N-morpholino) ethanesulphonic acid
(Me_3Si)	trimethylsilyl
NEM	N-ethylmaleimide
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
3PGA	3-phosphoglyceric acid
P-glycolate	2-phosphoglycolic acid
Pi	inorganic phosphate
PPGE	preparative polyacrylamide gel electrophoresis
R5P	ribose-5-phosphate
RuDP	ribulose-1,5-diphosphate
S.A.	specific activity

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SCMPT	S-(carboxymethyl)-phosphorothioic acid
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N', N'-tetramethylenediamine
Tris	tris(hydroxymethyl) aminomethane

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INTRODUCTION

The phenomenon of photorespiration intrigues plant scientists because it appears to reduce net photosynthesis. Photorespiration, defined as a light dependent O_2 uptake and CO_2 release, appears to be unnecessary since the sites of oxygen uptake are not linked to energy production and CO_2 release reduces net CO_2 fixation. No convincing rationale has been presented for the function of the phenomenon, nor has the fate of the accumulated products been unequivocally established.

Since the high O_2 in the present atmosphere is favourable toward photorespiration, the problem of CO_2 evolution is far from academic. As much as 90% of total carbon fixed may pass through the glycolate pathway and perhaps 50% is lost by CO_2 evolution, although much may be recaptured before leaving the leaf. Abolition of photorespiration could thus lead to increased net CO_2 fixation without change in the gross photosynthetic rate. This is observed in some species which have evolved unique morphology and biochemical pathways leading to lower rates of glycolate pathway activity and highly efficient CO_2 recapture.

Apparently the first commited step in photorespiration is P-glycolate biosynthesis by RuDP oxygenase, followed by P-glycolate hydrolysis by a specific chloroplast phosphatase. The oxygenase activity appears competitive with CO_2 fixation. It is possible that

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that atmospheric conditions during evolution of this carboxylase/ oxygenase protein favoured the double activity, and more recent changes in the gaseous environment have led to modifications in plant structure in attempts to overcome this superfluous and uneradicable activity.

It is thought that decreasing photorespiration in plants would be highly beneficial for substantially increasing dry matter production. Not only is it economically desirable but the world food situation is today approaching critical proportions and the humanitarian implications of even small increases in crop yield are staggering.

The research in this thesis describes some properties of P-glycolate phosphatase, an enzyme in the photorespiratory pathway. The substrate, P-glycolate, is a potent inhibitor of carbohydrate metabolism, and is therefore a potential regulator. The enzyme appears totally specific for its substrate, an unusual feature for phosphatases. Another regulatory role for the enzyme is its suggested function as a permease for the excretion of glycolic acid from the chloroplast.

The research described in this thesis is divided into four sections. The first section details the purification scheme and characterization of some physical properties of the enzyme. The second section describes research on the effect on activity of physiologically important parameters. This information is used in the isotopic, chemical, and kinetic experiments in the next section to examine the substrate specificity of P-glycolate phosphatase and investigation of the mechanism of enzymic hydrolysis. The fourth and final section describes regulatory phenomena, which may have physiological significance.

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LITERATURE SURVEY

Photosynthetic Carbon Fixation

During the late 1940's and early 1950's, Calvin and coworkers built up evidence for the series of enzyme catalysed reactions by which all green plants and algae fixed atmospheric CO_2 during the dark reactions of photosynthesis. In this photosynthetic reductive pentose phosphate pathway CO_2 is condensed with RuDP to yield two molecules of 3-PGA (the C-3 pathway) (40). The 3-PGA is reduced to sugar phosphate and used for sucrose or starch synthesis or for regeneration of the CO_2 acceptor, RuDP.

A second carboxylation reaction during photosynthesis, developed since 1965 (109,83), utilises PEP as the acceptor, and the initial product, oxaloacetate, is further converted to malate and aspartate. Initially the C-4 pathway was thought to be an altogether new CO_2 fixation method (84) but subsequent work (25,141,82,187) has shown that the carbon is eventually fixed into products of the C-3 carbon reduction cycle via decarboxylation of the C-4 acids and refixation using RuDP carboxylase. This series of reactions, which occur in a number of tropical C-4 plants, including sugar cane and maize, seem to promote CO_2 fixation efficiency because of its low KM (CO_2). The primary fixation occurs in the peripheral mesophyll leaf cells, whereas the C-3 pathway is confined to the internal bundle sheath cells.

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Photorespiration

Otto Warburg showed in 1920 that photosynthesis in Chlorella was inhibited by high partial pressures of oxygen. This effect has since been established in C-3 plants but not in C-4 plants and has been extensively reviewed (26,60,10,129,70,194,203,204). The O_2 inhibitory effect is readily reversible, thus not due to photooxidation phenomena. The inhibition is increased by increasing light intensities and O_2 concentration and by limiting CO_2 concentrations. Saturating CO_2 concentrations reduce the inhibition markedly. Both the net CO_2 uptake and the net O_2 output are affected and the process is not due to increased mitochondrial respiration. The latter is saturated at about 2% O_2, whereas the Warburg effect continues to increase up to 100% O_2. In recent years the term Photorespiration has been used in lieu of the "Warburg O_2 effect on Photosynthesis."

Photorespiration is defined as the light-dependent oxygen uptake and carbon dioxide release from photosynthetic tissue. The subject has been extensively reviewed (230,201,93,199). A variety of indirect methods have been used to measure photorespiration rates. Since internal recycling of both CO_2 and O_2 occurs, all methods underestimate its true rate. It is clear however, that under certain conditions the rate may be a significant fraction of gross photosynthesis. Net photosynthesis may be lower by as much as 50%.

The equilibrium established in a closed system between photosynthetic CO_2 uptake and photorespiratory CO_2 loss is designated the compensation point. Higher plants fall into two classes of

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photorespiratory activity. Plants containing only the C-3 carbon cycle have high rates of photorespiration with compensation points between 40-70 ppm of CO_2, and values as high as 155 ppm have been reported for the leaves of some trees. Those species possessing in addition the C-4 dicarboxylic acid pathway have low CO_2 compensation points of 1-5 ppm of CO_2. Photorespiratory O_2 uptake is difficult to demonstrate because of photosynthetic O_2 evolution, but Jackson and Volk (1970) have unequivocally shown that increased O_2 uptake does occur in the light during photosynthesis.

The Role of Glycolate in Photorespiration

The amount of carbon passing through the products of the glycolate pathway showed the same variation as photorespiration rates toward light intensities, oxygen and carbon dioxide concentrations, pH, and temperature (229,11,221,17,161,197,71). The glycolate pathway sequence in higher plants and algae was elucidated mainly by Tolbert and his coworkers (195,170,162,101,99,151,88,34,41). Pools of intermediates P-glycolate, glycolate, and glyoxylate are small, and most of the carbon appears in glycine and serine.

The sites of photorespiratory O_2 uptake and CO_2 release are still under investigation. The biosynthesis of P-glycolate involves the oxygenase activity of RuDP carboxylase (28,8,123,150). Oxygen uptake is also clearly associated with oxidation of glycolate to glyoxylate by glycolate oxidase. This reaction, along with other glycolate pathway enzymes, is localised in the microbody fraction peroxisomes (198). The oxidation of glyoxylate to oxalate, also catalyzed

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by glycolate oxidase (169), is probably only of significance in nitrogen deficient plants when transamination of glyoxylate to glycine is limited.

In vivo studies of Cossins and Sinha (1966) and Marker and Whittingham (1966) indicate the carboxyl group of glycine is the most immediate source of photorespiratory CO_2. Kisaki and Tolbert (1969,1970) and Kisaki et al. (1971) have shown the presence of glycine decarboxylase and serine hydroxymethyl transferase in leaf mitochondrial extracts which could account for the CO_2 release and serine formation. Criticism that this mechanism was insufficient (231) did not take into consideration that the serine-glycine interconversion could convert all of the carbon to CO_2.

During (^{14}C)-CO_2 photosynthesis, uniformly labelled glycolate is rapidly formed (24,185). Since then the mechanism of biosynthesis has been actively studied (122,199,231), and three hypotheses have been suggested: A. Direct reduction of CO_2 was suggested in 1960 by Warburg and Kripahl, and later by Zelitch (1965). However no supporting enzymic evidence has been found. B. Direct oxidation of a dihydroxyethylthiamine pyrophosphate intermediate of transketolase by H_2O_2, formed from the nonenzymic oxidation of reduced ferredoxin in a Mehler-type reaction (221,47,71). In vitro oxidation of the enzymatic thiamine pyrophosphate-C_2 intermediate readily occurs with any strong oxidant (H_2O_2,$Fe(CN)_6$) (30,90,69). Gibbs and coworkers have shown, with isolated chloroplasts and added transketolase, rates of glycolate production sufficient for photorespiration (71,72,183,158), but in vivo experiments to definitively test the mechanism are lacking. Rampant H_2O_2 production in situ

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may not occur. Andrews et al. (1971) demonstrated incorporation of (^{18}O)-O_2 into the carboxyl group of the products of glycolate metabolism in intact leaves. No incorporation would be expected of H_2O_2 into glycolate, since peroxide acts as an oxidant in the hypothesised reaction producing two moles of H_2O. C. RuDP oxidation by O_2 as discussed above is now favoured as the major if not only pathway of glycolate biosynthesis during photosynthesis. The reaction produces P-glycolate which is hydrolysed to glycolate by a specific phosphatase. P-glycolate is rapidly labelled during (^{14}C)-CO_2 fixation by algae and higher plants (23,8,230) and by isolated chloroplasts (106). Recently Bassham and Kirk (1973) using Chlorella photosynthesizing in (^{14}C)-CO_2 found in a subsequent period in 100% O_2 that glycolate synthesis was preceded by successive transient pools of RuDP and P-glycolate. The authors calculated that 50% of the total glycolate could be accounted for by this pathway from RuDP, but they underestimated the rate of P-glycolate hydrolysis so that this is a minimum value (112). Studies on RuDP oxygenase activity indicate that it is sufficient to account for all the glycolate synthesised (8,112).

Glycolate formation from RuDP explains the direct inhibition of photosynthesis by oxygen. RuDP carboxylase is competitively inhibited by O_2 with respect to CO_2 (29,150) and CO_2 is a competitive inhibitor of oxygenase activity (113,173).

In C-4 plants the lack of photorespiration and the absence of an inhibitory effect of O_2 on net CO_2 fixation is not due to the absence of the glycolate pathway (26) although the activities are generally lower

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than in C-3 plants. The most frequent explanation of this difference between C-3 and C-4 plants is refixation of the CO_2 by PEP carboxylase before it leaves the leaf. An additional explanation suggests that the bundle sheath chloroplasts concentrate CO_2, permitting it to compete more effectively with O_2 for RuDP carboxylase. Chollet et al. (1974) have outlined other evidence favouring this mechanism over the other two postulated.

Regulation of Carbon Metabolism During Photosynthesis

The enzymes involved in carbon metabolism are located in the soluble stromal fraction of the chloroplast. Mechanisms for general regulation of their activities requires knowledge of the changes in many of the physiological parameters such as the relative volume of soluble and lamellae space, buffering capacity, and effector concentrations. Fluxes in Mg^{++} concentration is one of these parameters. Dilley and Vernon (1965) first reported light-induced Mg^{++} and K^{+} efflux from broken chloroplasts. That this could lead to an increase in in vivo stromal concentration of Mg^{++} in the light has been established (121). Upon exposing intact chloroplasts to light an increase in Mg^{++} from approximately zero to 10mM in the stroma was calculated, due almost entirely to efflux from the lamellae. These results were confirmed by Hind et al. (1974) who estimated light-induced rises of 15mM. A word of caution has been that actual Mg^{++} concentrations could be lower due to specific binding proteins. The cation effuxes into the stroma are considered to be in response to concurrent acid proton influx.

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The implications of a change in Mg^{++} concentration in the stromal space relates to the activity of P-glycolate phosphatase as well as some of the other photosynthetic enzymes. Weissbach et al. (1955) first noted that purified RuDP carboxylase required Mg^{++} for activity. The stimulation of CO_2 fixation by the Mg^{++} requirement of the carboxylase has been extensively studied (97,98,209). RuDP oxygenase activity is also magnesium dependent, and recent evidence indicates that Mg^{++} alters the kinetic parameters of the carboxylase and oxygenase (12,113,173). The pH optimum shifts from pH 7.8 at 10mM Mg^{++} to more alkaline pH values with decreasing Mg^{++} concentration (18,191). The same shift has been observed for another key photosynthetic carbon reduction cycle enzyme - FDP phosphatase (159). Other chloroplast enzymes with possible regulatory roles which require Mg^{++} for maximum activity are ribose-5-phosphate isomerase, phosphoribulokinase, and alkaline pyrophosphatase (156,16,120,181,59,36).

The pH is the second physiological factor possibly exerting strong metabolic controls in the stroma. A small change in pH has a marked effect on the activity of many photosynthetic enzymes. Light-induced transport of protons across the thylakoid membrane has been shown by Neuman and Jagendorf (1964) leading to acidification of the thylakoid space (54). Subsequently the inner membrane of the chloroplast envelope was shown to be impermeable to protons (86) and alkalization of the stroma was demonstrated by Werdan et al. (1972). More recent results from these workers (87) indicate that a dark-light transition causes a change in the stroma from pH 7.2 to pH 7.8-9 and

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an accompanying acidification in the thylakoids of pH 6.2 to pH 5.6. An increase of 0.5 pH units could be very significant in the light activation of FDP phosphatase and RuDP carboxylase.

The regulation of CO_2 fixation through metabolite pool sizes, metabolite effector properties, and enzyme properties has been the subject of numerous reviews (16,160,210), especially in regard to light-dark transitions.

Phosphoglycolate Phosphatase

The presence of P-glycolate phosphohydrolase (E.C. 3.13.18.) was first detected in crude tobacco sap (170). The partially purified tobacco leaf enzyme (100 fold) required divalent metal ion for activity. The enzyme, treated with EDTA, was inactive, but activity could be restored with Co^{++}, Zn^{++}, Mg^{++}, Mn^{++}, or Ni^{++}. Zn^{++}, Cu^{++}, and Pb^{++} activated at 10^{-3}M. Fe^{++} and Ca^{++} were inactive. Activation was pH dependent and optima were observed from pH 4.5 to pH 6.3 depending on the nature and concentration of the cation. Substrate specificity toward 21 phosphomonesters and anhydrides increased with purification. The most purified enzyme preparation showed absolute specificity toward P-glycolate. Fluoride, cysteine, reduced glutathione, and p-chloro-mercuribenzoate were effective inhibitors.

P-glycolate phosphatase is a chloroplast enzyme (228,101). The enzyme is confined to photosynthetic tissue and activity increases rapidly on illumination of etiolated wheat seedlings. Levels also increase on illumination of dark-grown sunflower cotyledons (176) and Euglena gracilis (44). Chloroplast isolation and washing experiments which

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removed the enzyme were interpreted to suggest that P-glycolate phosphatase was in the chloroplast, possibly a loose association with the chloroplast surface, and that it may function as a glycolate permease in addition to hydrolysis of P-glycolate. The localization in the chloroplast was confirmed using nonaqueous isolation procedures (195,163) and aqueous isolation in a sorbitol medium (164,176). Washing the chloroplasts did not remove all of the P-glycolate phosphatase. Douce et al. (1973) confirmed that P-glycolate phosphatase was located in the chloroplast stroma by fractionation of spinach chloroplasts on sucrose density gradients.

The level of P-glycolate phosphatase in plants is subject to variation but is usually around 20 μmoles/min/mg chlorophyll (164) in C-3 plants and about one quarter this value in C-4 plants. The enzyme is mainly located in the bundle sheath chloroplasts of C-4 plants (164, 165), which is consistent with the lowered acitivity of glycolate metabolism in C-4 plants and its location in the bundle sheath cells is the same as that reported for RuDP oxygenase. Maximal P-glycolate phosphatase activities reported (170,228,164,4) are adequate to account for published rates of glycolate formation, but activities at higher physiological pH have not been measured.

Anderson (1969) purified the enzyme from tobacco leaves achieving a specific activity of 333 μmoles/min/mg protein. The Km of the purified enzyme at pH 6.3 was 7×10^{-5}M. He found that the enzyme, isolated from whole leaf homogenates, had many moles of bound tricarboxylic acids. These were citrate and isocitrate from tobacco leaves and cis-aconitate

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from wheat leaves; which are the tricarboxylic acids in preponderance in the vacuoles of these plants (207). This ionic association may be an artifact of isolation. However Anderson (1969) found removal of the tricarboxylic acids by gel filtration led to an initially active but highly unstable enzyme. Addition of tricarboxylic acids stabilised the enzyme. Cis-aconitate was a competitive inhibitor with a Ki of 2×10^{-3}M.

Anderson (1969) reported that P-glycolate phosphatase was highly active but unstable when isolated under reducing conditions (with mercaptoethanol or in N_2), but it could be stablished by oxidation with air. The oxidised enzyme was relatively heat stable, and failure to inactivate this phosphatase in plant tissue by killing in boiling alcohol of water (205) may have resulted in severe underestimation of the levels of P-glycolate in the tissue. However, the absence of a large pool of P-glycolate in the stroma is consistent with its rapid removal to prevent inhibition of carbohydrate metabolism. P-glycolate is a powerful inhibitor of pea leaf triose phosphate isomerase (6), with a Ki of 1.5×10^{-5} M for the chloroplastic and 4×10^{-6} M for the cytoplasmic enzyme. Obviously P-glycolate may regulate triose phosphate isomerase activity in the chloroplast. Wolfenden (1969,1970) and Johnson and Wolfenden (1970) found similar Ki values ($2\text{-}7 \times 10^{-6}$ M) for chicken and rabbit triose-P isomerases and suggested that the P-glycolate is acting as a transition state analogue.

Recently properties of a partially purified pea leaf P-glycolate phosphatase were reported (102). Highest activites were obtained using

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Mg^{++} at pH 8.3. Substrate and metal ion affinities were 30 and 60 fold weaker than previously reported and no specificity studies were performed. Maximum specific activity was 4% of that reported for the enzyme from tobacco leaves.

Orthophosphate Monoester Hydrolysis

Nonenzymic Hydrolysis

Since the literature on phosphate ester hydrolysis is voluminous, including mono-, di-, and triesters; phosphate anhydrides; alkyl and aryl substituents; and ortho, pyro, and triphosphoric acid esters; only those facts pertinent to enzymic hydrolysis of P-glycolate will be reported. Because phosphoryl transfer reactions are important in biochemistry, mechanistic studies of model systems have been reviewed extensively (49,94,33,37,23,175,218,227).

The pH-rate profile of monoalkyl phosphate hydrolysis (38) has a peak at pH 4 and indicates that the monoanion is the active species, and isotopic experiments indicated that phosphorus-oxygen bond fission occurs. The presence of a charge on the oxygen and a proton seemed essential for the mechanism and three possible routes were suggested: A. a preequilibrium transfer to give the zwitterion, B. a concerted proton transfer through a four-membered ring, which accompanies the P-O breaking, and C. avoidance of the energetically unfavourable four-membered ring by carrying out the proton transfer through a six-membered ring formed by incorporating a water molecule. The proton therefore acts to convert the alkoxide group to a better leaving group and protonation of the leaving group would explain the marked insensitivity of rates of hydrolysis to the nature of the leaving group

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group (37,105). Proton transfer is not possible for the dianion species, and nucleophilic attack by water would be expected to give greater rates with the neutral species than with the monoanion.

Dianions are relatively unreactive except in those cases where the leaving anion can be easily stabilised. Acyl phosphates, with carboxylate as a good leaving group, fall into this category (57), as do dinitrophenolphosphates (105,38). Hydrolysis rates of dianions are very sensitive to the pKa of the leaving group (105), which is consistent with a unimolecular elimination mechanism and monomeric metaphosphate formation. Thus the mechanism provides a rationale for general acid catalysis in the mechanism of phosphomonoesterases. The proton could be donated to the dianion by a dissociable acid group at the active site.

Metaphosphate ion probably never becomes completely free during the reaction due to its rapid capture by water or any alcohol present, and in some cases, the leaving group exerts limited specificity on the acceptors (38). The dissociative metaphosphate mechanism is also applicable to S-phosphate esters and phosphoramidates (137,21). Bronsted plots indicate the rate step for O-phosphate esters is diffusion; while for S-phosphate esters, proton transfer is involved (21). The high rates of S- and N- ester hydrolysis suggests why these compounds could act as kinetically competent intermediates during enzymic transfer. Also, a hydrophobic active site would minimize charge separation during metaphosphate formation, thus assisting catalysis.

Benkovic and Schray (1971) have reviewed model reactions involving nucleophilic attack of monoanions and dianions. They conclude that

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the associative bimolecular reactions cannot be ruled out in enzymic reactions, since additional groups on the enzyme could provide the rigid geometry required to stabilise the pentacovalent phosphorus intermediate. Phosphoryl oxygens could be protonated or complexed with metal ion to increase electrophilicity. Benkovic and Schray (1971) also indicate from experiments with model systems that metal ions could facilitate catalysis via the metaphosphate mechanism in a number of ways such as: A. a template for orienting substrates and enzymic catalytic groups, B. charge neutralisation, and C. chelation promoting metaphosphate expulsion.

The effect of vicinal carboxyl substituents in alkyl phosphomonesters is negligible. Phosphoglycolic acid is stable in weak acid at 37^{o} (103), phospholactic acid (208) and phosphoglyceric acids (133) are also of comparable stability to glycol phosphoric acid in weak acid. Intramolecular protonation is however, the accepted explanation for the extreme lability of salicyl phosphate as compared to its p and m analogues (20). The presence of a phosphoryl-enzyme intermediate in enzyme-mediated phosphotransferase reactions does not distinguish between dissociative and associative mechanisms.

Phosphomonoesterase Catalysis

The huge literature on this topic must again be confined to those results most directly pertinent to the mode of catalysis by the P-glycolate phosphatase of photosynthetic tissue. Phosphatases acting on simple substrates have been the subjects of numerous and comprehensive reviews. These include nonspecific acid phosphatase (89,174),

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nonspecific alkaline phosphatases from bacterial and mammalian sources (168,189,175,66), and glucose-6-phosphatase (192,39,146,147,46).

Historically, nonspecific phosphatases were divided on the basis of widely differing pH optima, and the terms acid and alkaline phosphatase were proposed by Davies in 1934. The first indication that other than generic differences were involved was the observation by Jenner and McKay (1931) that all nonspecific mammalian alkaline phosphatases required divalent metal ion. The mechanism of action of phosphatases has been the subject of very active research, since the introduction of isotopic labelling studies. This is possibly because of clinical interest, since plasma alkaline phosphatase levels increase dramatically in certain pathological conditions.

Transferase activity, established by isotopic labelling (136), followed the demonstration of phosphorus-oxygen bond cleveage for both classes of phosphatase (45). Stein and Koshland (1952) showed a single ^{18}O atom from (^{18}O)-H_2O was incorporated per phosphate released and that alkaline phosphate catalysed H_2O-Pi oxygen exchange. This inference of a Walden inversion type displacement and the known transferase activity led Morton (1955,1958) to propose a phosphorylated enzyme intermediate. Phosphoenzyme was first deteced by incubation of alkaline phosphatase with (^{32}P)-Pi and serine phosphate was isolated (19,61,62, 179,180). Barrett *et al*. (1969) and Neumann (1969) confirmed a kinetically important phosphoryl-enzyme intermediate existed by demonstrating that the ratio of the products for competing phosphoryl acceptors was independent of substrate. That the phosphoprotein was indeed the

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isolated intermediate was finally confirmed by Levine et al. (1969) and Reid et al. (1969) when kinetic analysis showed that the intermediate was also very stable. Failure to detect phosphoprotein at high pH was due to the greater stability of the Michaelis complex.

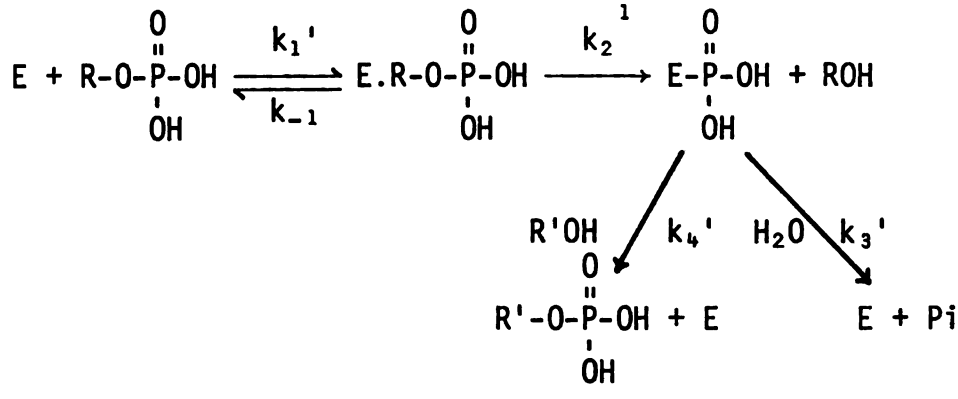
Indications that the acid phosphatases might not catalyse hydrolysis via a O-phosphorylserine intermediate were given by the action of diisopropylfluorophosphate (DFP). Alkaline phosphatase was weakly inhibited but the action was irreversible (51,139), yielding O-diisopropylphosphorylserine. Acid phosphatase was also inhibited, protected by substrate or competitive inhibtors from inhibition, but the inhibition was reversed by dialysis or by increasing the pH to 7.5 (74,73). Essentially no incorporation from (^{32}P)-DFP was found in the reactivated enzyme, indicating that the labelled serine found was not involved in the active site.

Igarashi et al. (1970) have isolated (^{32}P)-3-phosphohistidine from alkaline hydrolysates of crystalline rat liver acid phosphatase after incubation with (^{32}P)-Pi. They showed a single histidine residue per molecule by amino acid analysis and that histidine was important for catalytic activity. Microsomal glucose-6-phosphatase also involves a phosphohistidine intermediate in catalybis (65). More recently a phosphoryl-enzyme intermediate has been identified in the catalytic reaction of prostatic acid phosphatase (152). The properties suggest that phosphohistidine is involved here also. These studies show that the acid and alkaline phosphatases are most likely evolutionarily distinct. Furthermore, it appears that glucose-6-phosphatase, an

enzyme with substrate specificity, is more closely related to the non-specific acid phosphatases. This is consistent with its pH optimum range of pH 5.0-6.5 but inconsistent with its requirement for Mg^{++} ions.

Mechanistic differences are therefore likely between the two types of nonspecific phosphatases. Neumann (1968) has provided evidence supporting this prediction. She found that alkaline phosphatases hydrolyse S-substituted monoesters of phosphorothioic acid and O-substituted monoesters of orthophosphoric acid with identical kinetic parameters. O-substituted monoesters of phosphorothioic acid are not substrates but potent inhibitors. Acid phosphatases hydrolyse O-substituted monoesters of phosphorothioic acid but not S-substituted monoesters. The results suggest that two hydroxyl groups are essential for reactivity of alkaline phosphatases, whereas in acid phosphatases an oxygen linkage is essential for hydrolysis. It is therefore of interest that phosphoramidates are hydrolysed by glucose-6-phosphatase (154).

Early kinetic work is consistent with this reaction sequence in alkaline (114,222,95,3) and glucose-6-phosphatase (80,81,9,148, 182).



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Hsu et al. (1966) showed that the mechanism for potato non-specific acid phosphatase was similar to the above scheme, but presented evidence that two phosphoryl enzyme forms occurred and were interconvertible by isomerisation. Their reanalysis of earlier kinetic data with glucose-6-phosphatase and phosphoserine phosphatase (81) indicated that this isomerisation also occurred. Evidence that the rate determining step for glucose-6-phosphatase is the formation of phosphoryl enzyme through dissociation of the enzyme-substrate binary complex has been summarised (147).

Considerably more is known about the alkaline phosphatase from E. coli. This enzyme hydrolyses all phosphate esters at the same rate, and the addition of tris stimulates the rate of release of the alcohol but not phosphate (222). This data indicates that k_3^1 is rate determining. However, transient state kinetic studies (67,68) and the finding that enzyme dephosphorylation was more rapid than turnover at high pH (3) disputed this assessment.

Since then several explanations for this anomaly (202,115,167, 186,157,75) have lead to a presently accepted "flip-flop" hypothesis (76, 77,78,116). The enzyme is visualised as being in an asymmetric state with the active site of only one of the two subunits able to interact with the substrate, the other reacting preferentially with product. The sites are thought to alternate states during the catalytic sequence. However recent experiments with molecular hybrids, one active and one mutationally altered inactive subunit, which also showed "flip-flop" phenomena, cast doubt on this proposed mechanism (27).

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MATERIALS AND METHODS

Materials

Tobacco (Nicotiana tabaccum L. cv. Maryland Mammoth) seedlings were transplanted to the field in late May. The tobacco was fertilized regularly with commercial nitrogen fertilizer or 10mM urea as previous work on amount and specific activity of P-glycolate phosphatase had shown that both increase by application of abundant nitrogen. The leaves were used from July to late September. Greenhouse grown tobacco was less satisfactory as the specific activity was 2-3 fold lower in the crude homogenates. This phenomenon, plus flowering of the plants in the greenhouse, was attributed to the lower light intensities.

DEAE-cellulose (DE-52, microgranular) was obtained from Whatman and Sephadex G-200 from Pharmacia. TEMED, bis, and acrylamide were obtained from Canalco and the ampholines from LKB. P-glycolate is a product of General Biochemicals, and DHAP, FMN, NAD, NADH, Ribose-5-P, wheat germ lipase and DFP were obtained from Sigma Chemical Co. Deuterium oxide and Chelex 100 were purchased from Biorad. Glycolide is a product of American Cyanamid. Bis (Trimethylsilyl) trifluoroacetimide containing 1% (v/v) trimethylchlorosilane was obtained from Regis. Anhydrous phosphorous acid, ethylene glycol, and resublimed iodine were obtained from Fisher. Cyclohexylamine, triethylamine, chloroethanol, 2-methoxyethanol, guanidine HCl, bromoacetic acid, methyl glycolate, and

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2-cyanoethylphosphate were products of Eastman Kodak. Thiophosphoryl-trichloride was purchased from Alpha Products.

Glycidol-P was a gift from I. A. Rose, phosphonates a gift from I. F. Isbel and (^{18}O)-H_2O (61% atoms excess) a gift from R. H. Gerster. (1-^{14}C)-glycolate was from Radiochemical Centre, Amersham and (U-^{14}C)-citrate was obtained from Calbiochem. Chemicals used were analytical grade unless noted and used without further purification.

Methods

Protein Determination

Protein was determined by the method of Lowry *et al*. (1951), using bovine serum albumin as a standard. Purified protein solutions were analysed by the method of Warburg and Christian (1942).

Phosphoglycolate Phosphatase Assays

Routine assays were a modification of that previously described (5). The complete assay medium contained, in 0.5 ml, the following components: 20 µmole cacodylate buffer, pH 6.3; 2 µmole $MgCl_2$; 1 µmole P-glycolate (sodium or tricyclohexylammonium salt); and 0.1 unit P-glycolate phosphatase. The reaction at 30° was initiated with enzyme and terminated after 10 min with 0.2 ml 10% TCA. After centrifugation 0.5 ml was analysed for released phosphate as previously described (5). One unit of enzyme activity is defined as the amount of enzyme required to hydrolyse one µmole of P-glycolate per minute at 30°. The reaction was linear with time and enzyme concentration until about 40% of the substrate had been hydrolysed.

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A microphosphate analytical technique (4) was used to analyse nanomolar amounts of phosphate. Precise conditions are noted in the text.

Assays of glycolate released were obtained using (2-^{14}C)-P-glycolate (1.35 mc/mmole, Nuclear Research Chemicals). The assay was terminated by making the solution 0.05 M in HCl. An aliquot was added to a Dowex anion exchange resin column of AG 1-X4 (Biorad) (5 cm x 0.4 cm i.d.). Glycolate was eluted with 1.5 ml of 0.05 M HCl, neutralised with 75 µmole KOH, and the radioactivity determined in 19 ml of Bray's Scintillant. Counting efficiency was 70%.

High concentrations of glycolate (0.1 M) interfere with the P-glycolate-glycolate separation on Dowex-1. Standard (^{14}C)-glycolate was used to correct for retention of glycolate under the elution conditions used.

A spectrophotometric method, based on the technique of Schwabe (1970), who measured small pH changes associated with ATP hydrolysis, was tried. Although rates were linear for 60 seconds, inconsistencies with enzyme concentration and calibration difficulties precluded its routine use. The complete assay system contained 2 µmole barbital buffer at pH 7.5, 2.5 µmole P-glycolate, 3 µmole $CoCl_2$, 0.1 unit phosphatase in 3.0 ml. The reaction was initiated with enzyme and the initial velocity at 245 nm recorded for 60 seconds at 25^{o}. Blank rates from minus enzyme or minus P-glycolate or with boiled enzyme were negligible. This assay and another based on alkali production during the phosphatase reaction are discussed fully in the final section.

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Purification Steps for Phosphoglycolate Phosphatase from Tobacco Leaves

Homogenisation.--Leaves were homogenised within about 45 min after removal from the plant. Leaves larger than about 20 cm in length were washed, blotted dry, deveined, and weighed at room temperature. Homogenisation by a Waring Blendor was performed in a 4^{o}. The slurry, prepared over several minutes from successive additions of leaves, was strained through 4 layers of cheese cloth. The resultant green homogenate had a range between pH 5.7-6.0, and would loose 50% of the initial P-glycolate phosphatase activity over several days.

Acidification.--Normally acidification was carried out continuour with preparation of the homogenate. Aqueous HCl was added to the homogenate til a pH 4.8-5.0 was obtained, and stirring continued for 15 min at 4^{o}. The homogenate was centrifuged at 8000 x g for 15 min and the brown supernatant decanted and readjusted to pH 6.3-6.5 for maximum stability. This solution, called the acid supernatant, was stable for several weeks at 4^{o}. Longer periods of storage led to slow precipitation of white material, with only a small decrease in specific activity. If the solution was frozen at -20^{o}, the activity was stable for at least six months.

Acetone Precipitation.--The procedure was carried out in a large reach-in refrigerator at 4^{o}. Acetone was cooled to -20^{o} and added to the acid supernatant with continuous vigourous stirring. Addition was through a 1mm(i.d.) teflon tubing as a siphon, and the acetone (reagent

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grade) was permitted to run down the side of the beaker containing the supernatant. Each fractionation range was chosen from the results of a small pilot experiment. In general the cut between 0-25% acetone was discarded by centrifugation, and the enzyme found in the precipitate of the 25-40% acetone cut. After all the acetone had been added, solutions were stirred for a further 15 min at 4^{o}. The pellet was either dissolved in a small volume of 0.02M cacodylate buffer at pH 6.3 or was slowly dried at room temperature and pressure. This solid was ground with a mortar and pestle and stored in a dessicator at 4^{o}. On resuspension the powder dissolved almost completely and the solution was stable for several weeks at 4^{o} and for many months at -20^{o}. The enzyme was more stable in solutions formed from the acetone powder than by directly resuspending the acetone pellet. The latter solution lost activity and formed a precipitate slowly over a period of several weeks. In none of these acetone precipitates did the specific activity decrease markedly with storage time.

DEAE-cellulose Chromatography.--DEAE-cellulose was prepared and the column poured as described in the Whatman Laboratory Manual (Method 2). The elutant buffer, 0.02 M cacodylate at pH 6.3, was also used as equilibrant buffer, but with the addition of a KCl gradient. Prior to addition of the sample, buffer was passed through the column until both the pH and the conductivity were constant and identical in eluate and eluent, which usually required at least 10 column volumes. The sample was applied at rates between 0.3-0.5 ml/min, and the column was normally washed with at least one column volume of buffer before

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The eluted protein solutions were stable for several days at 4^{o}, but stability was increased by concentrating the solution by adding citrate. On occasion low levels (10mM) of citrate were present throughout the chromatography; however higher levels (50mM), which were necessary for maximal stabilisation, prevent P-glycolate phosphatase from binding to DEAE-cellulose at pH 6.3.

Molecular Sieving.--Separations utilised Sephadex G-200 columns (2.4cm x 50 cm) at 4^{o}. The gel was prepared in the eluting buffer of 0.02 M cacodylate and 0.02 M citrate at pH 6.3 by heating on a steam bath for 12 hours. It then sat for 2 days at 4^{o} before packing. All the gel was poured into a funnel above the column, allowed to settle, and then packed by elution with a 15-20 cm hydrostatic head to maintain a flow rate of 0.3-0.5 ml/min. The column was checked for homogeneity prior to use with 1 ml of 1% Dextran-2000, the criterion being a distinct narrow band on the column and a clean elution profile. The void volume was measured at the same time to calibrate the column. The sample was normally chromatographed in the upwards direction, using the same pressure as used to pack the column. Fractions of 2-10 ml were collected.

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The sample was applied in a volume of 1-3 ml and dilute solutions had been concentrated to this volume in Diaflo Ultrafiltration Apparatus (Amicon) using a PM 30 membrane. Phosphatase in the fractions after gel filtration was stable for several weeks at 4^{o} and this stability could be improved by concentration of the sample.

Preparative Polyacrylamide Gel Electrophoresis.--Preparative polyacrylamide gel electrophoresis (PPGE) was carreid out with commercial equipment (Canalco) at 4^{o} using the same system as described below for analytical polyacrylamide gel electrophoresis (PAGE). The running gel was 2-3 ml and the stacking gel was 0.5 ml. The sample, less than 1.0 ml, was carefully layered onto the gel using a syringe with a narrow gauge teflon tubing fitted over the needle. Electrophoresis was run at 5 ma and 300-400 V, and the current did not alter noticeably during the run. The phosphatase eluted after 90-120 min, some 30-45 min after the tracking dye. Coloured material, probably highly charged polyphenolic compounds, moved with the dye front, so that the dye (Bromophenol Blue) could usually be omitted. The fractions were eluted in reservoir buffer (0.018 M glycine, 0.0033 M tris at pH 8.3). Since the fractions were very dilute protein solutions and quite unstable, the enzyme was rapidly concentrated and stored at 4^{o} in 0.02 M cacodylate, 0.05 M citrate, pH 6.3. At concentrations of 1 mg/ml the enzyme was stable for at least six months.

Analytical Polyacrylamide Gel Electrophoresis

The system used for all analyses was modified from Davies (1964) and Williams and Reisfeld (1964). The solutions used were as follows:

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A1. 36.3 g tris, 48 ml 1M HCl, 0.23 ml TEMED, pH 8.9 in 100 ml

A2. 3.0 g tris, 48 ml 1M HCl, 0.23 ml TEMED, pH 6.8 in 100 ml

B. 20 g acrylamide, 1g bis in 100 ml

C. 0.56 g ammonium persulphate in 100 ml

D. 14 g glycine, 3.0 g tris, pH 8.3 in 1000 ml.

Gels were prepared by mixing 1 part A1, 1 part C, an amount of B to give the desired acrylamide concentration, and water to 8 parts total. The solution was deaerated on a water pump for 30 seconds, then 2 ml aliquots were allowed to gel at room temperature in 0.5 cm (i.d.) glass tubing. The stacking gels were prepared in the same manner as the running gels but using A2 instead of A1, and 0.25 ml was used per tube. The gels were stored at 4^{o} for at least 6 hr before use, and discarded after 4 days if not used. The reservoir buffer, D, was used in both the upper and lower chambers at 10 fold dilution. The gels were run vertically at 4ma per tube for 2-2.5 hr. Bromophenol blue was used as the tracking dye. Gels were removed immediately using a water-filled syringe with a long size 23 needle to loosen them from the glass tube and then stained.

Staining Procedures

Protein was stained with 1% Coomassie Brilliant Blue dissolved in 7% acetic acid and 33% methanol. The gel was shaken gently for 12 hr in the staining solution and then destained in 7% acetic acid and 33% methanol with about 0.2 g of Dowex-1 beads by shaking for 12 hr. Protein analysis on isoelectric focussing gels containing ampholines were stained and destained as reported below. Both gels were scanned at 660nm using the Gilford Linear Transport gel scanner.

P-glycolate phosphatase activity was located on the gels by a procedure based on the formation of a white lead phosphate formed simultaneously with the enzymic activity. Gels were stained for 15-60 min in the following mixture: equal volumes of 0.2 M cacodylate buffer at pH 6.3, 12mM $Mg(acetate)_2$, 12mM $Pb(acetate)_2$, and 25mM P-glycolate. The staining solution was prepared fresh each time and appeared slightly turbid presumably due to formation of the lead salt of P-glycolate. Lead is inhibitory of Mg^{++} activated enzymes, including P-glycolate phosphatase, but my empirically derived formulation gave satisfactory results to locate this enzyme. A white band indicated P-glycolate phosphatase on the gel, and it did not form in the absence of any of the above components nor did boiled enzyme give any staining. Occasionally very light staining occurred at the end of the gel beyond the tracking dye and may have been due to precipitated $PbCl_2$. The gel was destained in distilled water for several hours. Normally the appearance of the white lead phosphate band was sufficient, but better visualisation could be obtained by incubation of the gel in 5% aqueous ammonium sulphide for 2 min, and destaining again in distilled water. This treatment changes the band to black, PbS, but it does not alter the measured Rf. If however, the lead phosphate stain is very intense, the diffusion of $(NH_4)_2S$ into the band is hindered and leads to an artifactual double black band. Both stains could be measured at 660nm on the gel scanner due to their opacity. Usually about 0.2 units of enzyme was needed for a good gel band. The lead staining procedure produced quite broad bands probably due to diffusion of phosphate away from the enzyme. Gel scans

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gave a perfectly symmetrical and sharp peak however and Rf's measured from gel scans or visually using the band midpoint were identical.

The enzyme activity and protein Rf's were analysed as follows:

$$Rf = \frac{x_2}{x_1} \cdot \frac{l_1}{l_2}$$

where

l_1 - gel length after electrophoresis

x_1 - tracking dye position after electrophoresis

l_2 - gel length after staining

x_2 - enzymic activity/protein band after staining.

Measurements made using a millimetre ruler or using gel scan peaks gave identical results and similar accuracy. The stacking gel length was ignored in making the measurements.

Sedimentation Velocity on Sucrose Density Gradients

This technique is that of Martin and Ames (1961). Sucrose gradients of total volume 4.55 ml were prepared at 4^{o}. The gradients were linear, from 5-20% sucrose (w/v) in 0.02 M cacodylate, 0.02 M citrate buffer at pH 6.3. Each gradient was poured separately over a period of about 10 min; more rapid pouring resulted in nonlinear gradients. A solution of the enzymes was prepared as follows:

100µl purified P-glycolate phosphatase (62 unit/ml, SA of 38)

10µl malate dehydrogenase (pigheart, in $(NH_4)_2SO_4$ from Sigma)

10µl α-glycerophosphate dehydrogenase (rabbit muscle, in $(NH_4)_2SO_4$ from Sigma)

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10µl lactate dehydrogenase (rabbit muscle, in $(NH_4)_2SO_4$ from Sigma)

This mixture was dialysed for 6 hr against the cacodyate-citrate buffer and the additional lyophilised enzymes added:

0.2 mg catalase (beef liver, Worthington)

0.2 mg alcohol dehydrogenase (yeast, Worthington)

0.2 mg carbonic anhydrase (beef blood, Worthington).

Total volume was 170µl, and 100µl was layered onto one gradient and 100µl of phosphatase only was layered onto a second gradient. The gradients were run in the SE39 head of the Spinco LC2-B centrifuge at 5^{o} for 13 hr at 37,000 rpm. The gradients were collected after development in 4 drop fractions from the bottom of the tubes. No attempt was made to quantitate the enzyme recovery, and the activities expressed in Figure 7 are all relative. The enzyme assays are all to be found in "Methods in Enzymology."

SDS-gel Electrophoresis

The technique is essentially that of Shapiro et al. (1967) and analysis followed the procedures of Weber and Osborn (1969). The gel was prepared using equal parts of the following solutions:

A. 0.4 M sodium phosphate, pH 7.1, 0.4% SDS, 0.12% TEMED

B. 30% acrylamide, 0.65% bis

C. 0.28% ammonium persulphate

D. H_2O

The mixture was degassed and allowed to polymerise for 30 min at room temperature in 10cm x 0.4cm (i.d.) tubes and used within 4 days.

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The electrophoresis buffer was 0.1 M sodium phosphate at pH 7.1, containing 0.1% SDS.

The SDS-protein complex was prepared by dissolving 0.5 mg of protein in a 100µl solution of 0.1 M sodium phosphate at pH 7.1, 1% SDS, 1% mercaptoethanol, and 10% glycerol. This mixture was incubated at 100° for 10 min, cooled and 5µl of 0.05% Bromophenol blue was added. Electrophoresis toward the anode was performed at 8 ma per tube for 4 hours. Protein samples were 25-50 µg per tube.

The gels were stained in Coomassie Brilliant Blue R250 and destained as described for polyacrylamide gels. Rf's were calculated by methods also previously described.

Isoelectric Focussing on Polyacrylamide Gels

The technique of Malik and Berrie (1972) was modified to allow the native enzyme to be studied. The system without urea, and with FMN in place of riboflavin contained:

3.6 ml 28% acrylamide, 1.5 bis

1.0 ml 1.8mM FMN

0.08 ml 2N H_2SO_4

0.6 ml ampholine (LKB range pH 3-10)

1.0 ml 0.8% TEMED

11.1 ml H_2O

The gel mixture was degassed for 30 seconds on a water pump and allowed to polymerise at room temperature in 10cm x 0.4 cm (i.d.) glass tubing under fluorescent lighting for 30 minutes. Electrophoresis was

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performed at 4^{o} at a constant 300V for 8-14 hours to allow complete focussing of the proteins. The ampholines took 2-2.5 hr to form the pH gradient since the current dropped from an initial 15 ma to a constant 2-5 ma during this period. The upper reservoir was 0.2% H_2SO_4 and the lower cathode reservoir was 0.4% triethanolamine. From 10-20µl of enzyme was mixed with 30-40µl of a sucrose solution (100mg/ml) and applied by syringe below an acid lock of 100µl of sucrose solution (50mg/ml). Both sucrose solutions contained 30µl ampholine per ml sucrose solution.

The gels were analysed for enzymic activity, protein or pH gradient. The enzymic activity was detected using the lead phosphate stain. Protein was visualised by the rapid staining technique of Malik and Berrie (1972). The pH gradient was measured by slicing the gel into equal sections, usually 0.3-0.5 cm in length, and mascerating these slices in 1.0 ml of CO_2 free, distilled, deionised water overnight. The pH was read directly from a pH meter after 12 hours.

Calculation of Phosphoglycolate-Metal Ion Equilibria

The P-glycolate-magnesium complex concentration was calculated by a quadratic equation. Since the total Mg^{++} and total P-glycolate concentrations in the enzyme assay are known, the concentrations of free P-glycolate and Mg^{++} were calculated. It is assumed that only phosphate monoanion and dianion species were present in the pH range investigated (pH 5-9.5), that the P-glycolate carboxyl remained ionised fully, and that the amount of enzyme was negligible. The following species were defined symbolically:

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K1 = K_2, the ionisation constant for the P-glycolate phosphate dianion

K2 = $1/K_D$, dissociation constant for the P-glycolate-Mg complex

X = total P-glycolate concentration

Y = total Mg^{++} concentration

H = H^+ concentration

R = P-glycolate-Mg complex concentration

S1 = P-glycolate phosphate monoanion concentration

S2 = P-glycolate phosphate dianion concentration

M = free Mg^{++} concentration

The system was defined by the following equilibria in terms of the four unknowns: R, S1, S2, and M;

$X = S1 + S2 + R$

$Y = M + R$

$K1 = S2 \times H/S1$

$K2 = S2 \times M/R$

Solution of these equations leads to the following equation, which is directly soluble for R:

$$K1 \times R^2 - (K1 \times X + K1 \times Y + K2 \times H + K1 \times K2)R + K1 \times X \times Y = 0$$

Preparation of Standard Trimethylsilyl Derivatives

Two μmole of the compound, as the monovalent salt, was added to 200μl acetonitrile plus 100μl bis(trimethylsilyl)trifluoroacetimide containing 1% (v/v) trimethylchlorosilane in a pyrex tube. Tubes were sealed under a vacuum of less 10 micron (10^{-3} mm) Hg and heated in an

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oven for 35 minutes at 110° to facilitate the reaction. Samples were then stored at 4° until used.

Mass Spectrometry of Standard Trimethylsilyl Derivatives

Analysis of silylated derivatives was performed on 1-5µl aliquots with a LKB 9000 combined GLC and mass spectrometer using a 3% (w/v) SD-2401 column (1.4m x 3mm i.d.). Samples were eluted by a 50-200° temperature gradient which increased at 2-5°/min with a flow rate of 30 ml/min. The temperature of the ion scource was 290° and the ionizing voltage was 70eV. Mass spectra were obtained in the form of computer drawn, normalised bar graphs (193). Relative ion intensities were obtained from tabulations of bar graph data or by measuring peak heights from UV oscillographic recordings. Isotope incorporation was measured by the procedure of Thorpe and Sweeley (1967). The isotopic abundance in fragment ions of standard mass spectra was determined experimentally, rather than from probability theory based on empirical formulae. This value (f) was determined as the ratio of M + 2 (observed)/ M (observed) where M and M + 2 are the intensities of the ions at m/e = M and m/e = M + 2 in the standard compound. Samples containing ^{18}O were corrected for normal isotopic abundance as:

$$(M + 2)_{corr.} = (M + 2)_{obs.} - f \times M_{obs.}$$

Thus, $$\%\ ^{18}O = X(M + 2)_{corr.} \times 100/M_{obs.} + (M + 2)_{corr.}$$

where X is a factor to compensate for the presence of more than one oxygen atom in the ion fragment e.g.

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Preparation of (^{18}O)-KH_2PO_4

Two point five mmole PCl_5 was weighted into a small vented flask with a rubber stopper and placed in an ice bath. Then 0.235 ml of 2.59% atoms excess (^{18}O)-H_2O was slowly added and the HCl evolved escaped through the vent. The viscous liquid was evacuated to remove all the HCl and chlorine, and the clear syrup was titrated with 1M KOH to pH 4.4. This solution was made 80% (v/v) ethanol and KH_2PO_4 was allowed to precipitate at 2^{o} for 48 hours. The crystals were washed 3 times in 80% ethanol by centrifugation to remove any KCl, and dried under vacuum over silica at room temperature. The yield was 295 mg (93%).

Analysis of (^{18}O)-KH_2PO_4

The ^{18}O was analysed either after combustion to CO_2 or directly as the TMS derivative as described above. For the CO_2 analysis combustion tubes were prepared by sealing 8mm pyrex tubing at one end, and then washing them in chromic acid and distilled water before drying at 450^{o} for 6 hours. Tubes were stored under vacuum over silica until used. Five mg of KH_2PO_4 and 50 mg guanidine HCl were evacuated in a combustion tube for 1 hour to remove all traces of water. The tube was placed in a vacuum combustion apparatus and heated carefully by an O_2-gas flame, since overheating causes incorporation of silica oxygen. The CO_2 evolved was trapped in a liquid N_2 cooled 50 ml flask on the vacuum line. Samples were analysed on a Hitachi mass spectrometer at 70eV, 1.25-2.0 kV. Bleed into the system of background CO_2 was

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negligible. The ratio of m/e 46 to m/e 44 was calculated from averaged peak heights from 5-10 oscillographic spectra. Fractional excess (τ) ^{18}O was calculated from the ratio measured by the relation $\tau = R/2 - R$, where R is the ratio above, and corrected for the natural abundance.

Synthesis of Substrate Analogs

Several phosphate monoesters, chosen for their similarity to the substrate, P-glycolate, were synthesized by the method of Kirby (1963). Anhydrous phosphorous acid, 0.82 g (0.010 mole) was dissolved in 20 ml of the appropriate alcohol and 5 ml triethylamine. To this 3.8 g resublimed I_2 was added and swirled for 30 sec at room temperature to dissolve and react, and the mixture poured into 300 ml ice cold acetone. Cyclohexylamine (10 ml) was added and allowed to stand 15 min to precipitate the dicyclohexylammonium phosphate salt. The precipitate was washed extensively with ice cold acetone to remove I_2 and triethylammonium iodide and then dissolved in a minimum volume of absolute ethanol (50-150 ml) containing 0.5-1.0 ml cyclohexylamine. The salt was allowed to recrystallize on ice, refiltered, and washed with cold ethanol. The white crystals were dryed and stored over silica at 4^{o}. Yields were 40-70% based on H_3PO_3. The large excess of alcohol minimizes the formation of diester during the reaction.

The purity of the phosphate esters were examined by: A. Paper chromatography. Approximately 1 μmole was dissolved in 10μl H_2O and spotted onto Whatman No. 1 paper (10.5" x 30"). The chromatogram was developed for 3 hr by descending chromatography in methanol:formic acid:water (80:15:5) in a sealed glass jar that had been pre-equilibrated

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for 8-12 hr with the solvent. The chromatogram was dried and sprayed lightly with a solution containing 5ml 60% (v/v) perchloric acid, 10 ml 1M HCl, 25 ml 4% (w/v) ammonium molybdate, and 60 ml H_2O (14). It was oven dried at 90° for 1 min and exposed to UV light for 10 min. Organic phosphates gave blue spots and inorganic phosphate yellow blue. All synthesized compounds gave single spots indicating the absence of diesters and inorganic phosphate. Synthesized methyl-P had the same Rf as authentic monomethyl phosphoric acid (Victor Chemical Works). Rf's varied from 0.38 to 0.75 (Table 1). B. The melting points of selected compounds were analysed on a Fisher-Johns Melting Point Apparatus without correction (Table 1).

Synthesis of S-(carboxymethyl)-phosphorothioate, Barium salt

A slight excess of bromoacetic acid was added to an aqueous solution of trisodium phosphorothioate, and the product precipitated as the barium salt (2). After filtering, washing with ice water and absolute ethanol, a white flaky powder was obtained in 35% yield. The barium salt was converted to sodium prior to use by stirring the white solid with Dowex AG 50W-X4 (Na^+ form) til it dissolved and the resin was removed by centrifugation.

Synthesis of Trisodium phosphorothioate

Using the procedure of Yasuda and Lambert (1955), 75 ml PCl_3 was refluxed in 300 ml 3.3 M NaOH at 110-115° til the oily phase disappeared. The solution was cooled overnight in an icebath to precipitate NaCl and

TABLE 1.--P

Compound
Ethyl-P
Methyl-P
Methyl-P[b]
Phosphate
Isopropyl-P
2-Chloroeth
2-Hydroxyet
t-Butyl-P
P-Glycolate
methyl es
P-Glycolate
P-lactate[b]
Pyrophosph

[a] Solvent s
[b] Authentic
[c] Williams
[d] Kugel an
[e] Cramer et

TABLE 1.--Properties of Some Alkyl Phosphate Monoesters.

Compound	Rf[a]	Observed M.P. °C	Literature M.P. °C
Ethyl-P	0.60	173-176	169-170[c]
Methyl-P	0.70	178-182	160[c]
Methyl-P[b]	0.73		
Phosphate	0.52		
Isopropyl-P	0.52	205-209	209-212[c]
			212-214[d]
2-Chloroethyl-P	0.73		
2-Hydroxyethyl-P	0.68		
t-Butyl-P		177-179	168-170[c]
			191-193[e]
P-glycolate, methyl ester	0.65		
P-glycolate[b]	0.58		
P-lactate[b]	0.61		
Pyrophosphate[b]	0.38		

[a]Solvent system described in text.
[b]Authentic commercial compound.
[c]Williams and Naylor (1971).
[d]Kugel and Halman (1967).
[e]Cramer et al. (1962).

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the Na_3PO_3S. After filtering, this material was dissolved in a minimum volume of water at 40-45° and this Na_3PO_3S precipitated by the addition of 185 ml MeOH per 100 ml salt solution. This procedure was repeated and then the crystals were dehydrated by stirring in anhydrous methanol for 1 hour. The crystals were filtered, heated at 100° for 1 hour and stored over silica at 2°.

Since phosphorothioates are acid lablie, phosphate released enzymically had to be assayed by the method of Lowry and López (1946).

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PHOSPHOGLYCOLATE PHOSPHATASE:
PURIFICATION AND PROPERTIES

Introduction

Since the discovery of a chloroplast phosphatase specific for P-glycolate, P-glycolate has been suggested as an intermediate in glycolate and photorespiration. With the characterisation of P-glycolate as the product of RuDP oxygenase, an enzyme with sufficient activity to account for glycolate production during photosynthesis, this role was confirmed.

Anderson (1969) in this laboratory purified the enzyme from tobacco leaves to near homogeneity by a single, rather ambiguous, criterion. The highest specific activity he obtained was 333 μmoles/min/mg protein. From a single preliminary experimental determination of the retardation coefficient on Biogel P-60 he suggested a molecular weight of 20-30,000 daltons. He also detected the presence of endogenously bound citrate and isocitrate to the enzyme, which suggests that the enzyme may have a large net negative charge.

Results and Discussion

Purification

The data in Table 2 is a representative purification of P-glycolate phosphatase. The steps up to the third acetone fractionation are similar to those used by Anderson (1969). Use of an acidification

TABLE 2.--Pu

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TABLE 2.--Purification of P-glycolate Phosphatase.

	Total Activity µmoles/min	Yield %	Specific Activity µmoles/min /mg protein	Fold Purification
Homogenate	4030	100	0.314	1
First Acetone Fractionation	4230	105	6.30	20
Second Acetone Fractionation	3020	75	18.5	59
Third Acetone Fractionation	2860	71	31.8	101
DEAE-cellulose Chromatography	2420	60	100	314
Sephadex G-200 Chromatography	1200	30	152	484
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step as described in the methods enabled the third acetone step to be omitted, but acetone powders prepared after the acidification step were less stable, so the acidification procedure is not recommended.

In a typical elution profile from DEAE-cellulose (Figure 1) P-glycolate phosphatase elutes at about 0.15-0.20 M KCl. The active fractions are brown in colour as are those fractions eluting later from the column. The tendency for the enzyme to trail from the column accounted for the greater part of the activity lost at this step. Addition of 0.02 M citrate decreased the amount retained beyond the peak; but higher levels prevented the enzyme from binding to the DEAE-cellulose, the enzyme eluting in one column volume in 0.05 M citrate. Since citrate may be bound endogenously to P-glycolate phosphatase (4), the poor chromatographic resolution may be due to some variation in the numbers of citrate molecules bound to the protein.

P-glycolate phosphatase eluted from Sephadex G-200 in a symmetrical peak beyond the void volume (Figure 2A). The protein profile was often somewhat broader than shown, particularly for lower molecular weight material. The active fractions are usually slightly coloured but most of the coloured, non protein material, was retarded further in the column.

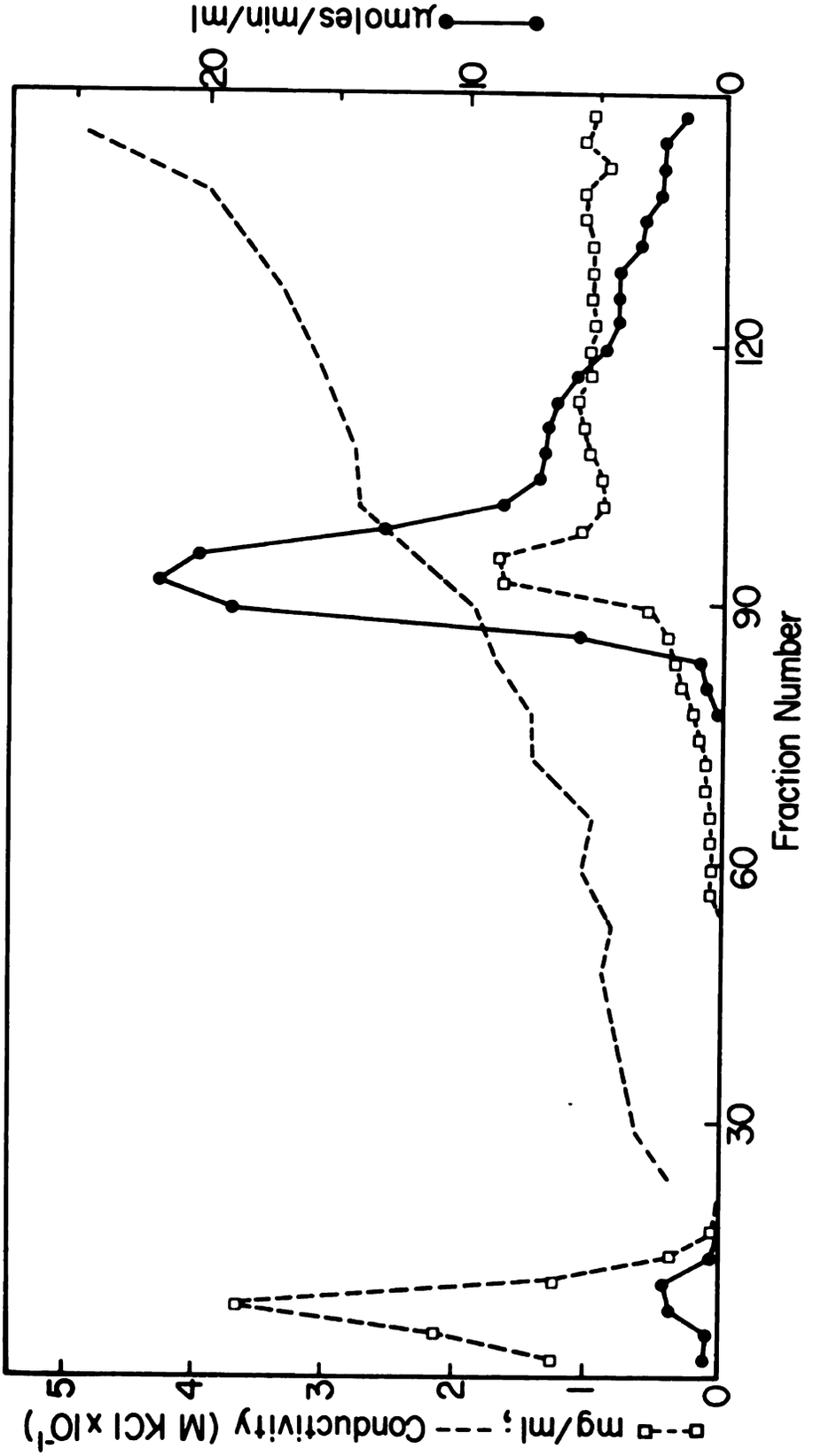
The best separations with PPGE were obtained using a running gel of 7% total acrylamide in columns about 4 cm in height (Figure 2B). Resolution was increased by higher acrylamide concentration and longer gels, but both these changes resulted in longer times before elution and consequently greater losses in activity. The enzyme was rather

Figure 1.--DEAE-cellulose column chromatography of P-glycolate phosphatase after acetone precipitation.

● , phosphatase activity;

□ , protein;

– – – – , KCl gradient.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mg/ml ; --- Conductivity (M KCl x 10⁻¹)
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3
4
5

Figure 2A.--Sephadex G-200 chromatography of P-glycolate phosphatase from pooled and concentrated DEAE-cellulose fractions.

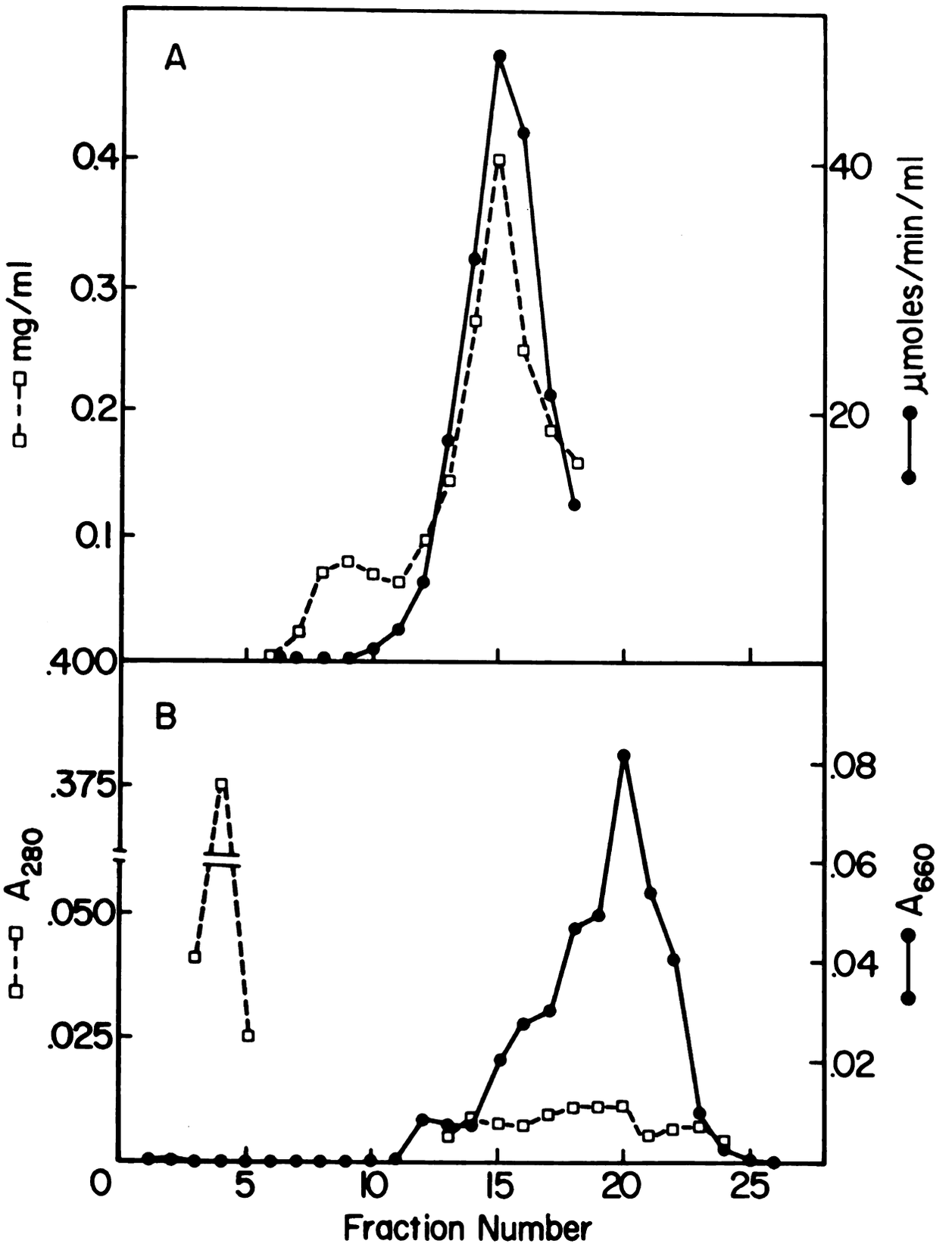
● , phosphatase activity;

□, protein.

Figure 2B.--Preparative polyacrylamide gel electrophoresis of P-glycolate phosphatase obtained after Sephadex G-200 fractionation.

● , phosphatase activity;

□, protein (280nm).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unstable during electrophoresis due to the high pH in the running gel and because electrophoresis removed from the enzyme most of the citrate needed for its stability (see Figure 6).

The P-glycolate electrophoresed in a rather diffused band. This was inherent rather than due to the method of collecting fractions, since both protein and enzyme appeared diffused. Pooled phosphatase fractions from PPGE had specific activities of 300 or greater. The preparations contained P-glycolate phosphatase which was 90-95% homogeneous by criteria outlined subsequently.

Purification of Phosphatase from Spinach Leaves

The properties of the enzymes from the tobacco and spinach leaves were very similar, and can be purified using essentially the same procedure. Specific activities were about the same for either source. In this section some modifications in the purification procedure used for the preparation from tobacco leaves are mentioned when using spinach leaves. The spinach enzyme required a higher percent of acetone to precipitate, but this may not be due to differences in the enzymes per se, but rather protein concentration or ionic strength. Dioxane was added to facilitate DEAE-cellulose chromatography. When chromatographed in the absence of 5% dioxane (Figure 3A) the enzyme appeared to elute nonspecifically with each protein peak. Addition of dioxane, thereby reducing the dielectric strength of the buffer, caused P-glycolate phosphatase to elute in a single peak (Figure 3B). This suggested that the nonspecific interactions were ionic in character and it is known that P-glycolate phosphatase has a very low pI. Dioxane

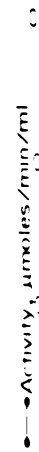
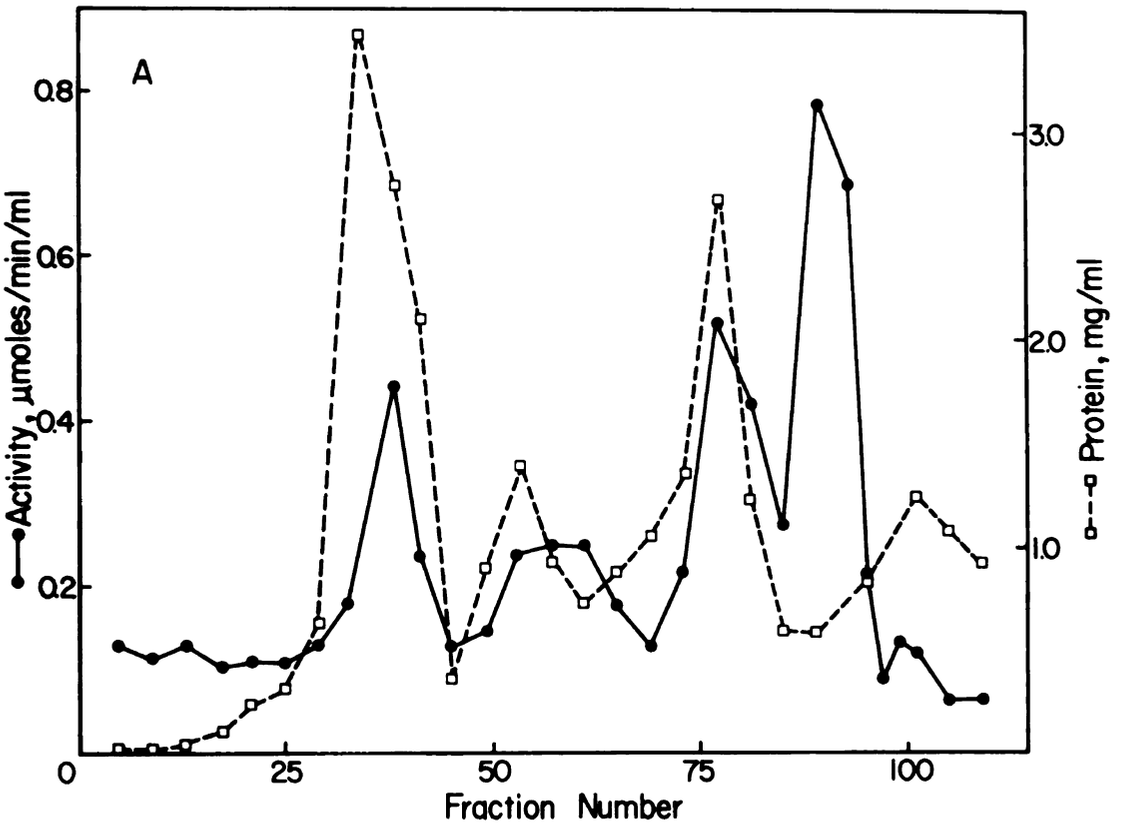


Figure 3.--DEAE-cellulose chromatography of spinach leaf P-glycolate phosphatase. The enzyme had been partially purified by acetone fractionation. Experiments were performed at 4° with 2.5 cm (i.d.) x 45 cm columns.

A. Equilibrant and eluant buffer was 0.02 M cacodylate at pH 6.3. The gradient was 0-0.4 M KCl.

B. Equilibrant and eluant buffer was 0.02 M cacodylate at pH 6.3 containing 5% p-dioxane (v/v). The gradient was 0-0.4 M KCl.


A
0.8
0.6
0.4
0.2
Activity, µmoles/min/ml
3.0
2.0
1.0
Protein, mg/ml
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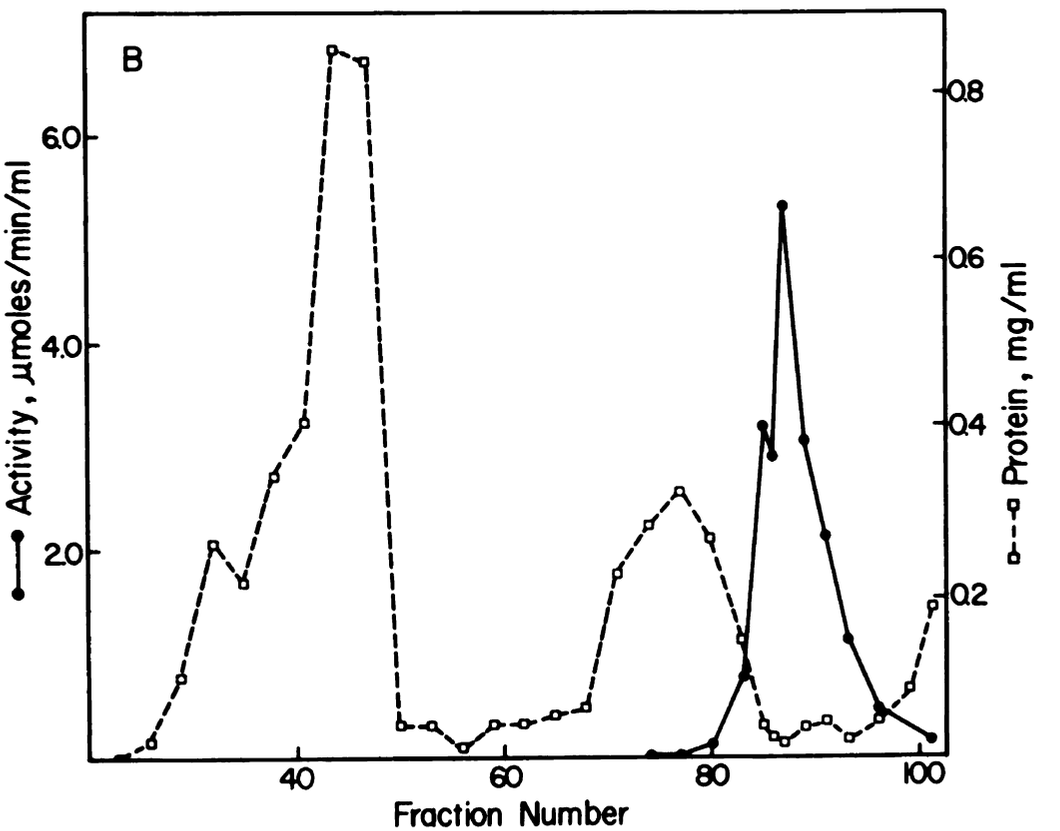
50
75
100
Fraction Number


B
6.0
4.0
2.0
Activity, µmoles/min/ml
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Protein, mg/ml
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was not found to have any beneficial or deleterious effects on enzyme stability. The phosphatase from DEAE-cellulose chromatography inevitably lost all activity within several days. Recoveries from subsequent purification procedures such as gel chromatography were usually zero.

Behavior of spinach P-glycolate phosphatase on gel chromatography was qualitatively similar to that from tobacco leaves. Low molecular fractions were found to activate or stablize the phosphatase from earlier fractions. When chromatography was performed with 5 mM citrate included in equilibrant and eluant buffers, the enzyme fractions were stable and were further not activated by small molecular weight fractions from the column. The unstable spinach enzyme, presumably lacking tricarboxylic acids, was strongly inhibited by inorganic phosphate; and the inhibition was reversed by the activating fractions from the column. The enzyme stabilized by citrate was not inhibited by inorganic phosphate.

The evidence suggests that tricarboxylic acids are required for stability of the spinach enzyme at both $0^{o}C$ and $30^{o}C$, while the tobacco enzyme requires tricarboxylic acids only at $30^{o}C$.

Criteria of Purity

A. Anderson (1969) had shown that P-glycolate phosphatase after filtration through Biogel P-60 had a specific activity of 333 and rechromatographed on DEAE-cellulose as a single peak. The specific activity of all fractions dropped to 212 as if inactivation of the enzyme was occurring during the procedure.

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B. My purified fraction from PPGE (7% T, 5% C) with a specific activity of 280 was electrophoresed on analytical polyacrylamide gels (9% T, 5% C). The stains for enzyme and for protein both showed single bands at an Rf of 0.620 (Figure 4). The protein stain was an intense band with a light diffused "halo" around it. This diffuseness may be due to inactive protein, since recoveries from PPGE had been in the 40-70% range.

C. An SDS-PAGE investigation of the enzyme gave one major band and a single contaminant accounting for about 5% of the total protein. The gel scan is presented later (see Figure 10).

D. The purified enzyme (SA of 250) was run on ampholine gels following storage for about 5 weeks at 4^{o}. Three distinct but very close protein bands were observed, all had enzymic activity.

From these above four studies P-glycolate phosphatase appeared to be about 95% pure at specific activities above 300 units per mg. The highest specific activity obtained was 468 units per mg protein. Preparations were somewhat contaminated with physiochemically identical material due to denaturation during the rather severe conditions of the final purification step. Upon aging the specific activity decreased and the physical properties of the molecule altered.

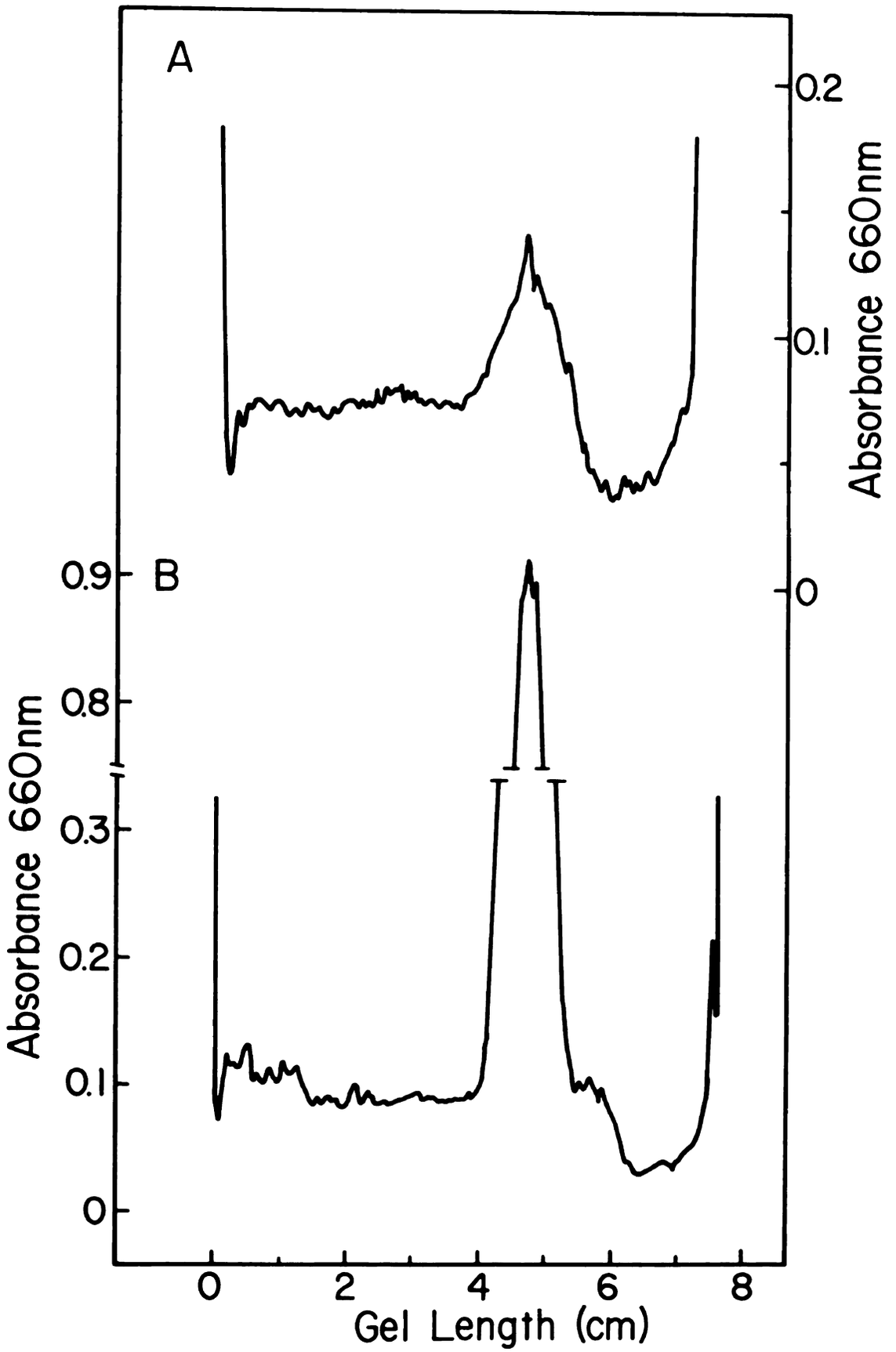
Molecular Weight from Ferguson Plots

Rodbard and Chrambach (1971) showed that a series of constant crosslinking gels (% C) with variable total acrylamide (% T) will generate mobility constants dependent on charge and molecular radius.

Figure 4.--Polyacrylamide gel electrophoresis of P-glycolate phosphatase.

A. Protein stain;

B. Lead phosphate enzymatic activity stain.


A
B
0.2
0.1
0
0.9
0.8
0.3
0.2
0.1
0
Absorbance 660nm
Absorbance 660nm
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A Ferguson Plot, the log Rf against % T is linear. The slope is defined as K_R, and the $\sqrt{K_R}$ is proportional to R, the molecular radius. Extrapolation of the line to the y axis gives the Rf at 0% T, a value dependent solely on charge. Antilog (y intercept) is defined as Y_o. P-glycolate phosphatase and several standard proteins of known molecular radius were run on separate gels. Slopes and intercepts of the Ferguson Plots in Figure 5 were analysed using an unweighted least squares computer programme (Table 3).

A replot of K_R against molecular weight was linear for all four standard proteins (hemoglobin behaving as a half-mer in the system), indicating that this empirical relationship (171) holds for these species. The molecular weight of the P-glycolate phosphatases, as obtained by interpolation of the graph, were 80,500 from tobacco and 92,800 from spinach leaves, assuming that the enzyme is globular. The K_R values obtained for the standards are in excellent agreement with those of Rodbard and Chrambach (1971) although they used a multiphasic system which improves the stacking of the proteins. They calculated an uncertainty of 5% for their data and my data has similar accuracy. As mentioned previously, the Rf values were measured with bromophenol blue as a marker for the moving boundary. This was adequate for analysis of molecular weight by the method described. Bromophenol blue is retarded in this system since it has a finite molecular weight. The $\pi\lambda$ boundary was detected as chloride as described in the methods or by incubating the gel briefly in 0.1 M $AgNO_3$. Values obtained from the empirical relationship for the molecular weights of the phosphatases

Figure 5.--Ferguson Plots for P-glycolate phosphatase and marker proteins.

A partially purified P-glycolate phosphatase preparation, free of contaminant nonspecific phosphatase was used and detected by the lead phosphate stain procedure. A protein stain was used to detect the other markers. Running gels varied from 3-12%T at 5%C; the stacking gel was 2.5%T and 2.5%C. The different species were:

■ , $\pi\lambda$ moving boundary

-----, bromophenol blue

△ , cytochrome c

● , P-glycolate phosphatase

○ , isocitrate dehydrogenase

□ , hemoglobin

▲ , lactate dehydrogenase.

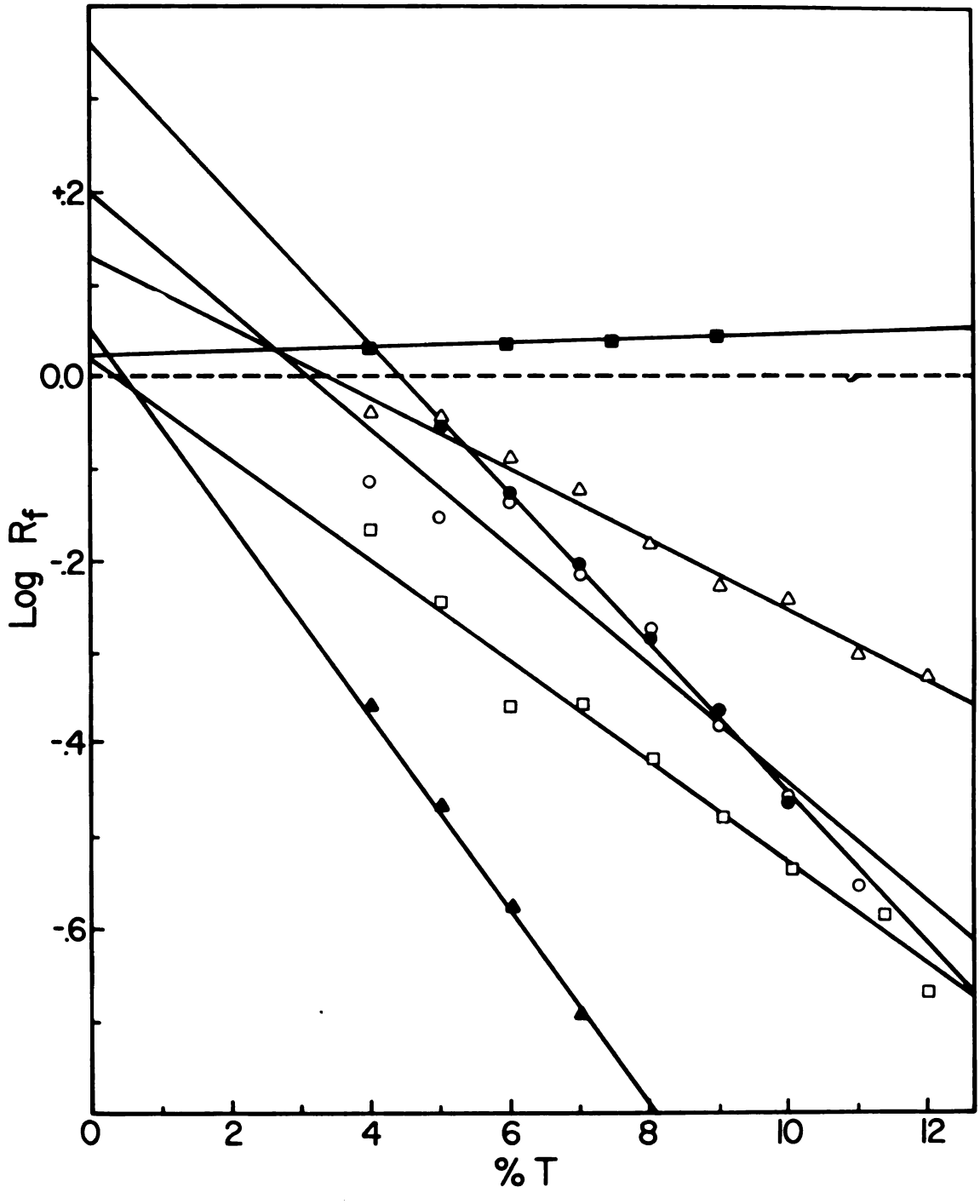
+.2
0.0
-.2
-.4
-.6
Log R_f
0
2
4
6
8
10
12
% T

TABLE 3.-

Cytochro

Hemoglobi

Isocitra
dehydroge

Lactate
dehydroge

P-glycol
phosphat

Chloride

Bromophe

TABLE 3.--Molecular Weight of Native P-glycolate Phosphatase from Tobacco and Spinach Leaves by Ferguson Plot Analyses.

	Source	K_R	Y_o	Corr. Coeff.	Mol. Wgt.	N
Cytochrome c	Horse Heart	-0.0385	1.352	0.990	12,384	9
Hemoglobin	Ox Blood	-0.0548	1.047	0.973	32,400	9
Isocitrate dehydrogenase	Pig Heart	-0.0643	1.586	0.961	64,000	8
Lactate dehydrogenase	Rabbit Muscle	-0.1050	1.130	0.975	137,000	6
P-glycolate phosphatase	Tobacco	-0.0742	1.987	0.993	80,500	4
	Spinach	-0.0811	2.289	0.999	92,800	6
Chloride	-	0.0024	1.051	0.874	35.5	6
Bromophenol	-	0.0000	1.000	1.000	670	9

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after correction of K_R for the $\pi\lambda$ boundary were identical to those obtained without correction.

Molecular weights calculated from interpolation of the curve drawn through the standards using a plot of the $\sqrt{K_R}$ against R gave values of 83,000 and 96,000 for the tobacco and spinach P-glycolate phosphatases (2.90 and 3.04 mμ for R). The values for the two plots are essentially identical (3%).

The mobility of the leading boundary was determined directly from the gels. The average of eight determinations gave a value $\mu f = -1.05 \times 10^{-4}$ $cm^2 \cdot sec^{-1} \cdot volt^{-1}$. The system appears to have mobilities about 200% of those in Rodbard and Chrambach's multiphasic systems, which may decrease peak diffusion owning to the shorter periods of time required for electrphoresis. The free mobilities of the proteins in my system were calculated directly from the relation: $M_O = Y_O \times \mu f$. These data allow calculation of the net valence on the molecule directly, with the necessary assumptions discussed in detail by Abramson et al. (1942) and outlined by Rodbard and Chrambach (1971). For tobacco P-glycolate phosphatase a net valence of -39.5 at pH 8.3 was calculated. The net valence for bromophenol blue calculated in the same way was -2.0, in fair agreement with the chemical formula (-1).

The lead phosphate stain developed for phosphatase allowed determination of the native molecular weight of the partially purified enzyme. Contaminating nonspecific phosphatases were judged to be absent by assaying the enzyme extracts with general phosphatase substrates and by the absence of multiple bands on the gels throughout the range of acrylamide concentrations.

The active form of the enzyme from spinach appears to be about 15% larger than that of the tobacco enzyme. Very similar results were obtained from Ferguson Plots for the two phosphatases using 3% C gels (data not shown). Thus the enzyme from two different plants appears to be similar but significantly different in molecular weight and possible causes of the difference will be discussed later.

The high Y_o values for P-glycolate phosphatases confirms the results obtained on DEAE-cellulose, to which the protein remained bound at pH 6.3 until 0.15 M KCl was added. Both observations indicate a strongly acidic protein. The net valence calculated at pH 8.3 indicates that at least 5% of the amino acid residues are negatively charged at this pH. Anderson (1969) found that large numbers of tricarboxylic acids remained bound with P-glycolate phosphatase during its purification. Therefore it was necessary to determine whether tricarboxylic acids remain bound to the enzyme during its electrophoresis and would account for the large charge of the protein. This possibility is ruled out in the next section.

Removal of Citrate from Phosphoglycolate Phosphatase during Electrophoresis

The electrophoresis of a mixture of (^{14}C)-citrate and P-glycolate phosphatase are shown in Figure 6. Free (^{14}C)-citrate moved with the moving boundary, slightly ahead of the tracking dye, and no radioactivity above background was detected elsewhere in the gel including the enzyme band. A single citrate bound per P-glycolate phosphatase should have been detectable in this system as shown by the analysis in Table 4, even if the activity spanned two gel slices.

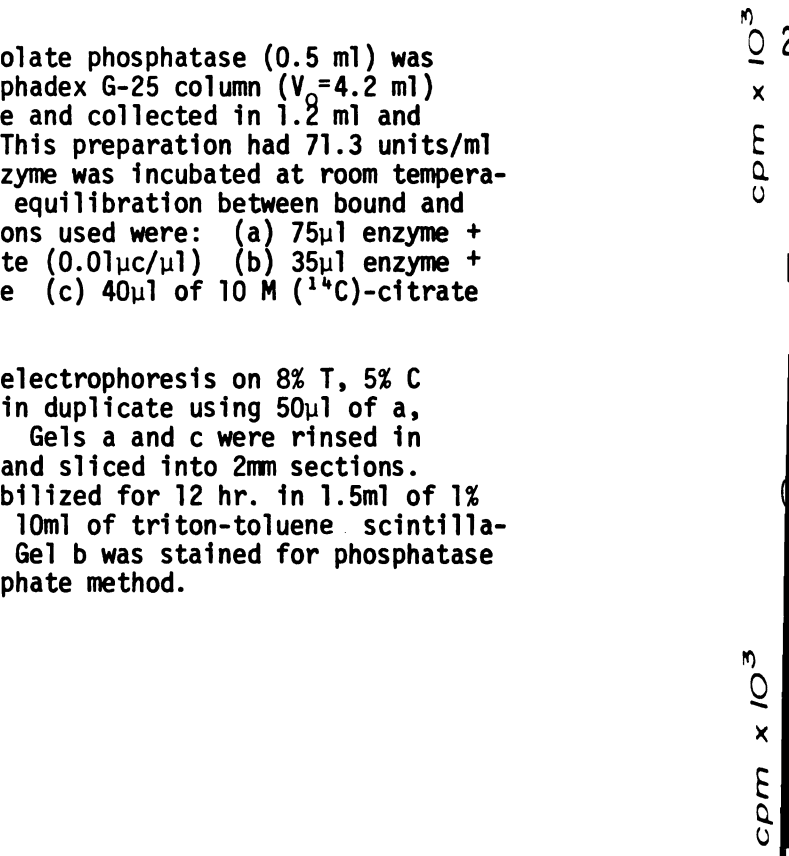
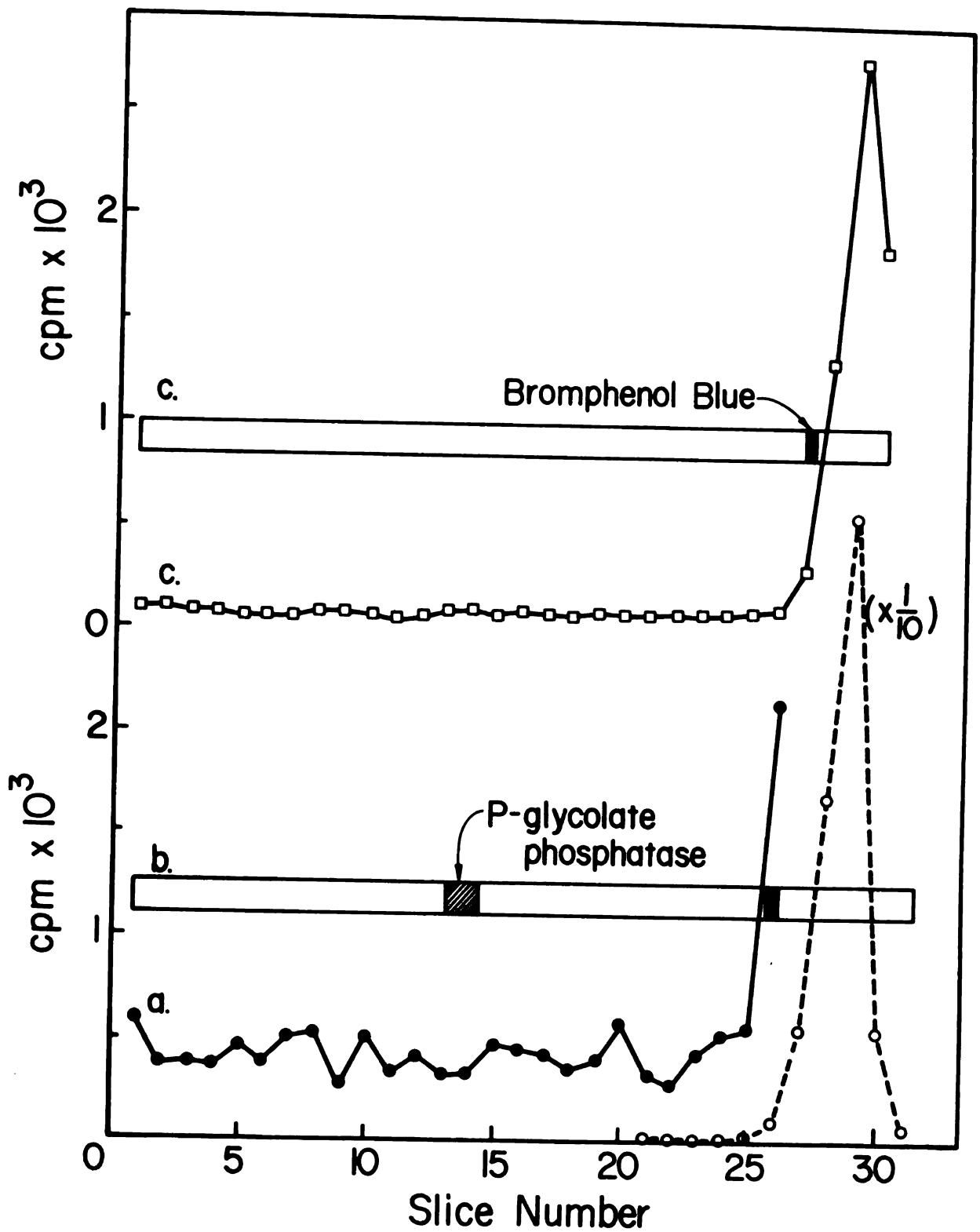


Figure 6.--Electrophoretic separation of P-glycolate phosphatase and exogenous (^{14}C)-citrate.

Partially purified P-glycolate phosphatase (0.5 ml) was passed through a small Sephadex G-25 column (V_o=4.2 ml) to remove any free citrate and collected in 1.2 ml and concentrated to 0.6 ml. This preparation had 71.3 units/ml and a S.A. of 40. The enzyme was incubated at room temperature for 1 hour to permit equilibration between bound and free citrate. The solutions used were: (a) 75µl enzyme + 25µl of 0.5mM (^{14}C)-citrate (0.01µc/µl) (b) 35µl enzyme + 5µl of 1.0mM cold citrate (c) 40µl of 10 M (^{14}C)-citrate (2×10^{-4}µc/µl).

Following equilibration, electrophoresis on 8% T, 5% C acrylamide gels were run in duplicate using 50µl of a, 20µl of b, and 20µl of c. Gels a and c were rinsed in distilled water, frozen, and sliced into 2mm sections. These fractions were solubilized for 12 hr. in 1.5ml of 1% SDS and counted by adding 10ml of triton-toluene scintillation fluid to the vials. Gel b was stained for phosphatase activity by the lead phosphate method.


cpm x 10^3
2
1
0
c.
Bromphenol Blue
c.
(x 1/10)
cpm x 10^3
2
1
P-glycolate
phosphatase
b.
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0
5
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(³⁵C)-cit

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TABLE 4.--Detection Limits for (^{14}C)-citrate on polyacrylamide gels.

(^{14}C)-citrate applied	= 2.775×10^5 dpm
(^{14}C)-citrate recovered	= 1.31×10^5 cpm
Counting efficiency	= 0.475
Moles citrate applied	= 6.25×10^{-9}
Moles phosphatase applied	= 8.38×10^{-11}
Recovery of one citrate/phosphatase	= 1.76×10^3 cpm
Average background/slice	= 0.40×10^3 cpm

This evidence suggests that no citrate remains bound to P-glycolate phosphatase during electrophoresis. Conditions used would detect less than 0.5 mole citrate/mole enzyme. It is possible however, that endogenous was bound so firmly that no exchange occurred during the period of equilibration. This does, however, seem unlikely for a noncovalent association, and citrate was fully ionized at the pH of both incubation and electrophoresis.

Molecular Weight Determination by Sucrose Density Gradient Centrifugation

Using the six standard enzymes of known moledular weight (Figure 7) for a calibration of weight against distance travelled (fraction no.) (Figure 8), linearity was established except for alcohol dehydrogenase which fell 11% below the line. A least squares programme

Figure 7.--Sedimentation velocity on a sucrose density gradient.

The experimental procedure is detailed in methods. The enzymic activities are:

▲ , carbonic anhydrase

● , malate dehydrogenase

○ , α-glycerophosphate dehydrogenase

△ , alcohol dehydrogenase

□ , catalase

✕ , P-glycolate phosphatase

■ , lactate dehydrogenase

The values for P-glycolate phosphatase are transposed vertically for clarity.

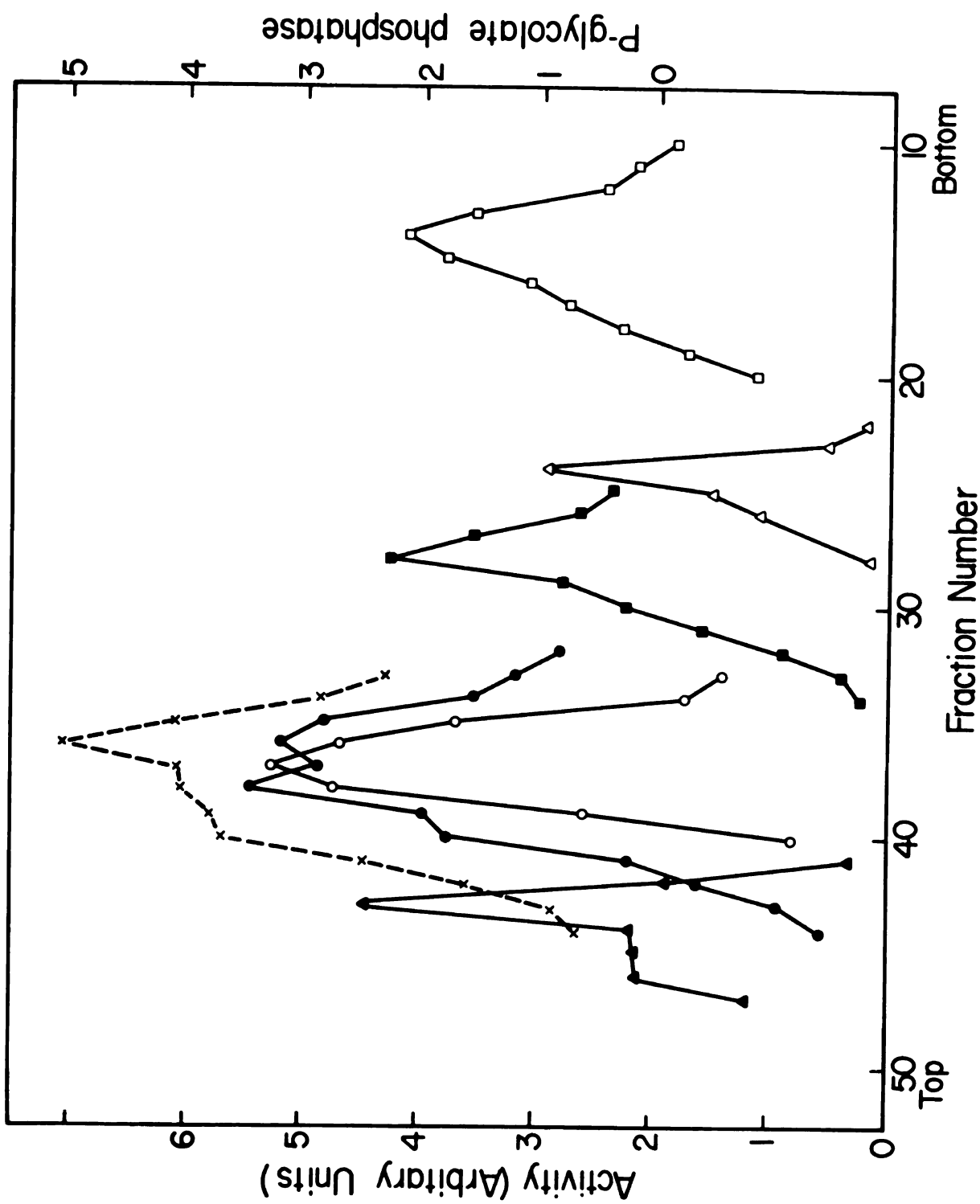
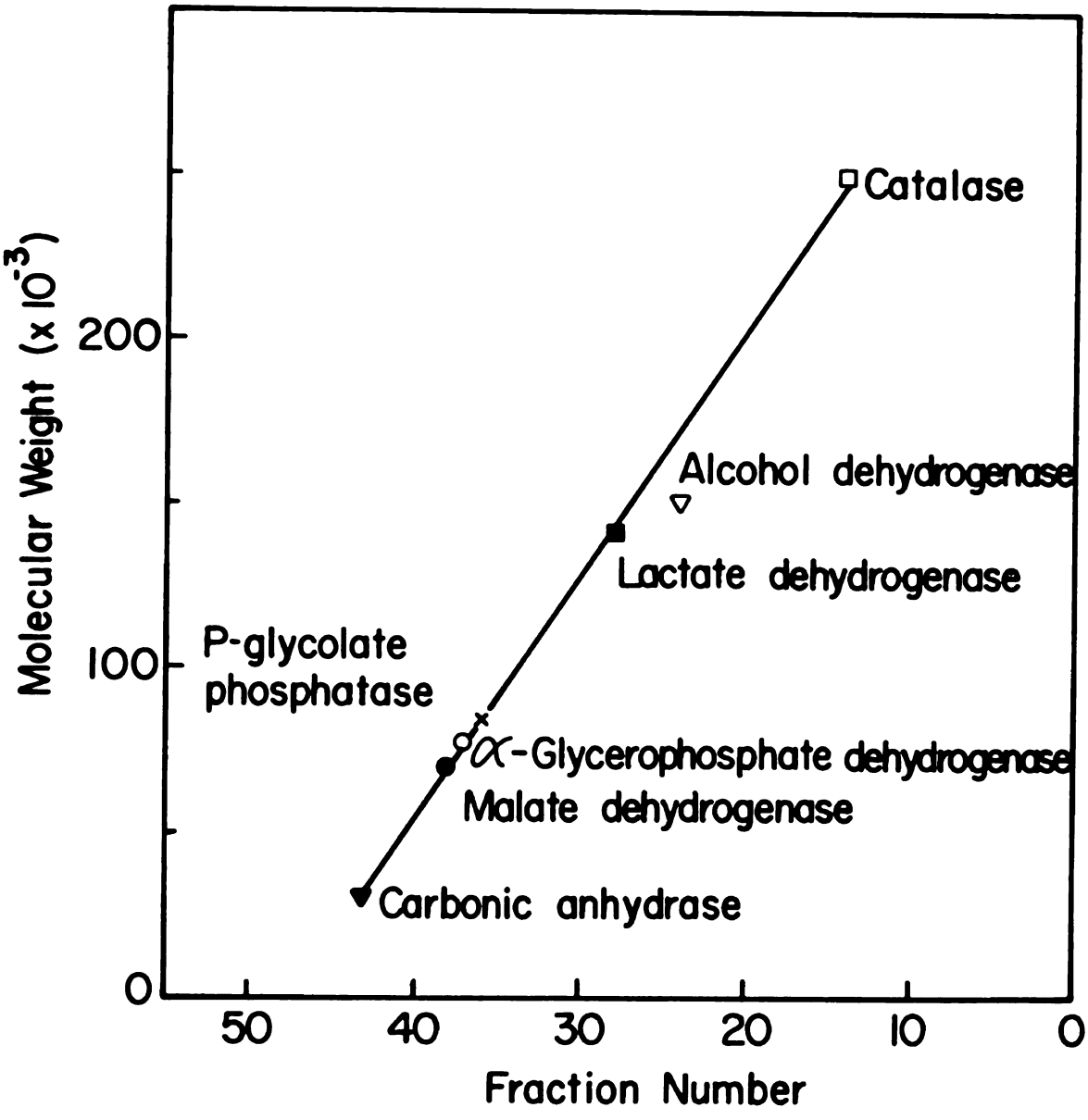

Activity (Arbitrary Units)
0
1
2
3
4
5
6
Top
10
20
30
40
50
Bottom
Fraction Number
P-glycolate phosphatase
0
1
2
3
4
5

Figure 8.--Calibration curve for molecular weight determination of P-glycolate phosphatase by sucrose density gradient sedimentation velocity.


Molecular Weight (x 10^{-3})
200
100
0
50
40
30
20
10
0
Fraction Number
Catalase
Alcohol dehydrogenase
Lactate dehydrogenase
P-glycolate phosphatase
α-Glycerophosphate dehydrogenase
Malate dehydrogenase
Carbonic anhydrase

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was used to determine the best line through the five remaining points (Slope = -7.504×10^3, Intercept = 354.3×10^3, Correlation Coefficient = 0.9999). The molecular weight of tobacco P-glycolate phosphatase was calculated at 86,300 daltons. This value for the native enzyme agrees well with the value of 81-83,000 obtained using PAGE, and a different set of protein standards.

As Martin and Ames (1961) noted, protein-protein interaction does not seem to be a serious problem. Peaks were sharp and in most cases symmetrical. The peaks of malate dehydrogenase and P-glycolate phosphatase appeared somewhat more diffuse. This could be interpreted as a weak interaction between the two species, especially since each appeared to have a shoulder under the peak of the other. However, when P-glycolate phosphatase was run in the absence of the standard enzymes, its peak was also diffuse although the shoulder was much less pronounced.

About 2% of the total P-glycolate phosphatase activity appeared in the lightest 4 to 5 fractions of the sucrose gradients (data not shown in Figure 7). This was enzymic activity since addition of TCA prior to enzyme assay abolished all phosphate release. The results suggest that some P-glycolate phosphatase activity had a molecular weight of about 20,000 or less.

The P-glycolate phosphatase used in these experiments had been purified by gel filtration on Sephadex G-200. Thus it is unlikely that the minor 2% low molecular weight activity represents a unique species of phosphatase contaminating the preparation. It is possible

that P-glycolate phosphatase has quaternary structure and undergoes a kinetically slow dissociation with retention of some activity in the subunits. In this case, 2% would be a rough approximation of the thermodynamic equilibrium between the species. The data, if interpreted in this fashion, would indicate at least four subunits for P-glycolate phosphatase. Another possibility would be some nonspecific "stickiness" of protein around the top of the gradient. Measurement of total protein throughout the gradient did indicate an increase in protein in this region near the top of the gradient. Martin and Ames do not comment on this phenomenon but inspection of their data shows very small increases in protein for the uppermost fraction only and not in the lower ones. Since they also showed that this top fraction only was contaminated with proteins in lower fractions due to frothing as it finally dripped from the tube, the real explanation for P-glycolate phosphatase activity near the top of the sucrose gradient is open to question.

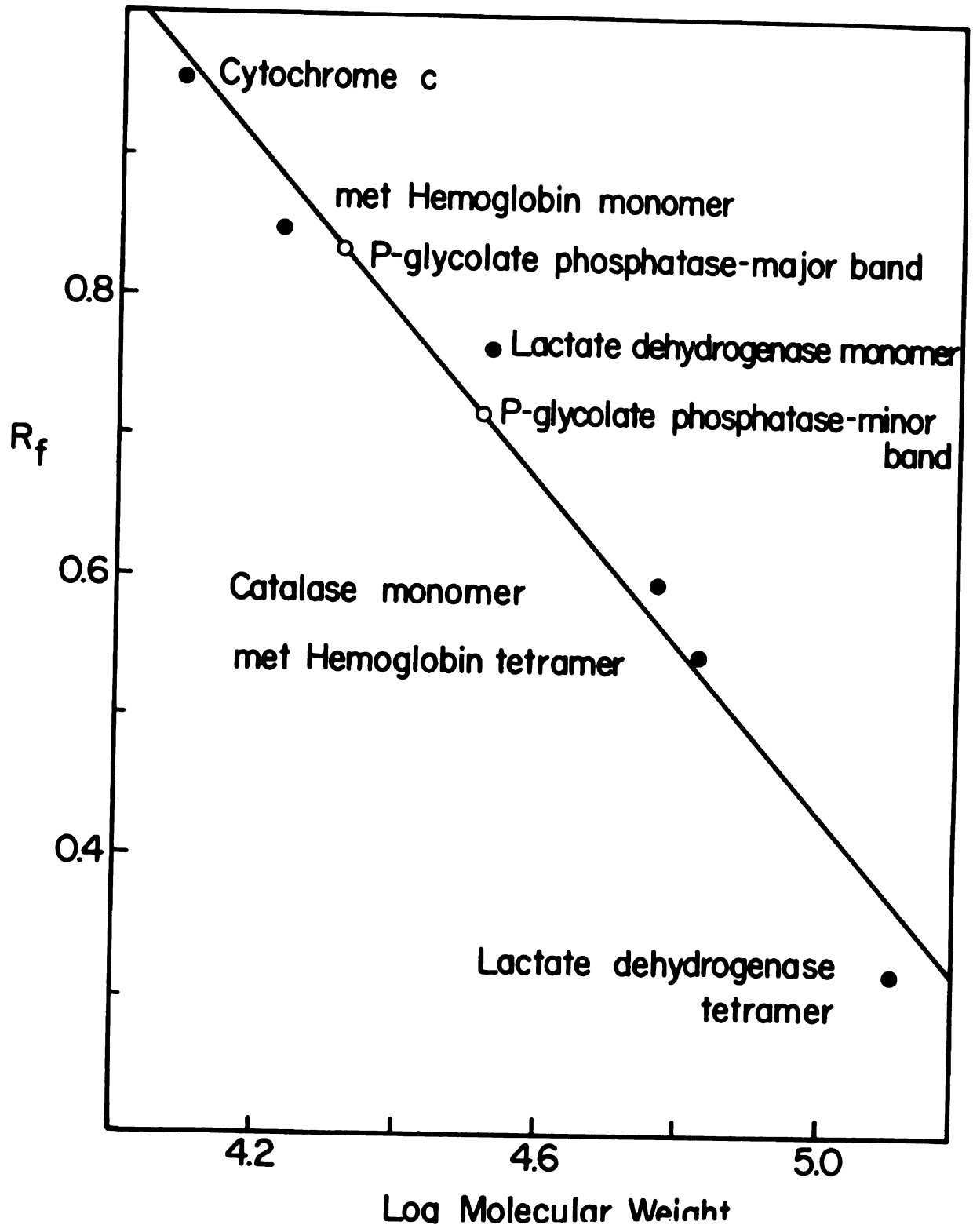
SDS-Polyacrylamide Gel Electrophoresis: Determination of Quaternary Structure

The Rf values (Table 5) of the standard proteins (Figure 9) were plotted against log MW using a least squares computer programme (Slope = -0.5839, Intercept = 3.3536, Corr. Coeff. = 0.9872). The Rf for P-glycolate phosphatase in the two experiments varied about twice that for the other proteins, possibly due to difference in treatment of the enzyme. In the first experiment the enzyme was prepared as described in methods, but in the second the protein was concentrated

TABLE 5.--Molecular Weight Determination of P-glycolate Phosphatase by SDS-gel Electrophoresis.

Protein	Mol. Weight of monomer	No. of subunits	Average Rf	Log. MW
Cytochrome c	12,384	1	.953 ± .017	4.0935
metHemoglobin	17,000	1	.846 ± .016	4.2305
		4	.541 ± .011	4.8330
Lactate dehydrogenase	33,750	1	.766 ± .004	4.5285
		4	.319 ± .014	5.1305
Catalase	58,000	1	.593 ± .003	4.7633
P-glycolate phosphatase	(major band)		.826 ± .029	
	(minor band)		.716 ± .007	

Figure 9.--Calibration curve for determination of the molecular weight of P-glycolate phosphatase by SDS-gel electrophoresis.


Cytochrome c
met Hemoglobin monomer
P-glycolate phosphatase-major band
Lactate dehydrogenase monomer
P-glycolate phosphatase-minor band
Catalase monomer
met Hemoglobin tetramer
Lactate dehydrogenase tetramer
R_f
0.8
0.6
0.4
4.2
4.6
5.0
Log Molecular Weight

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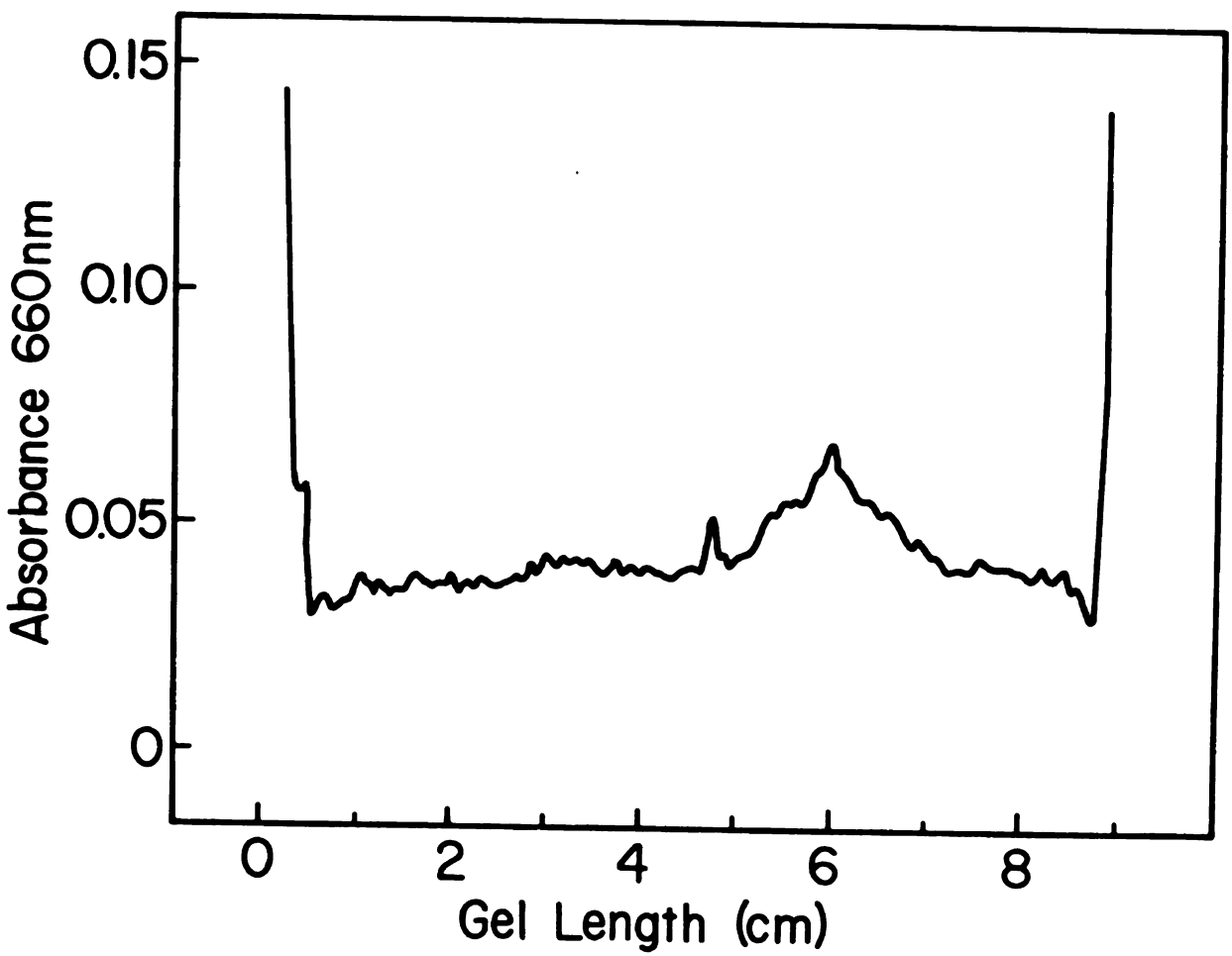
by precipitation in $(NH_4)_2SO_4$ and then incubated in 1% SDS for 12 hours at 30^{o}. The molecular weight of the major phosphatase band was 22,400 from the first experiment and 19,000 in the second. The molecular weight of the minor band was 32,700 and did not vary with the preparation procedure. The protein concentration in the first experiment was very low but after $(NH_4)_2SO_4$ concentration for the second experiment enough protein was present for a gel scan (Figure 10). The diffused nature of the bands, even for the standard proteins, was seen in this series of experiments.

The procedure should have fully dissociated the standard proteins, but apparently this did not occur, since hemoglobin and lactate dehydrogenase gave two distinct bands on the gels. From catalase several minor bands were present but were ignored. The slow moving bands were assigned to the tetramers of Hb and LDH since these values fitted well onto the line through the other points.

The P-glycolate phosphatase was highly purified (S.A. of 250) being homogeneous on analytical PAGE. Thus the major band was assumed to be P-glycolate phosphatase and the minor band to be a contaminant. Furthermore the molecular weight of the native enzyme was 81-83,000 and was not a simple multiple of the minor peak nor of a combination of both peaks. The most likely interpretation is that P-glycolate phosphatase is a tetramer in its native state, with similar or identical subunits having a molecular weight of 20-22,000 daltons each.

Figure 10.--Densitometric trace of SDS-gel of purified P-glycolate phosphatase.


0.15
0.10
0.05
0
Absorbance 660nm
0
2
4
6
8
Gel Length (cm)

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Isoelectric Focussing on Polyacrylamide Gels

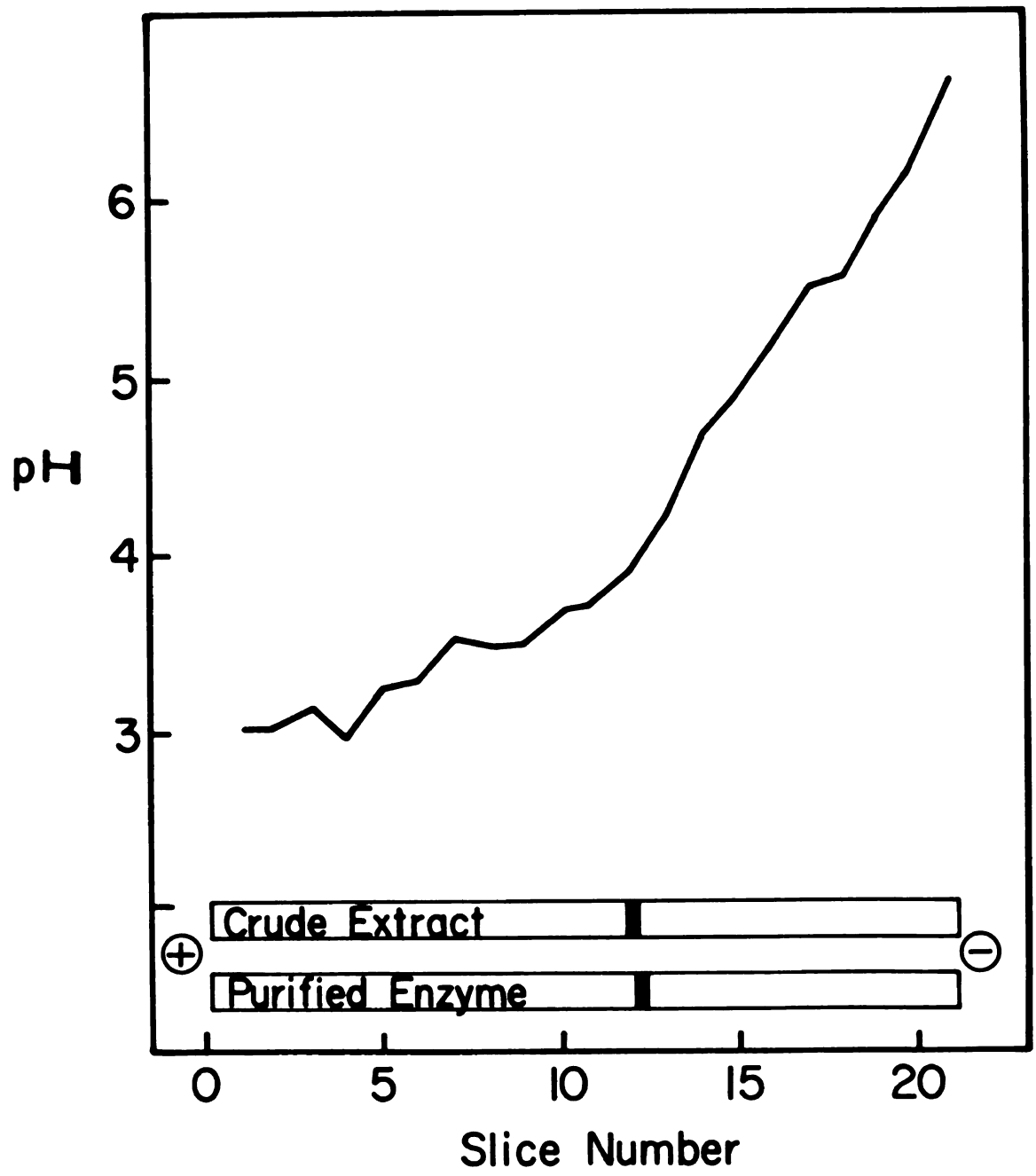
The isoelectric point of P-glycolate phosphatase in crude extracts with a S.A. of 10 or in newly prepared purified enzyme with a S.A. of 250 was similar, lying between pH 3.8 -3.9 (Figure 11). However after the purified enzyme had aged at 4^{o} for five weeks in 0.02 M cacodylate, 0.05 M citrate at pH 6.3 it had on isoelectric focussing a major band at pH 4.3 and two small bands at pH 4.8 and 5.3 enzyme activity. The process of aging is not understood but appeared to involve conformational changes involving masking of ionizable carboxyl groups.

The pI of P-glycolate phosphatase was lower than most other soluble proteins and the value obtained further adds to the observations (DEAE-cellulose binding and electrophoretic mobility and valence measurements) that it is a very acidic protein. The measured pI should also be the value with tricarboxylic acids removed, since they are neutral species at pH 3 and should have no tendency to move into the gradient towards the cathode.

The change of the pH gradient (Figure 11) for several batches of ampholines was very at low pH and increased sharply with linearly from pH 4-7. This characteristic of the gradient permitted more accurate determination of pI between pH 3-4 for P-glycolate phosphatase.

The loss of some charge on the aged phosphatase suggests the presence of carboxyl groups in an aqueous environment. At five pH units above the isoelectric point the net valence was -40, and must be due to carboxyl functions on the surface of the enzyme. The number of

Figure 11.--Determination of the Isoelectric Point of P-glycolate phosphatase by Isoelectric Focussing. The enzymic activity was located on the gels by the lead phosphate stain.


pH
6
5
4
3
Crude Extract
Purified Enzyme
⊕
⊖
0
5
10
15
20
Slice Number

protonated residues at pH 8.3 cannot be assessed, nor can the arrangement of charge on the surface of the molecule. The acidity of the protein is surprising in light of its ability to bind tricarboxylic acids tightly, but it suggests the charge is not evenly spread throughout the surface, since both species are negatively charged and would tend to repel each other.

CATION AND pH REQUIREMENTS FOR PHOSPHOGLYCOLATE PHOSPHATASE ACTIVITY

Introduction

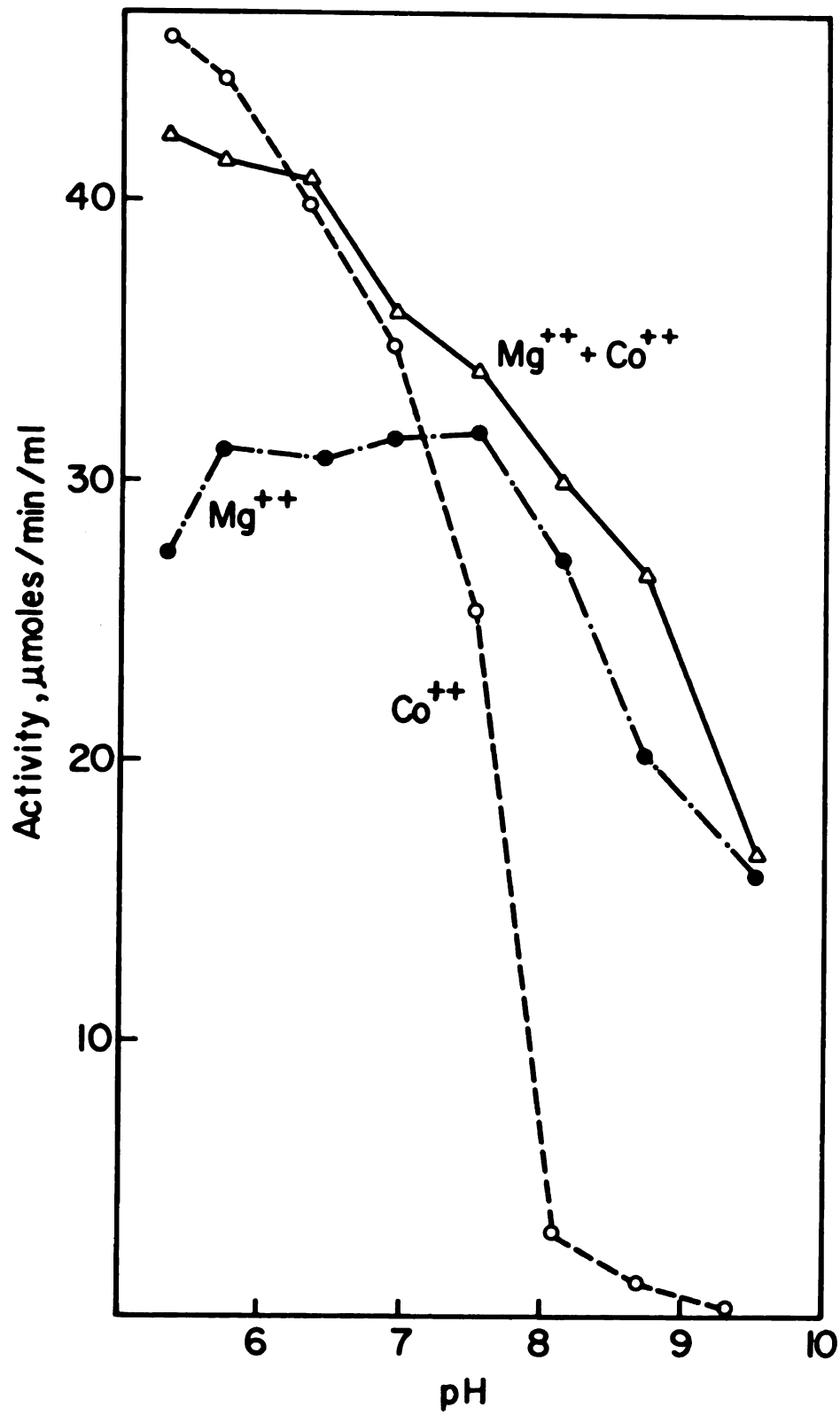
Richardson and Tolbert (1961) established a divalent metal ion requirement for P-glycolate phosphatase, and that the pH optimum varied from pH 5-6.5 depending on the cation present. Since the enzyme is localised in the chloroplast stroma, which, during steady state CO_2 fixation in the light may be at a pH 7.8-9.0, it has been necessary to evaluate the activity of the enzyme in this physiological range. Since Mg^{++} is present in the choroplast in high concentrations, it has been considered the physiological cation for this phosphatase. In this chapter are experiments designed to evaluate the interactions at different pH's between the enzyme, the various forms of the substrate, and divalent cations.

Results and Discussion

Effect of pH

pH Optimum of Phosphoglycolate Phosphatase.--The effect of pH on the velocity of the phosphatase reaction showed a striking difference in the presence of Co^{++} or Mg^{++} (Figure 12). With Co^{++} no activity was present above pH 8.0 and maximum activity was at lowest pH values (pH5). With Mg^{++} maximum activity occurred over a broad range from pH 5.5 to 7.5, and 50% of maximum activity was still present at pH 9.5.

Figure 12.--The effect of pH on the activity of P-glycolate phosphatase in the presence of Mg^{++} and Co^{++}. The pH optimum curves were assayed with the following buffers: below pH 6.5, MES; between pH 6.5 and 8.0, HEPES; and above pH 8.0, bicine. No buffer effects on phosphatase activity were observed upon interchanging these buffers in regions of overlapping buffer capacity. The assay system contained: 20 µmole buffer, 1 µmole cation, 1 µmole P-glycolate, and 0.1 unit phosphatase (S.A. = 40). The activity in the absence of cation was subtracted. No contaminating phosphatase activity was present in the preparation.


Activity, µmoles/min/ml
40
30
20
10
6
7
8
9
10
pH
Mg++ + Co++
Mg++
Co++

In the presence of saturating concentrations of both cations, activities were not additive throughout the pH range, but rather both cations were activating a single enzyme species. At low pH, Mg^{++} appeared to be somewhat inhibitory to Co^{++} activity, whereas at high pH Co^{++} had little or no effect on Mg^{++} activity. The data further rules out the possibility that at high pH Co^{++} formed an insoluble substrate complex thus causing the sharp loss of activity.

The range of activity from below pH 5 to above pH 9 indicates that both the phosphate monoanion and the dianion were substrates. Since phosphate monoanion-metal ion complexes are extremely weak, their presence must be insignificant (223) and thus substrate-cation complexes cannot be considered as substrates at low pH. The failure for Co^{++} activated enzyme to show activity at high pH also suggests that the uncomplexed P-glycolate is the true substrate.

At low pH the activity appeared proportional to the $K_{0.5}$ for the metal ion (Table 11, Experiment 1). This would explain the inhibitory effect of Mg^{++} in the mixed situation at low pH. At high pH, a possible explanation of the retention of Mg^{++} activity is the presence on the enzyme of an additional Mg^{++} binding site, which maintains active site integrity, probably by preventing ionisation of an active site residue. Additional evidence for this proposal is presented later. Also to be considered is the slightly increased activity at high pH with mixed cations. The hybrid enzyme would be more active due to the higher intrinsic activity of the cobalt enzyme.

Analysis of Logarithmic Plots at Different pH.--The plot of log Vmax against pH for three cations, as shown in Figure 13, can be resolved into a horizontal portion of low pH and a portion with a slope of -1 at its asymtote. The point of intersection indicates the pK of an ionizing group essential for activity in the enzyme-substrate comples, since in all cases it is far removed from the substrate pK_2 of 6.60. The protonated form of this unknown group is essential for activity.

The pKa values obtained for this protonated group were dependent on the nature of the cation. The Co^{++} enzyme pKa was observed at pH 7.50, the Mn^{++} enzyme at pH 7.75, and the Mg^{++} enzyme at pH 9.15. The values appear too high to be from ionization of the substrate or the substrate-cation complex.

Values for the Km (P-glycolate) are presented in Table 6. The Km was relatively constant, around 5×10^{-5} M, throughout the alkaline and neutral region and similar with Co^{++} and Mg^{++}, but increases very rapidly at low pH. However Km against pH curves are a complex function and difficult to interpret (43). The data can be further interpreted when plotted as log (Vmax/Km) against pH (Figure 14). The curves were horizontal over the mid portion pH range with slopes of +1 and -1 in the acidic and alkaline regions respectively. (Vmax/Km) is an apparent first order rate constant, indicative of the ability of the enzyme to combine with the substrate at low substrate concentrations. The pKa values given in Table 7, were determined directly from the intersections at the asymtotes, since the curves resolved into horizontal sections.

Figure 13.--Log Vmax against pH plots for P-glycolate phosphatase activity in the presence of different divalent cations. The data points are an average of two experiments in the case of Mg^{++} and Co^{++}, and a single experiment for Mn^{++}. The assay conditions were identical to those in Figure 12.

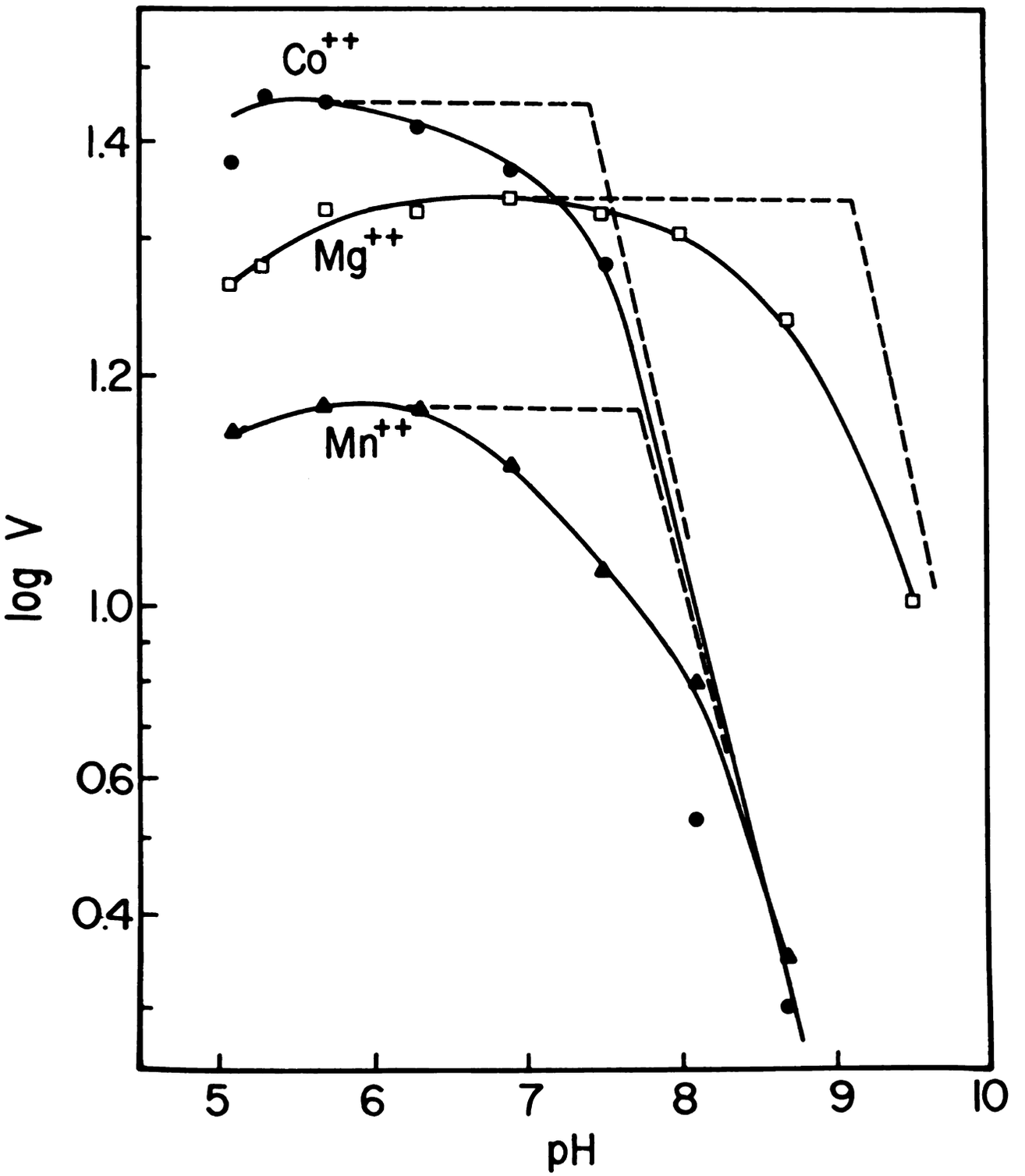

Co++
Mg++
Mn++
log V
1.4
1.2
1.0
0.6
0.4
5
6
7
8
9
10
pH

Figure 14.--Log (Vmax/Km) against pH for P-glycolate phosphatase activity in the presence of different divalent cations. Velocity data is that of Figure 13. The Km at each pH was obtained from a curve drawn through the data in Table 6. The same Km was used for each metal ion since Table 6 indicates that the Km did not depend on the nature of the cation. Furthermore, the data in Table 10 indicate that the Km was not altered by the concentration of cation.

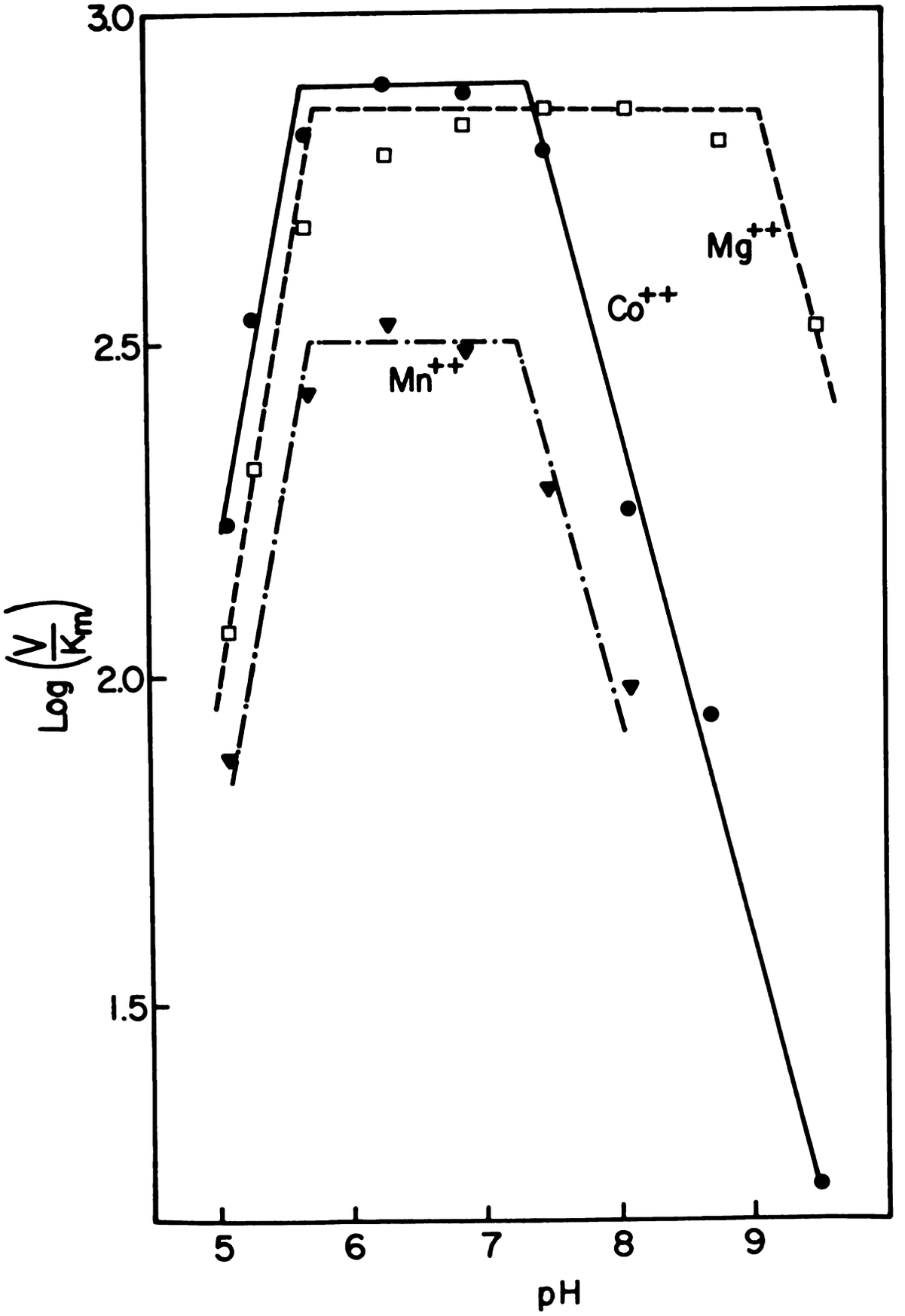

3.0
2.5
2.0
1.5
Log (V/Km)
Mg++
Co++
Mn++
5
6
7
8
9
pH

TABLE 6.--Variation of Km (P-glycolate) with pH.

pH	Km (x 10^{-5} M)	Cation	Cation (x 10^{-3} M)	Assay
5.4	10.9	Mg^{++}	15	(^{14}C)-glycolate
5.7	6.8	Co^{++}	5	Phosphate
6.3	5.3	Mg^{++}	5	Phosphate
7.5	6.4	Co^{++}	2	Spectrophotometric
8.1	4.2	Mg^{++}	5	Phosphate

TABLE 7.--Essential Ionizing Residues of P-glycolate Phosphatase Required for Substrate Binding and Activity.

Cation	From Log (Vmax/Km) Plots		From Log (V) Plots
	pK1	pK2	pKa
Co^{++}	5.70	7.40	7.50
Mn^{++}	5.70	7.30	7.75
Mg^{++}	5.75	9.10	9.15

Since the pK's obtained are again well separated from substrate pK values, they must again be occurring on the enzyme.

The data can be summarised to indicate that substrate binding requires an ionized residue on the enzyme with a pK ≃ 5.70 and an unionized residue, also involved in catalysis, whose pKa is affected by the cation, being 7.50 for Co^{++} and Mn^{++} and 9.10 for Mg^{++}.

Since at pH 5.7 both the carboxyl and a single phosphate oxygen of P-glycolate are fully ionized, it seems unlikely that another

negatively charged group is required for binding. If however a divalent cation was acting as an enzyme-substrate bridge, a carboxyl group of glutamic or aspartic acid would be applicable to the residue with the pKa of 5.7. Preliminary evidence for a carboxyl required for activity is presented later. The situation outlined has been well studied in pyruvate kinase and other enzymes (see review by Mildvan, 1971). Mandelate racemase (63) has also been identified as a Mg^{++} dependent enzyme, and further studies (64) have implicated a carboxyl group at the active site.

Alternatively the amino acid involved could be a histidine. Although a histidine pK this low is unusual it has been observed in ribonuclease (131,132). Irrespective of which possibility is correct, the pKa indicates a very hydrophobic environment for this particular residue, since the pKa for the anionic species is increased and that for the cationic species decreased relative to their values as free amino acids.

The identity of the residue at high pK values is unknown. It must remain protonated for activity and therefore is unlikely to be histidine in a hydrophobic environment. This is corroborated by the shift in pK to pH 9.1 in the presence of Mg^{++}. Further, mechanistic considerations for involvement of histidine in formation of a phosphoenzyme require the neutral form. Since studies later in this thesis and elsewhere (170) have implicated a sulphydryl with a structural role in activity, a sulphydryl group appears a possible candidate for this group.

The ionization could be due to ionization of a water molecule bound to the hydrated cation. (I thank C. H. Suelter for this suggestion.) All these cations have six molecules of water in the hydration sphere, however data from Sillén and Martell (1964) indicates that magnesium is considerably less acidic than either manganese or cobalt. The pK for all three ionizations would require to be lowered by three pH units (from 12.2 to 9.1 for Mg^{++}, from 10.5 to 7.5 for Mn^{++}, and from 9.9 to 7.5 for Co^{++}). Binding of the cation to the enzyme and possibly the substrate as well could neutralise the charge on the cation and facilitate the ionization. The protonated form could be directly involved in the reaction mechanism.

Determination of Ionization Constants for P-glycolate

Determination of the pK values for the ionizing groups of P-glycolate is essential to the quantitative analysis of the various species occurring in solution to decide on the active form of the substrate, to quantitate the spectrophotometric assay, and to interpret the mechanism of hydrolysis. From the titration curve of P-glycolate in Figure 15, pK values were assigned by comparison to those of known compounds (Table 8). These pK values were determined under changing ionic strength and substrate concentration, and no extrapolation to standard conditions was attempted. The conditions used were approximately those in enzymic assays. From the data P-glycolate is a slightly stronger acid in the physiological range then inorganic phosphate.

Figure 15.--pH Titration Curve of P-glycolate.
The tricyclohexylammonium salt of P-glycolate (100μmole) was dissolved in 10 ml of CO_2 free, distilled, deionised water. Equal volumes were titrated with 0.1035 M H_2SO_4 and 0.188 M NaOH at 25° on a pH meter (Radiometer). The titrants and pH meter were standardized against commercial standards (Mallinckrodt Chemical). The pK's were determined from the inflextion points in the titration curve.

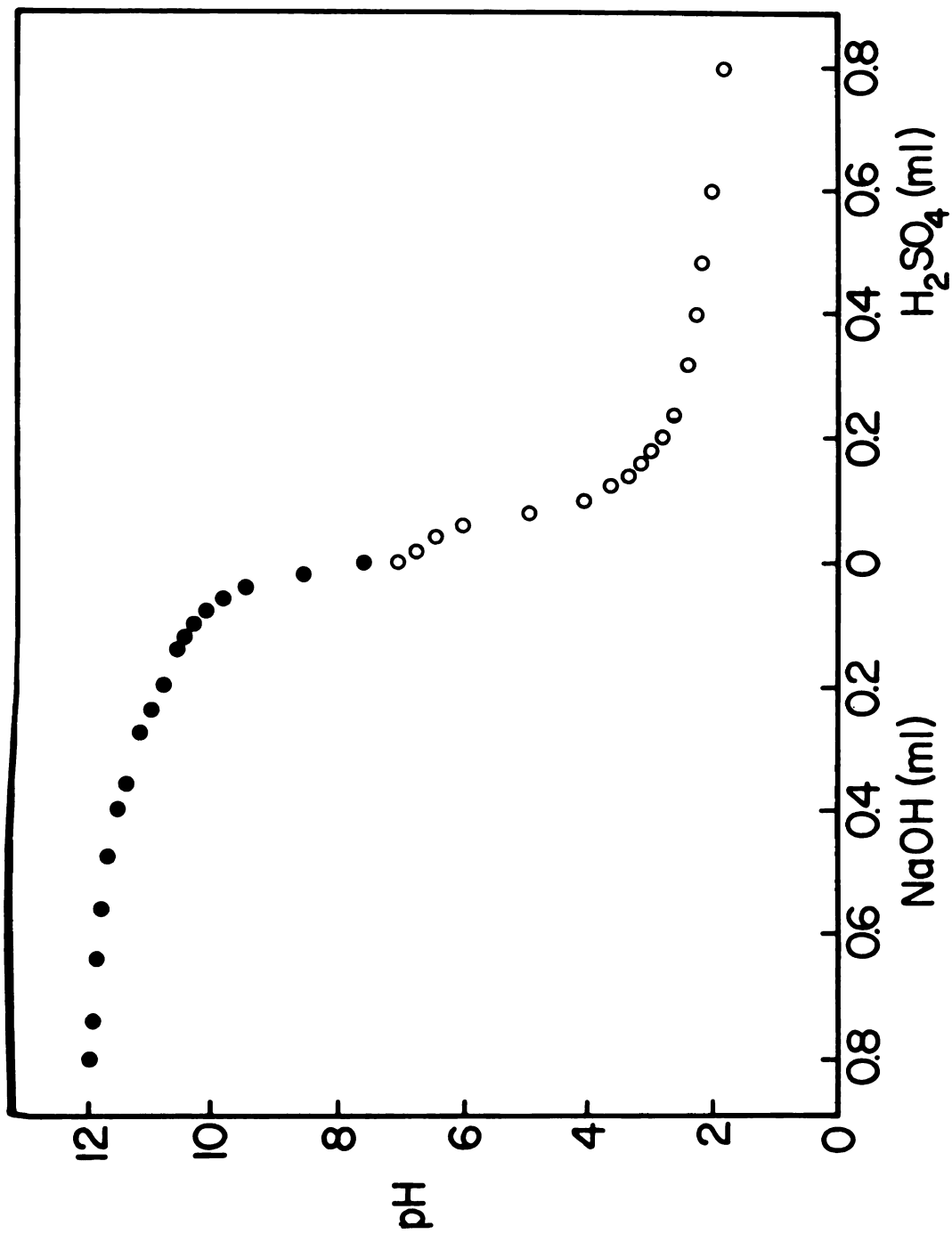

pH
12
10
8
6
4
2
0
0.8
0.6
0.4
0.2
0
NaOH (ml)
0.2
0.4
0.6
0.8
H_2SO_4 (ml)

TABLE 8.--P-glycolate, Its Ionization Constants and Their Assignment.

Experimental pKa	Assignment	Inorganic Phosphate
2.15	pK1, Phosphate hydroxyl	pK1 = 1.95
3.35	pKa, Carboxyl	
6.60	pK2, Phosphate hydroxyl	pK2 = 7.12
10.75	pKb, Cyclohexylammonium[a]	pK3 = 12.32

[a] pKb = 10.64 for cyclohexylammonium, Hall and Sprinkle (1932).

Phosphoglycolate-Cation Equilibria

A pK_2 of 6.60 was determined for P-glycolate by titration of the tricyclohexylammonium salt. The other important constant is the dissociation constant (K_D) for the phosphate dianion substrate-Mg^{++} complex. The K_D for P-glycolate-Mg^{++} was obtained from extrapolation of data from Sillén and Martel (1964) (Table 9). Because the K_2 is essentially the proton dissociation constant for the dianion-proton complex, a linear relationship between it and the K_D (cation) might be expected for a series of similar analogues. A value of 240 $mole^{-1}$ was determined for P-glycolate and a KD of 200 $mole^{-1}$ was found for methylphosphate complexes.

Determination of Species Concentration in the Phosphoglycolate Phosphatase Assay Mixture

With the values above calculation of all substrate-cation species over the complete range of enzymic activity can be achieved by

TABLE 9.--Ionization and Dissociation Constants for Magnesium Complexes with Phosphates and Carboxylates.

Compound	K_D (Mg^{++}) $moles^{-1}$	pK
$Citrate^-$	-	3.12
$Citrate^{--}$	40	4.76
$Citrate^{---}$	1580	6.39
P-glycolate	-	3.35
Phosphate	93	7.12
P-enolpyruvate	160	6.40
Glycerol-P	180	6.65
3- P-glycerate	280	7.00
P-glycolate	-	6.60
Methyl-P	-	6.80

solution of the quadratic equation presented on page 33. Although the determination of the dissociation constant was not very accurate, actual concentrations of species obtained and overall behaviour of the system were not critically affected by small variations in the K_D value.

Effect of Magnesium Ion Concentration on the Michaelis Constant for the Phosphate Ester Substrate

For these studies, the alternative substrate, ethylphosphate, was used rather than P-glycolate. Because the Km value for

ethylphosphate is higher than that for P-glycolate, the value can be more accurately determined since larger quantities of phosphate are released at the Km. The microphosphate assay was also used to improve accuracy. From the velocity of ethyl-P hydrolysis at 3 Mg^{++} concentrations (Figure 16) the kinetic parameters (Table 10) were obtained from reciprocal plots. These values and the concentrations of the species present at half maximal activity were calculated, assuming a K_D of 200 $mole^{-1}$ for the substrate.

The data provides evidence that free ethyl-P was the active species. The concentration of this species at half maximal enzyme saturation was independent of Mg^{++} concentration (within experimental error) while the other possible species; total ethyl-P varied 7.5 fold and ethyl-P-Mg complex varied 40 fold. The evidence is in agreement with the conclusions based on pH optimum studies on page 82.

Furthermore, ethyl-P-Mg was not significantly an inhibitor of the substrate, ethyl-P. The Km (ethyl-P) value did not increase appreciably over a 25 fold change in the substrate to ethyl-P-Mg ratio. The possibility that a charged phosphate is required is supported by the observation later in this thesis that phosphate, but not $MgHPO_4$, was an effective competitive inhibitor and that this complex cannot be rejected in terms of steric hinderance. Also, if divalent cation is bound to the active site it would not be expected to associate and dissociate with every catalytic turnover. This would explain why the substrate-cation complex appears to be catalytically inactive.

The data does not provide evidence for or against the participation of Mg^{++} at the active site. Divalent cations could be more

Figure 16.--Velocity-substrate concentration curves for the alternative substrate- ethylphosphate at different magnesium ion concentrations.

The complete system contained: 100 μmole cacodylate at pH 6.3; from 0.03 to 2.5 μmole ethylphosphate; 4, 20, or 100 μmole $MgCl_2$; and 0.1 unit phosphatase. Phosphate released in one minute was determined by the colourimetric assay at 310nm after extraction into isobutanol-benzene. Hydrolysis of the substrate was 20% or less.

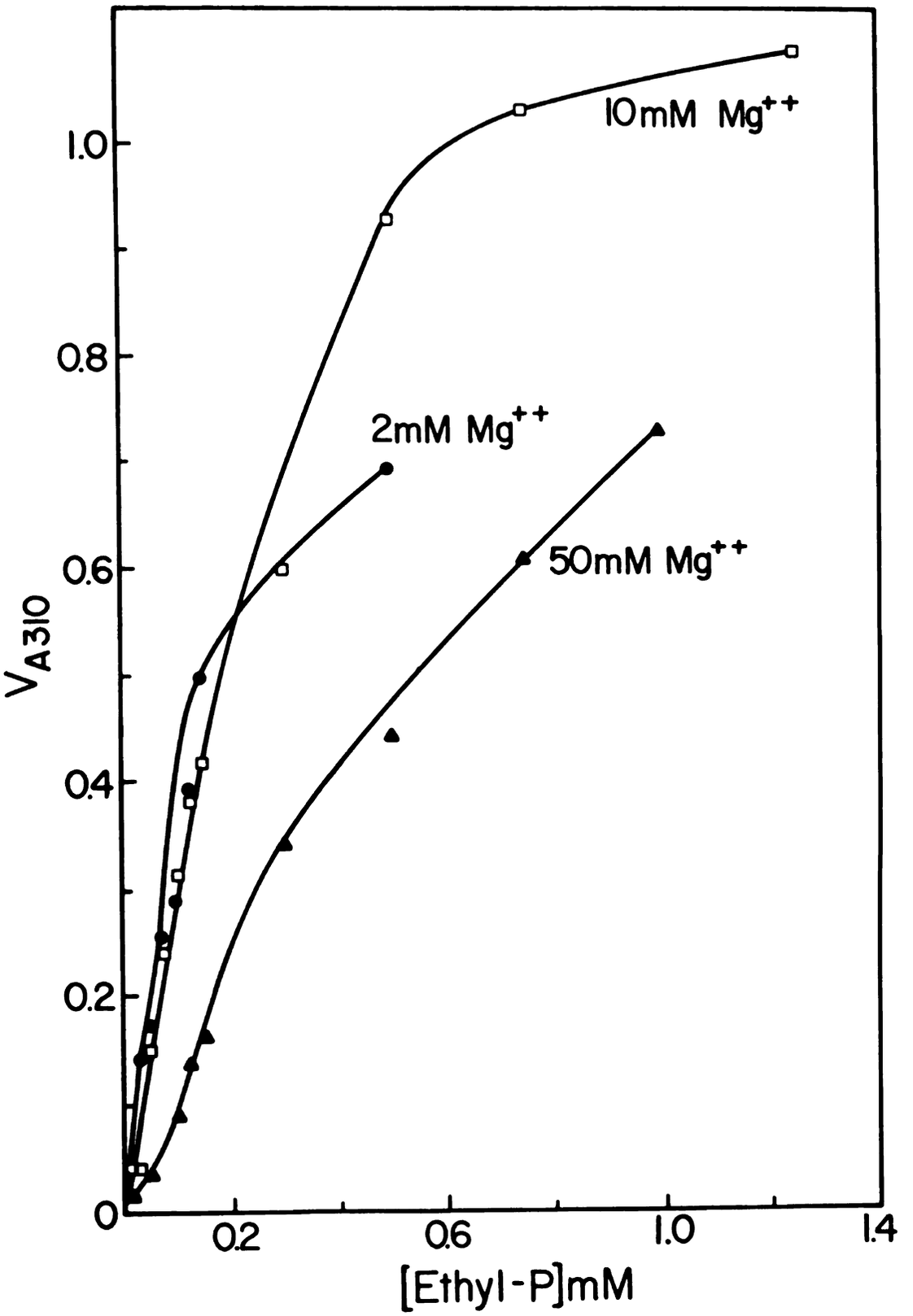

10mM Mg++
2mM Mg++
50mM Mg++
1.0
0.8
0.6
0.4
0.2
0
VA310
0.2
0.6
1.0
1.4
[Ethyl-P]mM

TABLE 10.--Effect of Magnesium Ion Concentration on Km (Ethylphosphate).

	Mg^{++} added (x 10^{-3} M)		
	2	10	50
Vmax (310nm)	0.815	1.28	1.53
Apparent Km (x 10^{-3} M)	0.15	0.45	1.10
Concentration at the Apparent Km of:			
A. Mg^{++} - Ethylphosphate complex	0.02	0.17	0.85
B. Free anion and dianion	0.13	0.28	0.25
A/B	0.16	0.61	3.40

tightly and permanently bound to the active site, could require prior binding of the substrate as a condition of its own binding, or play an entirely structural role in activity. The data could be interpreted to mean that free Mg^{++} and/or substrate-cation complex are activators of the enzyme.

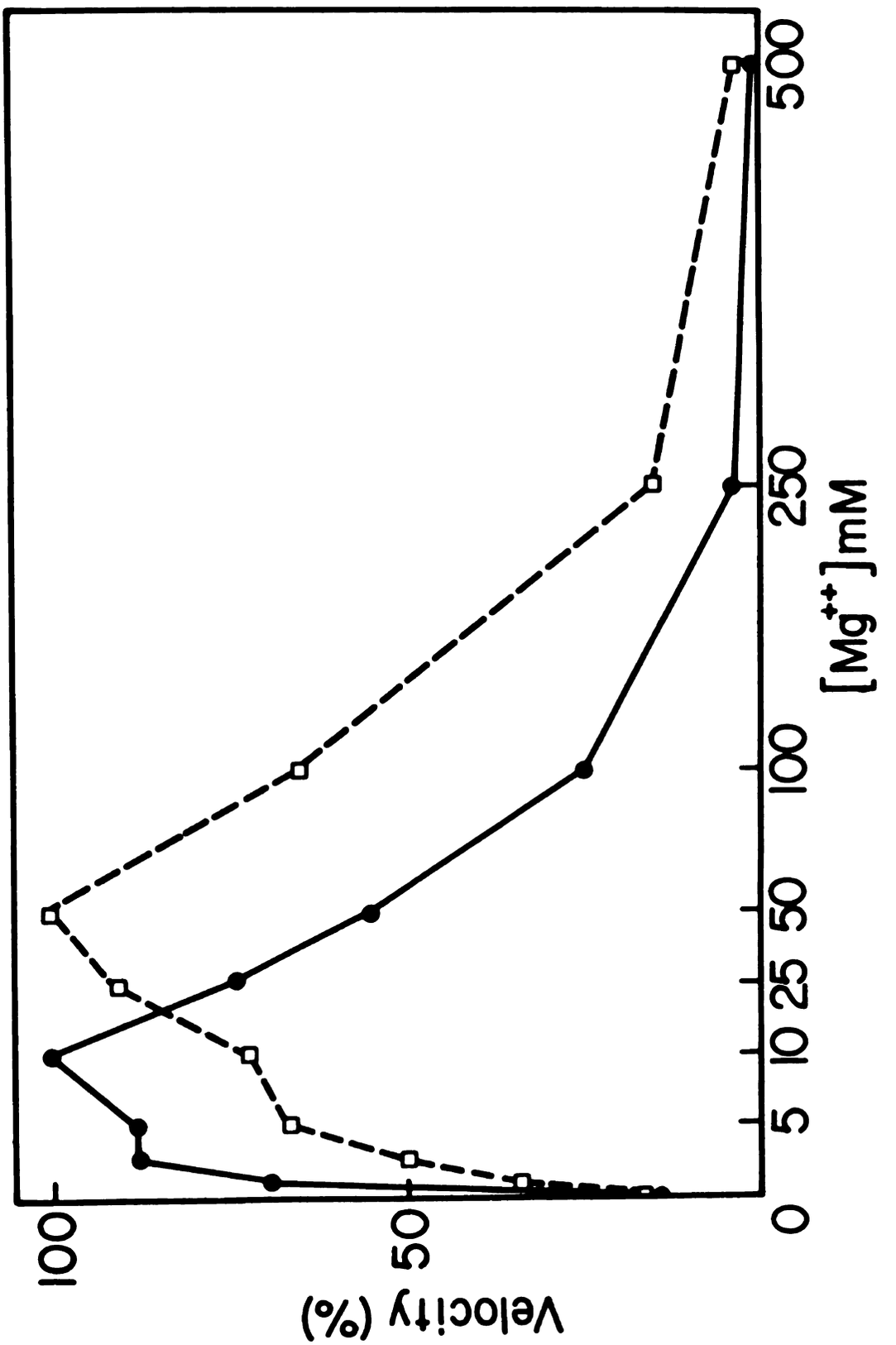
Effect of Magnesium Ion Concentration and pH on Reaction Velocity

Reaction velocity was determined at different Mg^{++} concentrations and at pH 5.5 where the phosphate monoanion of the substrate predominated and at pH 8.1 where the substrate was the dianionic phosphate (Figure 17). The phosphate monoanion and dianion forms were both substrates, their relative affinities must be similar, since in a

Figure 17.--Dependence of P-glycolate Phosphatase Activity on Magnesium Ion Concentration and pH. The assay was run for 10 min in 0.5 ml containing 10 µmole MES at pH 5.5 or 10 µmole bicine at pH 8.1, 1 µmole P-glycolate, 0.5 to 250 µmole $MgCl_2$, and 0.1 unit phosphatase. The two selected pH ensured that in each case, the concentration of phosphate monoanion or dianion of P-glycolate was greater than 90% of the total free substrate. During the assay hydrolysis was 10% or less. The maximum activity at pH 5.5 was approximately 40% of that at pH 8.1.

● , pH 8.1

□ , pH 5.5


Velocity (%)
100
50
0
5
10
25
50
100
250
500
[Mg++]mM

previous section Km was found to be essentially constant throughout a major protion of the Vmax-pH curve. Since Km did decrease slightly with pH, a possible preference for the phosphate dianion over the monoanion may exist. The decrease in activity at very high Mg^{++} concentrations is due to subsaturating levels of the free substrate. Calculations at each pH indicated that free substrate was still saturating at maximum activity, but became rate limiting at the next highest Mg^{++} concentration used. The difference in pH maxima can be explained on the basis of a preponderance of the phosphate dianion of the substrate at high pH, which results in a greater formation of the P-glycolate-Mg complex and hence free substrate becomes limiting at lower total Mg^{++} concentrations. At 100mM $MgCl_2$ there was essentially no free substrate, activity was maintained by complex dissociation as the substrate was removed. Since complex concentration was essentially identical at 100 and 500 mM $MgCl_2$, the complex can be ruled out as a substrate with more than 1-2% the activity of the free ester.

Effect of Divalent Cation on Saturation Velocities

These experiments were run over a cation concentration range of 2×10^{-5} to 2×10^{-3} M, within which range the M/Y ratio remained approximately constant, since M/Y = K2/(K2 + S2), S2 < K2 and S2 does not vary significantly at constant pH. Variation in P-glycolate concentration was also insignificant and saturating throughout. The data is plotted using free M^{++} concentration calculated on page 82, because it has been shown that P-glycolate-Mg was not kinetically active as a

substrate. Plotting of M rather than Y, changes the quantitative aspects but not the qualitative shape of the curve as is evident from the section on page . The background of activity in the absence of added cation was subtracted. The nature of this background will be discussed on page 112, but it did not interfere with the interpretation of cation effects.

The data was analysed using double reciprocal plots, Hill plots, and Eadie-Hofstee plots, with computer programs designed for these plots (I wish to thank C. P. Dunne and D. Bishop, who wrote these programs, for their assistance). The programs, in BASIC, were run on a CDC-6500 computer.

Variation of Mg^{++} concentration resulted in downward curving Lineweaver-Burke plots and concave upwards Eadie-Hofstee plots (Figure 18). The curvature at low pH was slight, but increased with increasing pH as determined by the Hill number (Table 11). On the other hand, saturation velocity experiments performed concurrently with Co^{++} gave linear reciprocal plots (Figure 19).

Several causes for curved reciprocal plots have been considered. The endpoint (10 min) assay would lead to such results if progressive inactivation of the enzyme occurred in the presence of Mg^{++} but not Co^{++}, or if the enzyme was unstable at low Mg^{++} concentrations but not at high Mg concentrations. These possibilities do not account for the data, since P-glycolate phosphatase was stable under my assay conditions throughout the cation and pH ranges used at 30^{o}. If the P-glycolate-Mg complex were a substrate these curves might also occur but it was proven not to be a substrate.

Figure 18.--Eadie-Hofstee plots for determination of P-glycolate phosphatase-magnesium binding affinity at different pH values. The assay contained 40 μmole of the appropriate buffer, MES, HEPES, or bicine; 1 μmole p-glycolate; from 0.101 to 1.0 μmole $MgCl_2$; and 0.1 unit phosphatase (S.A. = 40) in 0.5 ml. No contaminant nonspecific phosphatases were present in the preparation.

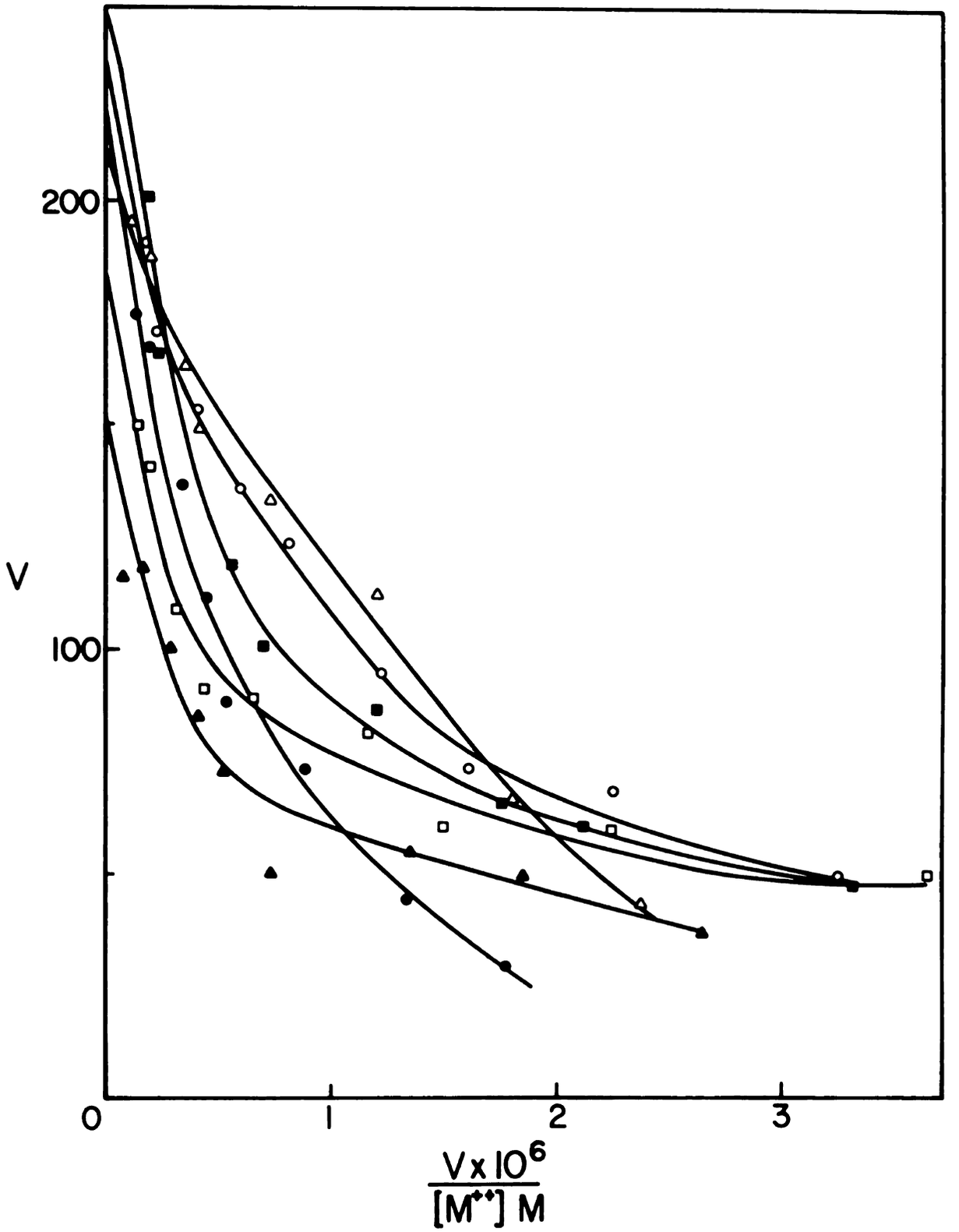
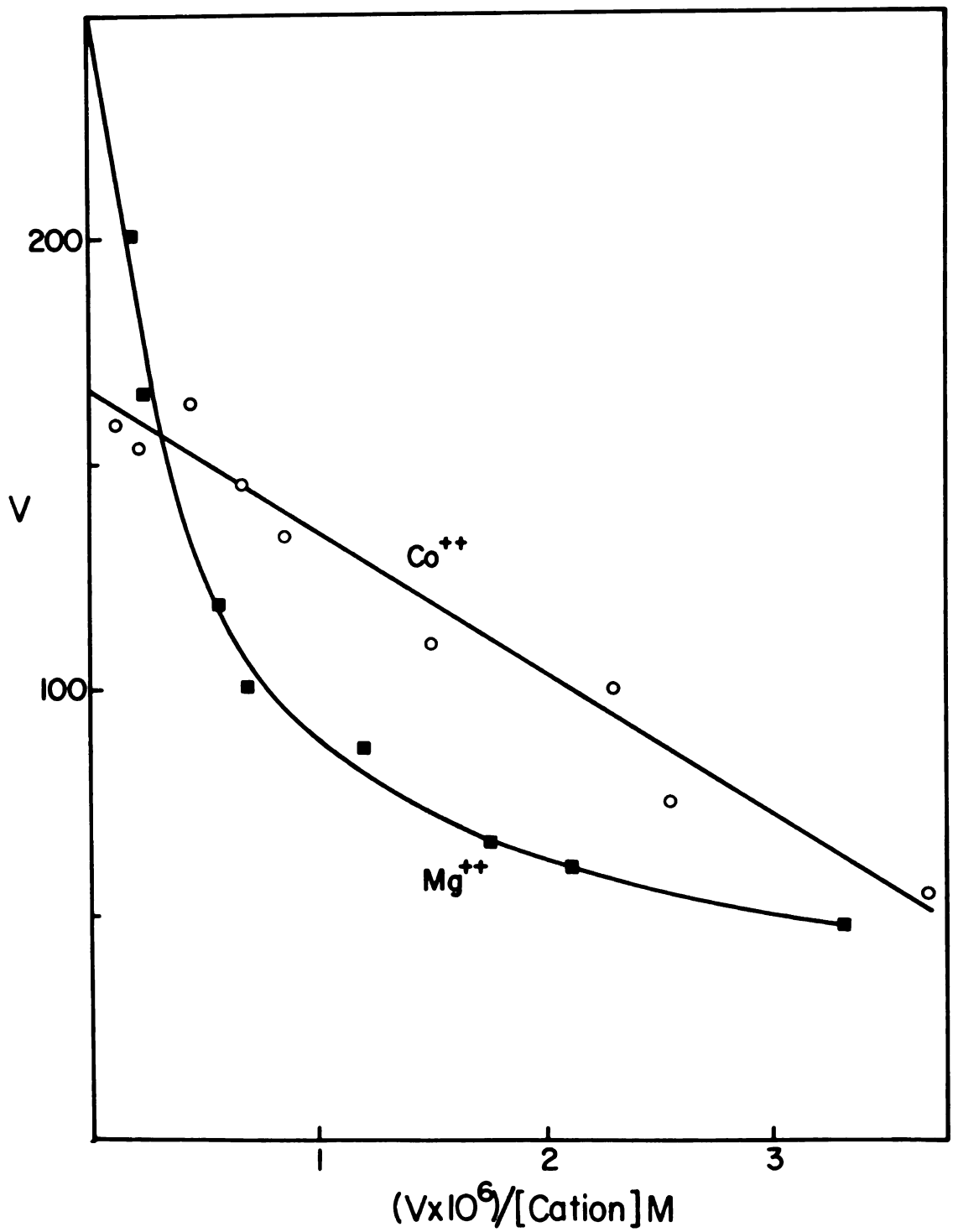

200
100
V
0
1
2
3
$\frac{V \times 10^6}{[M^{+\cdot}]\,M}$

TABLE 11.--Variation in Hill Number and Cation Affinity with pH.

Experiment Number	Cation	pH	N_H	$K_{0.5}$ ($\times 10^{-3}$ M)	
				lower limit	upper limit
1	Co^{++}	5.7	0.80	0.05	0.05
	Co^{++}	7.5	0.91	0.04	0.04
	Mg^{++}	5.7	0.85	0.07	0.07
	Mg^{++}	8.1	0.79	0.03	0.23
	Mg^{++}	8.7	0.45	0.02	0.18
2	Mg^{++}	6.3	0.61	0.05	0.26
	Mg^{++}	6.9	0.52	0.03	0.13
	Mg^{++}	7.5	0.45	0.025	0.15
	Mg^{++}	8.1	0.22	0.02	0.18
3	Mg^{++}	8.1	0.18		
			0.60[a]		

[a]This value is corrected as discussed in the text.

Figure 19.--Eadie-Hofstee plots for cobalt and magnesium ions at pH 7.5. The conditions used were those described in Figure 18.

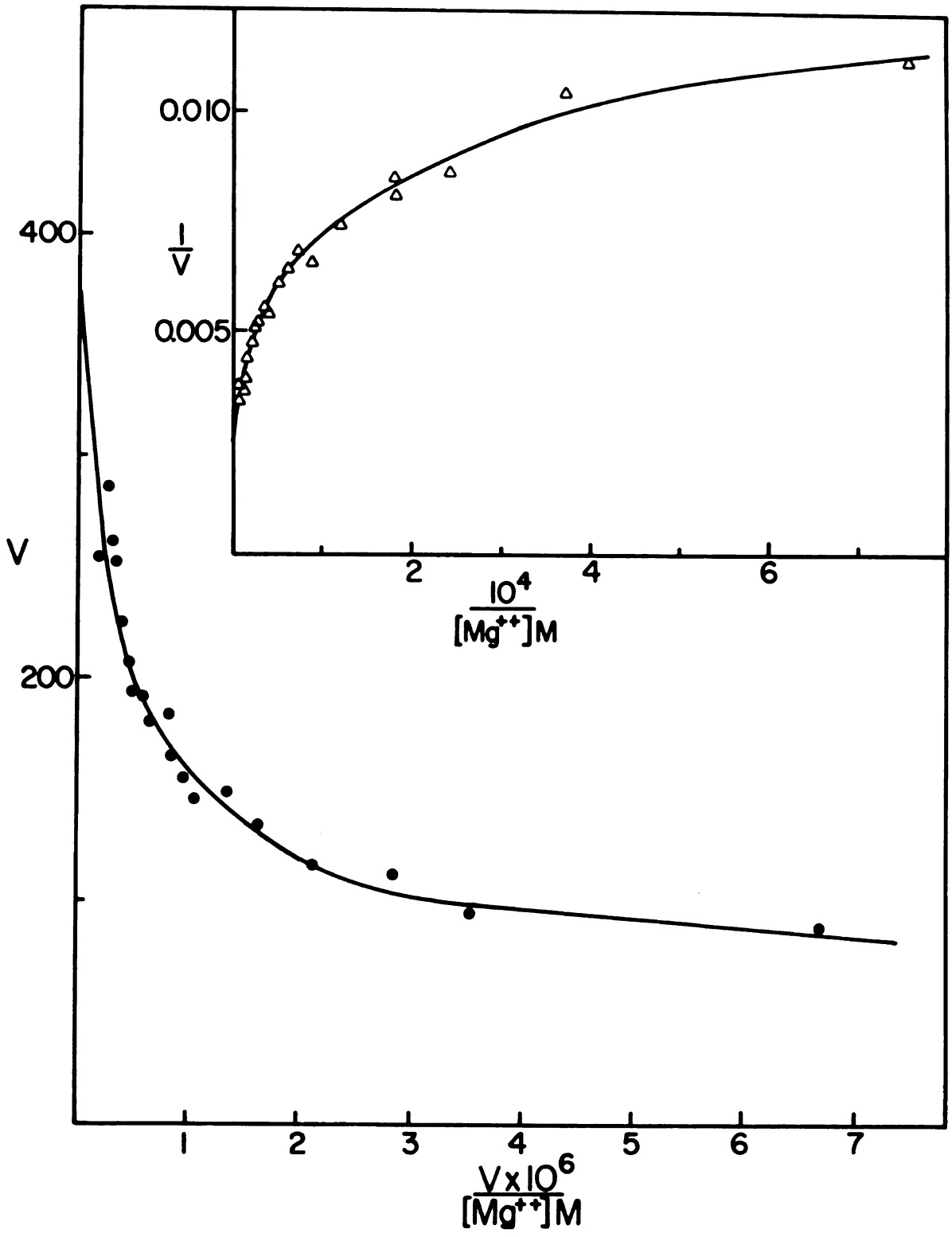

200
100
V
Co++
Mg++
1
2
3
(Vx10^6)/[Cation]M

Another situation leading to artifactual plots is the presence of two enzymes or two forms of a single enzyme having different kinetic properties. The results of earlier experiments ruled out the presence of two enzymes and the lack of variation of Km with pH or changing M^{++} concentration ruled out different forms of not only the substrate but also the enzyme i.e. the phosphate monoanion and dianion appear to have very similar or identical binding constants. Furthermore there was an intrinsic dependence on the type of cation.

Saturation kinetics with Mg^{++} at pH 8.0 were repeated using a different enzyme preparation. The results (Figure 20), confirm the previous observation that the Eadie-Hofstee plot had a concave upwards curvature and the Lineweaver-Burke plot was concave downwards. The simplest interpretation of these data is a two binding site model. There appears to be a site which binds either Mg^{++} or Co^{++} with similar high affinity. Velocity is assumed to be proportional to saturation of this binding site i.e. Michaelis-Menten kinetics. The second site may be specific for Mg^{++} as its affinity was dependent on both pH and Mg^{++} concentration. Since P-glycolate phosphatase is a tetramer, the data would be consistent with negative allosteric cooperativity, i.e. binding at any allosteric site causes decreasing affinity at the remaining sites on that multimer. The mechanism of Mg^{++}-stimulated, Mg^{++} dependent activity appears analogous to the K^{+}-stimulated, Mg^{++} dependent ATP'ase activity described in oat roots by Leonard and Hodges (1973).

The possibility that binding occurs at only one site and the interaction causes conformational and hence allosteric effects with Mg^{++},

Figure 20.--Eadie-Hofstee and Lineweaver-Burke plots of magnesium affinity for P-glycolate phosphatase at pH 8.1. Conditions were those reported in Figure 18.


0.010
1/V
0.005
2
4
6
10^4 / [Mg++]M
400
V
200
1
2
3
4
5
6
7
V x 10^6 / [Mg++]M

but not with Co^{++}, is very unlikely. Further, the fact that Co^{++} binds at high pH where its catalytic activity is negligible but where activity is high with Mg^{++} suggests additional sites for Mg^{++}. Finally the studies on the effect of EDTA to be reported on Page 118 also suggest the presence of two distinct types of binding sites for cations.

Quantitative values for the cation binding site affinities were not determined. First, the theoretical basis for such analysis is uncertain in studies involving velocities, the necessary assumptions having been discussed by Koshland (1970). Secondly, analysis would require abstraction of the parameters of the first binding site from the data. There is no valid procedure for doing this. The lower limiting slope in the Eadie-Hofstee plot provides a maximum value only, since it consists of the constant binding site parameter plus that for the situation of a single allosteric site filled per molecule. Similarly the upper limiting slope is also a function of two affinities, the constant site and the affinity constant for the binding of the fourth cation. If however, the admittedly invalid procedure of subtracting lower limiting slope velocities throughout the curve were adopted, the resulting Hill plot is linear over a greater range of values. This result thus provides support for the validity of the procedure, hence implies the existence of an invariant binding constant with a value close to the limiting value of the Eadie-Hofstee plot. The data further implies that the hypothesis of negative allosteric cooperativity is correct. The first binding constant has a small value compared to the invariant site, while the fourth is substantially higher.

These values for Figure 20 are shown in Table 12 and suggest that the decrease in allosteric affinity from binding the first to the fourth cation is greater than two orders of magnitude.

A third reason why quantitation of the binding constants was not done was the observation that the Hill numbers were not consistent between enzyme preparations i.e. the degree of cooperativity was dependent, to some extent, on the particular enzyme preparation (see Table 11). In all cases the pattern of decreasing Hill number with increasing pH was observed. The absolute variation is, almost certainly, an artifact of purification. As mentioned above, all preparations had some varying degree of basal activity in the absence of any added cation. Experiments reported below suggest that this activity was due to extremely tightly bound metal ion. Variation in the amount of contaminant bound cation might account for the variation in curvature of the reciprocal plots, and furthermore, quantitation would be meaningless unless all contaminant activity was initially removed.

The dependence of cooperativity on pH could be due to competition between Mg^{++} and protons for the residue at the binding site i.e. the change in Hill number with pH could represent an ionization curve of the allosteric binding site. Alternatively, the binding site affinity could be mediated through small pH induced conformational changes at the allosteric site. A different explanation for the pH dependence of the negative allosteric cooperative effect is obtained by assuming the P-glycolate-Mg complex is the allosteric activator. Reciprocal plots using P-glycolate-Mg as the variable effector remained non-linear. The increased allosteric interactions would be thus due to increased

TABLE 12.--Binding Constant Limits for Magnesium to P-glycolate phosphatase at pH 8.1. The values are the asymptotes of the curves in Figure 20.

Method	Lower Limit		Upper Limit	
	Vmax	Km	Vmax	Km
Eadie-Hofstee	113	4.1×10^{-6}M	376	4.9×10^{-4}M
Lineweaver-Burke	107	2.7×10^{-6}M	400	4.5×10^{-4}M

levels of the complex with pH for any given total Mg^{++} concentration. From the data in Table 10 it is possible that the complex could be the activator rather than free Mg^{++}. These two possibilities cannot be distinguished by the present data. Since the two species change in parallel, the physiological response would be identical.

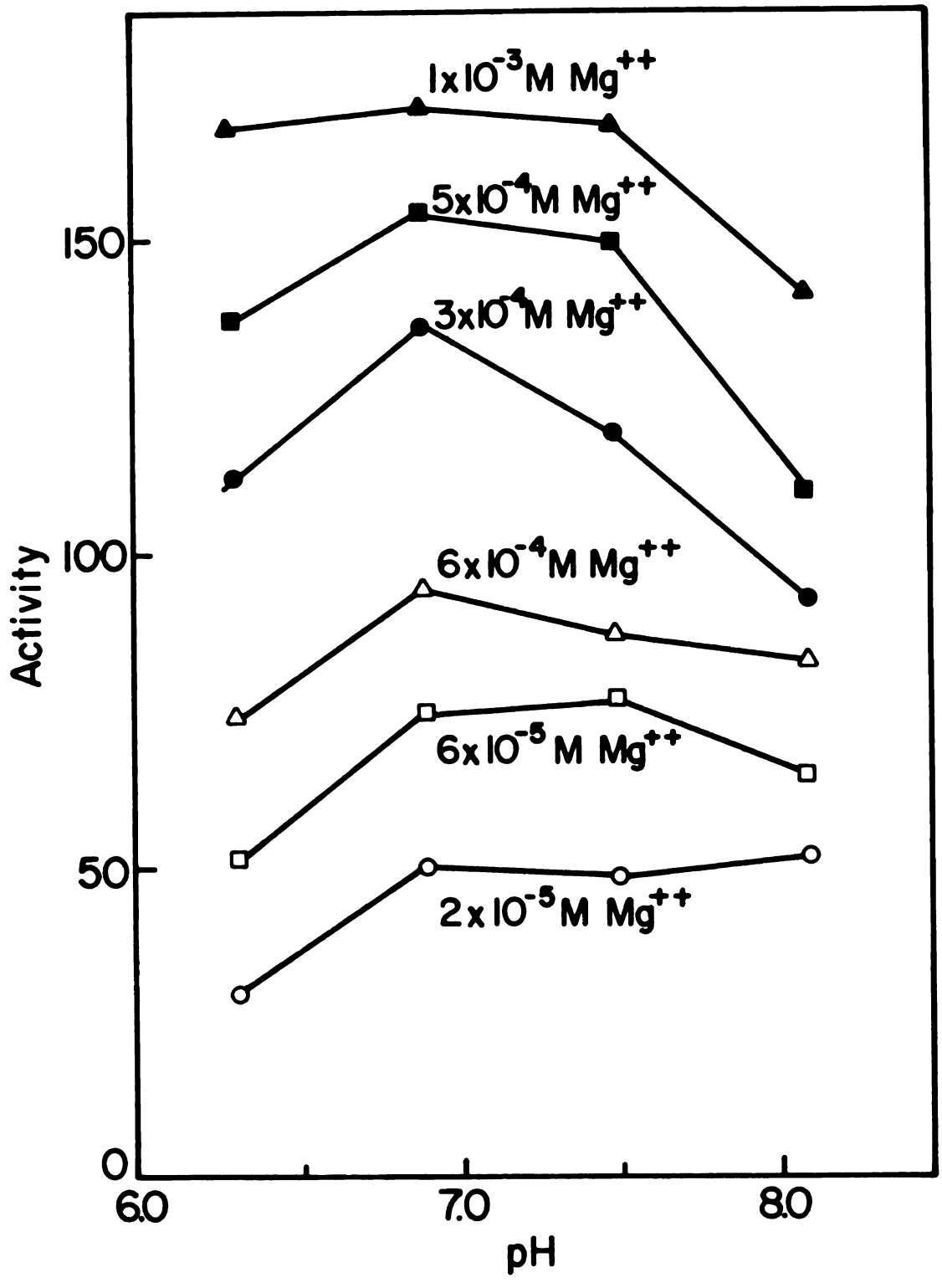
The inability to derive an accurate value for the invariant binding constant prevented accurate analysis of experiments designed to confirm the above suggestions, i.e. Co^{++} should activate the Mg^{++} saturated enzyme at low pH by direct competition for the activator site, giving a linear double reciprocal plot for the activation. At high pH, Mg^{++} should stimulate activity by competiton for the activator site and by binding to the unoccupied allosteric effector site. In this situation a curved double reciprocal plot was expected. However determination of residual activity due to the saturating cation requires knowing at least the ratio of the two metal ion $K_{0.5}$'s. Experiments at pH 7.5 with saturating (10mM) Co^{++} and varying Mg^{++}, and at pH 5.7 with saturating (20mM) Mg^{++} and varying Co^{++} and using $K_{0.5}$ values obtained from Figure 18 were not unequivocal.

The hypothesis provides a mechanism for the divalent metal ion effects on pH optima. As previously noted, Mg^{++} prevented the ionization of an active site residue which is required in the protonated form for the substrate binding and formation of the transition state. The pK of this residue is increased by Mg^{++} 1.6 pH units from that observed for Co^{++} and Mn^{++}. The additional allosteric site, by inducing a conformational change, may prevent this ionization and hence extend the range of activity on the alkaline side. The negative allosteric cooperativity is well suited to provide control. In effect, it causes a shift to alkaline pH at very low Mg^{++} concentrations where competency is becoming minimal. Decreased sensitivity of activity to fluctuations in substrate and effector concentrations (in this case Mg^{++} and pH) is a characteristic of negative cooperativity (119) (Figure 21). At total Mg^{++} concentration of 2×10^{-5} M, the activity optimum is above pH 8, but gradually shifts to lower ranges til at 1mM the maximum velocity occurs below pH 7.

The previous failure to detect multiple binding sites (4) seems due to saturation velocity studies being carried out only at low pH where the slight curvature could be easily overlooked. The Hill numbers in Table 11 further indicate a slight curvature in the presence of cobalt suggesting that the allosteric site is not absolutely specific, but that cobalt may interact very weakly. The interaction, however, does not prevent ionization of the active site residue at pH 7.5.

The lower limit binding constant also appears to reflect lessened competition between cation and hydrogen ion since the affinity of the

Figure 21.--Activity - pH curves for P-glycolate phosphatase. The effect of varying magnesium concentration on the pH optimum. The data presented is replotted from Figure 18.


1x10-3M Mg++
5x10-4M Mg++
3x10-4M Mg++
6x10-4M Mg++
6x10-5 M Mg++
2x10-5M Mg++
150
100
50
0
Activity
6.0
7.0
8.0
pH

cation increases with pH for both Co^{++} and Mg^{++} (Table 11). The variation, however, is slight, about 3 fold over 3 pH units and closely parallels the variation in substrate Km. Since the active species are the free cation and free P-glycolate and since Km contains a kinetic component, this is very likely fortuitous.

Evidence does not distinguish between an essential structural role and a catalytic role for the cation in activating the phosphatase. Since the cation is not expected to dissociate and reassociate with every substrate turnover it may be bound at the active site and play a role in the catalytic reaction.

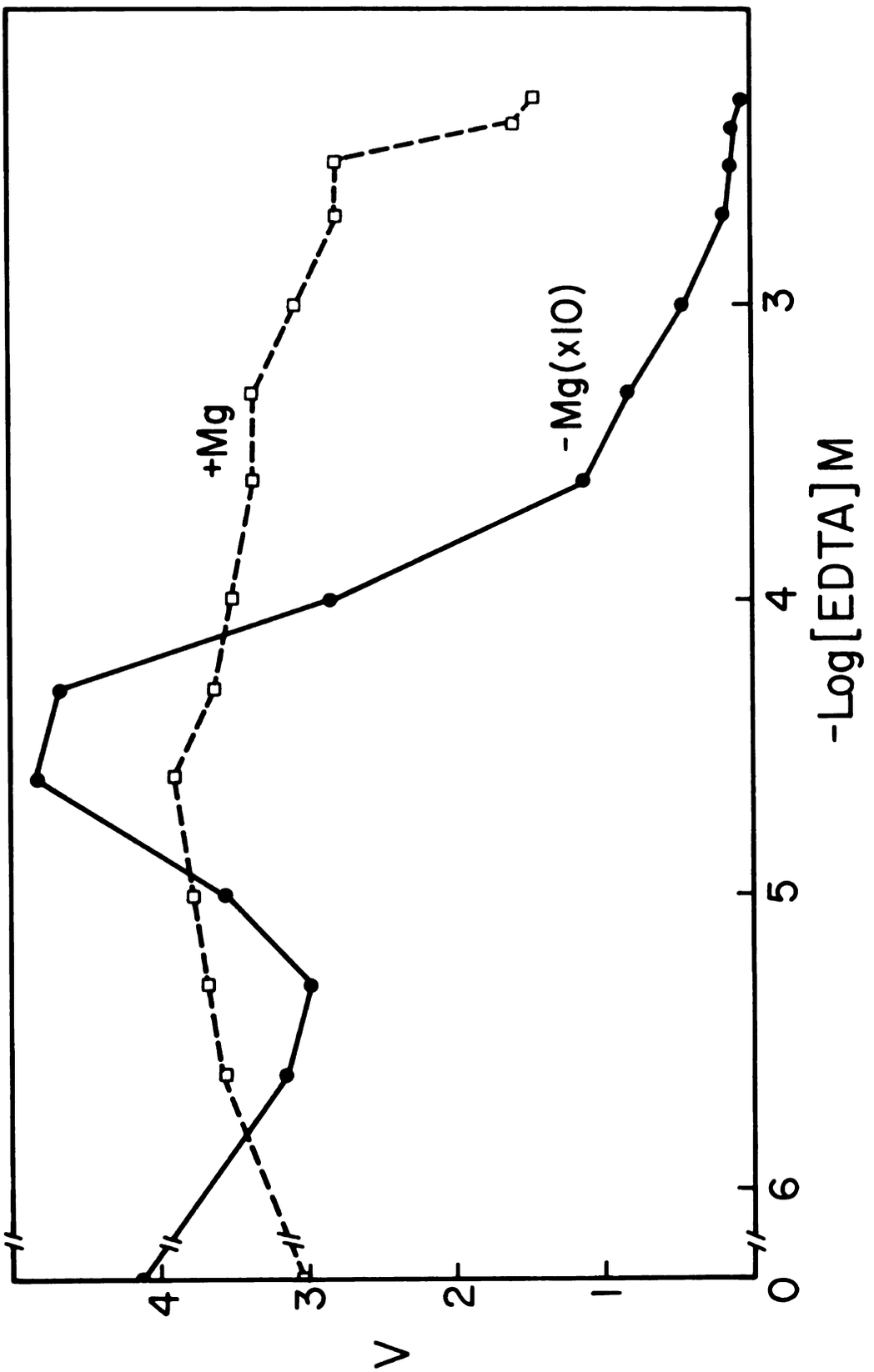
The mechanism proposed involves concerted protonation of the bridge oxygen with phosphorus attack by an enzyme nucleophile (serine hydroxyl or histidine imidazole). The divalent cation could (22) A. shield the phosphate O^- to prevent repulsion of the anionic nucleophile, B. withdraw electrons to make phosphorus a better site for nucleophilic attack, or C. change the pK and the reactivity of the nucleophile. The third alternative could act through a structural conformational change, and does so in the case of allosteric stimulation. Brunel and Cathala (1970) observed a slow exponential activation of bovine brain alkaline phosphatase by magnesium and suggested this was due to a conformational change. However P-glycolate phosphatase is activated immediately on adding either enzyme or cation to the otherwise complete assay medium, thus no similar evidence for a structural role exists. The alternative proposal would involve substrate protonation by a water molecule bound to the cation, thus forming a six-membered ring in the transition state complex rather than four, as proposed above.

Effect of EDTA, Dialysis and Resins

EDTA inhibits P-glycolate phosphatase (170) and the effect of increasing EDTA in the presence and absence should distinguish whether residual activity in the absence of added cation is due to slow catalysis from breakdown of the ES complex or to residual levels of tightly bound cation.

In the absence of added $MgCl_2$ the slow rate of hydrolysis upon adding EDTA decreased followed by a sharp increase with increasing EDTA until a final loss of all activity (Figure 22). The data is similar to that reported by O'Sullivan and Morrison (1963) for creatine kinase and by Bright (1965) for β-methylaspartase. Bright suggested the combination of highly electronegative cations at two distinct sites, acting as an activator at the most easily titrated site and as an inhibitor at the second site. This analysis is applicable to P-glycolate phosphatase. Competition between contaminant and divalent cation cannot explain the allosteric effects however since this would lead to sigmoid kinetics. The contaminant may affect the extent of the activation depending on the fraction of the enzyme containing it, and decrease the negative allostericity observed at high values. Moreover since the ratio of residual to maximally activated activity is constant throughout the experimental range, the contaminant activity is unlikely to be the cause of the variation in Hill number with pH. In fact, this would require the contaminant to be bound more tightly at low pH than at high pH, and evidence has been presented earlier that cations are bound with greater affinity at higher pH.

Figure 22.--Effect of EDTA on P-glycolate phosphatase activity. The system contained 10 μmole cacodylate at pH 6.3, 1 μmole P-glycolate, 2 μmole $MgCl_2$ (where indicated), 1.25×10^{-3} to 2.5 μmole Na_2EDTA, and 0.1 unit phosphatase in the presence of $MgCl_2$ and 1.0 units in the absence of $MgCl_2$.


+Mg
-Mg(x10)
V
-Log[EDTA]M
0
1
2
3
4
5
6

Several attempts to remove the contaminant were unsuccessful. P-glycolate phosphatase was exhaustively dialysed at 4^{o} against 1mM EDTA, with three changes of buffer during 96 hours. This procedure failed to reduce the level of residual activity. Passage of the enzyme through a 0.8 x 30cm Chelex-100 (Biorad) column caused a 99% loss of activity but some residual activity remained. For these experiments both substrate and buffer were purified on Dower AG-50W-X4 (Na^{+} form) to eliminate any source of contaminant.

MECHANISM OF PHOSPHOGLYCOLATE PHOSPHATASE--
ISOTOPIC, KINETIC, AND CHEMICAL STUDIES

Introduction

Richardson and Tolbert (1961) reported that P-glycolate phosphatase was specific, as it did not catalyse the hydrolysis of 21 other phosphate esters and anhydrides. Most phosphatases have broad specificity for substrate and only a few are known with a limited number of substrates. Because of the many phosphate esters in the photosynthetic carbon cycle, the need for a specific phosphatase in the chloroplast is obvious. This section contains experiments designed to evaluate the mode of hydrolysis, the rate limiting step in the reaction sequence, and the reasons for specificity. The proposed mechanism is evaluated in terms of information available for nonspecific phosphatases. The data in this chapter might permit designing a highly specific inhibitor for P-glycolate phosphatase which might be an effective herbicide with selectivity for C_3 plants, because any increase in P-glycolate levels will decrease CO_2 fixation through triose phosphate isomerase inhibition, thus reducing availability of CO_2 acceptor--RuDP.

Results and Discussion

Determination of Bond Cleavage Position During Hydrolysis by P-glycolate Phosphatase

To examine the possibility that absolute specificity was due to a unique hydrolytic mechanism, such as C-O bond cleveage or formation

of a cyclic intermediate, either the intramolecular acid anhydride or the dilactone, glycolide; the hydrolysis products in the presence of (^{18}O)-H_2O were analysed by mass spectrometry.

Trimethylsilyl derivatives of glycolate, phosphate, and P-glycolate were completely separable by gas-liquid chromatography (Figure 23), so that in a reaction mixture of (^{18}O)-H_2O and P-glycolate with pure enzyme the products, glycolate and phosphate were readily separated prior to mass spectrometry.

Mass Spectra of $(Me_3Si)_2$ -glycolate and $(Me_3Si)_3$ -PO_4

The mass spectrum of $(Me_3Si)_2$-glycolate (Figure 24a) contained no molecular ion at m/e 220. However, an intense ion at m/e 205 (M - 15), resulting from the loss of a methyl group, was present and thus isotopic analyses for ^{18}O were possible by comparing intensities at m/e 205 and m/e 207. The strong peaks at m/e 177 and m/e 161 arose from the loss of CO and CO_2 respectively from the m/e 205 ion by the migration of the OMe_3Si group or the Me_3Si group and then loss of the carboxyl group (130,55). This analysis is supported by the presence of metastables at m/e$\approx$ 153 and m/e $\approx$126 on the oscillographic trace, and the 50% and 100% loss of ^{18}O in the carboxy group of glycolate from these peaks (see Table 14).

The mass spectrum of $(Me_3Si)_3$-PO_4 (Figure 24b) had a strong molecular ion at m/e 314 and a very intense ion at m/e 299 due to one Me loss. This ion has been assigned the structure $(Me_3SiO)_2\ P(O)\text{-}\overset{+}{O} = SiMe_3$ (232). Little further degradation was observed at 70eV.

Figure 23.--Separation of glycolate, inorganic phosphate, and P-glycolate as trimethylsilyl derivatives by gas-liquid chromatography.

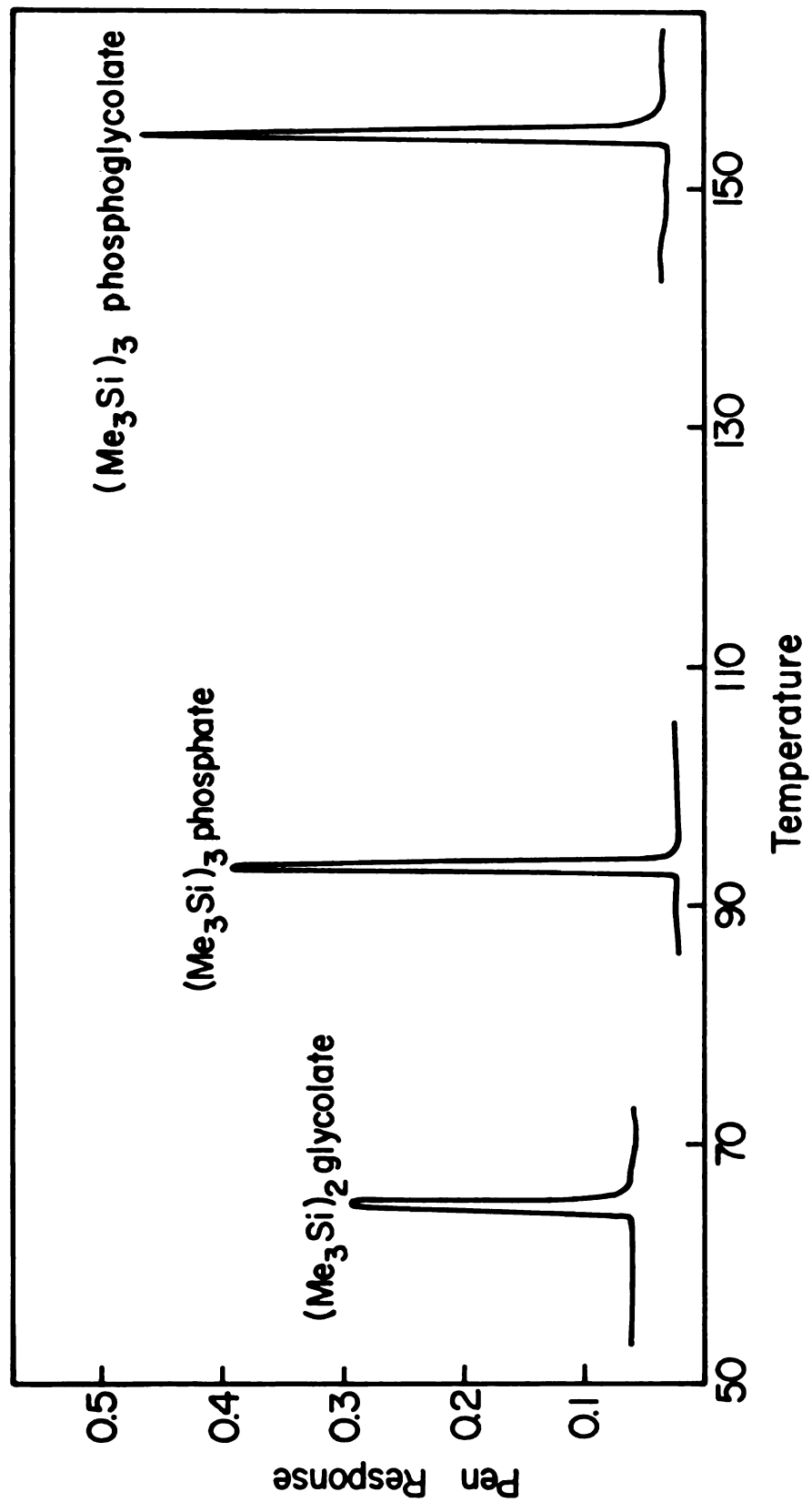
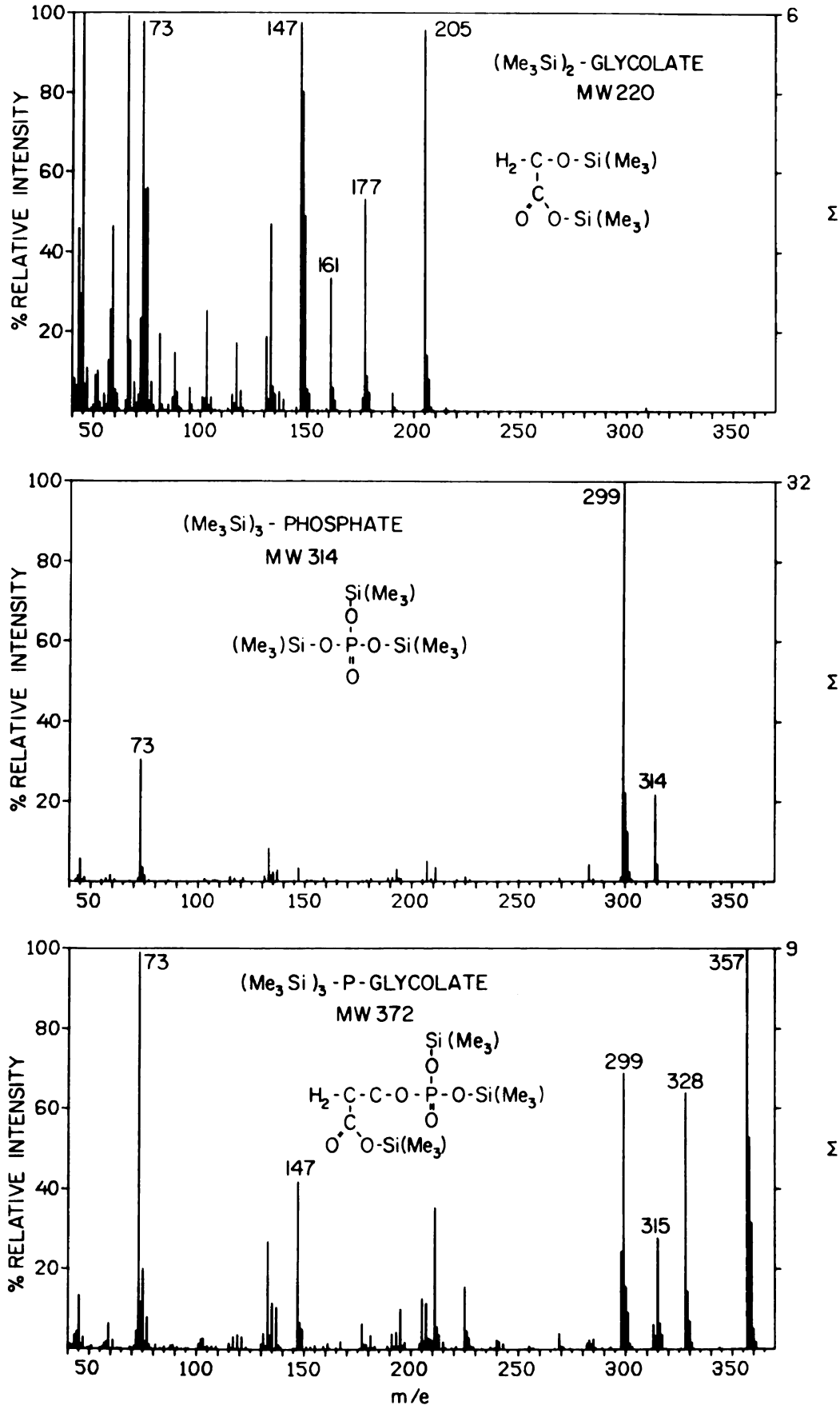

(Me3Si)3 phosphoglycolate
(Me3Si)3 phosphate
(Me3Si)2 glycolate
0.5
0.4
0.3
0.2
0.1
Pen Response
50
70
90
110
130
150
Temperature

Figure 24.--(a) Mass Spectrum of $(Me_3Si)_2$-glycolate.

(b) Mass Spectrum of $(Me_3Si)_3$-phosphate.

(c) Mass Spectrum of $(Me_3Si)_3$-P-glycolate.


% RELATIVE INTENSITY
100
80
60
40
20
73
147
205
177
161
(Me3Si)2 - GLYCOLATE
MW 220
H2-C-O-Si(Me3)
C
O O-Si(Me3)
6
Σ
50
100
150
200
250
300
350
% RELATIVE INTENSITY
299
(Me3Si)3 - PHOSPHATE
MW 314
Si(Me3)
O
(Me3)Si-O-P-O-Si(Me3)
O
32
Σ
73
314
% RELATIVE INTENSITY
73
357
(Me3Si)3 - P-GLYCOLATE
MW 372
Si(Me3)
O
299
328
H2-C-C-O-P-O-Si(Me3)
C O
O O-Si(Me3)
147
315
9
Σ
m/e

The mass spectrum of $(Me_3Si)_3$-P-glycolate (Figure 24c) has been previously described (122,123).

Mass Spectral Analysis of (^{18}O)-phosphate

The (^{18}O)-inorganic phosphate, prepared as described in methods, was analysed to confirm the validity of the trimethylsilyl derivative analysis. This procedure gave excellent agreement with both the theoretical value and that obtained by combustion of the sample to CO_2 (Table 13).

Combined GLC-MS proved to be a rapid and effective method for separating and analysing inorganic phosphate and phosphate containing compounds as their trimethylsilyl derivatives. The method has the advantages over combustion to CO_2 of A. rapidity and ease of analysis, B. greater accuracy of measurement, C. greater sensitivity for incorporation and thus possibility of using lower initial isotopic excess, and D. definitive assignment of the number of O atoms incorporated per molecule, as against an average value for CO_2. For example, this method would immediately distinguish between an isotope effect during incorporation into a single position from additional exchange into the molecule at another position.

Incorporation of (^{18}O)-H_2O into Products During the P-glycolate Phosphatase Reaction

The assay system in 0.5 ml adjusted to pH 6.3 contained 5 µmole P-glycolate, 12.5 µmole MES, 2 µmole $MgCl_2$, either 7.32 or 15.22% atoms excess (^{18}O)-H_2O, and 1 unit P-glycolate phosphatase with a S.A. of 53.

TABLE 13.--Analysis of (^{18}O)-Inorganic Phosphate.

Analytical Procedure	(^{18}O) Atoms Excess %
CO_2[a]	2.52
$(Me_3Si)_3PO_4$[b]	2.546
Theoretical	2.59

[a]In two different experiments with (^{18}O)-KH_2PO_4 the m/e 46: m/e 44 ratio was 5.62 and 5.58 x 10^{-2}. This value was corrected for a background of 0.24 x 10^{-2} for unlabelled KH_2PO_4.

[b]Two μmole (^{18}O)-KH_2PO_4 was derivatized and analysed for percent incorporation as described for standard inorganic phosphate in the methods section.

Controls contained 5 μmole phosphate or 5 μmole glycolate replacing the P-glycolate. Incubation at 30° for 30 min was stopped by addition of 50 μl 12 M perchloric acid (71%), and this was immediately neutralised with 50 μl 12 M KOH to prevent acid catalysed ^{18}O H_2O-carboxyl exchange. The solutions were cooled, centrifuged to remove protein and $KClO_4$, and the supernatants lyophilized to save the (^{18}O)-H_2O. The residue was dissolved in 1 ml H_2O and eluted through a Dowex AG-1-X4 column (4 cm x 0.5 cm i.d.) with 3.5 ml 0.1 M HCl. The eluates were lyophilized to dryness and trimethylsilylated derivatives prepared and analysed as previously described. Ratios of m/e 207 to m/e 205, m/e 169 to m/e 177, and m/e 163 to m/e 161 were analysed for enzyme or acid catalysed incorporation or exchange of (^{18}O)-H_2O into glycolate (Table 14). Phosphate was analysed as before using m/e 316 to m/e 314 and m/e 301 to m/e 299. Unhydrolysed P-glycolate was analysed for exchange by the ratio of m/e 359 to m/e 357.

TABLE 14.--Incorporation of (^{18}O)-H_2O into the Reaction Products of P-glycolate Phosphatase.

Expt. No.	Substrate	(^{18}O) atoms excess %			
		H_2O	P-glycolate	glycolate	phosphate
1	P-glycolate	7.32	-[a]	-	7.20(316)[b]
					7.36(301)
	phosphate		-	-	0(316,301)
2	P-glycolate	15.22	0(359)	0(207)	16.04(316)
					16.54(301)
	glycolate[c]		-	0(207)	-
	glycolate[d]		-	7.7(207)	-
			-	3.1(179)	-
			-	0(163)	-
	phosphate		-	-	0(316,301)
3	P-glycolate	15.22	-	0(207)	16.5(301)

[a]A dash indicates no analysis was made.

[b]The figure in parentheses indicates the m/e ratio of M + 2 used for the analysis.

[c]Nonenzymic incubation, 0.1 M HCl, 21 hr, 4°.

[d]Nonenzymic incubation, 1 M $HClO_4$, 0.5 min, 25°.

The results indicate that tobacco P-glycolate phosphatase catalysed the hydrolysis of the substrate by oxygen- phosphorus bond cleavage, since a single ^{18}O atom was incorporated into each phosphate produced, while the glycolate was not labelled. This is the mode of hydrolysis for both acid and alkaline nonspecific phosphatases and nonenzymic hydrolysis of alkyl phosphates in the pH 4-8 range. Thus the mode of hydrolysis does not explain the specificity of the enzyme.

At the concentrations used, no enzyme-catalysed exchange reactions were observed into the carboxyl, hydroxyl, or phosphate oxygens of substrate or products. Failure to observe $(^{18}O)\text{-}H_2O + PO_4 \rightleftharpoons H_2O + (^{18}O)\text{-}PO_4$ exchange may be due to the failure of phosphate to bind significantly at the pH and concentrations used or to a failure of bound phosphate to react with the enzyme to make the required transition state complex. Also no exchange into phosphate was observed during substrate hydrolysis, as there was no M + 4 peak diagnostic for such exchange.

The small discrepancy in incorporation could be due to a number of reasons A. mass spectral analytical and $(^{18}O)\text{-}H_2O$ dilution errors, B. error in original analysis of $(^{18}O)\text{-}H_2O$ excess, or C. a small isotope effect during hydrolysis, the longer bond of the ^{18}O atom is weaker and more easily broken. Errors due to use of an approximate formula i.e. neglecting the natural abundance of ^{17}O in the sample are negligible.

Substrate Specificity of Phosphoglycolate Phosphatase

A variety of alkyl phosphate monoesters were synthesized by procedures detailed in the methods section. Since specificity of phosphatases toward mono- or diesters appears to be absolute (see literature review) no attempts were made to utilise diesters. Because the carbon reduction cycle in the chloroplast is a phosphate ester pathway, the physiological requirement for a specific P-glycolate phosphatase is obvious. However, the reasons for this specificity are unknown. This study was designed to test the possibility that the active site recognition of the alkyl portion of the molecule was crucial. Perhaps a vicinal carboxyl is required for binding or steric hinderance of larger phosphate esters is possible, since P-glycolate is considerably smaller than the other sugar phosphates commonly found in the chloroplast. Steric hinderance was studied using the n-alkyl phosphate series. A series of substituted ethyl phosphates was also investigated for steric and electronic effects on binding and rates of hydrolysis, since substituted methyl phosphates are unstable. These studies permitted investigation of the requirement for a carboxyl group. The possibility that specificity was due to a unique hydrolysis mechanism was ruled out previously because P-glycolate phosphatase hydrolysed the O-P bond in the normal manner.

A very small range of short chain alkyl phosphates were hydrolysed by P-glycolate phosphatase. The relative activities are presented in Table 15. Evidence that these compounds were actually competing for the same active site as P-glycolate is shown in Table 16. Activities

TABLE 15.--Substrate specificity of Phosphoglycolate Phosphatase. Rates of hydrolysis of substrate analogs.

Substrate	Relative Activity %
P-glycolate[a]	100
Methyl-P	34.5
Ethyl-P	41.5
2-Chloroethyl-P	8.1
2-Hydroxyethyl-P	37.0
2-Methoxyethyl-P	1.9
n-Propyl-P	2.1
i-Propyl-P	1.7
P-lactate[a]	4.7
P-glycolate, methyl ester	2.2
2-Cyanoethyl-P[a]	2.4

[a]Commercial compounds. Assays contained 25 µmole cacodylate at pH 6.5, 2.5 µmole $MgCl_2$, 2.5 µmole substrate as the di- or tricyclohexylammonium salt in a volume of 0.5 ml. Between 0.1-0.5 units phosphatase was incubated with the sbustrates for 10 to 60 minutes to obtain accurate rates.

TABLE 16.--Mutual Inhibition by Various Substrates of Phosphoglycolate Phosphatase.

Substrate	Activity (A660)	Relative Activity %	% Inhibition of Additive Activities
P-glycolate	0.289	100	
Ethyl-P	0.131	45.3	
P-glycolate + Ethyl-P	0.277		34.1
2-chloroethyl-P	0.021	7.3	
P-glycolate + 2-chloroethyl-P	0.266		14.2

Note: Assays were run for 10 min and contained 20 μmole cacodylate at pH6.3, 2 μmole $MgCl_2$, 1 μmole P-ester, and 0.1 unit phosphatase in a volume of 0.5 ml.

in the presence of both substrates was reduced from that utilizing P-glycolate alone, as if the alternative substrate was competing for the same active site.

The kinetic parameters, Km and Vmax, were determined for those compounds which were hydrolysed sufficiently rapidly to permit analysis. Phosphate released was determined by the microphosphate assay and the data analysed by computer programs fitting the curve to Lineweaver-Burke and Eadie-Hofstee plots. Variation in the values of the parameters derived was less than 10% and average values are presented in Table 17.

During the course of these studies numerous other phosphate esters and their analogs with some resemblance to P-glycolate were analysed for hydrolytic activity and inhibitory capability. For the sake of completeness the list (Table 18) includes those compounds tested

TABLE 17.--Kinetic Parameters for Phosphoglycolate Phosphatase with Substrate Analogs.

Substrate	Km (x 10^{-3}M)	Vmax (units/mg enzyme)	pKa[a] (ROH)
P-glycolate	0.026	468	16
Ethyl-P	0.148	248	15.9
Methyl-P	0.212	221	15.4
2-hydroxyethyl-P	0.541	98	15.1
2-chloroethyl-P	3.31	31	14.3

[a]Ionization constant for alcohol product(14). Assays contained 100 µmole cacodylate at pH 6.3, 10 µmole $MgCl_2$, 2 x 10^{-2} to 2.5 µmole substrate (P-ester), and 0.03 to 0.1 unit phosphatase. Assays were run in 2.0 ml total volume for 1 minute.

TABLE 18.--Phosphate Esters and Related Compounds as Substrates and Inhibitors of Phosphoglycolate Phosphatase. None of the Compounds Listed was Hydrolysed at Greater than 2% of the Rate with P-glycolate.

Pyrophosphate	2-P-glyceric acid
Adenosine monophosphate	3-P-glyceric acid
Adenosine diphosphate	2,3-P_2-glyceric acid
Adenosine triphosphate	P-enolpyruvate
NADP	o-P-hydroxypyruvate
Guanylic acid	o-P-serine
Cytidylic acid	o-P-ethanolamine
	Carbamyl-P
Glucose-1-P	Acetyl-P
Glucose-6-P	
Fructose-6-P	o-Carboxyphenyl-P
Fructose-1,6-P_2	o-Nitrophenyl-P
Gluconic acid-6-P	p-Nitrophenyl-P
Ribose-5-P[a]	Phenolphthalein-P
Ribulose-1,5-P_2	
Glycoaldehyde-P	n-Propyl-P
Dihydroxyacetone-P	i-Propyl-P
Glycidol-P[a]	2-Cyanoethyl-P
Propanediol-P	2-Methoxyethyl-P
α-Glycerol-P	
β-Glycerol-P	P-glycolate, methyl ester
Phosphonoacetic acid	Creatine-P
2-Aminophosphonoacetic acid	Sulphoacetic acid
2-Amino,3-Phosphono-propionic acid	S-(Carboxymethyl)-phosphorothioic acid[a]

[a]These compounds were inhibitors of P-glycolate phosphatase. Their effects are discussed subsequently.

by Richardson and Tolbert (1961). None of these compounds were hydrolysed by P-glycolate phosphatase. The enzyme did not hydrolse other common phosphate esters of metabolism and photosynthesis. It did not hydrolyse phosphonoacetate, the C-P analog; S-(carboxymethyl)-phosphorothioate, the C-S-P analog; sulphoacetate, the phosphate analog; nor the C-N-P bond of creatine phosphate. The three analogs of P-glycolate which were inhibitory, although not hydrolysed, are discussed in detail later.

P-glycolate was the only physiologically important substrate for P-glycolate phosphatase. Other chloroplast phosphatases are also exceedingly specific; these are fructose-1,6-diphosphatase, sedoheptulose-1,7-diphosphatase, and inorganic alkaline pyrophosphatase which is present in high levels but is totally specific (61,38).

The active site of P-glycolate phosphatase appears to control the specificity through its strict steric requirements for binding, rather than any unique mechanism of hydrolysis involving a cyclic phosphate ring. The pocket at the active site must be short and exacting for the decrease in activity with substrates longer than 2 carbon atoms was very dramatic. Further, the size of the substituent on the second carbon atom from the phosphate was also critical. In comparing the 2-Hydroxyethyl derivative with the 2-Chloroethyl compound the Km increased 6 fold while the Vmax decreased 3 fold. The pocket appears also very narrow, since substitution at the first carbon atom effectively eliminates activity, as shown by the activity of P-lactate and the lower activity of isopropyl-P compared to both ethyl and n-propylphosphates.

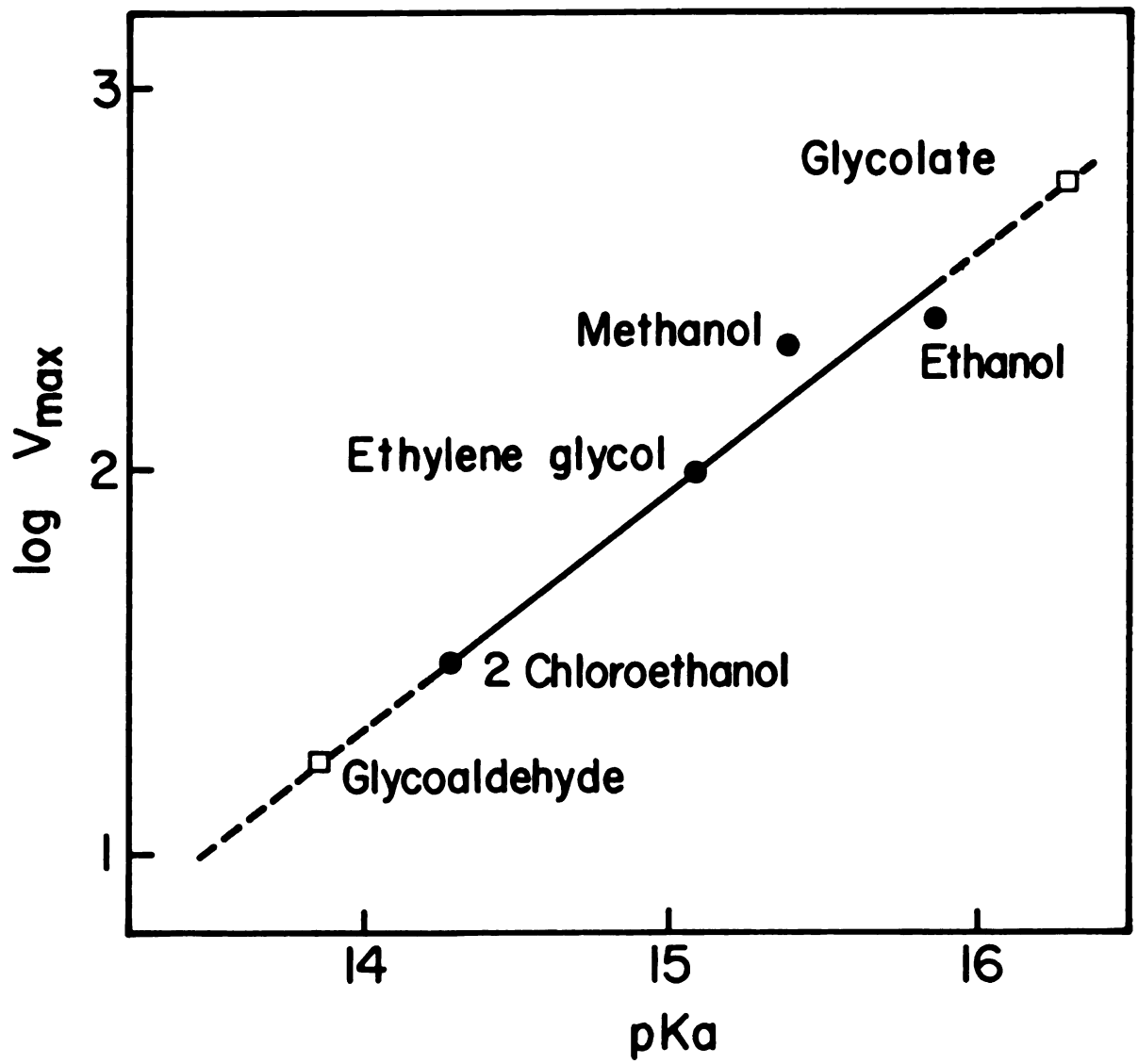
Attempts to rule out participation of α-hydrogens in the binding or reaction were unsuccessful due to the high spontaneous rates of

hydrolysis of tert-butylphosphate under assay conditions. However, the carboxyl seems to play a small role in binding since the Km for P-glycolate was 8 fold lower than for ethyl-P, while the rate was merely twice that of the latter. However the Km cannot be measured at a pH where unionized carboxyl is significant due to enzyme denaturation.

The Vmax measurement had a 150 fold range for the five substrates analysed. Since other data suggests that the reaction mechanism proceeds through a phosphoenzyme intermediate, dephosphorylation of this complex can be ruled out as the rate limiting step. End product inhibition studies on page 153 support this conclusion. Furthermore the reaction appears to obey the Brönsted Equation- a linear relationship between log Vmax and the pKa of the corresponding alcohol (Figure 25). This suggests that the rate limiting step is dependent on the ease of protonation of the substrate. This step is necessary for alcohol release and enzyme phosphorylation or could be a concerted reaction with the proton being supplied by the phosphoryl acceptor, presumably a serine or a histidine residue.

It would require a very hydrophobic environment for a single protonation step to be rate limiting. Although this appears to be true for P-glycolate phosphatase, the concerted mechanism can in no way be ruled out on present evidence. The proton, as previously mentioned, could also come from a cation bound water molecule. However, in the case, a highly charged environment would be required to lower the pK sufficiently. This mechanism would account for the seemingly anomalous failure of glycoaldehyde-P to act as a substrate. The carbonyl (or

Figure 25.--Brönsted Plot for relationship between the Vmax and the pKa of the corresponding alcoholic product from P-glycolate phosphatase action. The pKa values for glycolate and glycoaldehyde were unavailable but were assigned appropriate values (see text).


3
2
1
log V_{max}
Glycolate
Methanol
Ethanol
Ethylene glycol
2 Chloroethanol
Glycoaldehyde
14
15
16
pKa

diol hydrated form) function would give a substantially lowered pKa (155) for glycoaldehyde by delocalization of the alkoxide electron. Thus the extreme difficulty of protonation of glycoaldehyde-P would account for the low reactivity. However, because glycoaldehyde-P was not found to be inhibitory, it may not be bound strongly, possibly because it is hydrated and therefore larger. For example, methyl glycolate has a pKa of 14.8 and its phosphate ester would thus be expected to be a substrate. That it was not may indicate steric hinderance at the binding site.

This mechanism would predict that (^{18}O)-H_2O-Pi exchange would be catalysed, based on a pKa of 15.74 for water. That no exchange was observed could be explained if phosphate binding was unable to induce the correct transition state complex required for enzyme phosphorylation. Evidence for this is presented on page 174.

Kinetic Effects of Phosphorothioates

S-(carboxymethyl)-phosphorothioate (SCMPT) is a substrate analog of P-glycolate, being identical except the ester bridge oxygen is an electronically similar sulphur. Synthesis of this compound was described in the methods. Hydrolysis of SCMPT and P-glycolate by P-glycolate phosphatase, by nonspecific alkaline phosphatase from calf intestine (Nutritional Biochemical Corp.), and by a nonspecific acid phosphatase from wheat germ (Sigma Chem. Co.) is compared in Table 19.

The inhibition of P-glycolate hydrolysis by SCMPT ($HOOC-CH_2-S-PO_3H_2$) and trisodium phosphorothioate ($S = PO_3Na_3$) are shown in Figures 26 and 27. The compounds, substrate and product analogs, can

TABLE 19.--Hydrolysis of Phosphoglycolate and S-(carboxymethyl)-phosphorothioate by phosphatases.

Phosphatase	pH	Substrate	Activity (A660)
P-glycolate	6.3	P-glycolate	0.304
		SCMPT	0.008
Acid	4.0	P-glycolate	0.725
		SCMPT	0
Alkaline	8.7	P-glycolate	0.143
		SCMPT	0.462

NOTE: All activities were corrected for nonenzymic hydrolysis during the assay and analysis. The rate of hydrolysis of SCMPT was significant at pH 4.0. The complete assay system contained 20 µmole cacodylate at pH 6.3 or acetate at pH 4.0 or bicine at pH 8.7; 2 µmole $MgCl_2$, 1 µmole P-glycolate or SCMPT, and 0.1 unit phosphatase in a total volume of 0.5 ml. The assay ran 10 minutes.

Figure 26.--Lineweaver-Burke plots for S-(carboxymethyl)-phosphorothioate inhibition of P-glycolate phosphatase.

Assays were run for 2 min with (2-^{14}C)-P-glycolate. The complete system contained 10 μmole MES at pH 5.4, 7.5 μmole $MgCl_2$, 4.5 x 10^{-2} to 0.91 μmole P-glycolate (Sp.Act. of 0.03 μc/μmole), and 0.02 units phosphatase in a total volume of 0.5 ml. Hydrolysis did not exceed 10% of the substrate. Low pH was used to avoid inhibitor-magnesium complexes. Inhibitor concentration was calculated as total inorganic phosphate released by acid hydrolysis of an aliquot of stock solution.

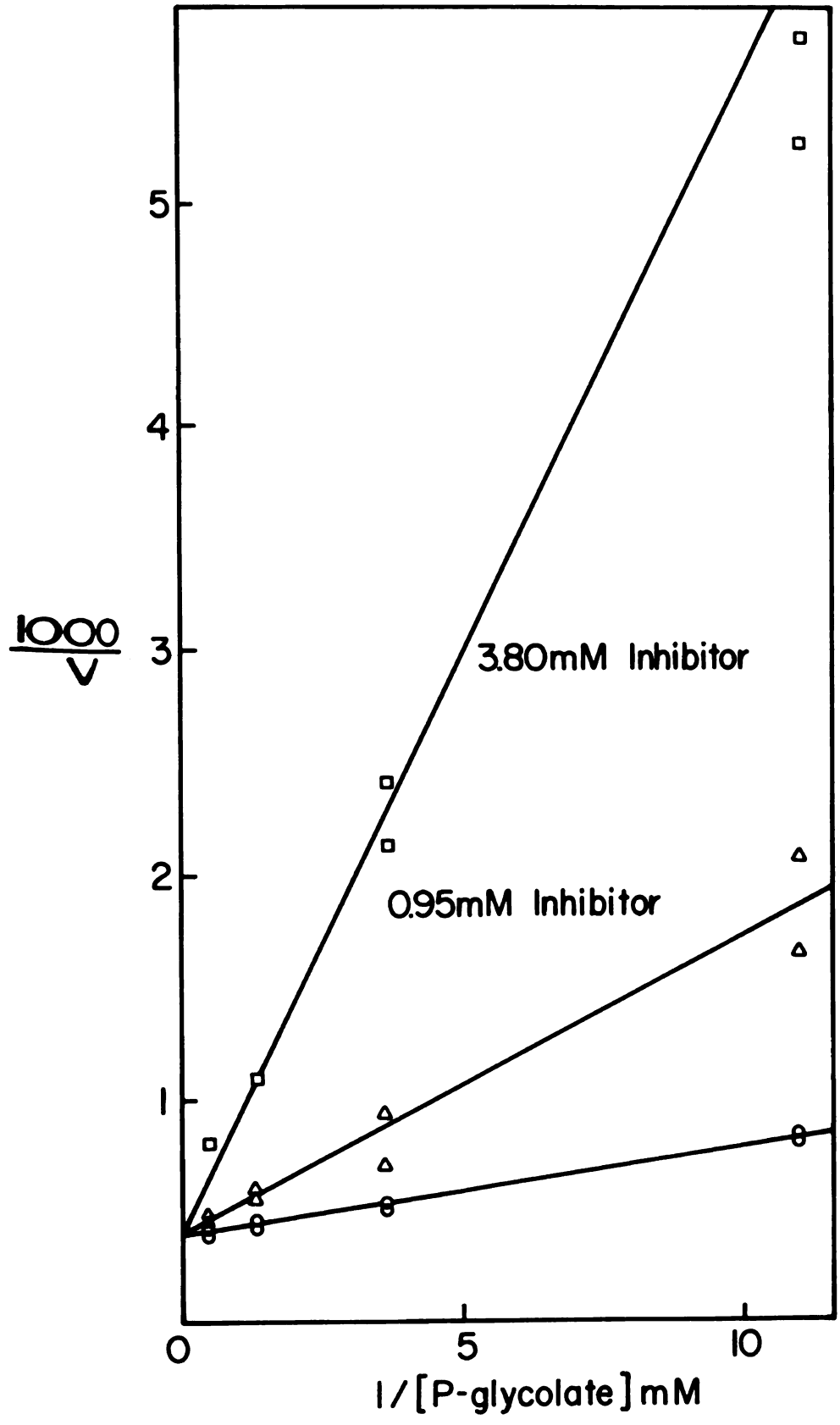
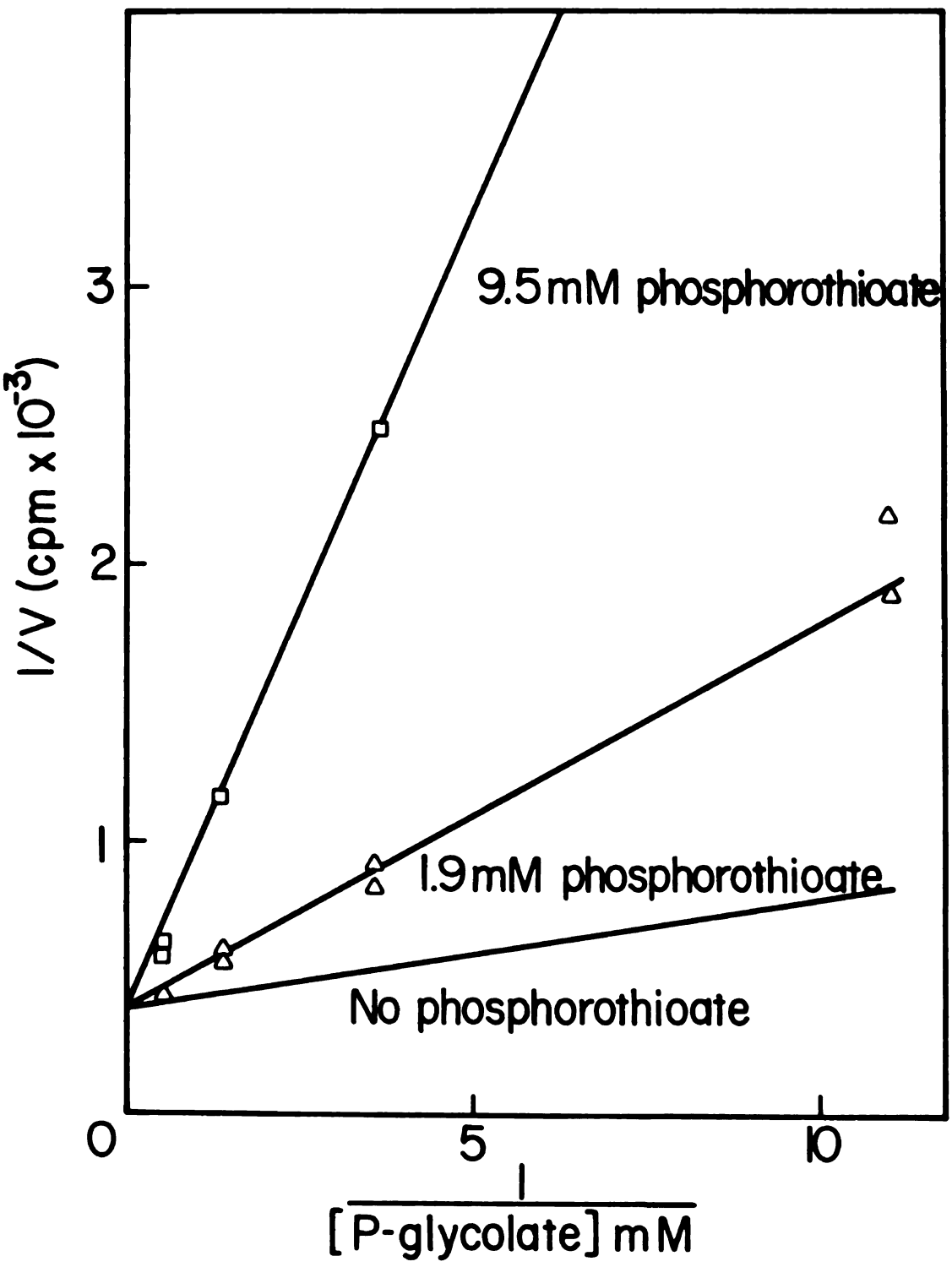

1000/V
5
4
3
2
1
0
5
10
1/[P-glycolate] mM
3.80mM Inhibitor
0.95mM Inhibitor

Figure 27.--Lineweaver-Burke plots for phosphorothioate inhibition of P-glycolate phosphatase. Assays were the same as in Figure 26.

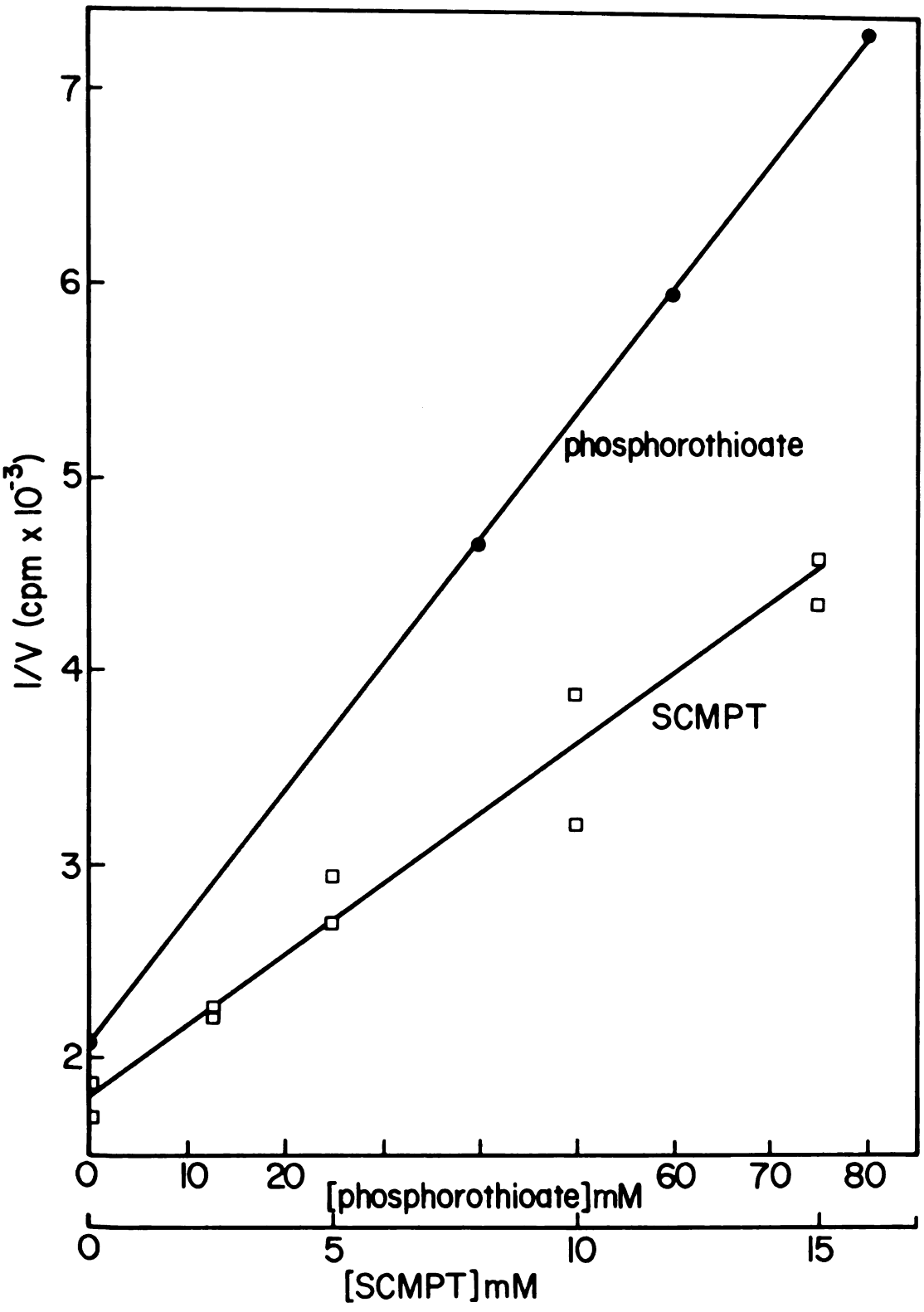

9.5mM phosphorothioate
1.9mM phosphorothioate
No phosphorothioate
1/V (cpm x 10⁻³)
3
2
1
0
5
10
1/[P-glycolate] mM

both be described as linear competitive inhibitors from this data and the data in Figure 28, a 1/v against inhibitor concentration (I) plot which is equivalent to a replot of slope in the Lineweaver-Burke plot against (I) (43). The inhibition constant was a measure of the true binding affinity and the Ki's were 0.382 $\pm$ 0.030 mM for SCMPT, and 0.822 $\pm$ 0.038 mM for phosphorothioate.

Neumann (1968) demonstrated that acid phosphatases will hydrolyse O-substituted monoesters of phosphoric acid and phosphorothioic acid, but not S-substituted monoesters of phosphorothioic acid. Alkaline phosphatases, on the other hand, hydrolyse O-substituted phosphoric monoesters and S-substituted phosphorothioic acid monoesters, but not o-substituted phosphorothioic acid monoesters.

Thus since P-glycolate phosphatase does not hydrolyse SCMPT it acts as an acid phosphatase. Neumann's suggestion that acid phosphatase requires one unionized phosphate hydroxyl for binding and that sulphur substitution lowers the ionization constant can be ruled out for P-glycolate phosphatase since the P-glycolate dianion is a substrate (unless protonation occurs after binding). Moreover SCMPT binds very tightly to P-glycolate phosphatase, as the Ki is very similar to the Ks' as determined by NEM inactivation (Ki is 0.38 at pH 5.4 and Ks' is 0.21 at pH 6.3) using the method of Mildvan and Leigh, (1964) (data not shown). The binding of SCMPT and P-glycolate appear to be very similar. The Km for P-glycolate approximately doubles during a change from pH 6.3 to 5.4. The failure of catalysis of SCMPT may be explained by the very low pKa of 10.40 for thioglycolic acid, preventing an appreciable rate of S-protonation and enzyme phosphorylation (Figure 25).

Figure 28.--Plot of reciprocal velocity against inhibitor concentration. The assay systems were those in Figures 26 and 27.


7
6
5
4
3
2
I/V (cpm x 10^{-3})
phosphorothioate
SCMPT
0
10
20
60
70
80
[phosphorothioate]mM
0
5
10
15
[SCMPT]mM

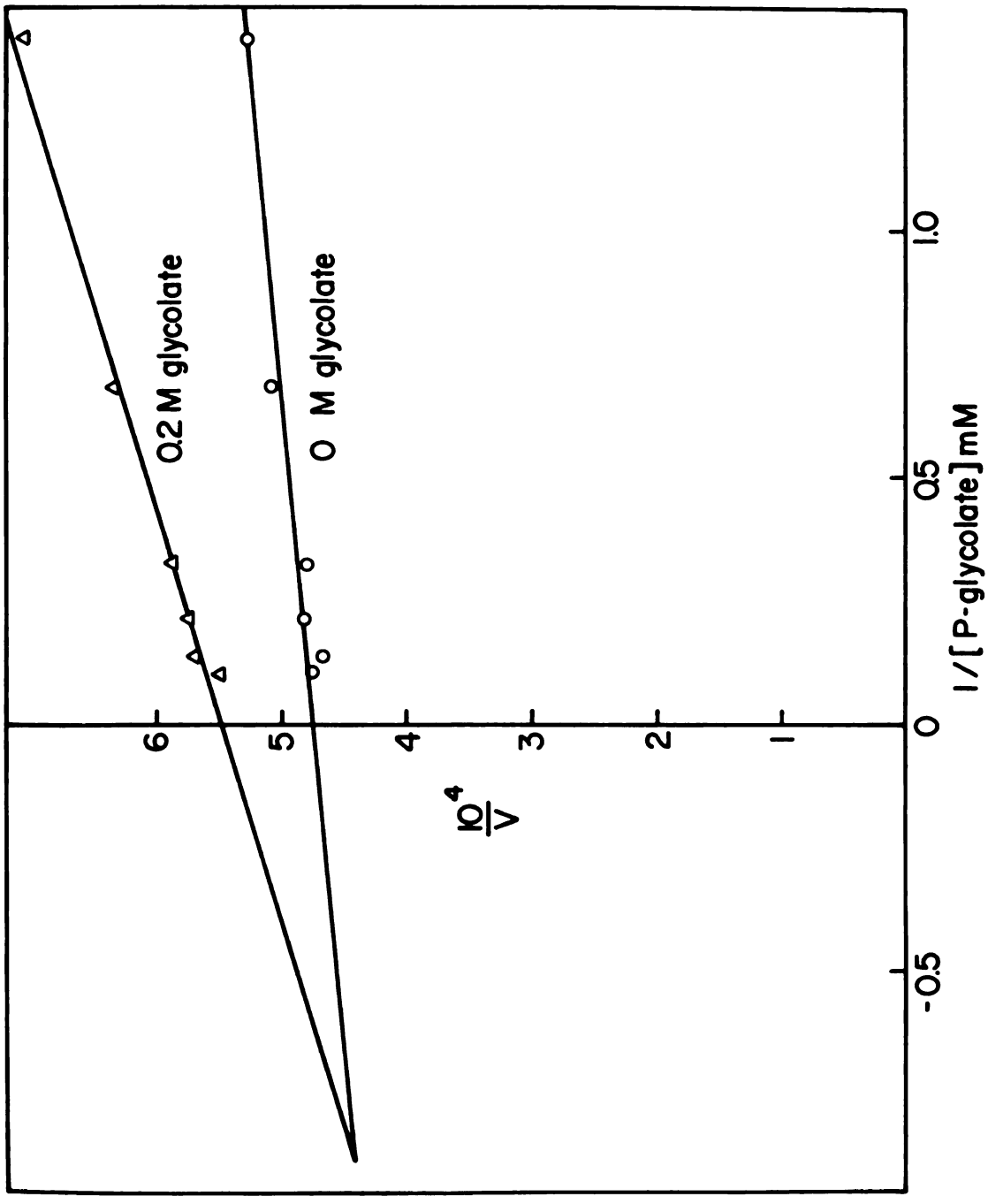
That substitution of sulphur for oxygen in the substrate does not effect the binding is suggested by the similarity of the Ki for phosphate (next section) and phosphorothioate (2.52 and 0.82 mM) and the identical inhibition patterns found. The differences could be attributed to the difference in pK_2, being 7.1 for phosphate and 5.75 for phosphorothioate (144). The dianion may be bound somewhat more tightly than the monoanion. The phosphate moiety is responsible for the larger portion of the binding affinity however since SCMPT is bound twice as tightly as phosphorothioate. This is also the conclusion from end product inhibition studies which show that the Ki for glycolate is some 35 fold higher than for phosphate.

The failure of phosphonoacetate to inhibit P-glycolate phosphatase may indicate that the bridge atom between carbon and phosphorus is essential to binding, but more likely reflects the gross steric differences between it and P-glycolate. This may be true for sulphoacetate also.

End Product Inhibition by Glycolate

The reaction rate as phosphate release gave linear double reciprocal plots in the presence and absence of 0.2 M glycolate. The lines intersected close to the vertical axis and indicated some degree of mixed inhibition which was however essentially competitive (Figure 29) since the change in Km was large (from 0.08×10^{-3} M to 1.11×10^{-3} M in the presence of 0.2 M glycolate) whereas the Vmax changed only slightly (from 0.210 to 0.182 A660). From the data a Ki value of 0.094 M for glycolate was calculated. That this inhibition by the

Figure 29.--Lineweaver-Burke plot for the inhibition by glycolate of P-glycolate phosphatase. The assay system contained 20 µmole cacodylate at pH 6.3, 10 µmole $MgCl_2$, from 1.0 to 10.0 µmole P-glycolate, and 0.1 unit phosphatase in a total volume of 0.5 ml. Glycolate concentration was as indicated and the assays were run for 1 min.


0.2 M glycolate
0 M glycolate
$\frac{10^4}{V}$
6
5
4
3
2
1
0
-0.5
0.5
1.0
1/[P-glycolate] mM

glycolate product can be considered competitive is indicated by a linear plot of the slopes of double reciprocal at various glycolate concentrations. Cleland (1971) has shown that this analysis is identical to that used in Figure 30A. A replot of the data from Figure 29 in this form gave a value for the Ki of 0.087 M for glycolate (Figure 30B).

End Product Inhibition by Phosphate

From the Lineweaver-Burke plot in Figure 31 inorganic phosphate is a competitive inhibitor of the substrate, P-glycolate. An average value for the Ki of 2.55 $\pm$ 0.25 mM was obtained. A replot of slope against inhibitor concentration (Figure 32) was linear and indicated competitive kinetics, as was the case also for glycolate. The degree of inhibition at pH 5.4 is much greater than at pH 7.1 for any given phosphate concentration. This may be interpreted to indicate that the $MgHPO_4$ complex, which is an important species at pH 7.1 (31%), but not at pH 5.4 (5%), is not an inhibitor of the phosphatase.

Hsu et al. (1966) have shown the mechanism catalysed by potato acid phosphatase is ordered; first the alcohol and then the phosphate is released. My data with P-glycolate phosphatase is competitive end product inhibition by both products and suggests that the P-glycolate phosphatase reaction is mechanistically rapid random equilibrium. This conclusion supports previous evidence that the dephosphorylation is very rapid and not rate limiting. However in the next section on transferase activity, the probably role of a phosphoenzyme intermediate is not consistent with random release of products. The mechanism is therefore thought to be compatible with an ordered release of products,

Figure 30A.--Plot of reciprocal velocity against glycolate concentration. Determination of the mode of inhibition. Assay system contained 20 µmole cacodylate at pH 6.3, 10 µmole $MgCl_2$, 2.5 µmole P-glycolate, 0 to 150 µmole glycolate, and 0.1 unit phosphatase in 0.5 ml, and were run for 1 minute.

B.--Plot of reciprocal velocity against glycolate concentration. Recalculation of the inhibition constant for the end product of the reaction, glycolate. The data is replotted from Figure 29.

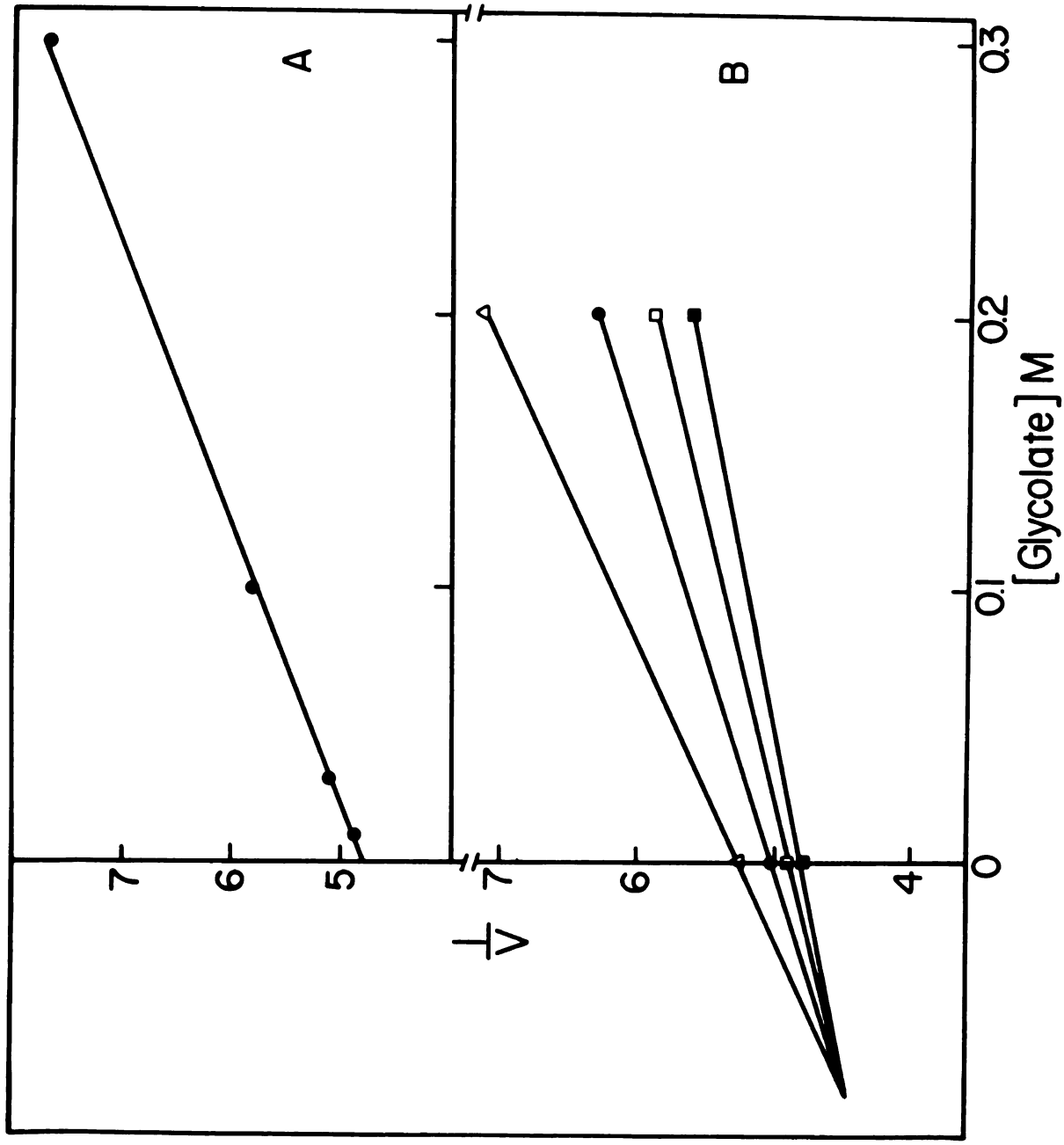

A
B
7
6
5
4
0
0.1
0.2
0.3
1/V
[Glycolate] M

Figure 31.--Lineweaver-Burke plot for inorganic phosphate inhibition of P-glycolate phosphatase. The assay contained 10 µmole MES at pH 5.4, 12.5 µmole $MgCl_2$, 5.0 or 12.5 µmole Pi at pH 5.4, and 0 to 2.29 µmole (2-^{14}C)-P-glycolate (Sp. Act. of 0.325 µc/µmole) in a volume of 0.5 ml. The reaction was terminated with 25µl 1 M HCl. Total hydrolysis was 10% or less. The reaction was run at low pH to prevent interference by the phosphate dianion-magnesium complex.

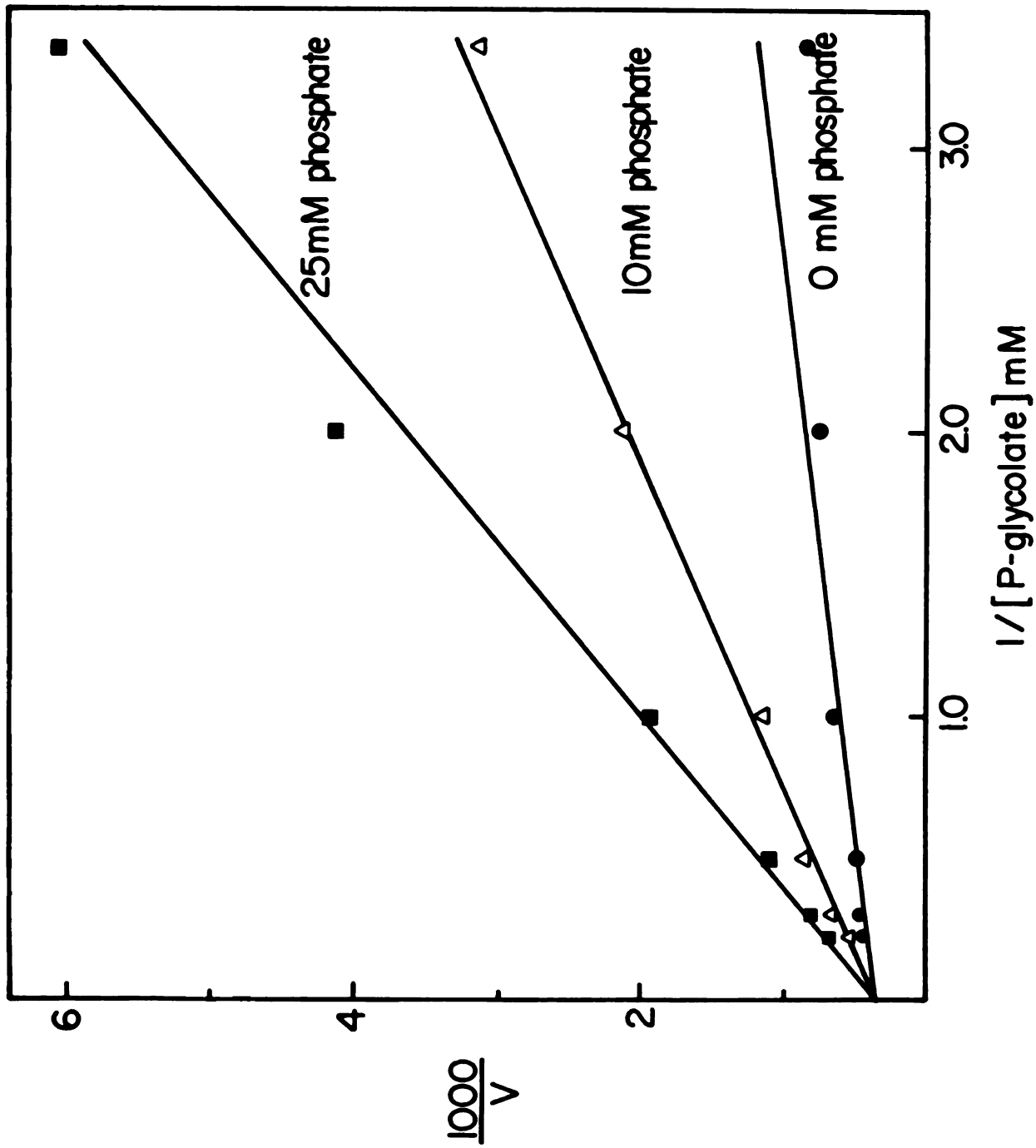
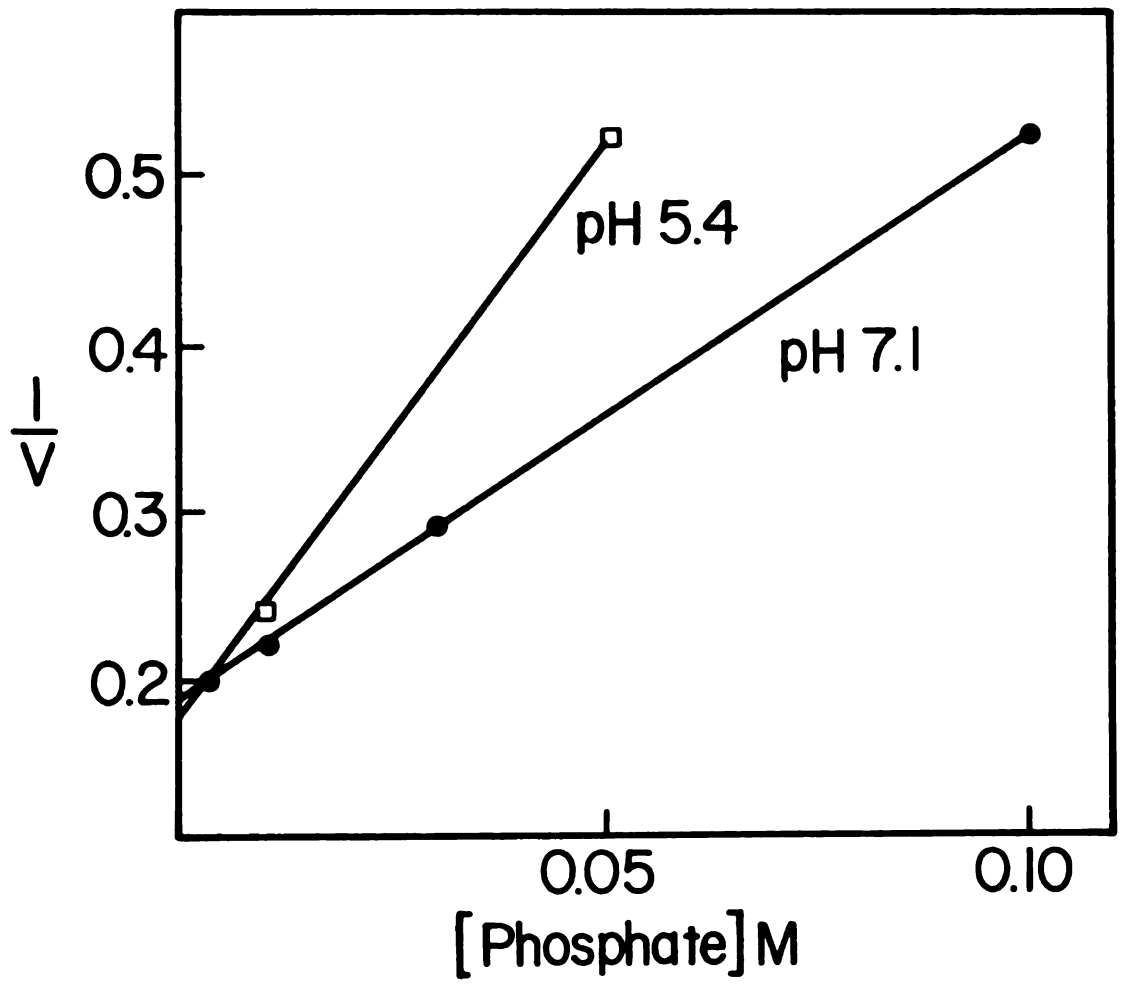

25mM phosphate
10mM phosphate
0 mM phosphate
1000/V
6
4
2
1.0
2.0
3.0
1/[P-glycolate] mM

Figure 32.--Plot of reciprocal velocity against phosphate concentration. Determination of the mode of competitive inhibition at pH 5.4 and 7.1. The data at pH 5.4 is replotted from Figure 31 at 2mM P-glycolate. The assay at pH 7.1 contained 15 µmole HEPES at pH 7.1, 12.5 µmole $MgCl_2$, 1 µmole P-glycolate, and Pi as indicated. Other conditions were similar to those for Figure 31.


0.5
0.4
0.3
0.2
1/V
pH 5.4
pH 7.1
0.05
0.10
[Phosphate] M

with the pattern being masked by the very rapid dephosphorylation of the enzyme.

Transphosphorylation

The amount of both glycolate and free inorganic phosphate released during enzyme hydrolysis was measured in the presence and absence of possible phosphate acceptors. The ratio of glycolate to phosphate had been shown to be unity in the absence of any acceptors (170). For these experiments I used (^{14}C)-P-glycolate and measured (^{14}C)-glycolate in half the solution and used the rest of the solution for Pi determination.

A time course for enzyme activity with 2 M ethylene glycol as Pi acceptor in addition to water is shown in Figure 33. During the reaction time up to 5 min the hydrolysis of any 2-hydroxyethyl-P would be negligible due to enzyme saturation with P-glycolate. Calculations from this figure confirm that hydrolysis in the absence of acceptors other than water released one Pi and one glycolate per P-glycolate hydrolysed. After 5 min 0.60 μmole phosphate was released and 0.61 μmole glycolate was formed. In the presence of ethylene glycol the rate of Pi formation was decreased but the rate of glycolate formation was unchanged. The difference in Pi release is assumed to be Pi transfer to the alternate acceptor.

P-glycolate phosphatase has phosphotransferase acitivty with a range of acceptors (Table 20). Because of the required high concentration of acceptors for transphosphorylation, it is doubtful that the reaction is of physiological significance. The transferase activity

Figure 33.--Transphosphorylation activity of P-glycolate phosphatase with ethylene glycol as the phosphoryl acceptor. The complete system contained 20 µmole cacodylate at pH 6.3, 10 µmole $MgCl_2$, 3.0 µmole ($2^{-14}C$)-P-glycolate (S.A. of 0.0038 µc/µmole), 161 mmole ethylene glycol, and 0.1 unit phosphatase in a volume of 0.7 ml. The reaction was stopped with 0.1 ml of 0.5 M HCl and equal aliquots analysed for ($2^{-14}C$)-glycolate. Counting efficiency was 70%.

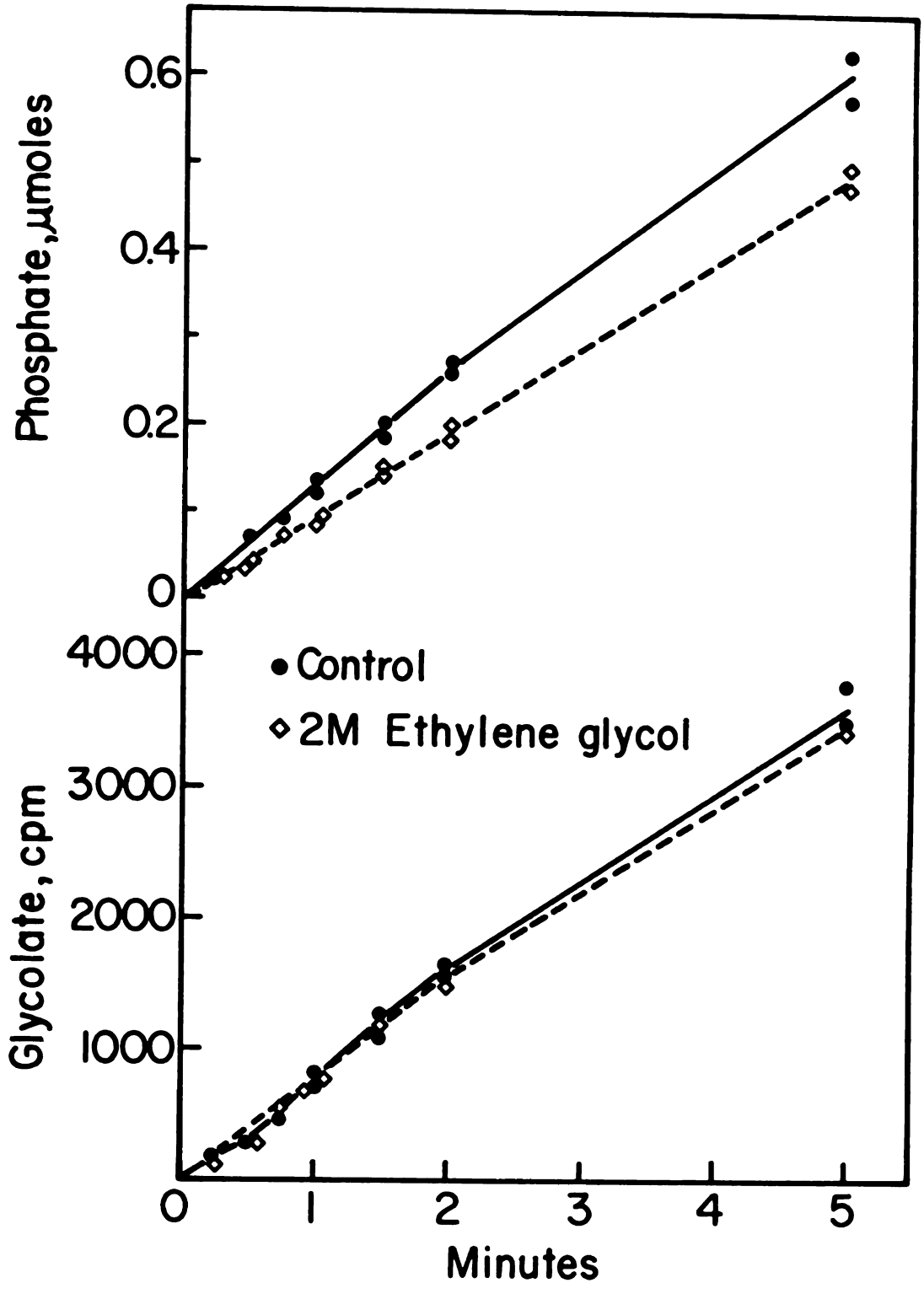

Phosphate, µmoles
0.6
0.4
0.2
0
Control
2M Ethylene glycol
Glycolate, cpm
4000
3000
2000
1000
0
1
2
3
4
5
Minutes

TABLE 20.--Acceptor Specificity of P-glycolate Phosphatase Transferase Activity.

Acceptor	Molar	Relative Pi Released	Relative Glycolate Released	Pi/Glycolate
H_2O only	55.5	100	100	1.00
Plus				
Ethylene glycol	2.42	74 ± 2	101 ± 1	0.73
Glycolate	0.167	72 ± 2	77[a]	0.94
Ethanol	4.16	93 ± 1	127 ± 2	0.73
n-Propanol	6.0	1 ± 1	8 ± 2	0.13
Tris	0.50	33 ± 4	126 ± 19	0.22
Glyceraldehyde	0.083	85 ± 1	88 ± 4	0.96
Glucose	0.618	81 ± 1	91 ± 1	0.88

Note: The experimental conditions are those of Figure 33. Duplicates were analysed after 3 and 6 minutes of incubation. During this time the rate in the presence of all acceptors was linear. The ratios observed are conservative since in several cases the anticipated transferase product is also a substrate, and all alkyl phosphates are hydrolysed slowly in 0.05 M HCl. Experiments performed at saturating P-glycolate concentrations and Dowex separations were made immediately on completion of the assay to minimize these errors.

[a]This measurement is discussed in methods.

does not rule out metaphosphate ion formation, although from the specificity in acceptors this would not be expected. The ion is expected to be so unstable in aqueous solution that transferase activity would be dependent only on the hydroxyl group concentration. For example, on a molar ratio, the 0.5 M tris used would be expected to be phosphorylated at about 3% of the rate of Pi release (water is 55.5 M). However, tris was observed to be phosphorylated 150% faster than water.

As previously noted, glycolate inhibition was partially noncompetitive. This deviation can be ascribed to transphosphorylation and/or noncompetitive inhibition, these being kinetically equivalent events. Thus the data from transferase activity and end product inhibition studies is consistent with a phosphoenzyme intermediate being required in the reaction pathway. An ordered release of products from the enzyme can be expected since glycolate must leave the active site before the phosphoryl moiety is exposed. However, the specificity of the acceptor is substantially broader than that of the substrate, suggesting a substantial alteration in the active site during the reaction.

Diisopropylfluorophosphate Inhibition

DFP is an inhibitor of enzymes containing serine at the active site by forming a stable diisopropylphosphoserine residue on an equimolar basis and inhibiting completely and irreversibly. If P-glycolate phosphatase also has a phosphorylated form, inhibition by DFP might be observed. DFP (Sigma Chem. Co.) was prepared as an aqueous solution as

needed and used immediately, since the reagent hydrolyses slowly in solution and one product, fluoride, also inhibits P-glycolate phosphatase (170). Assays for enzymic activity were terminated with 0.1 ml 5 M NaOH rather than 0.1 ml 10% TCA, as routinely used, and then stood for 30 minutes for hydrolysis of excess reagent. Only then were samples removed from the hood and analysed.

DFP weakly inhibited P-glycolate phosphatase (Table 21). The high concentration of DFP and weak inhibition are similar to its inhibition of mammalian alkaline phosphatases (139,51) and acid phosphatases from several sources (74). P-glycolate phosphatase was inhibited 68% in 2.5 hr at 20^{o} at pH 6.3 with 3 mM DFP. Alkaline phosphatase was inhibited 12% in 15 min at 37^{o} at pH 7.6 and 10.1 with 10 mM DFP, whereas acid phosphatase may be somewhat more susceptible to 10 mM DFP as it was inhibited 97% in 2 hr at pH 5.0. Thus the kinetics of inhibition appear more closely allied to those of acid phosphatases. This appears reasonable as other properties of P-glycolate phosphatase seem similar to the acid phosphatases.

The ability of both the substrate and a competitive inhibitor, citrate, to protect the enzyme against inactivation (Table 21) indicates that the mode of action is at the active site, suggesting that a phosphoenzyme may be formed. However, properties of the suspected P-enzyme intermediate differ from that of the acid phosphatases, which when inactivated by DFP at pH 5.0, would regain 100% of their original activity at pH 7.5 (74). P-glycolate phosphatase was inactivated at pH 8.0 although at a slower rate than at pH 5.4 (Table 22). It was not reactivated at pH 8.0 when first incubated at pH 5.4 (data not shown). This

TABLE 21.--Effect of Phosphoglycolate and Citrate on the Rate of Diisopropylfluorophosphate Inhibition of P-glycolate Phosphatase.

Incubation Mixture	Activity µmoles/min	% Control
Phosphatase	0.360	100
Phosphatase + 3mM DFP	0.115	32
Phosphatase + 3mM DFP + 0.20M citrate	0.372	103
Phosphatase + 3mM DFP + 0.05M P-glycolate	0.285	79

Note: The enzyme (0.4 units) in 50µl of 2 µmole cacodylate at pH 6.3 plus the cited additions was incubated for 150 minutes at 20°. The assay for 3 min was initiated by adding 1 µmole P-glycolate, 3.5 µmole $MgCl_2$, and 10 µmole cacodylate at pH 6.3 to a final volume of 0.5 ml.

TABLE 22.--Effect of pH on P-glycolate Phosphatase Inactivation by Diisopropylfluorophosphate.

pH	DFP (3mM)	Activity Remaining µmoles/min	
		5 minutes	65 minutes
5.4	-	0.329	0.319
5.4	+	0.101	0.002
8.0	-	0.324	0.235
8.0	+	0.299	0.006

Note: Incubations contained 0.3 µmoles DFP, 3.5 µmole MES at pH 5.4 or bicine at pH 8.0, and 2 units phosphatase in 100µl. Aliquots of 16µl were removed in duplicate for assay.

could be due to steric hinderance by the bulky isopropyl groups for this enzyme specific for two carbon alkylphosphates.

Thus the pH range of DFP inhibition susceptibility correlates for both acid and P-glycolate phosphatases with pH activity curves. The data supports the hypothesis that formation of phosphoenzyme is essential to the reaction. On this basis P-glycolate phosphatase can be considered an acid phosphatase which has evolved to operate at a higher pH and with greatly increased specificity.

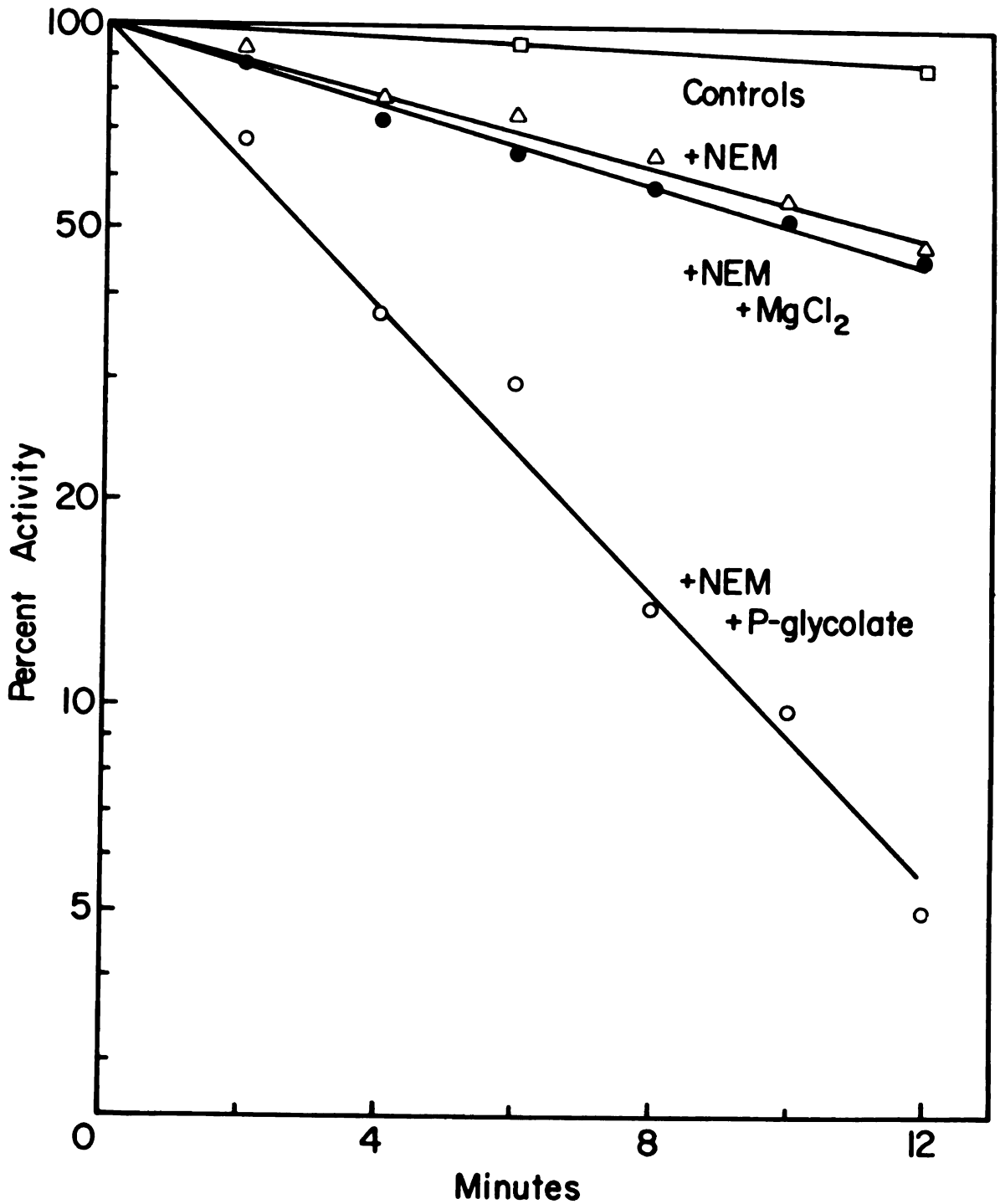
Studies of Sulphydryl Groups with N-ethylmaleimide

The rate of inactivation of P-glycolate phosphatase by NEM, an alkylating agent for sulphydryl groups, was followed by removal of aliquots from an incubation mixture and assaying the remaining enzymic activity by dilution of the NEM to a point of ineffectiveness. In all cases the NEM concentrations were in excess and the rates followed pseudo first order kinetics. Mixtures at pH 6.3 where the enzyme is most stable were incubated in a corked test tube at 30^{o} and the reaction usually initiated with enzyme. Any substrate hydrolysis during the incubation with NEM was subtracted from assay activity by removing an equal aliquot at the same time and stopping the activity with TCA. The same procedure was followed with incubations containing inorganic phosphate.

The rate of inactivation of P-glycolate phosphatase in the presence of substrate, Mg^{++}, and NEM at pH 6.3 is shown in Figure 34. NEM inactivation occurred more rapidly in the presence of substrate than in its absence, whereas $MgCl_2$ did not affect the rate of inactivation.

Figure 34.--Kinetics of P-glycolate phosphatase inactivation by N-ethyl-maleimide.

The incubation mixture contained 0.25 µmole NEM, 0.625 µmole $MgCl_2$, 1.5 µmole P-glycolate; 1.0 µmole cacodylate at pH 6.3 and 0.6 units phosphatase in 25 µl total volume. Three µl aliquots were removed at intervals and activity assayed for 1.5 minutes. The controls consisted of buffered enzyme, enzyme plus $MgCl_2$, and enzyme plus substrate. All incubations and assays were at 30°.


100
50
20
10
5
0
4
8
12
Percent Activity
Minutes
Controls
+NEM
+NEM
+MgCl2
+NEM
+P-glycolate

As controls, this enzyme was stable at 30° in the absence of NEM, and P-glycolate and $MgCl_2$ did not affect this stability. Under some conditions where the enzyme shows heat instability, P-glycolate has no noticeable effect, but Mg^{++} stabilises the activity. On the other hand, Anderson (1969) had shown that instability of P-glycolate phosphatase from dilution can be prevented by P-glycolate but not by Mg^{++}.

Incubation by 10 mM NEM appears to follow pseudo first order kinetics. The rate of inactivation on adding 25 mM $MgCl_2$ is essentially unchanged. Thus any conformational change from saturating divalent metal ion are without effect on the essential sulphdryl or sulphydryls, suggesting that these sulphydryls are not involved in cation binding. The data also suggests that the mechanism of cation activation is not due to changing the reactivity of an active site sulphydryl.

On the other hand, the rate of inactivation by NEM was stimulated 4 fold by the addition of 0.3 M P-glycolate. Although about 50-80% of the substrate was hydrolysed during the incubation with NEM, the P-glycolate concentration remained two orders of magnitude above the Km level.

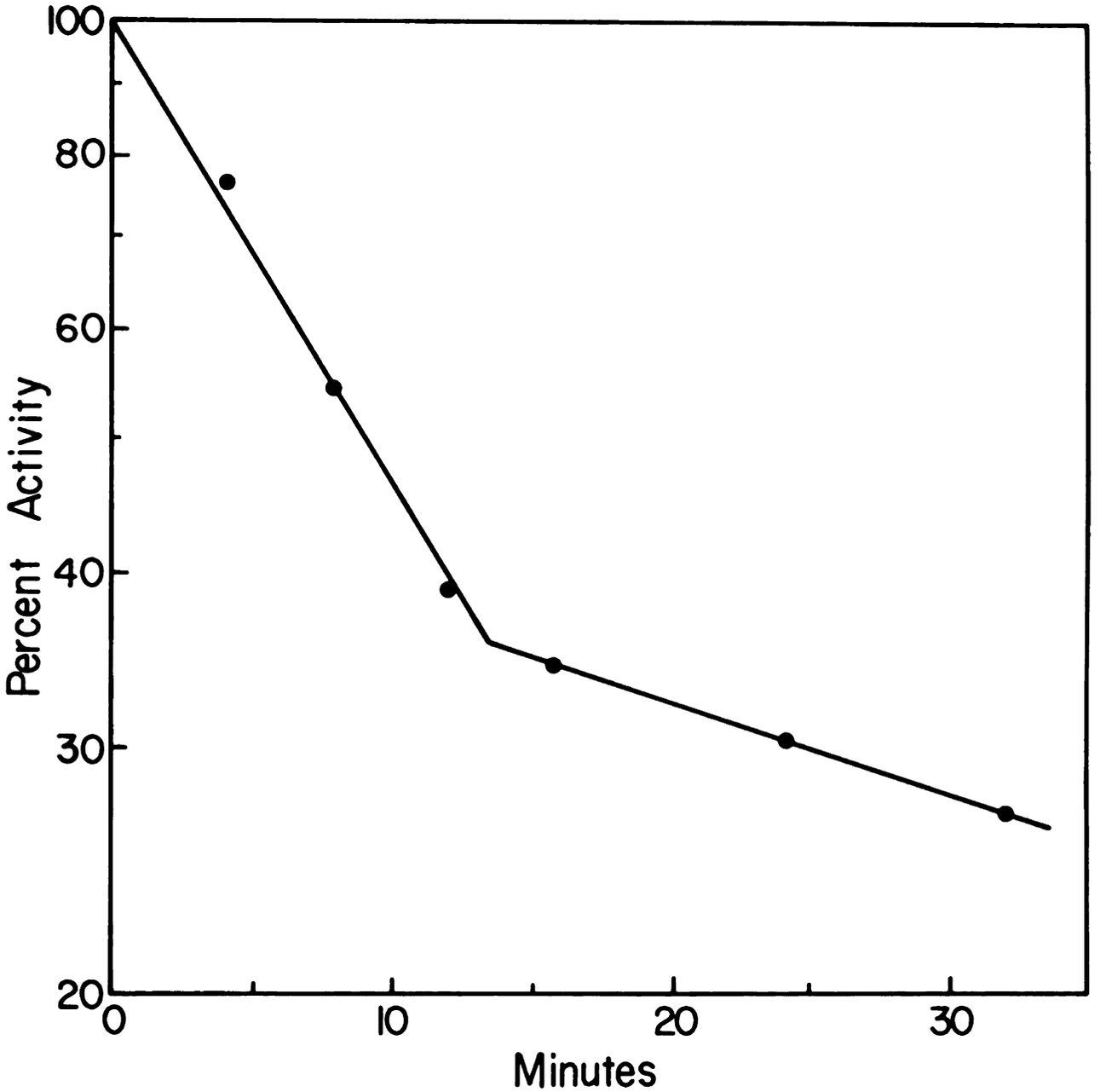
Vallee et al. (1971) observed that inactivation of aspartate aminotransferase by tetranitromethane occurred by attack on an essential tyrosyl in the presence of both substrates but was negligible with each substrate separately. They termed this inactivation of the catalytically poised conformation as syncatalytic modification, and suggested the inactivation occurred during or after formation of the ketimine intermediate of the pyridoxyl enzyme.

My data indicates that P-glycolate phosphatase also undergoes syncatalytic modification. The simplest reaction sequence consistent with available experimental results would be binding of the substrate to the enzyme-metal complex. This would induce a conformational change poising the ternary complex for protonation and enzyme phosphorylation. Following release of the alcoholic product the enzyme dephosphorylates rapidly. Although the concentration of the first intermediate can be expected to be many times greater than that of the phosphoenzyme, this does not, a priori, rule out the latter as the syncatalytically modified form. This data also does not distinguish between the possibility that the conformational change observed on binding substrate is due to exposure of a new sulphydryl or merely causes the sulphydryl(s) already susceptible to NEM to move into an environment where maleylation can occur more rapidly. In fact, in another section, it is shown that there is no specificity for alcohols which might serve as phosphoryl acceptors in transferase reactions. This suggests that the active site had opened up during substrate binding, which may expose a reactive sulphydryl group.

Further studies (Figure 35) suggest that an additional sulphydryl is maleylated in the presence of substrate. The longer time period followed here shows that inactivation in the absence of substrate leads to a maleylated enzyme with about 30% the activity of the native enzyme, rather than a totally inactive enzyme. The second rate in Figure 35 is equal to those of the controls in Figure 34. In the presence of substrate, however, the inactivation proceeds rapidly to zero (Figure 34)

Figure 35.--Kinetics of inactivation of P-glycolate phosphatase by NEM.

The incubation conditions were identical to those described in Figure 34. The incubation mixture contained 0.25 units phosphatase in 15 µl of solution.


100
80
60
40
30
20
Percent Activity
0
10
20
30
Minutes

without a break in the exponential curve, indicating that a previously inaccessible sulphydryl is also being maleylated.

The data in Figure 36 provides evidence that the transition state is the complex undergoing syncatalytic modification. Glycolate, at twice the Ki concentration, caused an increased rate of inactivation by NEM. Since glycolate is a competitive inhibitor, binding at the active site, it induces the same conformational change as P-glycolate. No phosphoenzyme is formed under these conditions, thus ruling out the possibility that this intermediate is responsible for syncatalytic modification. It appears that the interaction of the alcohol portion of the substrate with the enzyme is essential, although it is very weak compared with the interaction of the phosphate moiety. Inorganic phosphate, at 4 times its Ki concentration did not cause a conformational change detectable by NEM inactivation, despite its affinity being 35 fold greater for the active site than glycolate. The failure of phosphate to induce the transition state is consistent with the absence of Pi - H_2O exchange by P-glycolate phosphatase. Although phosphate binds to the active site, no transition state complex and hence no phosphoenzyme can be formed. This observation probably also explains why no pyrophosphatase activity is found with P-glycolate phosphatase, since it might be expected to bind at the phosphate moiety subsite.

EDTA prevented essentially all hydrolysis of P-glycolate, presumably by removing the cation present. When EDTA plus P-glycolate were incubated with enzyme and NEM syncatalytic modification still occurred (Table 23). Thus binding but not turnover seems necessary for this effect. The results further substantiate that cation is required for catalytic activity at pH 6.3.

Figure 36.--Kinetics of inactivation of P-glycolate phosphatase by NEM.

The conditions used were the same as those described in Figure 34. Glycolate concentration was 0.2M.

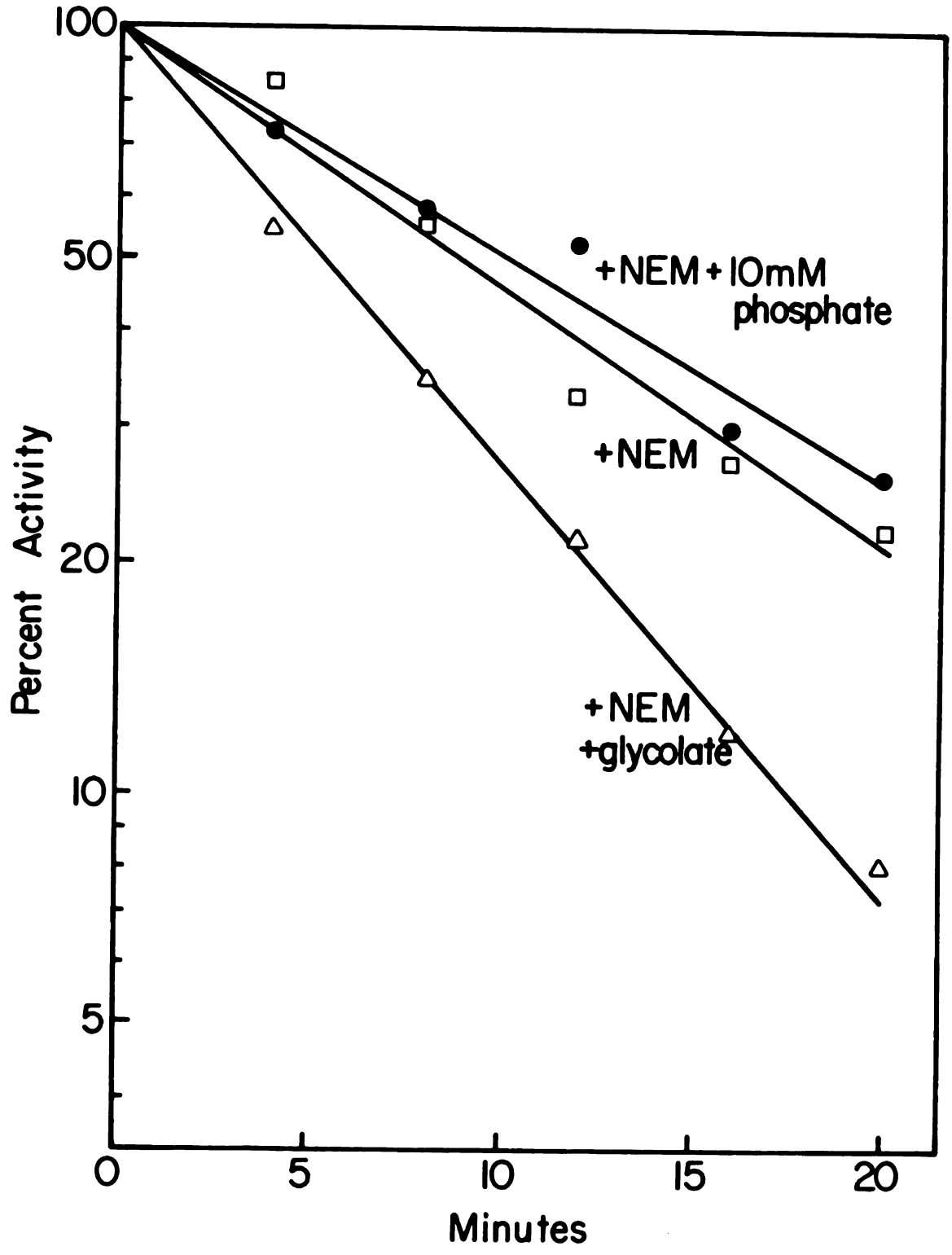

100
50
20
10
5
0
5
10
15
20
Percent Activity
Minutes
+NEM + 10mM phosphate
+NEM
+NEM +glycolate

TABLE 23.--Determination of Rate Constants for Inactivation of P-glycolate Phosphatase by NEM with EDTA.

System	k_1 (min^{-1})
Enzyme + 5mM EDTA + 5mM NEM + 2mM P-glycolate	0.0242
Enzyme + 5mM EDTA + 5mM NEM	0.0184
Enzyme + 5mM EDTA	0.0030

Note: Incubation conditions were those of Figure 34. Total incubation volume was 50 µl.

The mechanism of involvement of the sulphydryl in hydrolysis cannot be inferred from these studies. Normally substrate protection against inactivation has been regarded as evidence for involvement of an active site residue. However in this case, no inference as to whether the essential sulphydryl(s) is at the active site can be made. Assuming the partially modified sulphydryl is partially buried in the free native enzyme, addition of the large maleyl group may "freeze" the enzyme into the transition state conformation, thus preventing binding of further substrate molecules.

The kinetic parameters of the enzyme after treatment with NEM as described in Table 24 were obtained by computer fitting of the curves. The inactivation of sulphydryl groups by NEM had no significant effects on the allosteric interaction of the enzyme and Mg^{++} ion. This result is perhaps expected since, if sulphydryl was involved in cation binding, Mg^{++} would have provided some protection against inactivation by NEM. The data does not rule out the possibility that cation is bound to NEM inaccessible sulphydryl.

TABLE 24.--Effect of NEM on Cation Saturation Kinetics.

Pretreatment[a]	Vmax	$K_{0.5}$ ($\times 10^{-3}$M)	Hill Number (N_H)
None	157	0.14	0.45
NEM	75	0.15	0.50
NEM + P-glycolate	106	0.25	0.23

[a]P-glycolate phosphatase was inactivated by NEM in the presence and absence of P-glycolate. Phosphatase (1.8 units) was incubated with 1 μmole NEM for 13 min at 30° in 120 μl total volume, pH 6.3. The solution was quenched by adding 5 μmole 2-mercaptoethanol in 5 μl. Activity remaining was 42%. Incubation with P-glycolate contained 0.6 units phosphatase, 0.75 μmole NEM, 2.5 μmole P-glycolate in 75 μl volume, for 4 min then quenched as above. Remaining activity was 64%. Unmodified enzyme and the two partially inactivated preparations were assayed in the following system: 20 μmole bicine at pH 8.1, 1 μmole P-glycolate, 1 x 10^{-2} to 1.0 μmole $MgCl_2$, and 0.1 unit phosphatase for 10 min in the case of native and NEM treated enzyme, and 25 min in the case of the NEM and P-glycolate treated enzyme.

A sulphydryl group(s) plays an essential role in P-glycolate phosphatase activity. It appears to be unrelated to cation activation but their modification and related conformational changes probably increase the Km for the substrate. It appears that the substrate and cation induced conformational changes are essentially independent of each other. However this does not provide any insight into whether the binding of substrate is ordered or random. It seems unlikely that a high concentration of cation would protect against syncatalytic modification, but this experiment has technical problems due to rapid depletion of substrate, so control rates would not be under analogous conditions.

SOME STUDIES ON THE PHYSIOLOGICAL ROLE OF PHOSPHOGLYCOLATE PHOSPHATASE

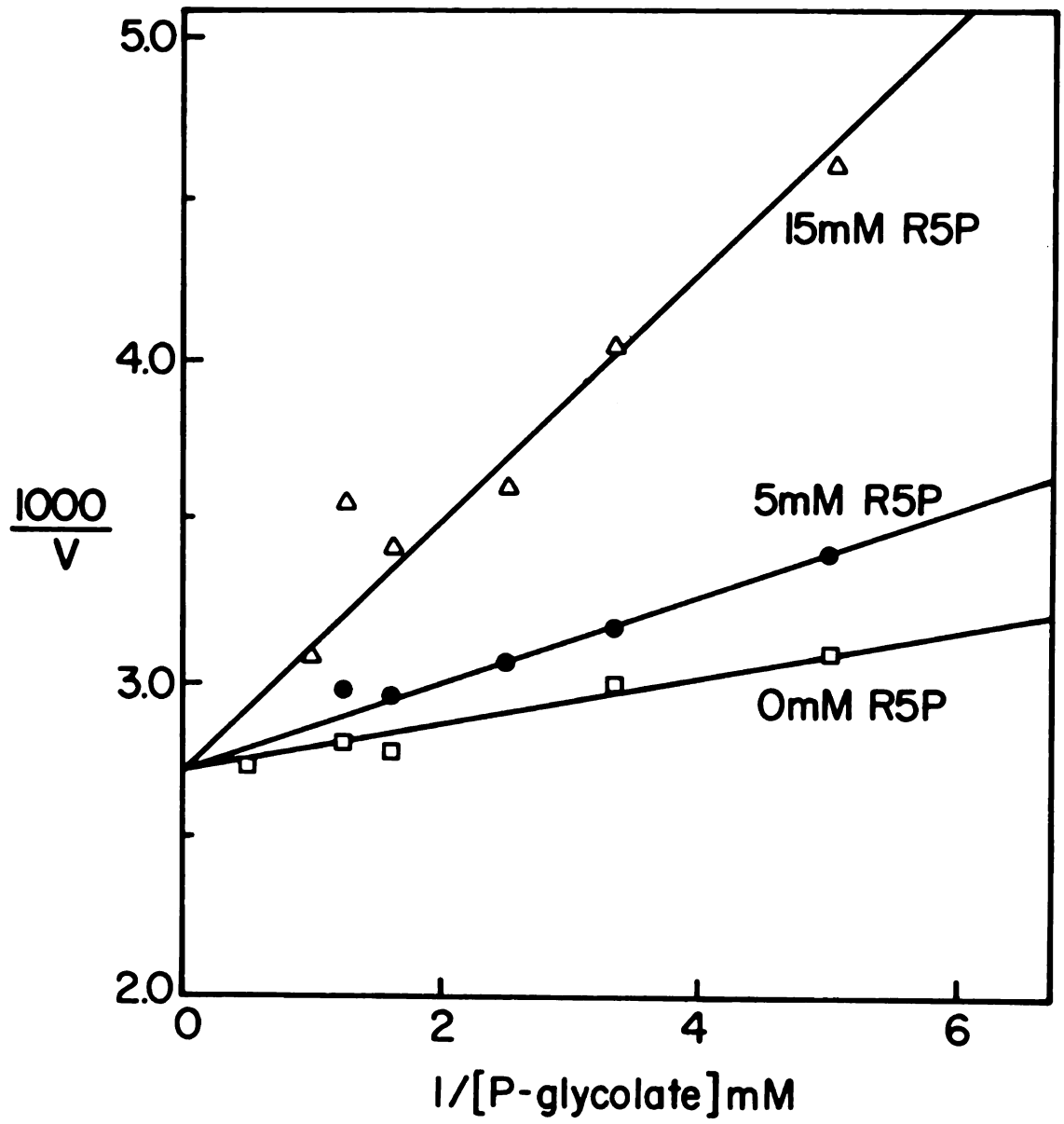
Results and Discussion

Ribose-5-Phosphate Inhibition

Ribose-5-phosphate is a competitive inhibitor of P-glycolate phosphatase (Figure 37), with a Ki of 5.68 mM at 5mM R5P and 3.43 mM at 15 mM R5P. The mode of inhibition is thus nonlinear competitive and not identical to that by end products. Although R5P inhibits competively it is unlikely, for steric reasons, that the interaction is direct binding to the active site. This pentose phosphate, which is not a substrate, is much larger than is considered possible for a substrate and has no special features which might make it more analogous to the substrate, P-glycolate, than the numerous other monophosphates which were also not hydrolysed by the enzyme. In the mode of action the R5P is probably binding elsewhere on the molecule, thereby causing a conformational modification of the active site in such a way to cause an apparent increase in Km. The nonlinearity could then be explained as some cooperativity between the sites as the saturation increased; whereas active site competition, not involving conformational changes beyond those required for formation of the transition state complex, would not be expected to involve site-site interactions.

That R5P, alone of all the endogenous sugar phosphates tested, inhibits P-glycolate phosphatase at physiological levels, suggests it

Figure 37.--Lineweaver-Burke Plot for ribose-5-phosphate inhibition of P-glycolate phosphatase. The assay contained 20 µmole MES at pH 5.4, 10 µmole $MgCl_2$, 2.5 or 7.5 µmole R5P as indicated, 5 x 10^{-2} to 1.0 µmole (2-^{14}C)-P-glycolate (S.A. of 0.0145 µc/µmole), and 0.1 unit phosphatase in a volume of 0.5 ml. Assays were run for 1 min and stopped with 25 µl of 1 M HCl. Hydrolysis was 12% or less. A background at each substrate concentration was subtracted after being carried through the complete procedure in the absence of enzyme. The low pH was chosen to avoid consideration of magnesium complexes.


5.0
4.0
3.0
2.0
1000/V
15mM R5P
5mM R5P
0mM R5P
0
2
4
6
1/[P-glycolate]mM

may be a significant mode of regulation. R5P is the most effective inhibitor of RuDP oxygenase which catalyses P-glycolate formation (173) and R5P inhibition of P-glycolate phosphatase indicates that it is the effector for both these sequential reactions.

Inactivation by Glycidol Phosphate

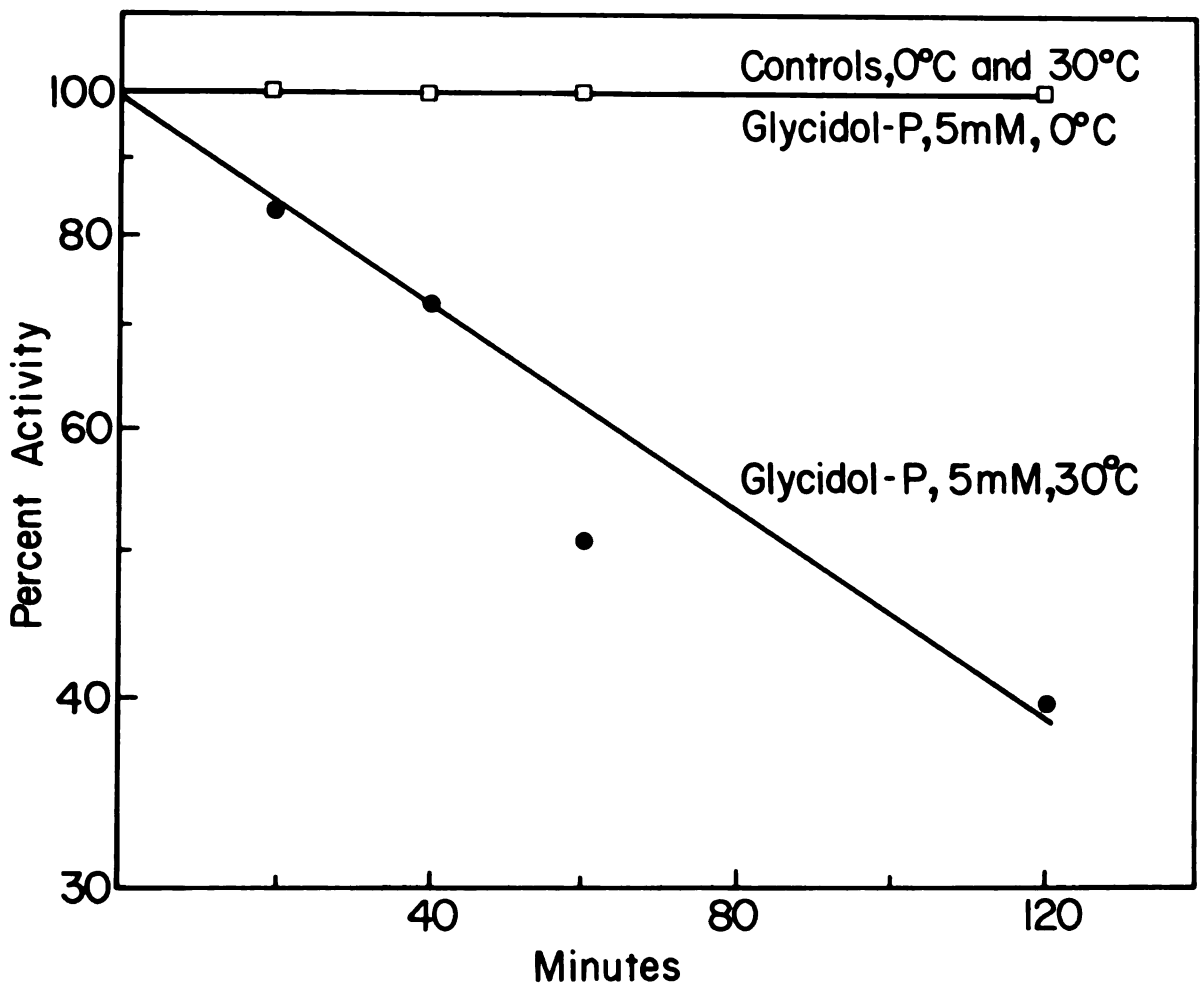
P-glycolate is an effective inhibitor of triose phosphate isomerase with a Ki of 2×10^{-6} M, and is suggested to be a transition state analog (225). Glycidol-P (2,3-epoxypropanolphosphate) is an irreversible, active site directed inhibitor of triose phosphate isomerase and enolase (172) and 3 -P-glycerate phosphatase (163).

I found (Figure 38) that glycidol-P is also an inhibitor of P-glycolate phosphatase although the reaction is not rapid. Slow inhibition may be due to steric hinderance, as glycidol-P is an analog of n-propyl-P which does not interact very well with P-glycolate phosphatase. The inactivation was pseudo-first order at 30° but no inactivation was observed at 0° after 2 hours.

The mode of action cannot be ascertained, but is most likely alkylation through the epoxide group. Miller and Waley (1971) identified an ester linkage to a glutamic acid residue at the active site of triose phosphate isomerase. Schray et al. (1973) reached the same conclusion with enolase. Assuming the glycidol-P inhibits at the active site of P-glycolate phosphatase, the result is consistent with the presence of a carboxyl group required for activity as suggested by the studies on log (Vmax/Km) against pH, but the potential role of substrate or magnesium in affecting the inactivation has not been studied.

Figure 38.--The effect of glycidol-P on the activity of P-glycolate phosphatase.

The incubation system contained 8 μmole cacodylate at pH 6.3; 0.25 μmole glycidol-P, and 5 units of phosphatase in a total volume of 50 μl. Duplicate aliquots of 2 μl were removed for assay at 30°.


Controls,0°C and 30°C
Glycidol-P,5mM, 0°C
Glycidol-P, 5mM,30°C
Percent Activity
100
80
60
40
30
40
80
120
Minutes

Effect of Deuterium Oxide

Because water is involved in the phosphatase reaction the isotope effect from D_2O was measured (Figure 39). A significant rate effect was observed, decreasing linearly from 100% H_2O to 100% (extrapolated) D_2O. The rate effect was almost identical at pH 5.8 and pH 8.2. The data suggests that water, per se, is not involved in the rate limiting step, since a much greater isotope effect would be expected for deuterium for hydrogen substitution. This is consistent with the hypothesis that phosphoenzyme hydrolysis is very rapid. On the other hand, the changes are linear and invariant over a 400 fold change in hydrogen concentration. However, involvement of ionized species cannot be completely ruled out, as the variation in isotope effect is 10%, and the actual environment at the active site is not known. The lack of any marked effect is suggestive that the substrate protonation involves an active site residue or bound water rather than water of the medium. Since the effect is small and independent of pH, the effect can also be explained by a change in the bulk milieu properties which causes some small perturbation in the enzyme system.

Spectrophotometric Assay

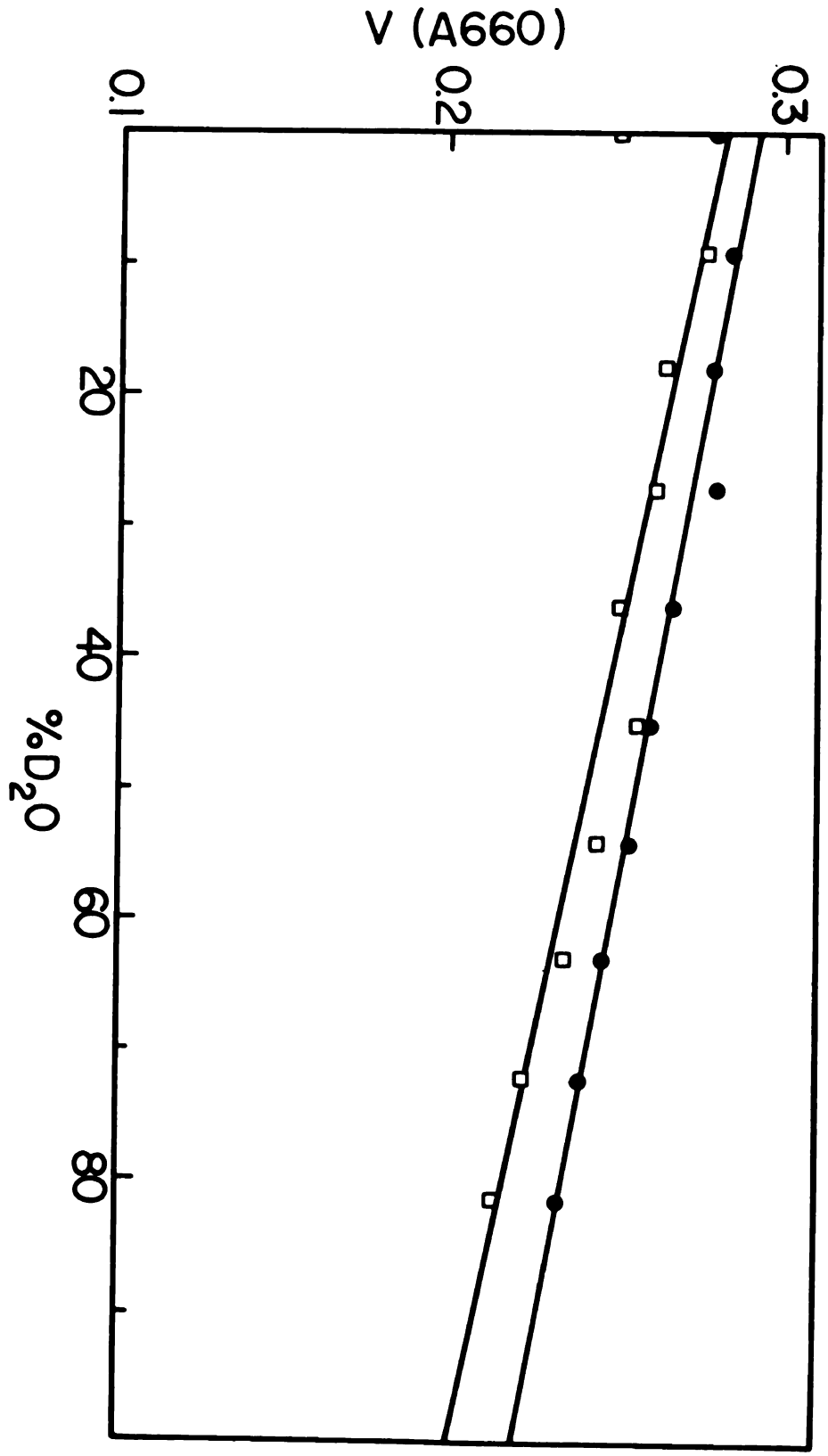
This assay was developed on the basis of the difference in pK_2 of P-glycolate and inorganic phosphate which results in OH^- production during P-glycolate hydrolysis and the stoichiometry depends on the pH of the medium. The pk's of Pi and P-glycolate are 7.12 and 6.60 respectively. Thus at pH 7.5 phosphate is 70.5% dianion and the phosphate moiety on P-glycolate is 88.8% doubly ionized. The carboxyl

Figure 39.--The effect of increasing concentrations of D_2O as the solvent medium on the activity of P-glycolate phosphatase.

The system contained 2 μmole P-glycolate, 20 μmole cacodylate at pH 8.2 or pH 5.8 and 0.1 unit phosphatase, in 0.5ml volume, deuterium oxide as indicated. The range of D_2O concentrations did not affect the measured pH.

● , pH 8.2 $k_1H/k_1D = 1.295$

□ , pH 5.8 $k_1H/k_1D = 1.387$


V (A660)
0.1
0.2
0.3
20
40
60
80
%D2O

group of the glycolate remains fully ionized at this pH. Therefore hydrolysis of 1 mole of P-glycolate will require 0.183 mole of hydrogen ion to maintain the pH of the solution. This alkali production in a buffered system can be visualized spectrophotometrically as an increase in absorbance of a barbital buffer (178). The absorbance change has been quantitated for other systems from a knowledge of the barbital concentration and molar extinction coefficient, but this has not been done for the phosphatase. Rather the assay has been used to monitor whether there was a change in reaction mechanism which might alter the production of OH^- ions. Although more alkali production should be present at lower pH's, no buffers were available to detect it spectrophotometrically.

That the assay is a true measure of P-glycolate phosphatase activity was obtained by comparing it with measurements of phosphate release. The ratio was invariant during purification of the enzyme from spinach leaves (Table 25). Furthermore, activities by both assays were stimulated similarly by various divalent cations (Table 26). The Michaelis constant was also determined by the spectrophotometric method in assays in which the substrate concentration was varied from 3.5×10^{-5} M to 1.67×10^{-3} M, $CoCl_2$ was 10^{-3} M, and the initial pH was 7.5. The value for the Km of 6.4×10^{-5} M is in excellent agreement with those obtained from the phosphate release or (2-^{14}C)-glycolate release assays (Table 6).

Alkali Production (pH Stat)

Alkali production was measured using the Radiometer pH titrator, set at pH 6.0. The titrant was 10 mM Hcl and it titrated 5 μmole for

TABLE 25.--Ratio of Activities by the Spectrophotometric and Phosphate Assays During Purification of P-glycolate Phosphatase.

Extract	Pi released μmole/min/ml	Δ A245/ min/ml	Ratio Pi/A245
Homogenate	4.9	4.20	1.17
Acid supernatant	2.4	2.06	1.16
Sephadex G-25	2.7	2.32	1.16
Acetone 40-60%	3.6	3.38	1.07
Acetone 60-80%	0.6	0.52	1.15

Note: Five g spinach leaves were homogenised in water, and centrifuged at 20,000 x g for 10 min after acidification. Small molecular weight species were separated from the enzyme by passage of the acid supernatant through a Sephadex G-25 column and this extract was subject to acetone fractionation.

TABLE 26.--Comparison of the Spectrophotometric and Phosphate Assays of P-glycolate Phosphatase with Various Divalent Cations.

Cation (10^{-3}M)	Spectrophotometric Assay at pH 7.5		Pi release at pH 6.3[a]	
	Δ A245/min/ml	% Maximum	μg/min/ml	% Maximum
Co^{++}	0.089	100	79	100
Mn^{++}	0.076	85	54	68
Mg^{++}	0.058	65	36	46
Ca^{++}	0.008	9	4	5

[a]From Richardson and Tolbert (1961).

full scale displacement. Total assay volume was 2.0 ml held at 25°. The unbuffered solution was initiated with enzyme, and after an initial rapid titration to adjust this extract, a steady rate of alkali production was observed. Quantitation of the system was difficult since it requires knowledge of the buffering capacity of the extract, substrate, and product. Theoretically, at pH 6.0 the hydrolysis of 1 mole of P-glycolate should produce 0.205 mole of hydroxide ion.

Representative traces are shown in Figure 40. Trace A shows the full assay system for the hydrolysis of 5 μmole of P-glycolate. Extrapolation of the trace gave 0.312 μmole of hydroxide produced, corresponding to 1.52 μmole P-glycolate or 30% hydrolysed. Because buffering occurs, it is possible that this production was due to total hydrolysis. Traces B. C. and D show that all components were required; in the absence of P-glycolate (trace B) no alkali was produced, in the absence of added $MgSO_4$ (trace C) the rate of alkali production was very slow, while the boiled extract (trace D) was also inactive. Traces E, F, and G indicate that the activity was proportional to the volume of the enzyme extract added, but smaller total pH change with larger aliquots appear to be due to the greater volume of buffer added. Results are analysed in Table 27, and indicate that the alkali production in each trace was about the same, suggesting total hydrolysis.

The activity of P-glycolate phosphatase can be detected in crude extracts of spinach by the pH stat titration. However the sensitivity and accuracy are very poor compared to the spectrophotometric assay. Phosphate release remains the method of choice since it is most

Figure 40.--pH Stat-titrimetric assay for P-glycolate phosphatase activity. Enzyme extract "A" was the Sephadex G-25 treated preparation from Table 25. Enzyme "B" was the 40-60% acetone fraction from Table 25. The components of the various traces are:

A. 5 µmole P-glycolate, 5 µmole $MgSO_4$ and 400 µl enzyme "A",

B. 5 µmole $MgSO_4$ and 400 µl enzyme "A",

C. 5 µmole P-glycolate and 400 µl enzyme "A",

D. 5 µmole P-glycolate, 5 µmole $MgSO_4$ and 400 µl boiled enzyme "A",

E. same as A, but with 50 µl enzyme "B",

F. same as A, but with 100 µl enzyme "B",

G. same as A, but with 200 µl enzyme "B",

All traces were initiated with enzyme.

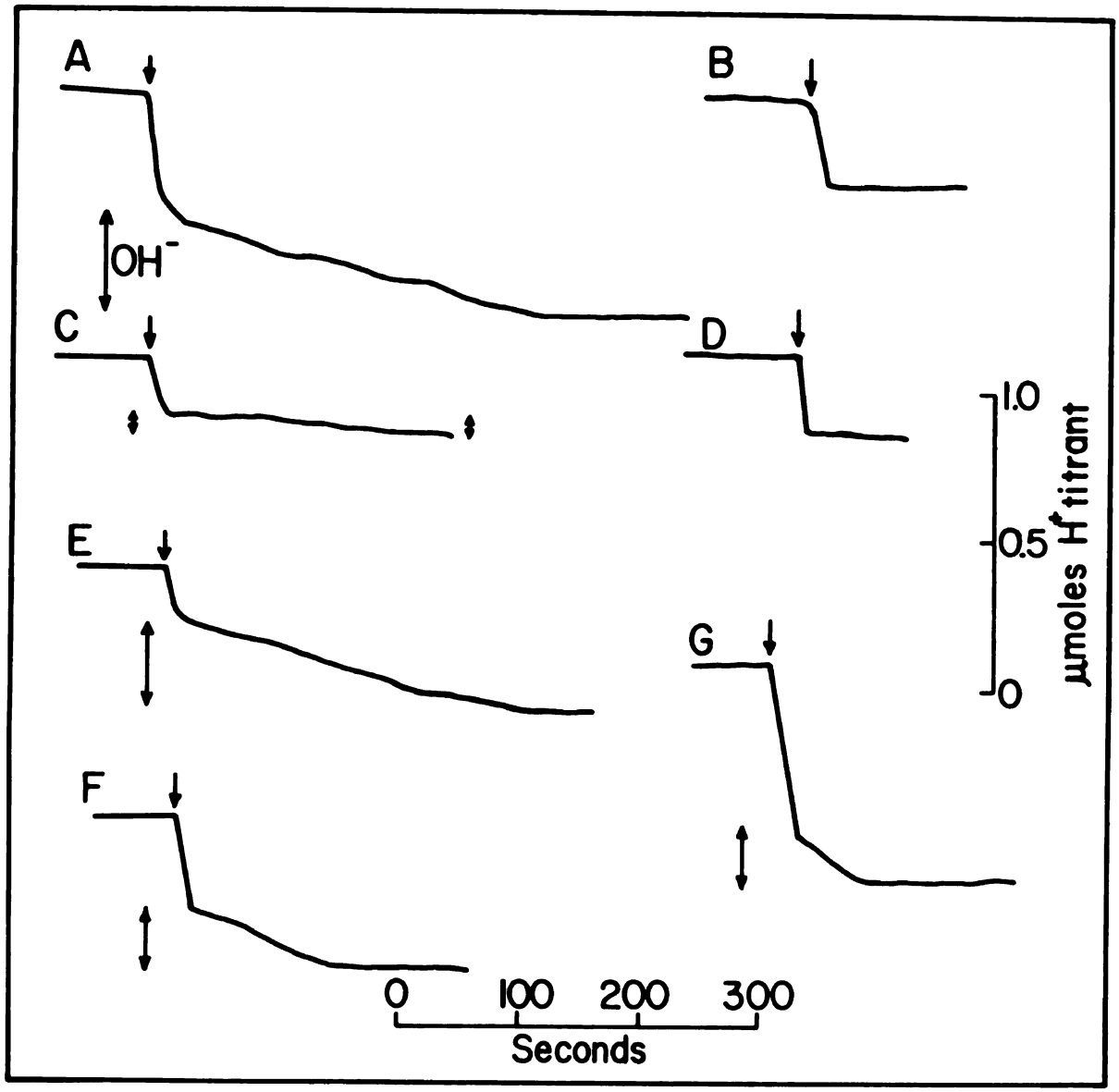

A
B
OH⁻
C
D
E
G
F
1.0
0.5
0
μmoles H⁺ titrant
0
100
200
300
Seconds

TABLE 27.--pH Stat Titrimetric Analysis of P-glycolate Phosphatase Activity.

Trace[a]	Rate (Arbitrary units)	Duration (sec)	Rate x Duration
A	4 ± 1	333 ± 5	1340
A1[b]	4 ± 1	290 ± 5	1160
E	5 ± 1	270 ± 5	1350
F	8 ± 1	135 ± 5	1085
G	13 ± 1	80 ± 5	1040

[a]Trace designated from Figure 40.

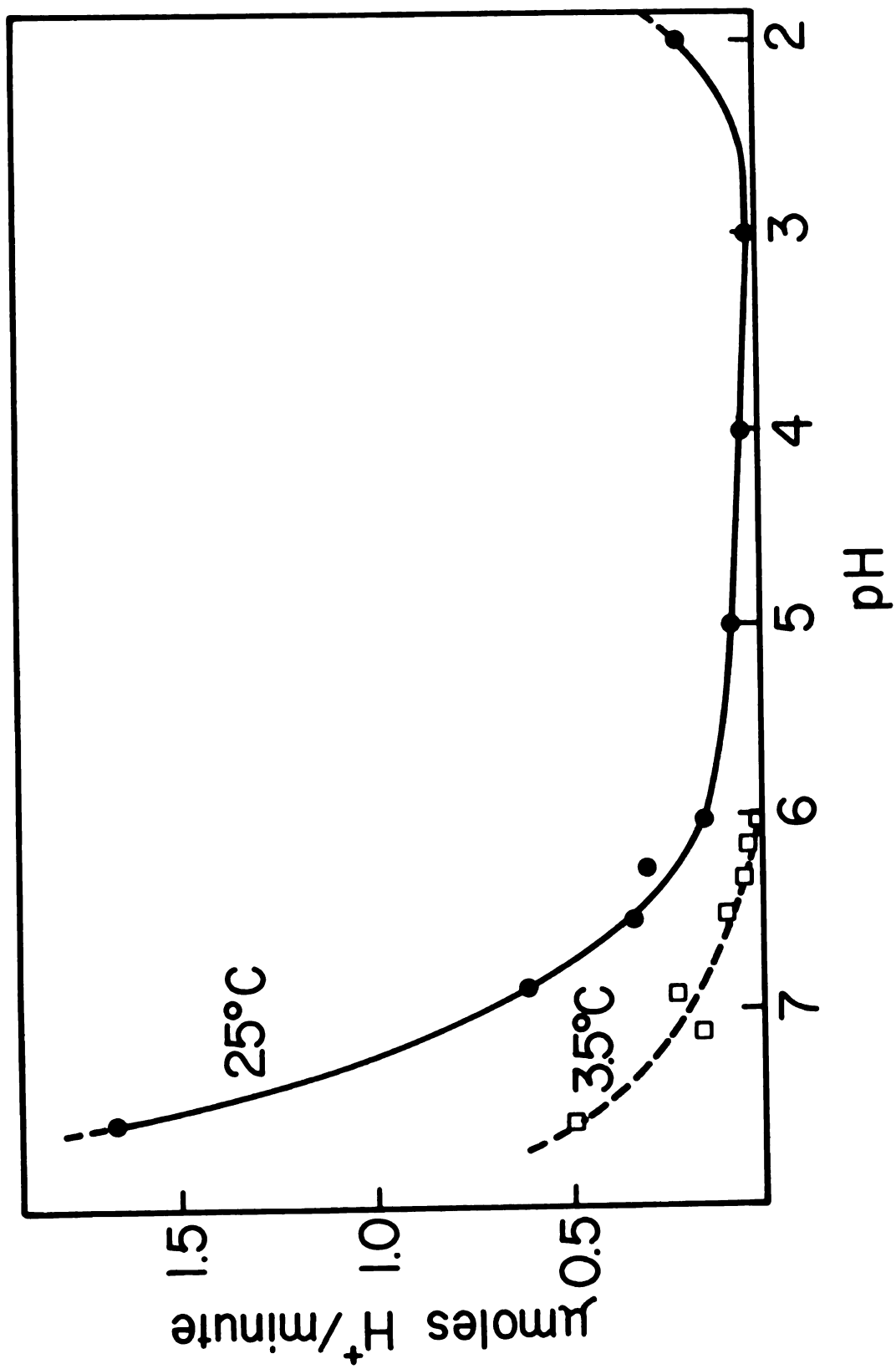
[b]Duplicate of Trace A.

easily calibrated, most sensitive (except for ^{14}C-glycolate release), most rapid, and most adaptable to semi-automated procedures. However the alkali titration method was developed during considerations of the mechanism of P-glycolate phosphatase hydrolysis and because pH control in the chloroplasts during photosynthesis is a significant research area which may relate to this phosphatase.

Nonenzymic Hydrolysis of Glycolide

Glycolide ($C_4H_4O_4$), is the cyclic dilactone of glycolic acid, and hydrolses to produce two equivalents of acid. The rate of spontaneous hydrolysis was measured with the Radiometer pH Stat (Figure 41). Glycolide is comparatively stable below pH 6.0, but the rate increases rapidly above neutrality. The position of the bond fission is presumed to be at the carboxyl carbon, probably by nucleophilic attack of the

Figure 41.--Nonenzymic hydrolysis of glycolide. The pH-Stat was standardised to titrate 5 µmole full scale (0.5ml syringe, 10mM NaOH-freshly prepared). The glycolide was dissolved in acetone (11.6 mg/ml) as a 0.1M solution. Hydrolysis at 0°C was about 1% per hour in acetone. 20 µl of this solution was injected into 5 ml of distilled, deionised water to initiate the reaction. The solution was adjusted to the required pH and temperature prior to injection. Injection of acetone had no effect on the pH.


μmoles H+/minute
0.5
1.0
1.5
25°C
35°C
2
3
4
5
6
7
pH

hydroxide ion from the pH profile. The increase in rate at pH 2 must be due to a different mechanism and different products since the glycolate carboxyl is unionized at this pH. Addition of plant extracts at several different pH values had no effect on this rate of spontaneous hydrolysis. Although these results were preliminary they suggest that no lactonase for glycolide was present in spinach leaves.

This study, as well as those on alkali production, was carried out as part of a hypothesis that glycolate may be excreted from the chloroplast in the form of a stable lactone. Chang and Tolbert (1970) showed that the alga, Chlamydomonas, excretes isocitric lactone, which was stable, rather than the acid; at the same time when they are rapidly excreting glycolate. The neutral compound, rather than the acid, might be more easily transported through the lipid bilayer. The hypothesis was however ruled out by Andrews et al. (1971) who showed no exchange of the ^{18}O- carboxyl group in glycine and serine which had been incorporated into glycolate during photorespiration in 100% oxygen. Cyclization and hydrolysis would remove 50% of the ^{18}O incorporated into a glycolide intermediate. Furthermore, my (^{18}O)-H_2O incorporation studies have ruled out P-glycolate phosphatase as a cyclizing enzyme.

Lipase Inactivation

The instability of P-glycolate phosphatase from spinach leaves was the main problem in its purification. This may be caused by loss of lipid or another constituent, since the enzyme was labile to freezing and thawing in crude preparations. Therefore acid supernatant from spinach leaves was incubated at 30^{o} with various lipases and liquids or emulsions of lipids.

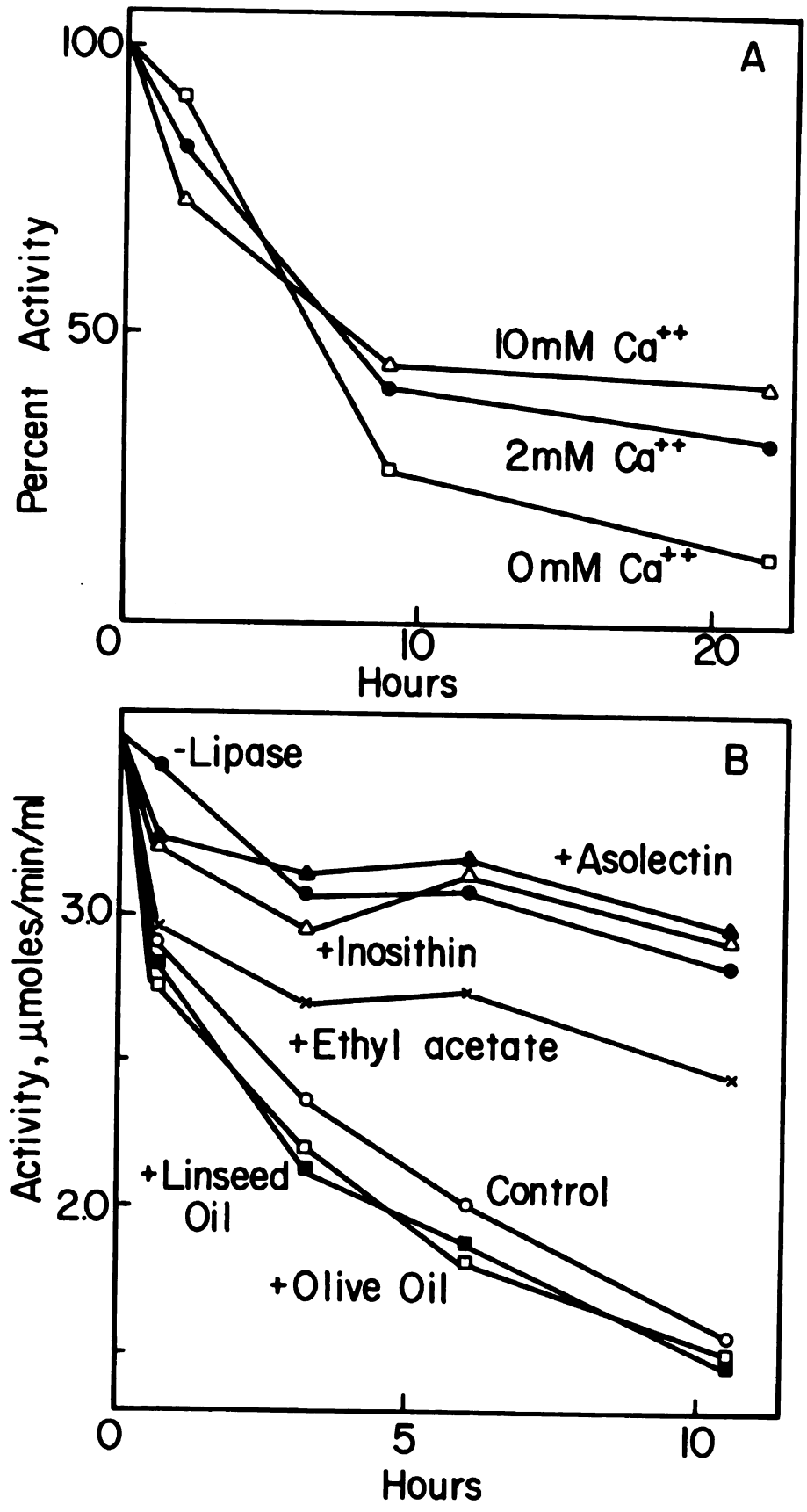
The P-glycolate phosphatase was inactivated by wheat germ lipase (EC 3.1.1.3) (Figure 42A) but not by phospholipase C from Closteridium welshii or by Crotalus adamanteus venom (phospholipase A activity). Trypsin destroyed activity slowly when incubated with the phosphatase in 50 mM $CaCl_2$. Although the wheat germ lipase was not highly purified, the increase in initial rates of inactivation by increasing calcium suggests that the lipase is indeed the active component since Ca^{++} is known to activate lipase. The data also suggests that Ca^{++} seems to destabilize the lipase because the extent of the inactivation is decreased by higher Ca^{++} concentrations.

A further indication that the lipase was involved in the inactivation of P-glycolate phosphatase was a protective effect from lipase substrates (Figure 42B). Lipase hydrolyses short chain fatty acid esters but not long chain esters (187). Thus the protective effect of ethyl acetate on the phosphatase inactivation may be due to a substrate competitive effect on the lipase. Other good lipase substrates (data not shown), such as triacetin, Tween 80 and Tween 20 also protected. Lipase substrates had no marked effect on the stability of the phosphatase in the absence of lipase. On the other hand, the plant oils, rich in long chain triglycerides such as triolein, were completely ineffective against lipase inactivation. The protective effects of the phospholipid mixtures, asolectin and inosithin, could be due to other causes also as they stabilize P-glycolate phosphatase in the absence of the lipase, as does Triton X-100 (data not shown).

DEAE-cellulose chromatography is known to remove lipid from proteins in some situations. Thus it is possible that this is the

Figure 42A.--Inactivation of P-glycolate phosphatase by wheat germ lipase and calcium ions. Incubation mixtures contained 0.1 mg lipase, 3 units phosphatase, 20 µmole cacodylate at pH 7.5, and calcium as indicated in 1.4 ml at 30°.

Figure 42B.--Effect of lipase substrates and lipid extracts on P-glycolate phosphatase inactivation by lipase. The control incubation contained 0.5% lipid, 10mM Ca^{++}, 0.3 mg lipase, 3 units phosphatase, and 20 µmole cacodylate at pH 7.5 in 1.4 ml at 30°C. Other additions and deletions are indicated in the figure. Aliquots (25 ul) were removed for assay.


A
100
50
0
Percent Activity
10mM Ca++
2mM Ca++
0mM Ca++
0
10
20
Hours
B
-Lipase
+Asolectin
+Inosithin
+Ethyl acetate
+Linseed Oil
Control
+Olive Oil
Activity, µmoles/min/ml
3.0
2.0
0
5
10
Hours

cause of the extreme instability of the spinach enzyme following DEAE-cellulose treatment. The enzyme from tobacoo leaves, however, is comparatively stable throughout purification and lipase does not inactivate fractions obtained after DEAE-cellulose chromatography. This seems to be a distinct difference between P-glycolate phosphatase from the two plants, which otherwise are very similar in properties. The spinach enzyme has a molecular weight 10% greater than the tobacco enzyme and it is tempting to suggest that this may be due, to some extent, to bound lipids. The nature of these lipids is unknown and no attempts were made to reactivate the phosphatase by adding back lipid mixtures.

CONCLUDING DISCUSSION

P-glycolate phosphatase is the first tetrameric phosphatase to be reported. It seems likely that the complexity of the enzyme is related to its strict physiological role and specificity. Whether the subunits are active individually or not is unknown, however, assuming that there are four active sites per molecule; P-glycolate phosphatase has a turnover number in the region of 8×10^3 sec^{-1}. The value falls in the midrange for phosphatases. The isoelectric point is very low and lends credence to the possibility that the enzyme is associated with other proteins or membranes *in vivo*. It is possible that the tricarboxylic acids which stabilize the enzyme displace the protein from these associations during homogenization of the leaves. The presence of tricarboxylic acids from P-glycolate phosphatase released from isolated intact chloroplasts has not been determined.

No evidence exists that P-glycolate phosphatase acts as part of a permease system, nor has the need for such a system been demonstrated. As P-glycolate phosphatase is readily solubilized from chloroplasts it must be in the soluble stroma of the chloroplast and at the site of synthesis of its substrate, where it can catalyse hydrolysis most rapidly and effectively. The protein concentration in the stroma is very high and very different physically from the dilute systems used for experiments. Therefore an association between RuDP carboxylase/oxygenase and P-glycolate phosphatase cannot be ruled out.

An additional property which suggests P-glycolate phosphatase is a stromal enzyme is the mode of regulation. The negative allosteric cooperativity toward Mg^{++} appears to be a mechanism whereby the pH range is extended from around 6 to more physiological values around 8. Thus the possibility that the in vitro pH optimum around pH 6.3 is an artifact due to release of the enzyme from a membrane or protein association is unlikely. Rather, Mg^{++} concentration in the chloroplast stroma, which increases greatly in the light, is probably the primary regulant of P-glycolate phosphatase activity, similar to Mg^{++} regulation of RuDP carboxylase/oxygenase activity.

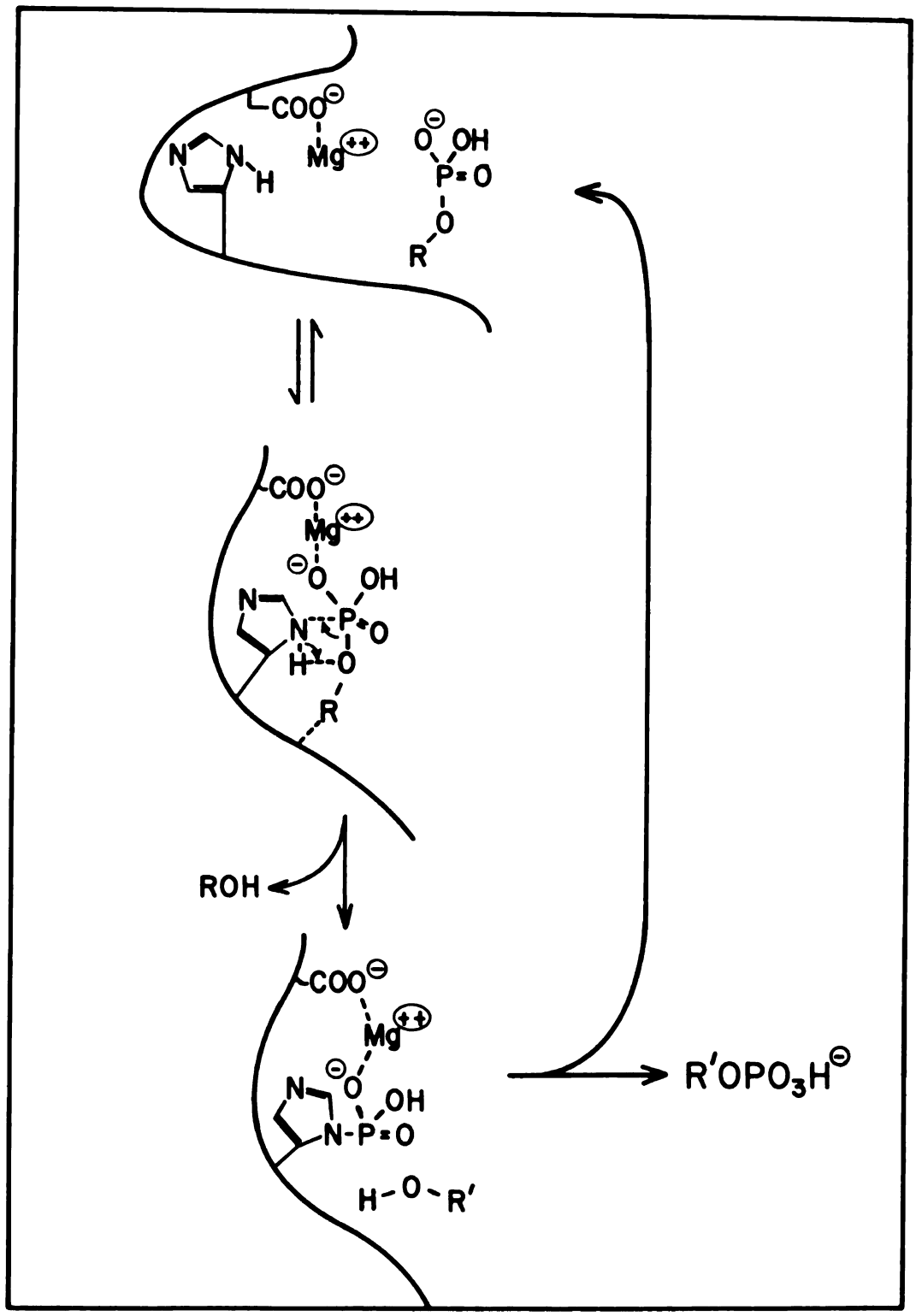
Substrate specificity, DFP inhibition, pH optimum, and kinetic studies suggest that P-glycolate phosphatase, like glucose-6-phosphatase, falls into the classification of an acid phosphatase. Non-specific acid phosphatases have a pH optimum around pH 4-5 and have no cation requirement. The fact that all alkaline phosphatases as well as both glucose-6-phosphatase and P-glycolate phosphatase have a divalent cation requirement suggests that phosphohydrolase activity in neutral and alkaline conditions is dependent on the presence of cations. The demonstration that Mg^{++} has a role in extending the activity pH range of P-glycolate phosphatase by an allosteric mechanism provides some evidence in favour of these speculations. Furthermore, it is implied that the alkalization of the pH optima of these specific acid phosphatases is due to evolutionary response to changing physiological conditions, perhaps concurrently with the evolution of photophosphorylation and the development of proton gradients with primeval "chloroplasts." The complex interactions between the enzyme, its substrate

and cofactor, seem designed to promote maximum activity under all physiological conditions.

The available evidence from this thesis is incorporated into a tentative mechanism for the action of P-glycolate phosphatase (Figure 43). However, none of the active site groups have been positively identified. All evidence is consistent with a role for divalent metal ion at the active site, and the carboxyl group at the active site involved in binding is tentatively identified on the basis of the pK observed and glycidol-P inhibition. Histidine is suggested as the residue through which the phosphoryl transfer occurs on the basis of similarities between P-glycolate phosphatase and nonspecific acid phosphatases and glucose-6-phosphatase for which histidine has been positively identified, and because DFP inhibition studies suggest that the residue is not serine. Since the second ionizing residue has not been identified with any particular binding function, nor with the sulphydryl group required for activity, it is absent from the scheme. Because binding the substrate exposes a sulphydryl, that group, at least, is not involved in binding. The mechanism is drawn as proceeding through a metastable pentacovalent phosphorus intermediate. This predicts apical interaction with the imidazole nitrogen, protonation and pseudorotation to permit the alcohol to leave apically. There is no evidence favouring this mechanism over a metaphosphate intermediate however.

Figure 43.--Tentative mechanism for the action of P-glycolate phosphatase.

R is a small alkyl group since approach to the active site is very sterically hindered. Methyl and larger alkyl groups interact on binding to produce a conformational change and a stable Michaelis Complex. The rate limiting steps are then considered to be the phosphorylation of the enzyme and the diffusion of the alcohol from the active site. Since large acceptors, such as glucose, as well as water, are then able to hydrolyse the complex, presumably the phosphoenzyme is "frozen" into the open conformation. Transfer of the phosphoryl group to this acceptor permits isomerization back to the sterically restricted active site, and this step appears to be non-rate limiting.


COO⊖
Mg⊕⊕
N
N
H
O⊖
OH
P=O
O
R
ROH
H-O-R'
N-P=O
R'OPO3H⊖

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