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Kinetic and Binding Studies on L-∝-Glycerophosphate Dehydrogenase

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KINETIC AND BINDING STUDIES ON L-α-GLYCEROPHOSPHATE DEHYDROGENASE

by

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DEDICATION

The author would like to dedicate this work to his mother

Dorothy B. Rosevear

for her love, understanding, encouragement and most of all her confidence, and to

Mary and Sterling Montgomery
and
Peggy and John Stancil

for their help throughout the years.

P.R.R.

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P.R.R.

ABSTRACT

The properties of the coenzyme, NAD, binding site of chicken muscle L-∝-glycerophosphate dehydrogenase were studied. Adenosine monophosphate, adenosine diphosphate and adenosine diphosphoribose were shown to inhibit the enzyme competitively with respect to NAD. The presence of adenosine, pyrophosphate and ribose regions in the coenzyme binding site of the enzyme was suggested by the inhibitor constants obtained for these compounds.

Disodium monoalkyl phosphates, n-butyl to n-dodecyl phosphate, inclusive, were also shown to inhibit the L-∝-glycerophosphate dehydrogenase catalized reaction competitively with respect to NAD. A positive chain length effect was observed in the binding of these compounds to the enzyme, suggesting the presence of a hydrophobic region in the coenzyme binding site of the enzyme. Multiple inhibition studies demonstrated the simultaneous binding of the inhibitor pair adenosine monophosphate and disodium n-heptyl phosphate. However, mutual exclusion was observed in the multiple inhibition studies with inhibitor pairs of disodium n-heptyl phosphate and disodium n-undecyl phosphate, and adenosine diphosphoribose and disodium n-heptyl phosphate. These results suggested the presence of a hydrophobic region in or nearby the ribose binding region of the coenzyme binding site of the enzyme.

Fluorescence quenching was used to study the properties of the binding of coenzyme-competitive inhibitors to L-α-glycerophosphate dehydrogenase. The binding of adenosine diphosphoribose and 3-aminopyridine adenine dinucleotide to chicken muscle α-glycerophosphate dehydrogenase in the absence of substrate was observed to be anticooperative and in the presence of substrate noncooperative. However, the binding of either adenosine monophosphate or disodium n-dodecyl phosphate was found to be noncooperative whether in the presence or absence of substrate. Results indicate that the binding of coenzyme-competitive inhibitors that occupy an area in the coenzyme binding site smaller than adenosine diphosphoribose cannot provide the steric requirements necessary for the normal type of cooperativity seen with the analog of NAD, 3-aminopyridine adenine dinucleotide. The change from anticooperativity in the absence of substrate to noncooperativity in the presence of substrate for 3-aminopyridine adenine dinucleotide and adenosine diphosphoribose results from decreased interactions between the NAD binding region on each subunit through formation of the ternary complex.

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INTRODUCTION

Enzymes compose the largest and most highly specialized class of protein molecules. They are responsible for the catalysis of the various chemical reactions that constitute the intermediary metabolism of cells. These enzymes are divided into six major classes; one of which is the oxido-reductases. Dehydrogenases are a subclass of the oxido-reductases and catalyze the dehydrogenation of their substrates with a molecule other than oxygen as a hydrogen acceptor (1). L- α -glycerophosphate dehydrogenase (L-α-glycerol - 3 - phosphate: NAD+ oxidoreductase EC 1.1.1-8) catalyzes the reversible oxidation of L- α -glycerophosphate by oxidized nicotinamide-adenine dinucleotide (NAD) forming dihydroxyacetone phosphate and NADH (2), as shown in the following reaction:

α-Glycerophosphate + NAD $\rightleftharpoons$ Dihydroxyacetone Phosphate + NADH + H^+

This enzyme is a cytophasmic enzyme abundant in insect flight muscle and several mammalian tissues (2). Its function in the cytoplasm is to regenerate NAD through the reduction of dihydroxyacetone phosphate. In certain tissues it also acts cooperatively with mitochondrial L- α -glycerophosphate oxidase to couple the reoxidation of NADH from the cytoplasm to the respiratory chain. This latter function is commonly known as the α-glycerophosphate cycle (2).

An ordered mechanism of the formation of the ternary complex was demonstrated, in L- α -glycerophosphate dehydrogenase, where the binding of the coenzyme, NAD in this case, is the first step (3).

The binding of the coenzyme is followed by the binding of α-glycerophosphate through its ester bond (3). It has been indicated that thiol groups, histidyl residues and zinc metal are involved in the active site (4, 5).

This complex protein was found by Ankel et al. (6) to contain one mole of adenosine diphosphoribose per mole of enzyme and, more recently, Cellers et al. (7) have found another non-protein component bound to L-α-glycerophosphate dehydrogenase that, as yet, remains unidentified.

The amino acid compositions of both rabbit and chicken muscle L-α-glycerophosphate dehydrogenase were found to be quite similar and are listed in Table 1 (8, 9). The molecular weight of the rabbit muscle enzyme was determined to be 76,800g·atoms by velocity centrifugation. However, values for the molecular weight of this enzyme have been reported ranging from 60,000g·atoms to 78,000g.atoms depending on source of enzyme and the method used for purification (8, 9).

Binding of pyridine nucleotide coenzymes to dehydrogenases occurs in most cases at a ratio of one mole of coenzyme per subunit. Kim and Anderson (10) have observed, through the use of fluorescence titration of rabbit muscle L-α-glycerophosphate dehydrogenase with NADH, the binding of two moles of NADH per mole of enzyme (78,000g·atoms). Peptide mapping of trypsin digests of L-α-glycerophosphate dehydrogenase showed the presence of two identical subunits of 39,000 molecular weight (10). The nature of the binding of the coenzyme, nicotinamide-adenine dinucleotide, to rabbit muscle L-α-glycerophosphate dehydrogenase was investigated by Kim

and Anderson (11). Adenylic acid, adenosine diphosphate and adenosine diphosphoribose were shown to be coenzyme competitive inhibitors of L-α-glycerophosphate dehydrogenase. The binding of N'-alkylnicotinamide chlorides larger than the N'-pentyl derivative were also shown to be competitive with respect to coenzyme and their binding facilitated through nonpolar interactions with this enzyme (11). It was observed that the effectiveness of binding the adenine derivatives increased with the size of the derivative (11). The facilitated binding of the N'-alkylnicotinamide chlorides suggested the presence of a hydrophobic region, in the coenzyme binding site, adjacent to the nicotinamide binding region. Through the use of these derivatives, Kim and Anderson (11) suggested that the binding of NAD to α-glycerophosphate dehydrogenase occurred through interactions involving an adenosine moiety, a pyrophosphate grouping and a positively charged nicotinamide ring region. N-alkylammonium chlorides were shown to be binding to the "pyridinium ring" region of the NAD binding site through the competitive nature of this binding and multiple inhibition studies with adenylic acid (12). Again, as the alkyl chain length was increased the binding to the inhibitor increased indicating nonpolar interactions with a hydrophobic region on the enzyme. A linear relationship between the logarithm of the reciprocals of the inhibitor constants was observed (12). This linear relationship relates the binding constant, $1/k_1$, to the free energy change produced by the binding of additional methylene groups. The free energy change per methylene group was

TABLE 1

Amino Acid Composition of Rabbit and Chicken Muscle L-α-Glycero-phosphate Dehydrogenase.

	Rabbit Muscle Calculated Subunits (8) Residue	Chicken Muscle Calculated Subunits (9) Residue
Lysine	30	20
Histidine	9	6
Arginine	8	6
Aspartic acid and asparagine	28	24
Threonine	13	14
Serine	12	11
Glutanic Acid and glutamine	41	33
Proline	16	13
Glycine	40	30
Alanine	34	22
Half-cysteme	10	10
Valine	32	25
Methionine	8	8
Isoleucine	27	22
Leucine	30	23
Tyrosine	4	4
Phenylalanine	15	11
Tryptophan	2	2

calculated from the plot of log $1/k_1$ versus the number of carbon atoms as demonstrated by Equation 1.

$$\Delta\Delta F = 2.3RT\ pK_1;\ \text{Eq. 1}$$

The free energy change per methylene group was found to be 0.59 kcal/mole (12). This value lies within the range, 0.36-0.95 kcal/mole, depending on the closeness of the two hydrophobic groups, suggested for interactions through dispersion forces only (13). Finally, a homologous series of aliphatic carboxylic acids, formic to dodecanoic acid, inclusive, was also found to be coenzyme competitive inhibitors with respect to NAD (14). Data obtained from multiple inhibition studies suggested the binding of these compounds to the pyrophosphate region in the coenzyme binding site. Further, the binding was again shown to be facilitated by increasing the chain length of the carboxylic acid, suggesting the presence of a hydrophobic region in the NAD coenzyme binding site. A linear relationship between the logarithm of the reciprocals of inhibitor constants to chain length of alkyl substituents was observed (14). The free energy change per methylene group, 0.383 kcal/mole, was again found to lie in the range suggested for interactions through dispersion forces (14). Multiple inhibition between n-decylammonium chloride and n-decanoic acid gave rise to an interaction constant, α, equalling 1.63 (14). This indicates that the hydrophobic region is located between the "pyridinium ring" region and the pyrophosphate region. Based on this evidence it was suggested that the hydrophobic region was in or nearby the ribose binding region in the active site

of the enzyme. Likely candidates for this hydrophobic region in L-α-glycerophosphate dehydrogenase are the nonpolar amino acids; tryphotphan, phenylalanine, tyrosine, leucine and valine.

In summary, L-α-glycerophosphate dehydrogenase has four regions in the coenzyme binding site responsible for the binding of one molecule of NAD. These regions consist of a negatively charged region for interaction with the "pyridinium ring" region, a hydrophobic region for interaction with the ribose, a positive region for interaction with the pyrophosphate moiety and a region for interaction with the adenosine of NAD. The effect of these regions on the ability of the enzyme to bind its coenzyme, NAD, was shown through inhibition studies with adenine derivatives (11). Kim and Anderson (11) found that adenosine, adenylic acid, adenosine diphosphate, and adenosine diphosphoribose were all competitive inhibitors of rabbit muscle L-α-glycerophosphate dehydrogenase with inhibitor dissociation constants of 4.28×10^{-3}M, 2.01×10^{-3}M, 6.67×10^{-4}M and 1.79×10^{-4}M, respectively. Clearly, as the size of the derivative increased, allowing for interactions with regions other than the adenine region, in the coenzyme binding site, the dissociation constants decreased. This step wise increase in binding reinforces the theory of the four specific regions for the binding of one molecule of NAD in the coenzyme binding site of L-α-glycerophosphate dehydrogenase.

No useful discussion of enzyme mechanisms can be restricted to just kinetic properties. Direct equilibrium binding of substrates

and effectors must also be considered. The generally applicable methods for studying substrate binding to protein molecules are equilibrium dialysis, steady-state dialysis, ultracentrafugation, ultrafiltration and gel filtration (16). Sometimes other physical properties such as light absorption or fluorescence emission can be used to distinguish free and bound substrate or free and liganded enzyme (16).

For a multisubunit protein, the binding of one molecule of ligand to one subunit can influence the binding of another ligand molecule to the same protein molecule. Monod et al. (16) introduced the term "allosteric protein" to describe proteins in which such effects occur. These allosteric effects were first observed in hemoglobin but can be readily extended to include any multisubunit molecule. There are two classes of allosteric effects. First, interactions between identical ligands which is termed "homotropic" effects and second, interactions between different ligands are termed "heterotropic" effects. General properties of allosteric systems are that most allosteric proteins are polymers or contain several identical units and the allosteric interactions are frequently correlated with alterations in quaternary structure of the protein. Three types of effects which can be seen in an allosteric system are described as noncooperativity, positive cooperativity and negative cooperativity.

In the noncooperative system, the binding of the first molecule of ligand to the protein molecule exerts no influence on the binding of the second ligand molecule to the protein molecule. Positive

cooperativity is characterized by the enhanced binding of a second ligand. Finally, negative cooperativity is observed when the binding of one ligand to the protein molecule decreases the affinity of the protein molecule for the second. These effects, positive and negative cooperativities, are observed as deviations from classical Michaelis-Menten kinetics and can be distinguished from one another through the use of Lineweaver-Burke (17), Hill (18), Klotz (19) and Scatchard (20) plots. Characteristic of noncooperativity is a linear double reciprocal plot, a Hill coefficient of one and linear Klotz and Scatchard plots. Positive cooperativity gives a concave Lineweaver-Burke plot, a Hill coefficient greater than one, a concave Klotz plot and downward curved Scatchard plot. In contrast to the above, negative cooperativity gives a convex Lineweaver-Burke and Klotz plot, a Hill coefficient less than one and a Scatchard plot that curves upward.

Models for the cooperative ligand binding to proteins have been put forth by Monod, Wyman and Changeux (16) and Atkinson (21). The Monod, Wyman and Changeux Model postulates that a protein composed of subunits must maintain symmetry during conformational changes (16). From this postulate they deduce that the protein can exist only in two forms of which all the subunits are in the same form. Atkinson proposed a model that is significantly different from that of Monod *et al.* (16). In his model the binding of the ligand at one site can either increase or decrease the affinity of ligand binding at a second site. Further, Atkinson's model assumes progressive

changes in ligand site interactions rather than the "all or none" change proposed by Monod et al. (22).

Both noncooperative and positive cooperative can be accounted for by the concerted model of Monod et al; however, negative cooperativity can only be accounted for by a sequential allosteric model like that of Atkinson (23).

STATEMENT OF PROBLEM

The binding of the coenzyme, NAD, to the coenzyme binding site of NAD-dependent dehydrogenases is important in determining the mechanism of these dehydrogenases. Recently, Kim and Anderson (11) have shown that compounds structurally analogous to the purine or pyridinium portions of the NAD molecule were effective coenzyme inhibitors of rabbit muscle L-α-glycerophosphate dehydrogenase. Studies using these purine or pyridinium analogs suggested that the binding of the coenzyme to the coenzyme binding site occurred through interactions involving the adenosine moiety, the pyrophosphate grouping and the positively charged "pyridinium ring" region (11). These studies also indicated the presence of a hydrophobic region in the NAD binding site. Further studies by Kim and Anderson (11) using carboxylic acids showed the presence of a hydrophobic region, in the NAD binding site, between the pyrophosphate and the positively charged "pyridinium ring" region. The nonpolar amino acids, tryptophan, phenylalanine, tyrosine, leucine and valine are likely to be the major components in the composition of the hydrophobic region.

An homologous series of monoalkyl phosphates, n-butyl phosphate to n-dodecyl phosphate, inclusive, will be synthesized according to the procedure of Kirby (24) with minor modifications. Their interactions with the NAD coenzyme binding site of rabbit and chicken muscle L-α-glycerophosphate dehydrogenase will be studied. The phosphate moiety of the monoalkyl phosphate is expected to bind to

the pyrophosphate region of the NAD binding site. If the hydrophobic region in the coenzyme binding site is adjacent to the pyrophosphate region the binding of the monalkyl phosphates could be expected to be facilitated through the interaction of the nonpolar amino acids, in the NAD binding site, and the hydrophobic region of the monoalkyl phosphates. Coenzyme competitive inhibition of α-glycerophosphate dehydrogenase should be seen if the monoalkyl phosphates bind in the coenzyme binding site. Multiple inhibition analysis will be used to study the binding of the monoalkyl phosphate to the pyrophosphate binding region in the coenzyme binding site.

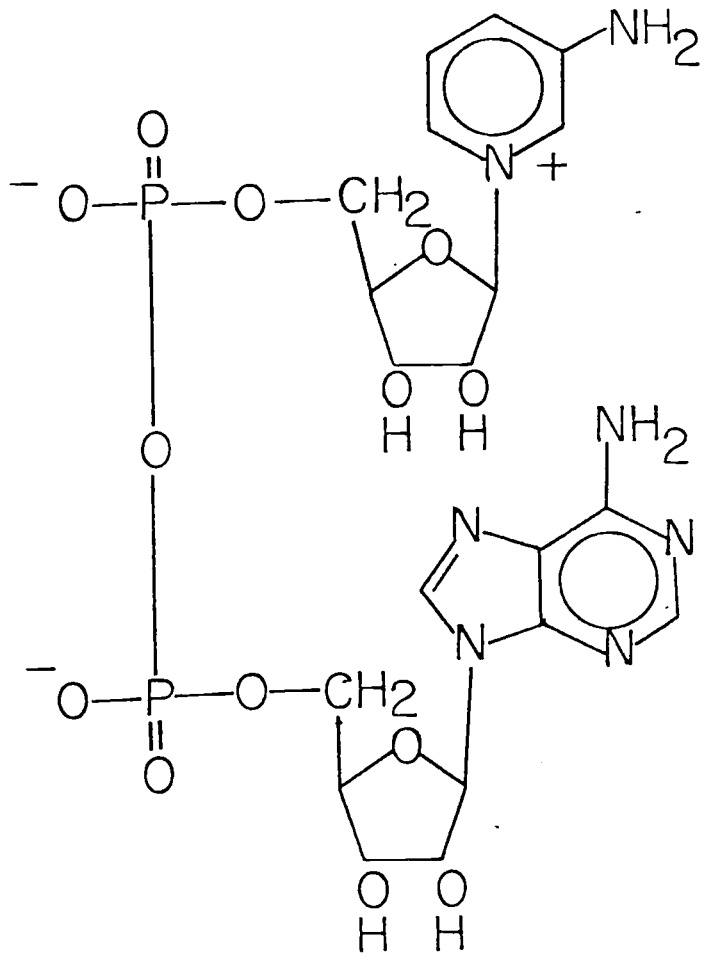
Kim and Anderson (14) have shown the presences of this hydrophobic binding region in rabbit muscle L-α-glycerophosphate dehydrogenase. A similar hydrophobic region might also be expected to be found in chicken muscle L-α-glycerophosphate dehydrogenase due to the general similarity of dehydrogenases. Assuming that the chicken muscle form of the enzyme has the same four regions for the binding of one molecule of NAD in the coenzyme binding site as rabbit muscle α-glycerophosphate dehydrogenase, then coenzyme competitive inhibition should be observed using adenine derivatives as inhibitors. Coenzyme inhibition studies will be carried out using adenosine monophosphate, adenosine diphosphate, adenosine diphosphoribose, and 3-aminopyridine adenine dinucleotide. If the chicken muscle α-glycerophosphate dehydrogenase has the same four regions for the binding of one molecule of NAD, then a stepwise increase in the binding constant should be observed on going from adenosine monophosphate to

3-aminopyridine adenine dinucleotide. This stepwise increase would correlate with a greater portion of the coenzyme analog bound to the coenzyme binding site.

L-α-glycerophosphate dehydrogenase, like most dehydrogenases, is a multisubunit protein capable of binding one coenzyme per subunit. It would be intereating to determine how the binding of one coenzyme molecule affects the tertiary conformation of this enzyme and the binding of the second coenzyme molecule. Several dehydrogenases have shown negative cooperative interactions between coenzyme binding sites in the absence of substrate (25, 21, 26). Equilibrium binding cannot be directly observed in the presence of both substrate and coenzyme since the enzymatic reaction would occur. Therefore, it has been cumstomary to look at an abortive complex of the enzyme. An abortive complex of the enzyme is formed when both the oxidized form of the coenzyme and product are used and thus no enzymatic reaction is capable of taking place. Direct equilibrium binding of substrates and effectors to rabbit and chicken muscle L-α-glycerophosphate dehydrogenase will be studied. Adenosine monophosphate, adenosine diphosphoribose, and 3-aminopyridine adenine dinucleotide (AAD) will be used as the coenzyme. AAD will be chemically synthesized from nicotinamide-adenine dinucleotide through the Hofmann hypobromite reaction and purified by means of ion exchange chromatography. The structure of AAD is given in Fig. 1. AAD has been shown to be a competitive inhibitor of several NAD dependent dehydrogenases (15). Since these adenosine derivatives are coenzyme

FIGURE 1

The Structure of 3-Aminopyridine Adenine Dinucleotide (AAD)



competitive inhibitors, the actual reduced substrate, in this case α-glycerophosphate, may be used to study the binding of the coenzyme analog rather than the oxidized product, dihydroxyacetone phosphate. Coenzyme binding studies on rabbit and chicken muscle α-glycerophosphate dehydrogenase will be performed employing protein fluorescence quenching. Adenosine monophosphate, adenosine diphosphoribose, 3-aminopyridine adenine dinucleotide, and disodium n-dodecyl phosphate will be used to study the conformational changes occurring when compounds are bound with and without substrate, α-glycerophosphate. Through these coenzyme binding studies, insight is hoped to be gained into the conformational changes that might take place in the binding of NAD to the coenzyme binding site both in the presence and absence of α-glycerophosphate. Results obtained using the different coenzyme competitive inhibitors could possibly determine the region, in the coenzyme binding site, that is responsible for the conformational change which occurs from binding one molecule of coenzyme analog.

EXPERIMENTAL

Equipment

Ion exchange of the monoalkyl phosphates was carried out on a Dowex AG 50-X10, sodium form, 20-50 mesh, cation exchange resin purchased from Dow Chemical Company. The fractions were collected in a Buchler refrigerated automated fraction collector. The conductance of the fractions was determined on an Industrial Instrument's conductivity bridge, Model RC 16B2 and conductivity cell. Pooled fractions were lyophilized on a Virtis Model 10-010 automatic freeze dryer equipped with a Cenco Hyuac 7 vacuum pump. NMR spectra were recorded with either a Varian T-60 or T-100 spectrometer, and IR spectra were recorded with an Unican SP-1000 IR spectrophotometer.

Phosphate determinations were performed spectrophotometrically using a Hitachi Perkin-Elmer Model 139 UV-visible spectrophotometer assuming that the error in the change in transmittance was 0.2% and independent of transmittance.

Kinetic studies were performed at ambient temperature using a Hitachi Perkin-Elmer Model 204 fluorescence spectrophotometer with a model 150 Xenon power supply and a Hewlett-Packard Mosely 7004A x-Y recorder. The fluorescence spectrophotometer was calibrated using NADH. Fluorescence intensity between zero and 100 was made to correspond with zero and 3×10^{-5}M NADH. All pH measurements were determined at ambient temperature using a Corning

Digital 110 pH meter and Thomas Model 4909-B15 glass electrode or a Radiometer pH meter, type PHM 4b with a G-200-B glass electrode.

Separation of NAD and AAD was carried out on a Dowex AG1-X8, chloride form, 200-400 mesh anion exchange resin purchased from Sigma Chemical Co. Fractions were collected in a Buchler Fracto-Mette Model 200 automated fraction collector. The absorbence of the fractions were monitored on a Hitachi Perkin-Elmer Model 139 UV-visible spectrophotometer.

Lithium was determined by flame emission on the Beckman Model DB-G grating spectrophotometer with a flame emission-atomic absorption module model 1301-AA. Acetylene and compressed air were used as the fuel and oxidant, respectively. Chloride ion was determined by an Orion's ion specific electrode for chloride.

Coenzyme binding studies were carried out in the laboratory of Dr. Bruce M. Anderson, Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, on an Aminco-Bowman spectrophotofluorometer equipped with a Xenon lamp model #901C-11, a Pacific Laboratories photometric recording photometer model 110 fitted with an RCA 6903 photomultiplier tube and a Mosely Autograf Model 135A X-Y recorder. All studies were performed at ambient temperature. Data treatment was handled on a Digital Equipment Corporation POP 11 computer.

Materials

n-Butanol, n-pentanol, n-hexanol, and n-dodecanol were obtained from J. T. Baker Chemical Co. n-Octanol was obtained from Matkson

Coleman and Bell and n-nonanol, n-decanol, and n-undecanol were obtained from Aldrich Chemical Company. Rabbit muscle L-α-glycerophosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, E. C. No. 1.1.1.8) crystalline suspension in 2.0M Ammonium sulfate solution, pH6, containing 100 micrograms of EDTA per ml with specific activity 100-200 units/mg protein, adenosine 5^1-diphosphoribose, adenosine monophosphate, D,L-α-glycerophosphate, and bovine serum albumin were obtained from Sigma Chemical Co. Chicken muscle L-α-glycerophosphate dehydrogenase was supplied by Dr. Gregory Lee from National Institute of Environmental Health Science. Elon (p-methylamino phenyl sulfate) was obtained from Eastman Kodak. Denterium oxide for nmr use was purchased from Mallinlkrodt Chemical Works. All other chemicals were reagent grade.

Methods

Preparation of the Disodium Salt of Monoalkyl Phosphates - The dicylcohexylammonium salt of the monoalkyl phosphates, n-butyl phosphate through n-dodecyl phosphate, inclusive, were synthesized according to Kirby (24) with minor modifications. Alcohols, n-butanol through n-dodecanol were stored over night over $Na_2\ SO_4$ to remove water. Crystalline phosphorous acid (H_3PO_3) was stored for several days over $CaCl_2$ under vacuum. 0.70 moles of the corresponding alcohol was added to 25 ml (0.175 moles) triethylamine. To this was added 4.10g (0.05 moles) of crystalline phosphorous acid. This reaction mixture was stirred until the H_3PO_3 was completely dissolved and then 19.0g (0.15 moles) solid iodine was added. The reaction

mixture was stirred for ten minutes during which time the reaction mixture became hot and thickened. After ten minutes the solution was poured and mixed into 1500 ml of acetone at room temperature. With stirring, 50 ml (0.44 moles) of redistilled cyclohexylamine was added. The resulting suspension was allowed to stand for 15 minutes. The precipitate was filtered through glass fritted funnels by gravity and resuspended in 500 ml of room temperature acetone by stirring. The resulting suspension was filtered by gravity, resuspended in 250 ml acetone at room temperature with stirring and again filtered by gravity. The precipitate was recrystallized from 200 ml of hot absolute ethanol and allowed to stand overnight at -20°C before filtering by gravity in a medium fritted funnel. The second recrystallization was carried out in 150 ml of absolute ethanol and the resulting precipitate dried over $CaCl_2$ under vacuum. Yields of the dicyclohexylammonium salt of monoalkyl phosphates were approximately six grams. Ion exchange of the dicyclohexylammonium salts of the monoalkyl phosphates was carried out on Dowex AG50-X10, 20-50 mesh, catron exchange resin. The resin, 1.9 meq. per ml of bed volume in water, was washed with 1N NaOH until the pH of the eluant was greater than 9. Deionized water was then used to wash the column (25 x 2cm) until the pH of the eluant was less than 7.5. The dicyclohexylammonium salts, approximately 100 meq., were dissolved in deionized water, placed on the column, and washed with deionized water. 20 ml fractions were collected and the conductance was monitored to determine fractions containing the disodium salt of the monoalkyl

phosphate. Fractions containing the monoalkyl phosphate were pooled and lyophilized. The lyophilized sample was then stored over $CaCl_2$.

Identification of the dicyclohexylammonium and disodium salts of the monoalkyl phosphates were performed using nmr, IR and descending paper chromatography. Standard monoalkyl phosphates were provided by Dr. Bruce M. Anderson, Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University. NMR spectra of the alkyl phosphates were run in deuterium oxide using TMS (tetramethylsilane) as an external standard. Since TMS was used as an external standard, the chemical shift values are only approximate. The principle use of the nmr spectra was to determine the presence of unreacted phosphorous acid and sodium phosphite. These spectra were also used to assure completeness of ion exchange of the cyclohexylammonium ion. Infrared spectra of the monoalkyl phosphates taken as KBr pellets were used to determine the presence of phosphite in the sample. Descending paper chromatography of the disodium monoalkyl phosphates was carried out on 24 cm by 48 cm Wattman #1 paper with acetone: water: ammonia hydroxide (60:35:5) as the solvent system. Standards chromatographed consisted of approximately 0.01M solutions of monobasic sodium phosphate, sodium pyrophosphate, sodium phosphite and standard monoalkyl phosphates. After allowing six hours for development, the wet chromatogram was blown dry and sprayed with Hanes and Isherwood's phosphate spray (28) consisting of 5 ml of 70% formic acid, 10 ml of 1N HCl, 25 ml of 4% W/V Ammonium Molybdate and 55 ml of deionized water. The chromatogram was then placed in an oven

at 75°C for 15 minutes and finally developed for 15 minutes under UV light.

Concentrations of aqueous solutions of the disodium salts of the monoalkyl phosphates were determined through phosphate analysis by a modification of the method of Fiske and Subbarow(27). This modification consisted of using 1% Elon (p-methylamino phenyl sulfate) in 3% sodium bisulfite as the reducing agent. The formation of molybdenum blue was measured spectrophotometrically to provide a determination for phosphate.

Inhibition Studies - Rabbit and chicken muscle L-α-glycerophosphate dehydrogenase used in the kinetic studies were prepared fresh daily in 0.05 M Tris-Cl (Tris Hydroxy methyl) amino methane chloride) Buffer, pH 7.85, and 1% bovine serum albumin. All kinetic studies were performed at ambient temperature.

The inhibition studies were carried out as a function of varying coenzyme concentration at constant inhibitor concentration. L-α-glycerophosphate dehydrogenase activity was followed by the reduction of NAD in a 3.0 ml reaction mixture containing 18 mM Tris-Cl Buffer, pH 7.85, 33 mM D,L-α-glycerophosphate, 2 g enzyme, and NAD concentrations varying from 2.87×10^{-6}M to 1.72×10^{-5}M. Concentrations of inhibitors are included in the specific descriptions of the individual experiments. Reactions were initiated by the addition of enzyme and initial velocities were obtained by measuring the linear increase in fluorescence intensity at 460 nm for one minute using 352 nm as the excitation wavelength. Inhibition of the

chicken muscle enzyme was studied at three concentrations of disodium monoalkyl phosphates, n-butyl phosphate through n-dodecyl phosphate, adenosine monophosphate, adenosine diphosphate, and adenosine diphosphoribose. However, inhibition on the rabbit muscle enzyme was studied using only the monoalkyl phosphates, n-butyl phosphate through n-dodecyl phosphate. All data obtained were plotted according to Lineweaver and Burke(17).

Determination of Inhibitor Interaction Constants

Yonetani and Theorell (29) have described a graphical method for the measurement of the inhibitor interaction constant, α, which is a measure of the interaction between two inhibitors binding to the same enzyme active site. Initial velocities were measured by the linear increase in fluorescence intensity. The reactions were measured at ambient temperature in 3.0 ml reaction mixture, containing 18 mM Tris-Cl Buffer, pH 7.85, 2 g chicken muscle L-α-glycerophosphate dehydrogenase, 33 mM D,L-α-glycerophosphate and 4.5×10^{-5}M NAD. Five different concentrations of one inhibitor with a constant concentration of a second inhibitor were used to measure initial velocities. This was repeated several times with different constant concentrations of the second inhibitor. Inhibitor pairs used were disodium n-heptyl phosphate with adenosine monophosphate, adenosine diphosphate, adenosine diphosphoribose and disodium n-undecyl phosphate. Data obtained in these experiments were plotted as the ratio of initial velocity in the absence of inhibitors (Vo)

over initial velocity in the presence of inhibitors (Vi) versus the concentration of the first inhibitor.

Preparation of 3-Aminopyridine Adenine Dinucleotide

3-Aminopyridine adenine dinucleotide (AAD) was synthesized using the method of Fisher et al. (15). 0.077 ml of ice cold bromine was dissolved in 100 ml of 0.05 M NaOH. This solution was added to 400 ml of 2.5 mM β-NAD and allowed to stir at room temperature for three hours. The pH was then adjusted to 7.0 with concentrated HCl. This neutralized reaction mixture was applied to a 3.8 x 37 cm column containing approximately 400 ml of AG-1X-8, chloride form, 200-400 mesh, anion exchange resin. The column was washed with deionized water until it was free of 260 nm absorbing material. A linear gradient (1 liter of 1N LiCl into 1 liter deionized water) was applied to the column and 5 ml fractions were collected. Optical density was monitored at 260 nm and all major peaks were chromatographed on cellulose F TLC plates. The peak corresponding to 3-aminopyridine adenine dinucleotide was pooled and lyophilized. The lyophilized sample was extracted twice with 200 ml of absolute ethanol and centrifuged at 2000 rpm for 15 minutes at 4°C. The supernant was discarded and the sample dried in vacuum over P_2O_5. Concentrations of aqueous solutions of 3-aminopyridine adenine dinucleotide were determined at 257 nm where its molar absorptivity equals $19.5 \times 10^{-3} M^{-1} cm^{-1}$.

Coenzyme Binding Studies

Rabbit and chicken muscle L-α-glycerophosphate dehydrogenase was dialyzed with two changes of buffer, in 600 ml of 0.01 M Tris-Cl Buffer, pH 7.85, for two hours at 4°C. The dialyzed enzyme was filtered through a Millipore filter and concentrated by ultrafiltration until the enzyme concentration was approximately 3.5 mg/ml based on $E_{280}^{1\%}$ of 5.15. The enzyme was titrated in a 2.0 ml volume contained in a 1.0 x 1.0 cm quartz cuvet both in the presence and absence of substrate, L-α-glycerophosphate. The reaction misture contained 3.3×10^{-4}M Tris-Cl Buffer, pH 7.85, 1.4×10^{-5}M L-α-glycerophosphate dehydrogenase and 30 mM D,L-α-glycerophosphate when titrated in the presence of substrate. The titrant ligand, adenosine monophosphate, adenosine diphosphate, adensine diphosphoribose, or disodium n-dodecyl phosphate was added with a microsyringe and the titrant ligand concentration was adjusted to about 100 - 200 times the enzyme site concentration depending on how loosely the particular ligand was bound. All titrations were performed at ambient temperatures. Protein fluorescence quenching was measured at 330 nm with 287 nm as the exciting wavelength.

Data Analysis - Both the concentration of titrant ligand and the change in fluorescence, ΔF_q, were normalized back to the original reaction mixture volume, 2.00 ml. Since the ligand bound to the enzyme reduces the contraction of free ligand, particularly at high enzyme concentration, the concentration of free ligand was calculated from Equations 2 and 3 where

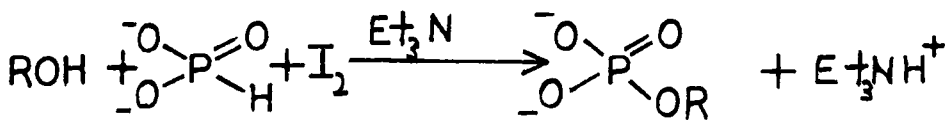
$$[\text{Ligand}]_{\text{free}} = [\text{Ligand}]_{\text{total}} - [\text{Ligand}]_{\text{bound}} \quad ; \text{ Eq. 2}$$

$$[\text{Ligand}]_{\text{free}} = [\text{Ligand}]_{\text{total}} - \frac{\Delta F}{\Delta F_{\text{sat}}} \text{ (number of sites) } [\text{Enzyme}]; \text{ Eq. 3}$$

ΔF is the change in fluorescence upon addition of titrant and ΔF_{sat} is the total fluorescence change at ligand saturation of binding sites. ΔF_{sat} is most conveniently determined by appropriate linear extrapolation of a double reciprocal plot of $1/[\text{ligand}]_{\text{total}}$ versus $1/\Delta F$. Data obtained were plotted according to the methods of Lineweaver-Burke (17), Hill (18), Klotz (19) and Scatchard (20).

RESULTS

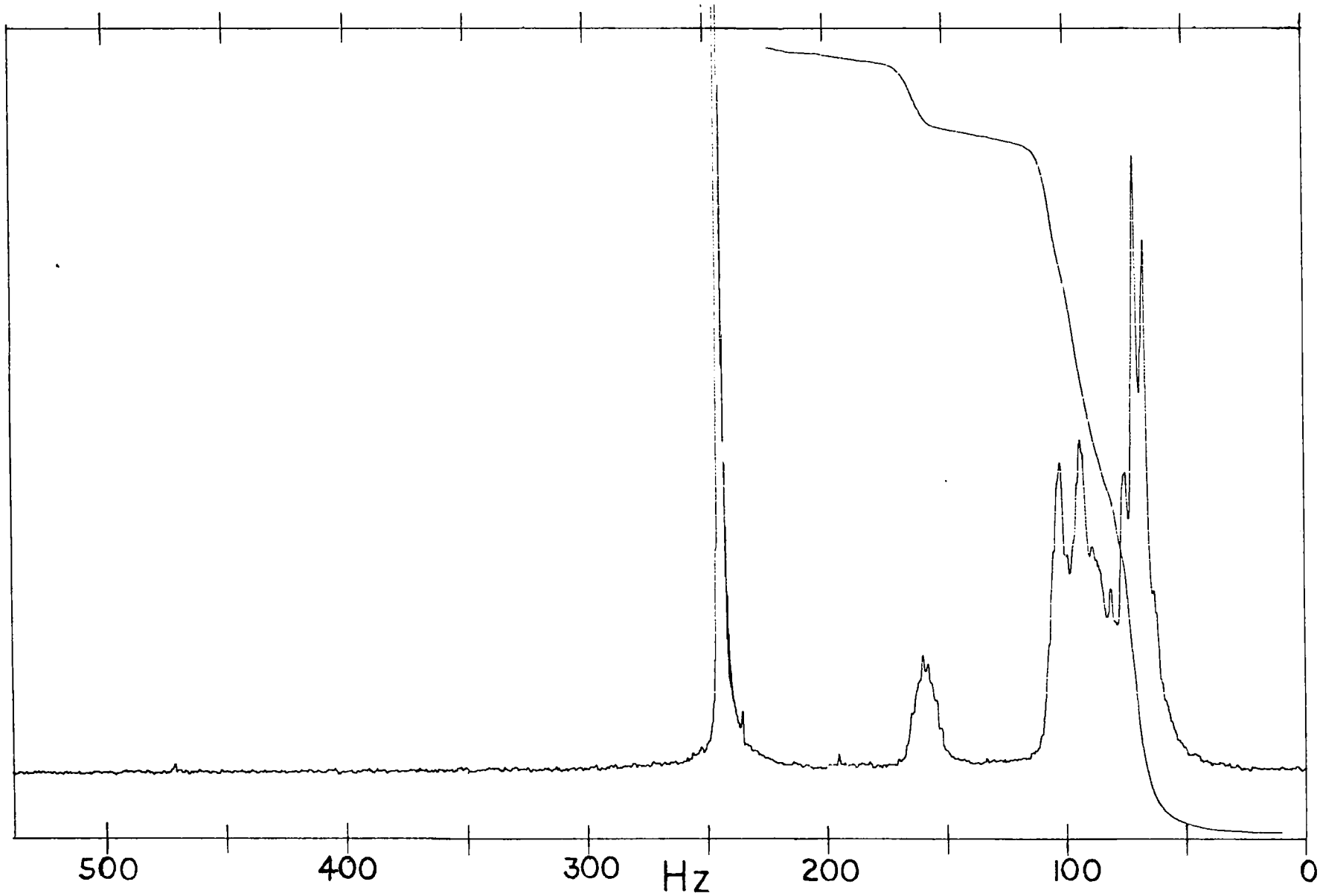
Dicyclohexylammonium salts of monoalkyl phosphate, n-butyl phosphate through n-dodecyl phosphate were synthesized by the procedure of Kirby (24) with minor modifications. The oxidation of phosphite anion by iodine in the alcohol solvent, shown in Equation 4 where R equals n-butyl, n-pentyl, n-hexyl, n-heptyl, n-octyl, n-nonyl, n-decly, n-undecyl, and n-dodecyl, respectively, gives quantitative yields of the corresponding monoalkyl phosphate.

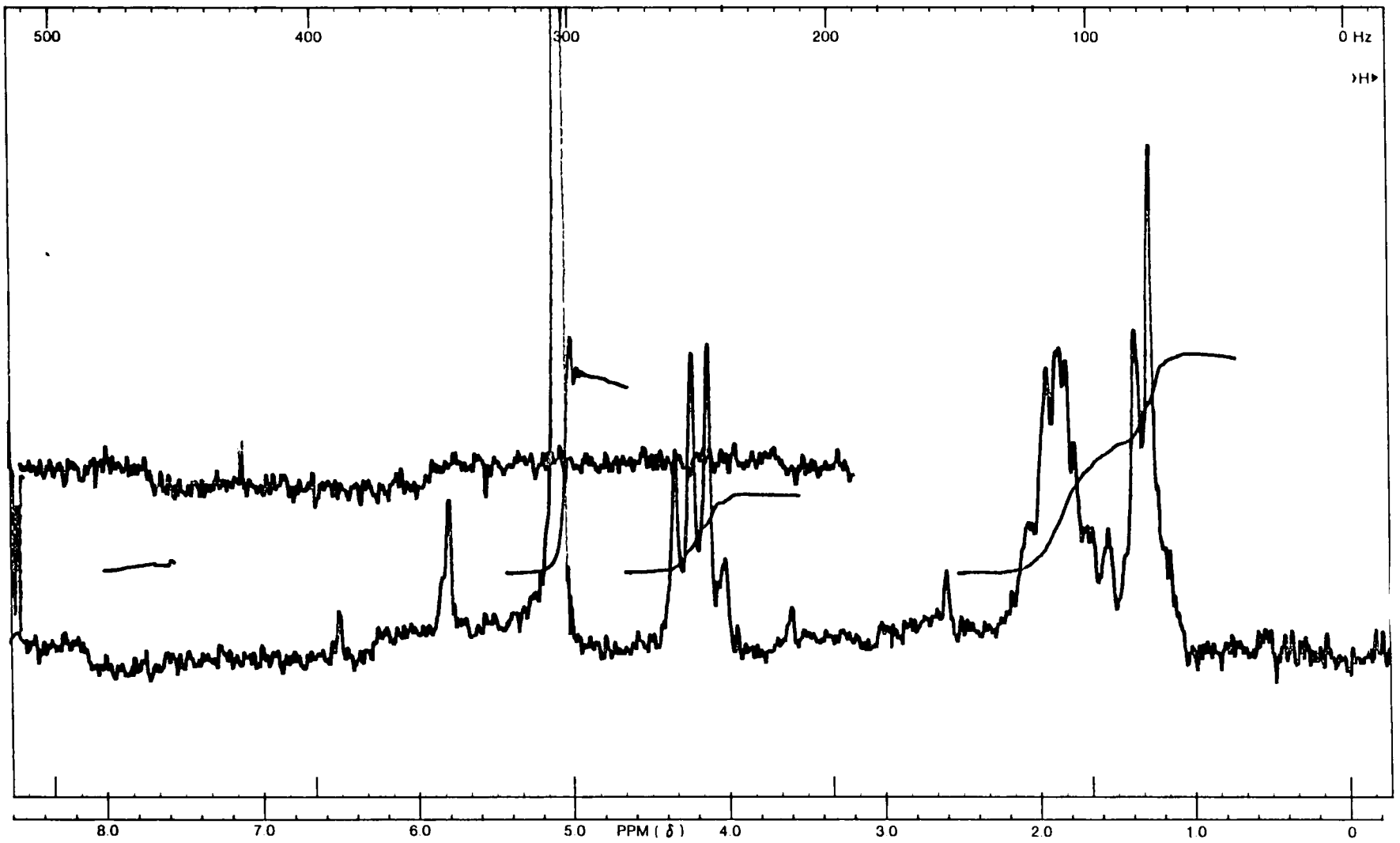


; Eq. 4

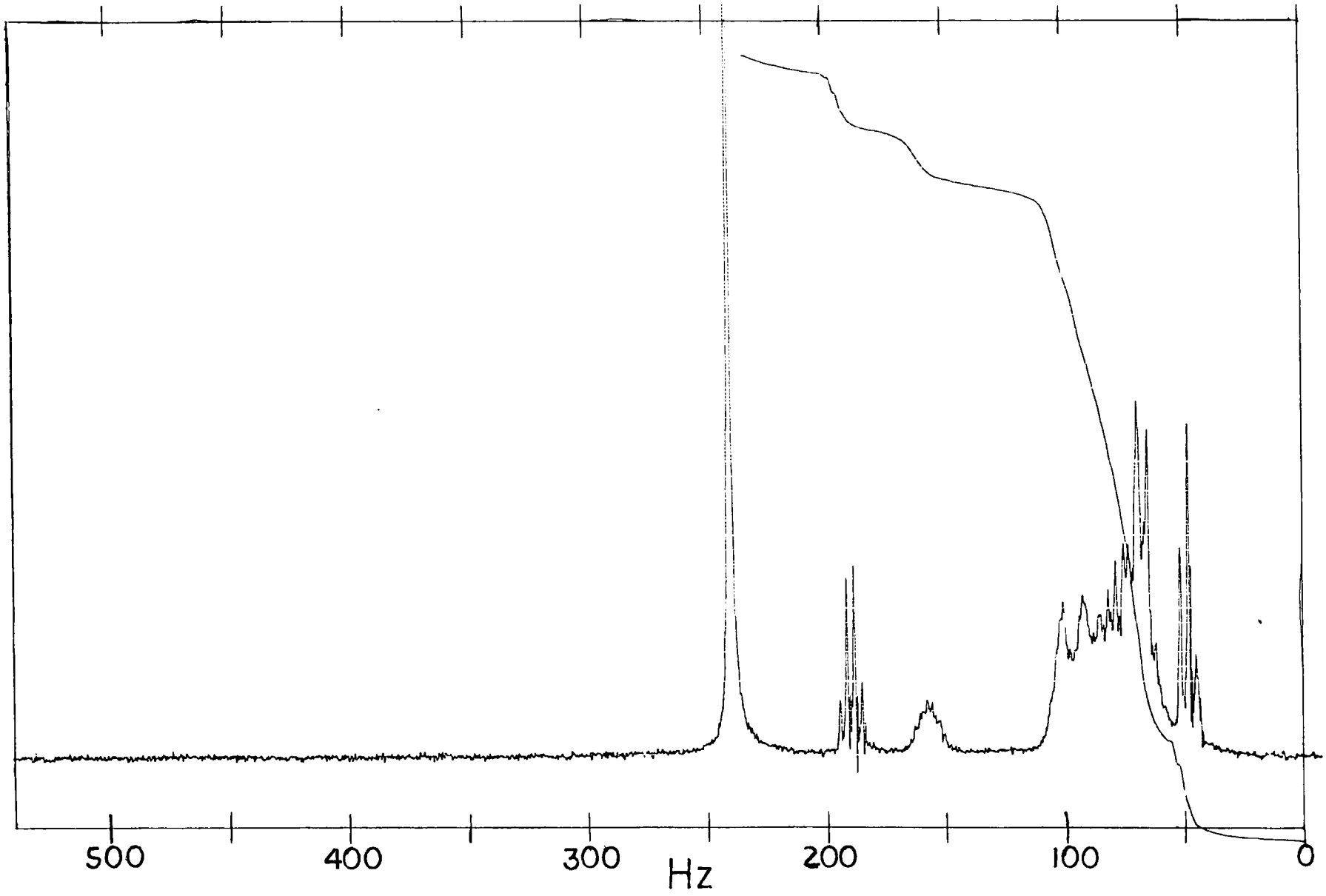
The major side products, triethylammonium iodide and excess alcohol were removed by gravity filtration and acetone washing.

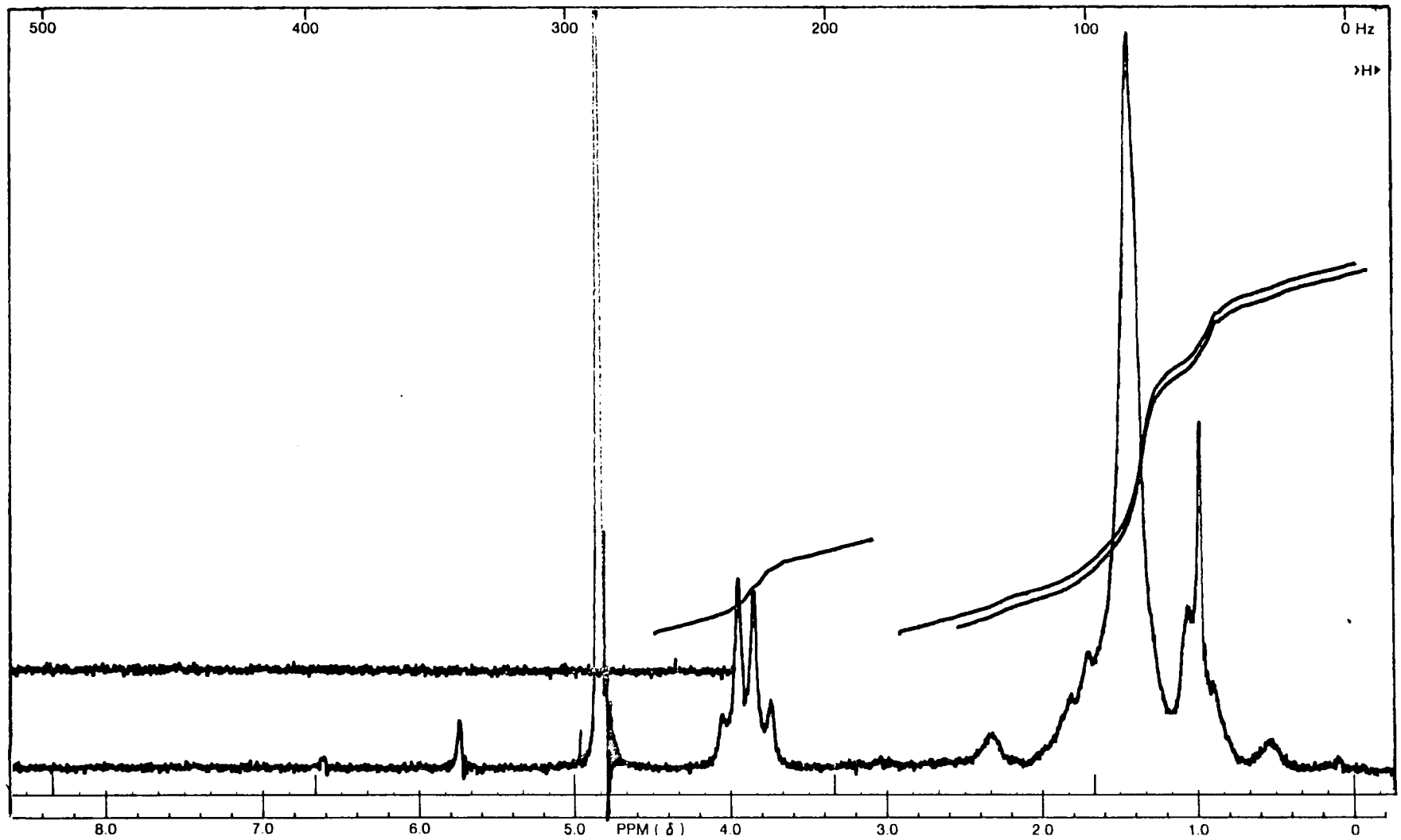
Successful synthesis of the dicyclohexylammonium salt of the monoalkylphosphates was dependent on drying the alcohols overnight over anhydrous sodium sulfate. The percentage yields after two recrystallizations from absolute ethanol were approximately 50%. Identification of the monoalkyl phosphates were based on nmr, IR, and descending paper chromatography. Comparison of the nmr spectra of known dicyclohexylammonium n-butyl phosphate, Figure 2, with the disodium salt of n-butyl phosphate, Figure 3, synthesized using a modification of the Kirby procedure (24), showed that, except for the absence of the cyclohexyl group at a chemical shift of approximately 3.5 in the disodium salt, the spectra are identical.

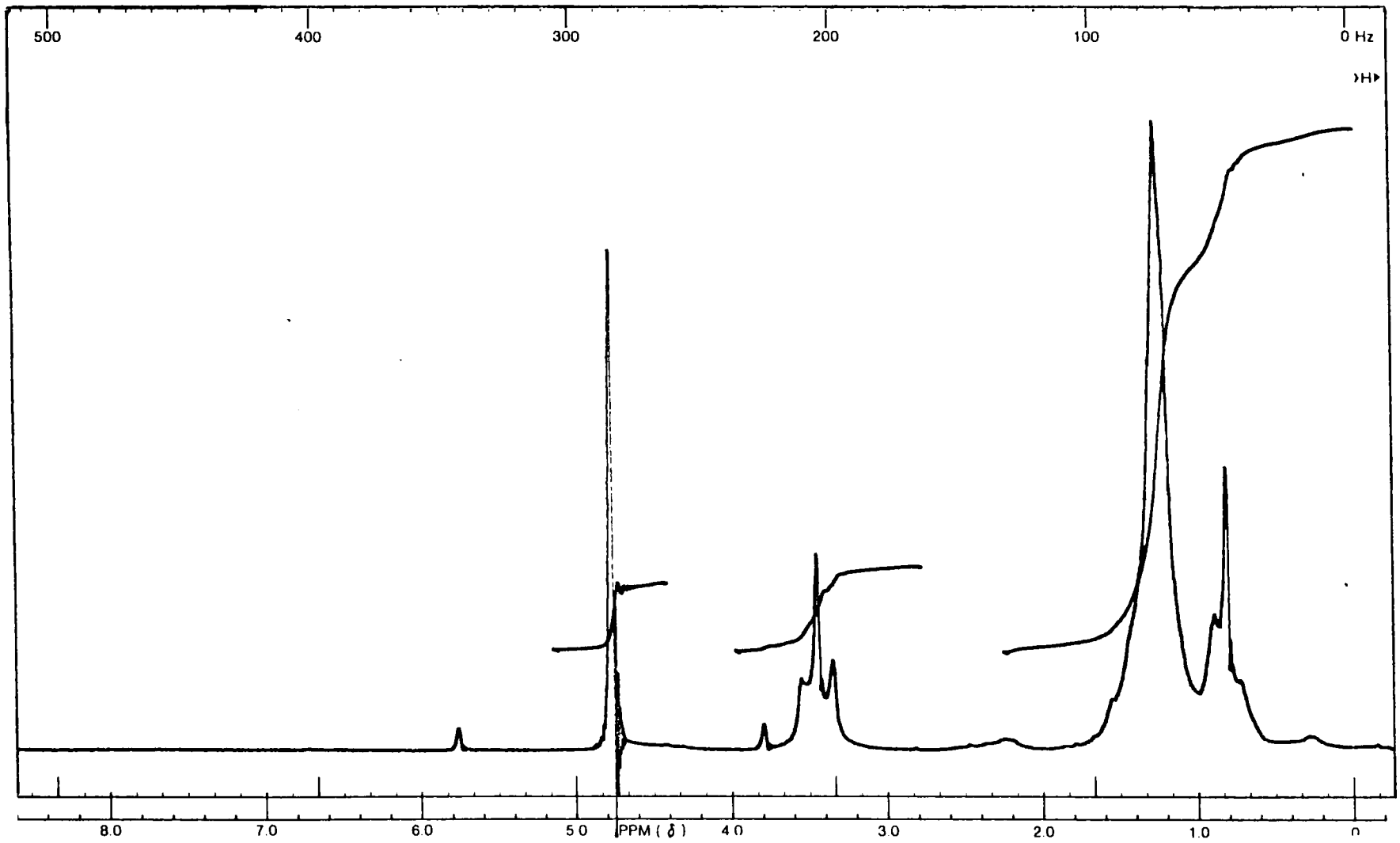

500
400
300
Hz
200
100
0


500
400
300
200
100
0 Hz
8.0
7.0
6.0
5.0
PPM (δ)
4.0
3.0
2.0
1.0
0

This evidence along with descending paper chromatography of the monoalkyl phosphates using inorganic phosphates, phosphites, pyrophosphates and known monoalkyl phosphates was used to confirm the identification of these compounds. However, it was found that complete chromatographic separation of the shorter chain monoalkyl phosphates from the inorganic phosphates could not be obtained. Longer chain monoalkyl phosphates gave better separation from the inorganic phosphates. Known monoalkyl phosphates, characterized by C, H, and P analysis, and nmr, were found to have identical R_f values to the corresponding monoalkyl phosphates synthesized by the Kirby procedure (24) with minor modifications. Chain lengths where known monoalkyl phosphates were not available were characterized through comparison of the nmr spectra of the monoalkyl phosphate and its starting alcohol. The nmr spectra of the dicyclohexylammonium salt of n-heptyl phosphate, Figure 4, and the corresponding disodium salt, Figure 5, were compared with the spectrum of n-heptyl alcohol, Figure 6. These spectra demonstrated that the hydrocarbon chain of the alcohol was unmodified in the monoalkyl phosphate with one exception. The methylene group adjacent to the hydroxyl group in the alcohol was split into a triplet whereas the same methylene group adjacent to the phosphate was found slightly farther downfield and split into a quartet. This was expected since phosphorus also has a magnetic quantum number, I, of one half and would also be expected to split the methylene protons (30). Spectra, shown in Figures 4 and 5,


500
400
300
Hz
200
100
0

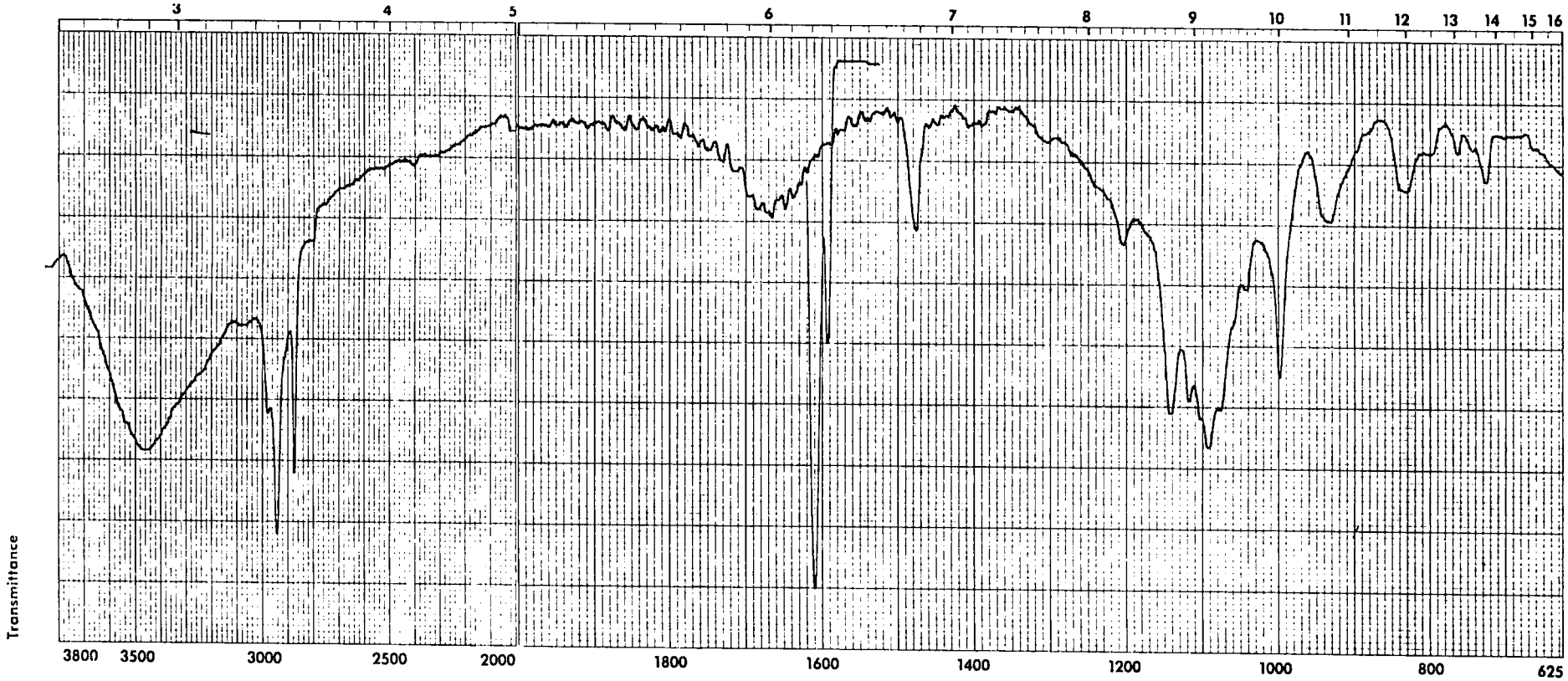

500
400
300
200
100
0 Hz
8.0
7.0
6.0
5.0
PPM (δ)
4.0
3.0
2.0
1.0
0


500
400
300
200
100
0 Hz
›H‹
8.0
7.0
6.0
5.0
PPM (δ)
4.0
3.0
2.0
1.0
0

were used to determine the completeness of ion exchange of the cyclohexylammonium ion for the sodium ion The cyclohexyl group, Figure 4, was found at a chemical shift of approximately 3.2.

Low solubility of the long chain monoalkyl phosphates in the common deuterated solvents used for nmr work made it extremely difficult to get concentrations of the sample high enough to obtain reasonable spectra. Infrared spectra of the long chain alkyl phosphates were used for identification of these compounds. The infrared spectrum of disodium n-dodecyl phosphate is shown in Figure 7.

Inorganic phosphites were found to be possible contaminates of the monoalkyl phosphates when the reaction did not proceed to completion due to the presence of water in the reaction mixture. All three techniques, nmr, IR, and descending paper chromatography were used to determine if any inorganic phosphites contaminated the monoalkyl phosphates. Since phosphites contain a P-H bond and phosphorus has a magnetic quantum number of one half the presences of a P-H bond would lead to a doublet in the nmr spectrum (30). The coupling constant, J, of this doublet is equal to 460 cps (30). This large J value can not be attributed to anything other than a P-H bond. The P-H bond also has a characteristic infrared absorption at 2450 cm^{-1}. The absence of an absorption at this frequency indicated that the monoalkyl phosphates were free of contaminating inorganic phosphites.


3
4
5
6
7
8
9
10
11
12
13
14
15
16
Transmittance
3800
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
625

Inhibition Studies on Rabbit and Chicken Muscle L-α-Glycerophosphate Dehydrogenase Using Disodium n-Monoalkyl Phosphates - Disodium salts of the monoalkyl phosphates, n-butyl phosphate through n-dodecyl phosphate were found to be effective inhibitors of the L-α-glycerophosphate dehydrogenase - catalyzed oxidation of L-α-glycerophosphate. Inhibition obtained with the monoalkyl phosphates, when studied as a function of varying NAD concentration, was observed to be competitive with respect to NAD in every case. Initial velocity measurements made in the presence of disodium n-nonyl phosphate, plotted according to Lineweaver and Burk (17), for the chicken muscle L-α-glycerophosphate dehydrogenase, are shown in Figure 8. The competitive nature of the inhibition caused by disodium n-nonyl phosphate is representative of the relationship observed in the inhibition of all monoalkyl phosphates on both forms, chicken and rabbit muscle, L-α-glycerophosphate dehydrogenase. Inhibitor dissociation constants for each of the nine disodium monoalkyl phosphates were calculated from independent experiments and plotted according to the method in Figure 8. The inhibitor dissociation constants obtained on the chicken and rabbit muscle L-α-glycerophosphate dehydrogenase are listed in Table 2. As the alkyl chain length of the monoalkyl phosphates was increased, the inhibitor dissociation constants were found to decrease. This chain length effect on the inhibition by disodium monoalkyl phosphates is shown in Figure 9.

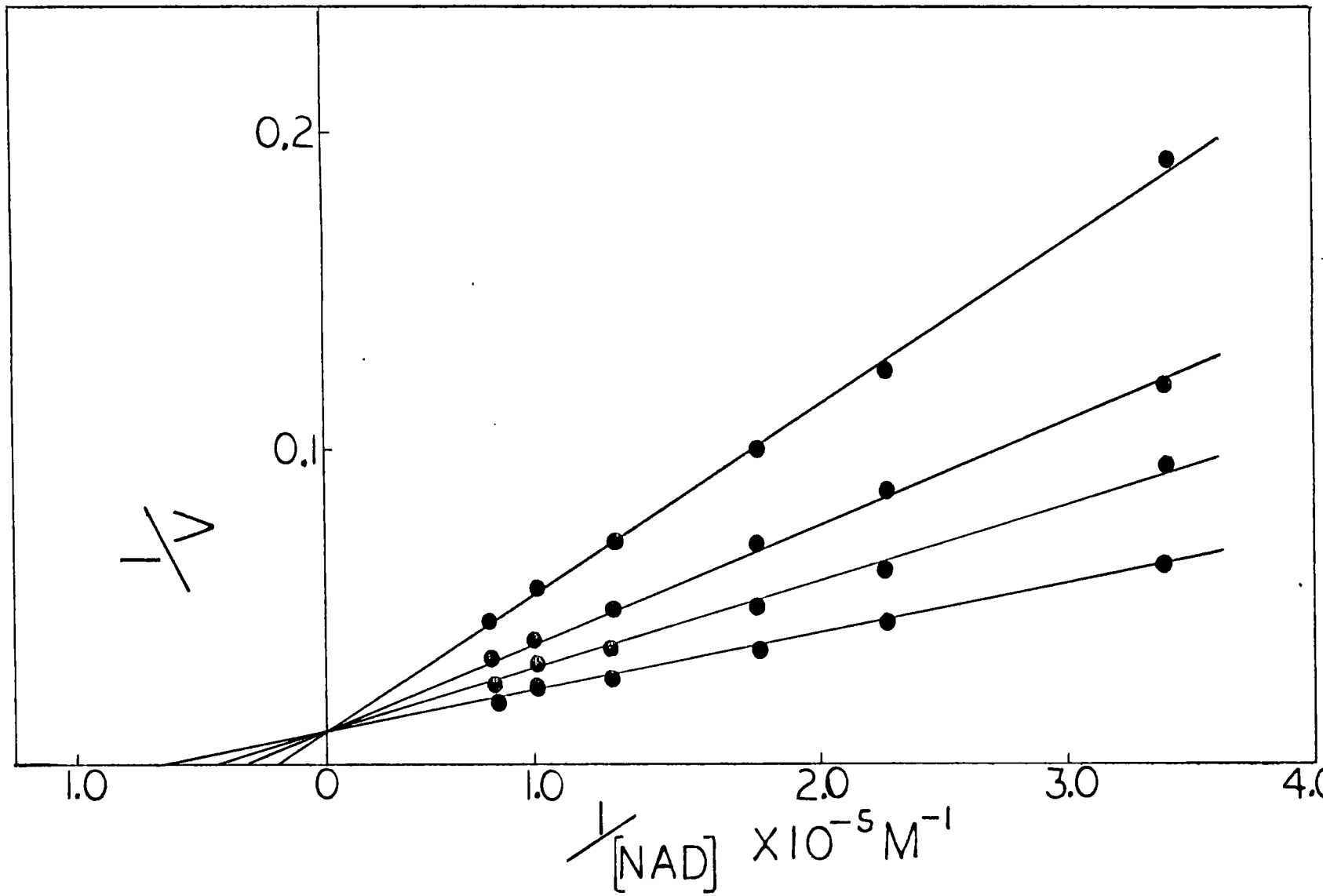
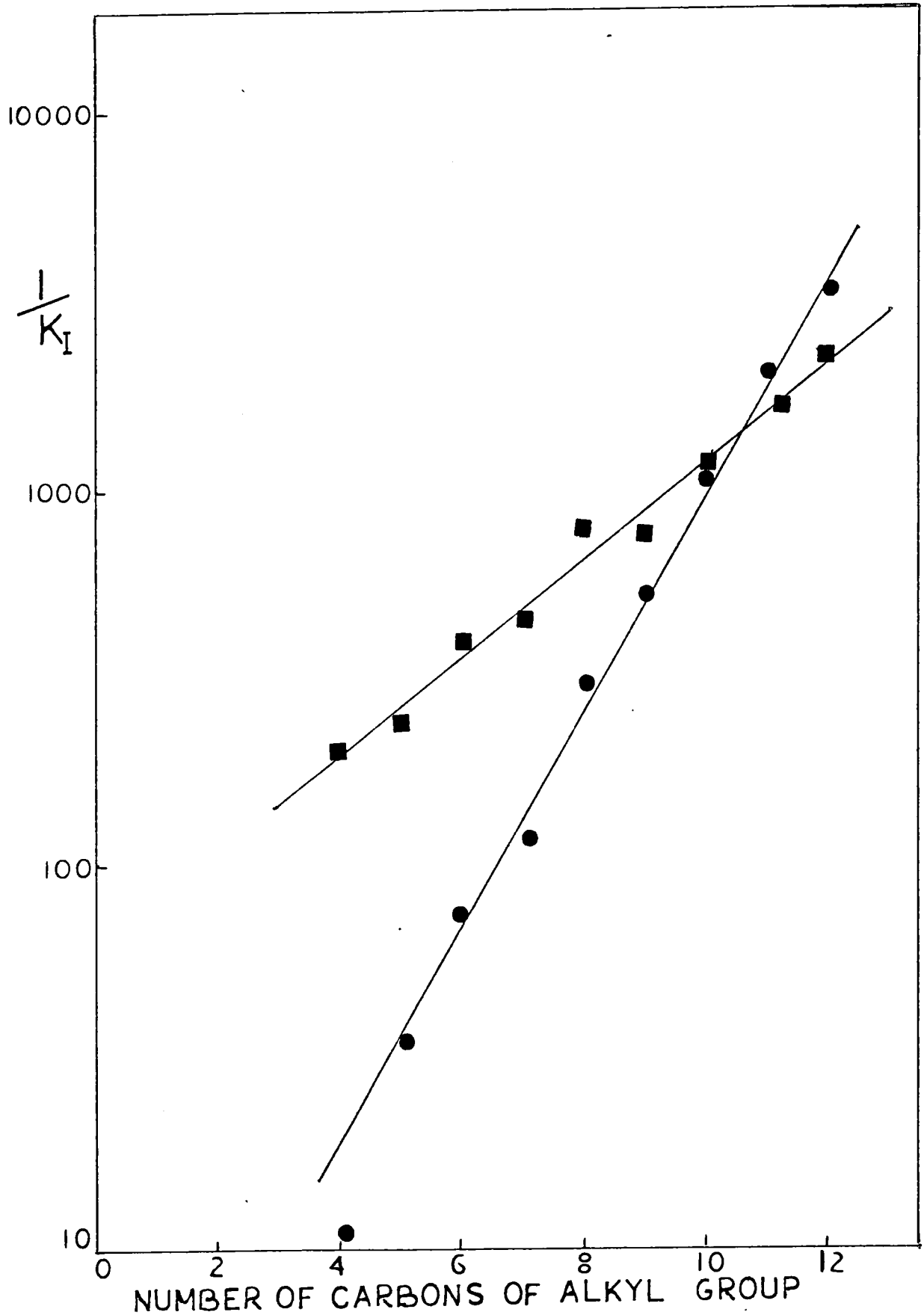

0.2
0.1
1/v
1.0
0
1.0
2.0
3.0
4.0
1/[NAD] X10⁻⁵ M⁻¹

TABLE 2

Inhibition of Chicken and Rabbit Muscle L-α-Glycerophosphate Dehydrogenase by Disodium Monoalkyl Phosphates.

Disodium Monoalkyl Phosphate	K_I (M)	
	Rabbit Muscle	Chicken Muscle
n-butyl phosphate	9.9×10^{-2}	4.6×10^{-3}
n-pentyl phosphate	2.7×10^{-2}	4.2×10^{-3}
n-hexyl phosphate	1.2×10^{-2}	2.4×10^{-3}
n-heptyl phosphate	8.0×10^{-3}	2.1×10^{-3}
n-octyl phosphate	3.1×10^{-3}	1.4×10^{-3}
n-nonyl phosphate	1.8×10^{-3}	1.3×10^{-3}
n-decyl phosphate	9.2×10^{-4}	9.1×10^{-4}
n-undecyl phosphate	4.6×10^{-4}	5.9×10^{-4}
n-dodecyl phosphate	2.8×10^{-4}	4.2×10^{-4}


10000
1000
100
10
1/K_I
0
2
4
6
8
10
12
NUMBER OF CARBONS OF ALKYL GROUP

Inhibition by Adenosine Derivatives on Chicken Muscle, L-α-Glycerophosphate Dehydrogenase - The use of adenosine derivatives as inhibitors in the α-glycerophosphate dehydrogenase - catalyzed oxidation of L-α-glycerophosphate was studied with adenosine monophosphate, adenosine diphosphate, and adenosine diphosphoribose. Each inhibitor was studied in an individual experiment as a function of varying NAD concentration. The inhibition obtained with these adenosine derivatives was found to be competitive with respect to the coenzyme, NAD. Initial velocity measurements made in the presence of adenosine diphosphoribose, plotted according to Lineweaver-Burk (17) and shown in Figure 10, exemplified the competitive inhibition obtained with adenine derivatives.

Inhibitor dissociation constants obtained for the adenosine derivatives, adenosine monophosphate, adenosine diphosphate, and adenosine diphosphoribose are shown in Table 3.

Multiple Inhibition Studies on Chicken Muscle L-α-Glycerophosphate Dehydrogenase - The possibility of simultaneous binding of two inhibitors, which are structurally analogous to different portions of the NAD molecule, was investigated using multiple inhibition analysis previously described by Yonetani and Theorell (29). Inhibition resulting from the presence of two competitive inhibitors, I_1, and I_2 was expressed by these authors in the following equation:

$$\frac{1}{V_i} = \frac{1}{V_m} + \frac{K_m}{sV_m}\left(1 + \frac{i_2}{K_{EI_2}}\right) + \frac{K_m}{sV_mK_{EI_1}}\left(1 + \frac{i_2}{K_{EI_2}}\right) i_1$$

Where i and i_2 equal the concentrations of I_1 and I_2, K_{EI_1} and K_{EI_2} represent the inhibitor dissociation constants for I_1 and I_2, respectively.

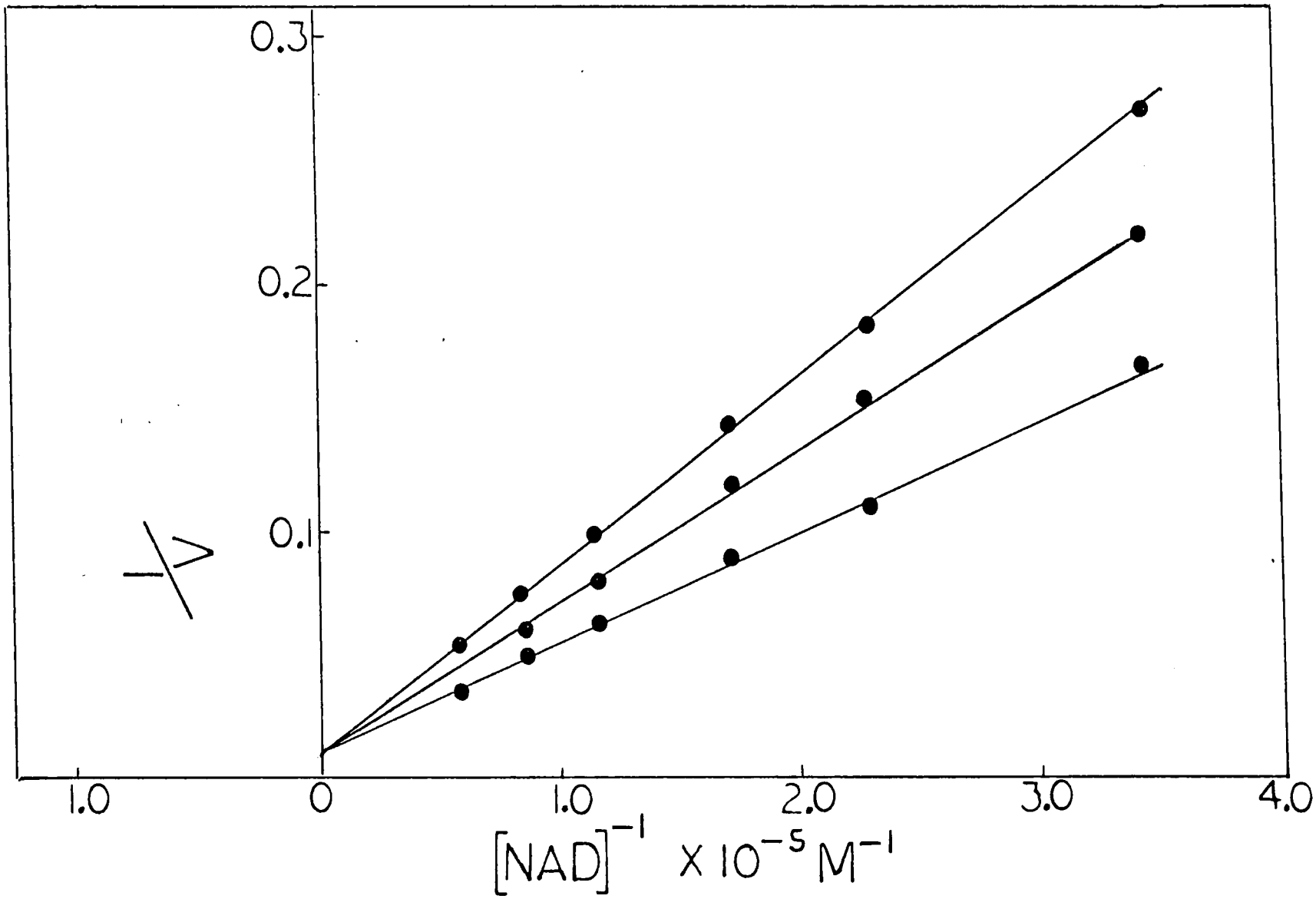
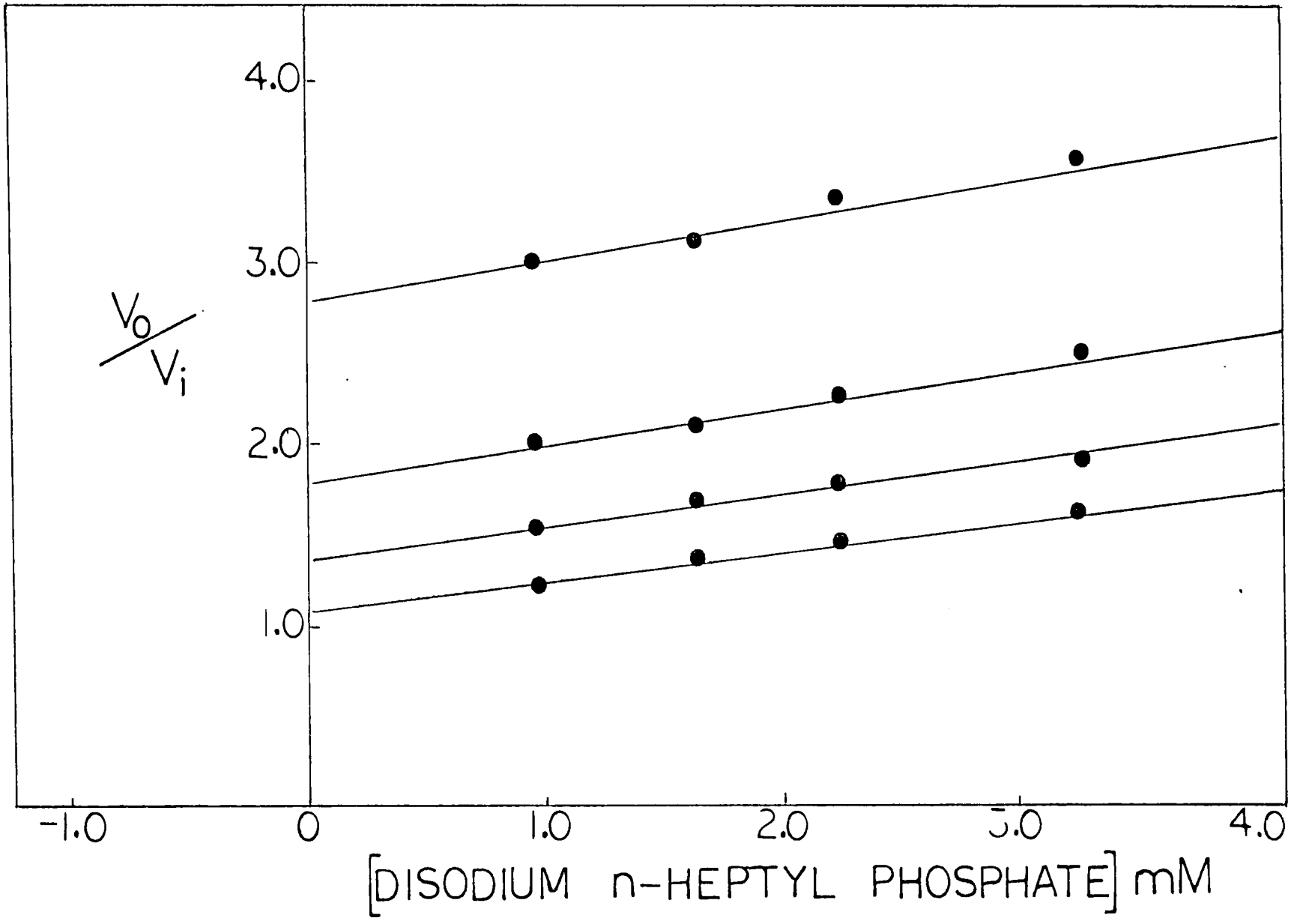

0.3
0.2
0.1
1/v
1.0
0
1.0
2.0
3.0
4.0
[NAD]$^{-1}$ X 10^{-5} M^{-1}

TABLE 3

Inhibition by Adenosine Derivatives on Chicken Muscle L-∝-Glycerophosphate Dehydrogenase.

Adenosine derivative	K_I (M)
Adenosine monophosphate	4.4×10^{-3}
Adenosine diphosphate	1.34×10^{-3}
Adenosine diphosphoribose	4.5×10^{-4}

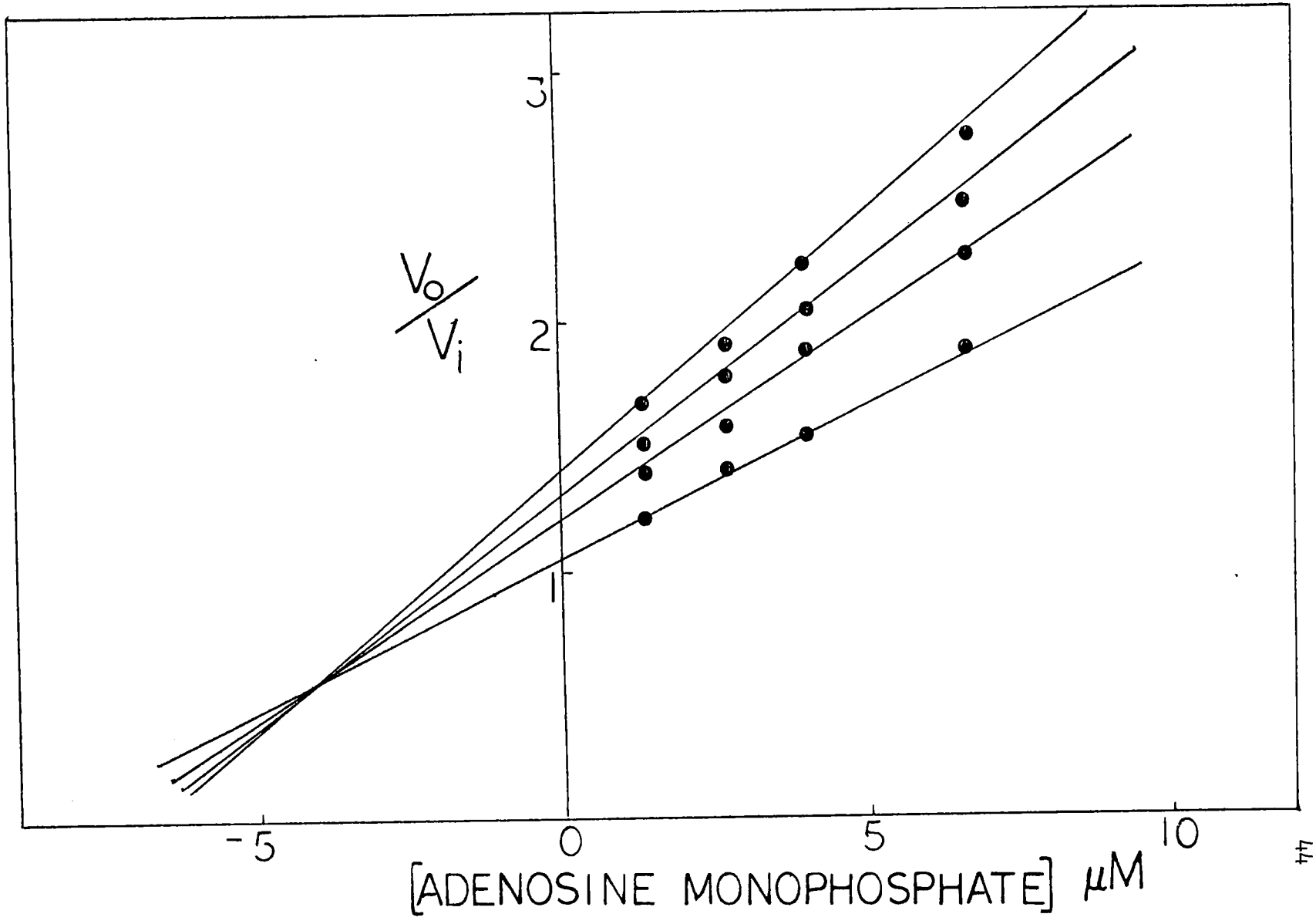
α is defined as an interaction constant (29) which is a measure of the interactions existing between I_1, and I_2 in the EI_1I_2 complex. A plot of $1/V_i$ versus i_1 at different fixed concentrations of I_2 yields a series of straight lines that intersect at an abscissa value of $-\alpha K_{EI_1}$. Using the value of K_{EI_1}, determined from inhibitor studies of I_1 alone, the interaction constant, α, can be calculated. The interaction constant can also be determined if one plots the ratio of the initial velocity in the absence of inhibitors (V_o) to the initial velocity in the presence of inhibitors (V_i) against the concentration of I_1. Inhibitor pairs used were disodium n-heptyl phosphate with adenosine monophosphate, adenosine diphosphate, adenosine diphosphoribose, and disodium n-undecyl phosphate. The multiple inhibition observed with the inhibitor pair disodium n-heptyl phosphate and adenosine diphosphoribose, plotted as the ratio, V_o/V_i against disodium n-heptyl phosphate concentration, resulted in a series of parallel lines (Figure 11). Each of the four lines was generated from five reaction mixtures containing varying disodium n-heptyl phosphate concentrations at a constant concentration of adenosine diphosphoribose. The concentration of disodium n-heptyl phosphate was varied from zero to 3.84×10^{-3}M and the concentration of adenosine diphosphoribose used were: line 1, zero; line 2, 1.75×10^{-4}M; line 3, 3.93×10^{-4}M; line 4, 6.11×10^{-4}M. A parallel line relationship was also observed in the multiple inhibition of L-α-glycerophosphate dehydrogenase with inhibitor pairs composed of


4.0
3.0
2.0
1.0
V_o / V_i
-1.0
0
1.0
2.0
3.0
4.0
[DISODIUM n-HEPTYL PHOSPHATE] mM

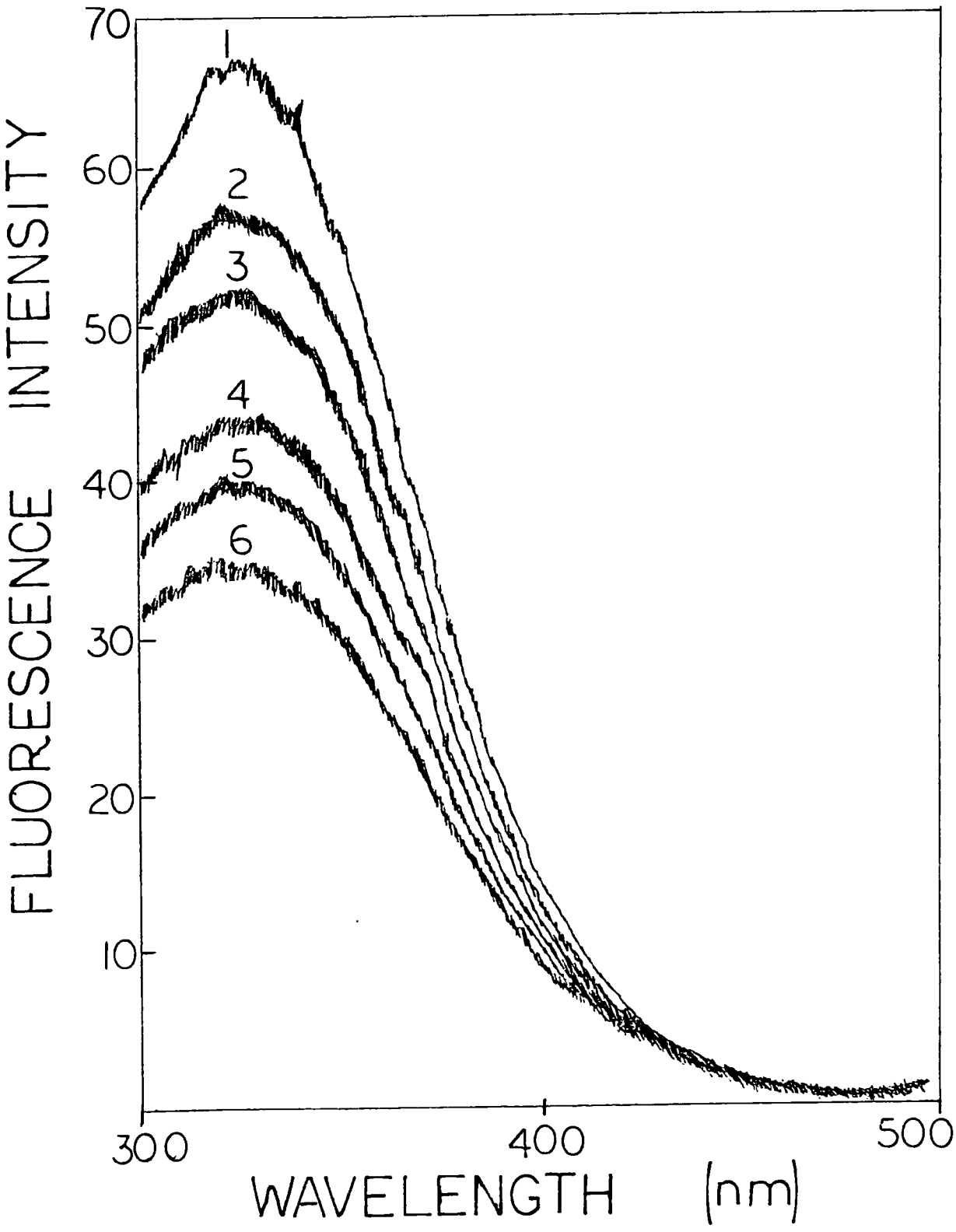
disodium n-heptyl phosphate and adenosine diphosphate, and disodium n-heptyl phosphate and disodium n-undecyl phosphate.

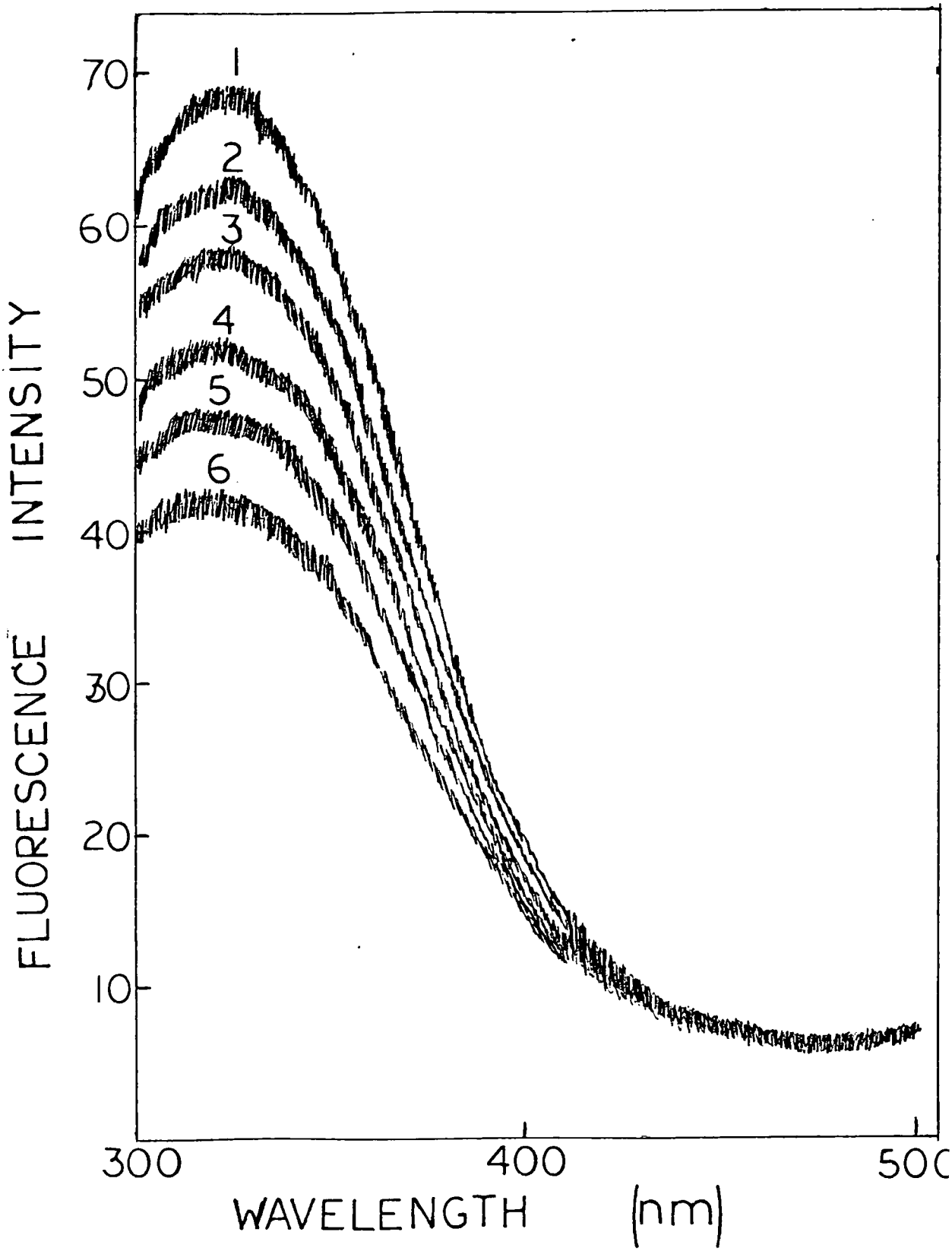
Multiple inhibition by inhibitor pairs, in many cases, results in a converging line relationship. This converging line relationship was observed in the multiple inhibition of L-α-glycerophosphate dehydrogenase by the inhibitor pair, disodium n-heptyl phosphate and adenosine monophosphate. The concentration of adenosine monophosphate used varied from zero to 8.16×10^{-3}M and the concentrations of disodium n-heptyl phosphate used were as follows: line 1, zero; line 2, 1.60×10^{-3}M; line 3, 2.24×10^{-3}M; line 4, 3.84×10^{-3}M. In Figure 12, the lines converge at a point equal to $-\alpha K_I$ for adenosine monophosphate. The interaction constant, α, obtained by dividing the value of $-\alpha K_{I_1}$ by the known K_{I_1} value for adenosine monophosphate was 0.98. Inhibitor interaction constants calculated from the multiple inhibition studies using the various inhibitor pairs are listed in Table 4.

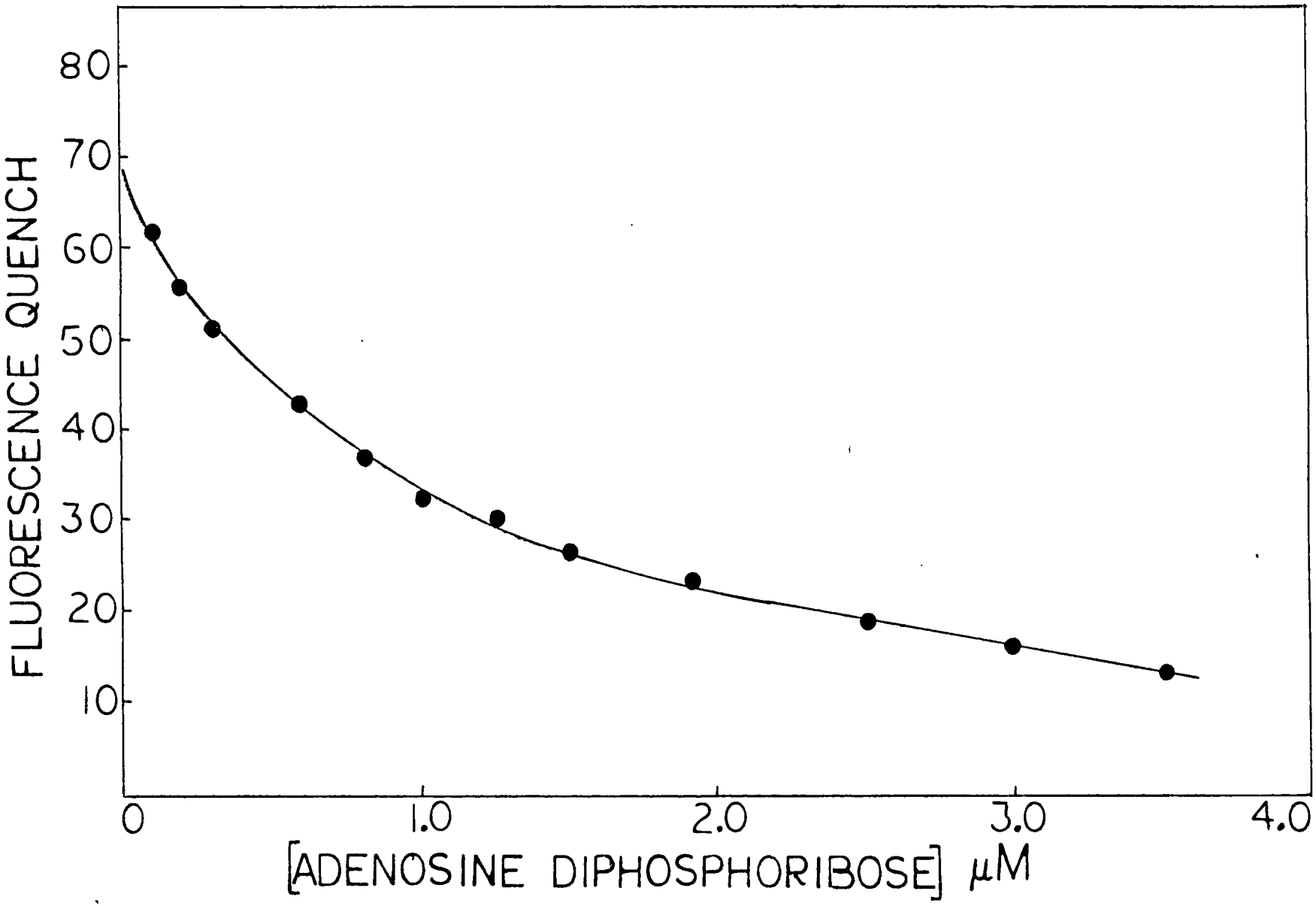
Coenzyme Binding Studies - The binding of competitive inhibitors to the coenzyme binding site of rabbit and chicken muscle L-α-glycerophosphate dehydrogenase was studied using the technique of fluorescence titration. The coenzyme competitive inhibitors used in these binding studies were 3-aminopyridine adenine dinucleotide, adenosine diphosphoribose, adenosine monophosphate and disodium n-dodecyl phosphate.


3
2
1
V_o/V_i
-5
0
5
10
[ADENOSINE MONOPHOSPHATE] μM

Fluorescence titration spectra showing changes in the fluorescence as a function of the amount of adenosine diphosphoribose bound by chicken muscle L-α-glycerophosphate dehydrogenase and the same in the presence of saturating α-glycerophosphate are shown in Figures 13 and 14, respectively. Spectra were recorded from 300 nm to 600 nm with excitation at 287 nm. Concentrations of adenosine diphosphoribose used were: line 1, zero; line 2, 4.3×10^{-8}M; line 3, 8.7×10^{-8}M; line 4, 1.7×10^{-7}M; line 5, 2.6×10^{-7}M; line 6, 3.9×10^{-7}M. There is an isoemissive point at about 480 nm for the enzyme adenosine diphosphoribose complex which shifts to about 460 nm in the enzyme adenosine diphosphoribose α-glycerophosphate complex. The existence of an isoemissive point in the system of α-glycerophosphate dehydrogenase and stoichiometrically bound coenzyme competitive inhibitor was proof that the quantum yields of the two components are independent of their proportions (31). Therefore, occurrence of isoemissive points in these spectra suggests a relatively simple change in the fluor environment and the formation of a single fluorometrically distinquishable species with ligand binding. Protein fluorescence quench signals are, therefore, useful probes in the study of ligand binding by the enzyme. Fluorescence was measured at 330 nm with excitation at 287 nm. Figure 15 shows the fluorescence titration of 1.87×10^{-5}M L-α-glycerophosphate with adenosine diphosphoribose plotted as fluorescence quench (F_q) versus the concentration of


FLUORESCENCE INTENSITY
70
60
50
40
30
20
10
1
2
3
4
5
6
300
400
500
WAVELENGTH (nm)


70
60
50
40
30
20
10
1
2
3
4
5
6
FLUORESCENCE INTENSITY
300
400
500
WAVELENGTH (nm)


FLUORESCENCE QUENCH
80
70
60
50
40
30
20
10
0
1.0
2.0
3.0
4.0
[ADENOSINE DIPHOSPHORIBOSE] μM

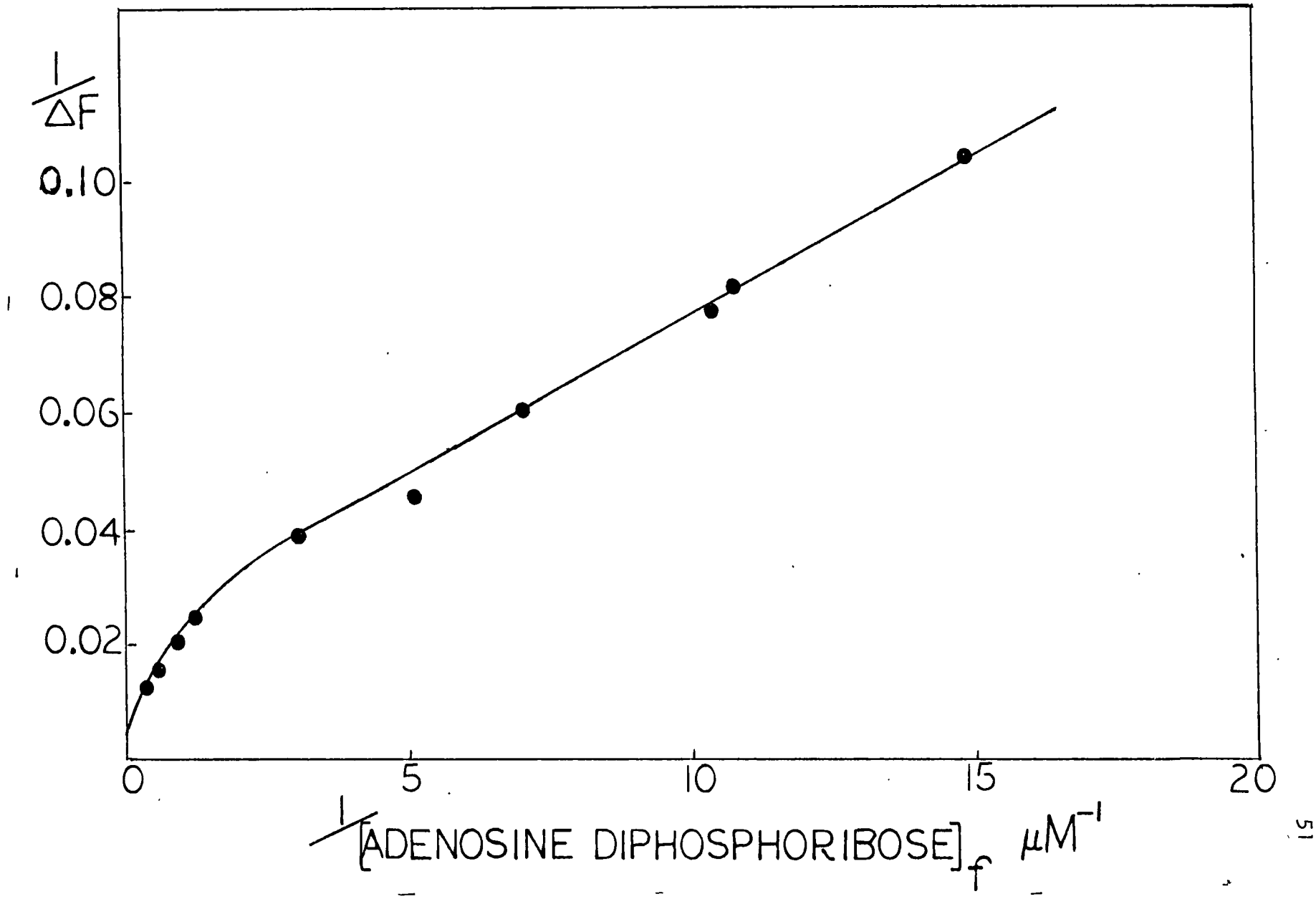
adenosine diphosphoribose. The change in fluorescence upon the addition of a known increment of ligand was used to calculate the free and bound concentrations of ligand with the use of Equations 2 and 3. Several graphical methods have been developed to determine the type of cooperativity that is observed upon the binding of a second ligand molecule to a protein molecule already having a bound ligand. The simplest graphical method used, for determining cooperativity, was the double reciprocal plot (32) expressed by Equation 5 where $\bar{\upsilon}$ is the average number of ligand molecules bound to the enzyme molecule which is also proportional to the inverse of the change in fluorescence, $1/\Delta_F$, n the total number of ligand molecules capable of binding to one enzyme molecule, K_a the association constant for the binding of the ligand to the protein molecule and $[A]$ the concentration of free ligand.

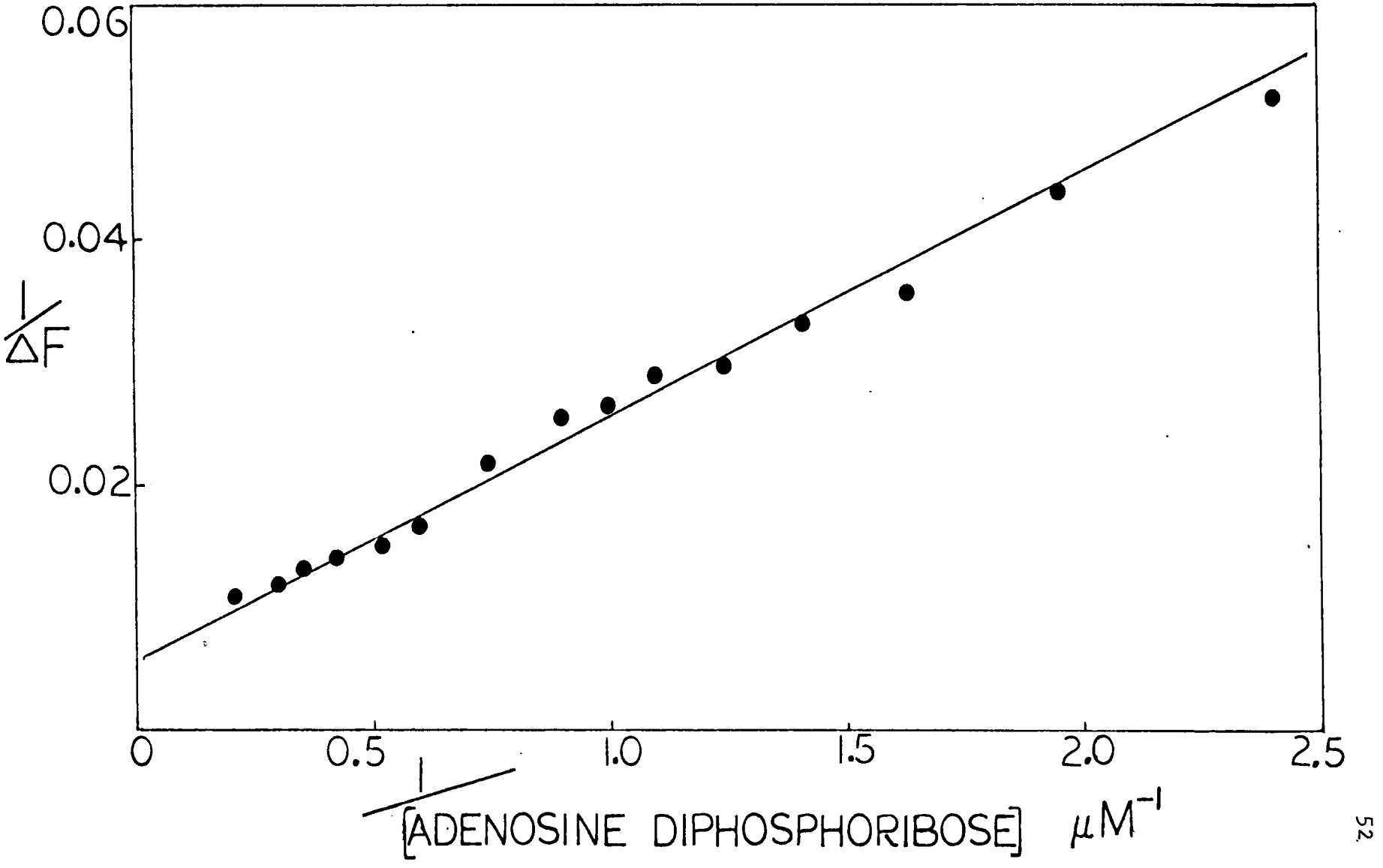
$$\frac{1}{\bar{\upsilon}} = \frac{1}{n} + \frac{1}{nK_a\,[A]} \quad ; \quad \text{Eq. 5}$$

The titration data of L-α-glycerophosphate dehydrogenase titrated with adenosine diphosphoribose both in the absence and presence of substrate, α-glycerophosphate, is shown in Figures 16 and 17, respectively.

Scatchard (20) employed a different type of plot, for detecting cooperative interactions, based on Equation 6, a modification of Equation 5.

$$\frac{\bar{\upsilon}}{A_f} = K_a\,(n - \bar{\upsilon}) \quad ; \quad \text{Eq. 6}$$


1/ΔF
0.10
0.08
0.06
0.04
0.02
0
5
10
15
20
1/[ADENOSINE DIPHOSPHORIBOSE]f μM⁻¹

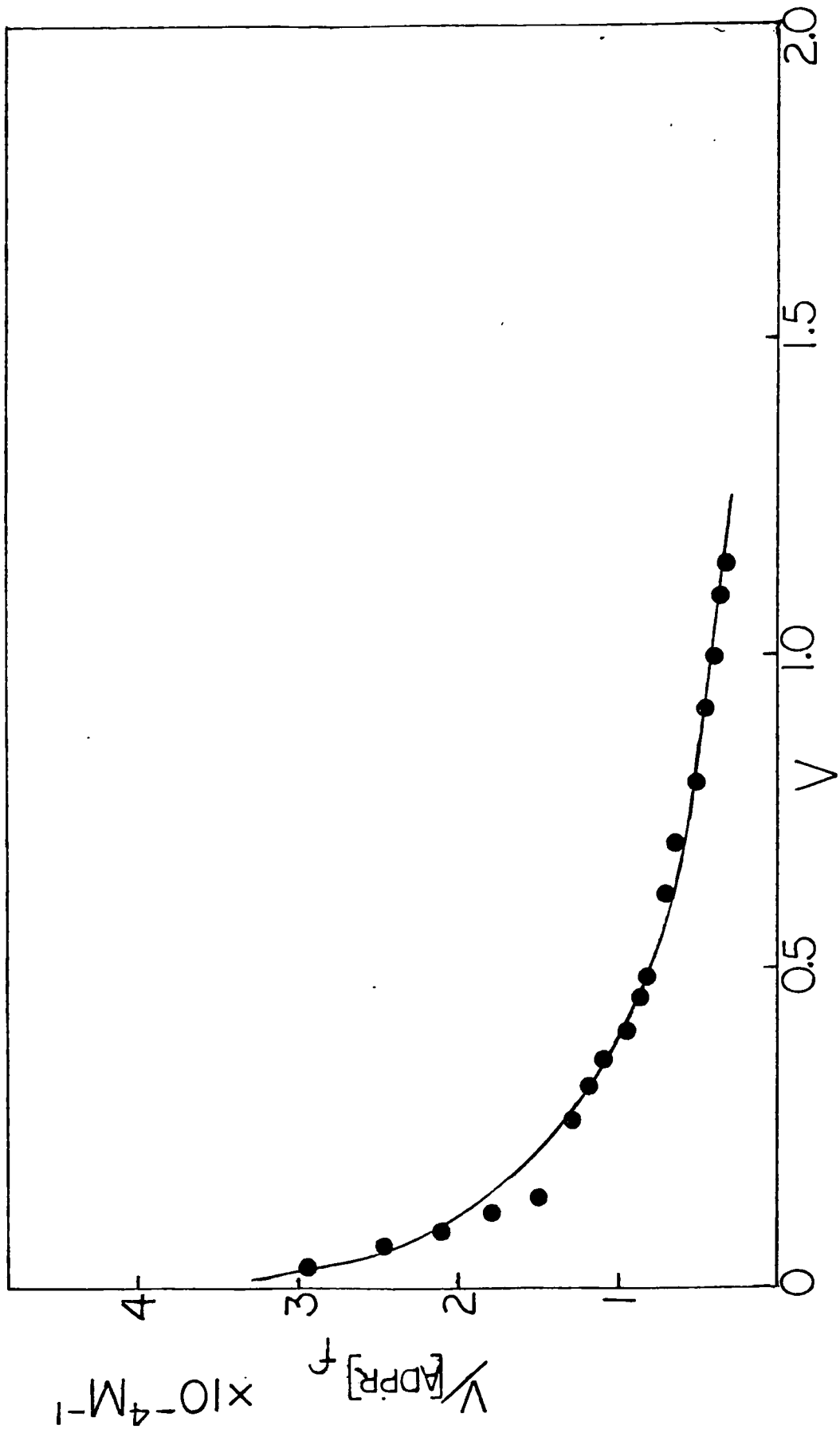

0.06
0.04
0.02
1/ΔF
0
0.5
1.0
1.5
2.0
2.5
1/[ADENOSINE DIPHOSPHORIBOSE] μM⁻¹

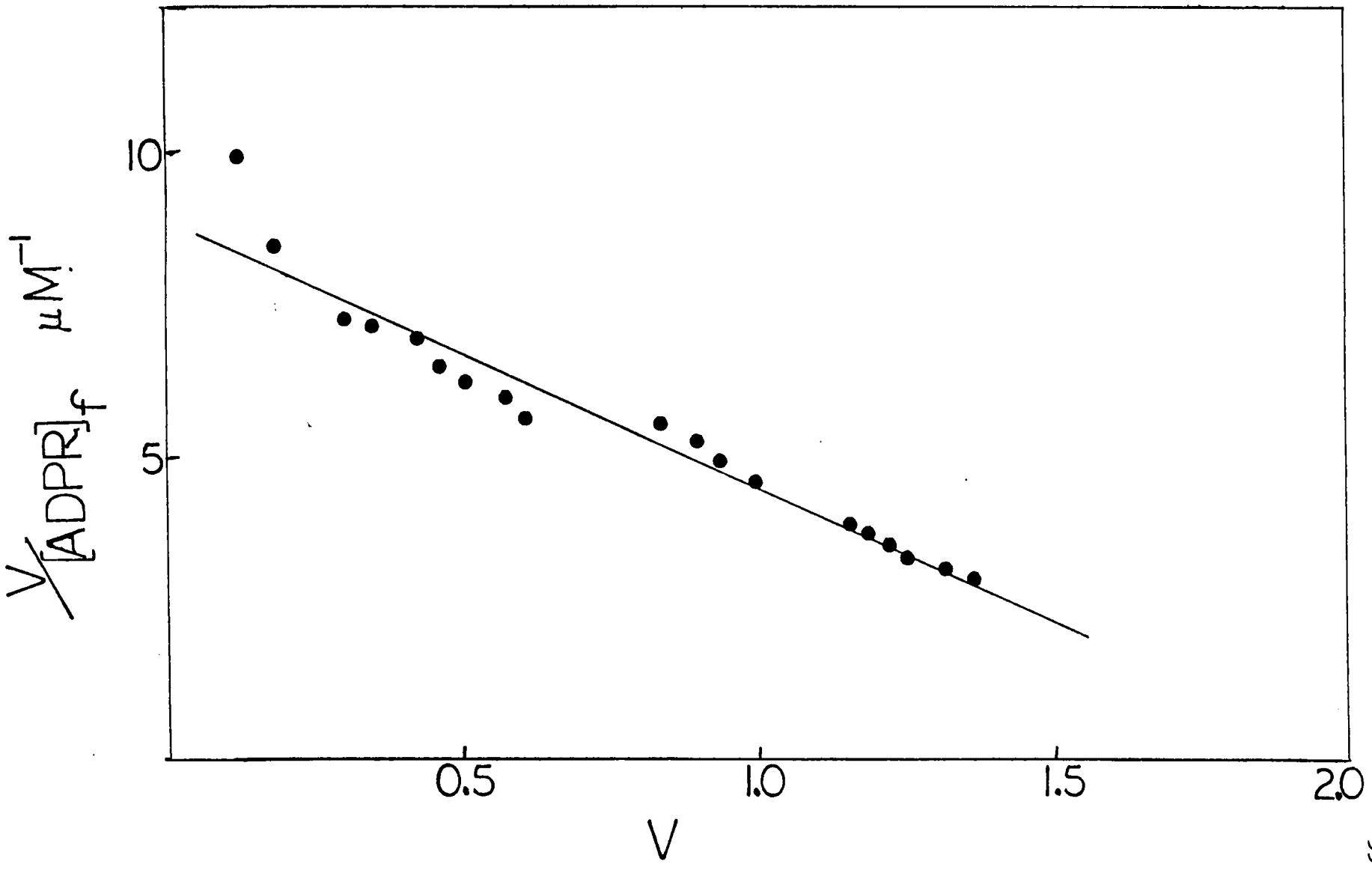
Equation 6 could also be written as shown in Equation 7 where $[S]_b$ equals the concentration of bound substrate, $[S]_f$ the concentration of free substrate, $[E]_t$ the total enzyme concentration, K_b the binding constant, and n the total number of ligand molecules capable of binding to one enzyme molecule.

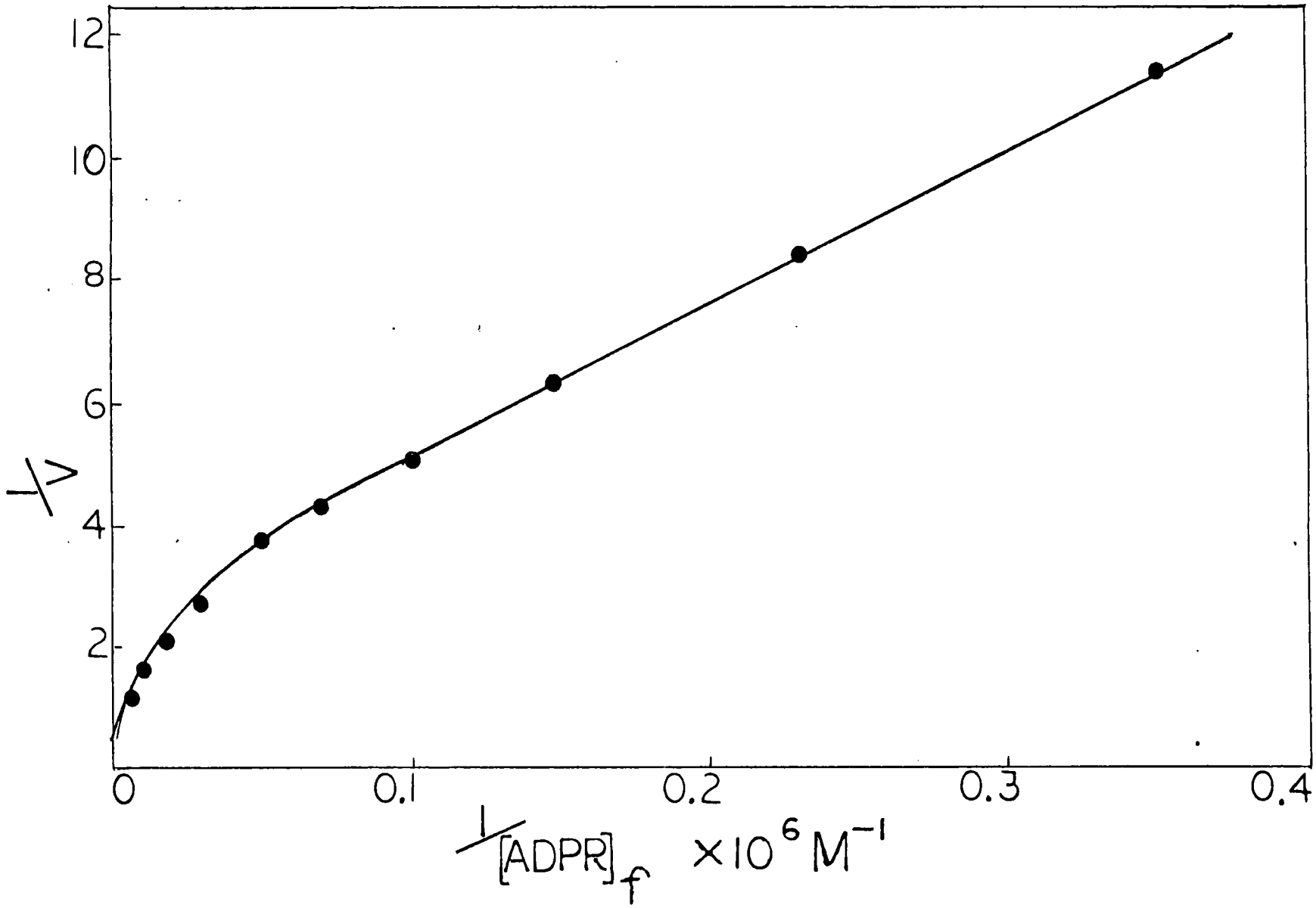
$$\frac{[S]_b}{[S]_f \; E_t} = -\frac{1}{K_b} \quad \frac{[S]_b}{[E]_t} + \frac{N}{K_b} \; ; \text{ Eq. 7}$$

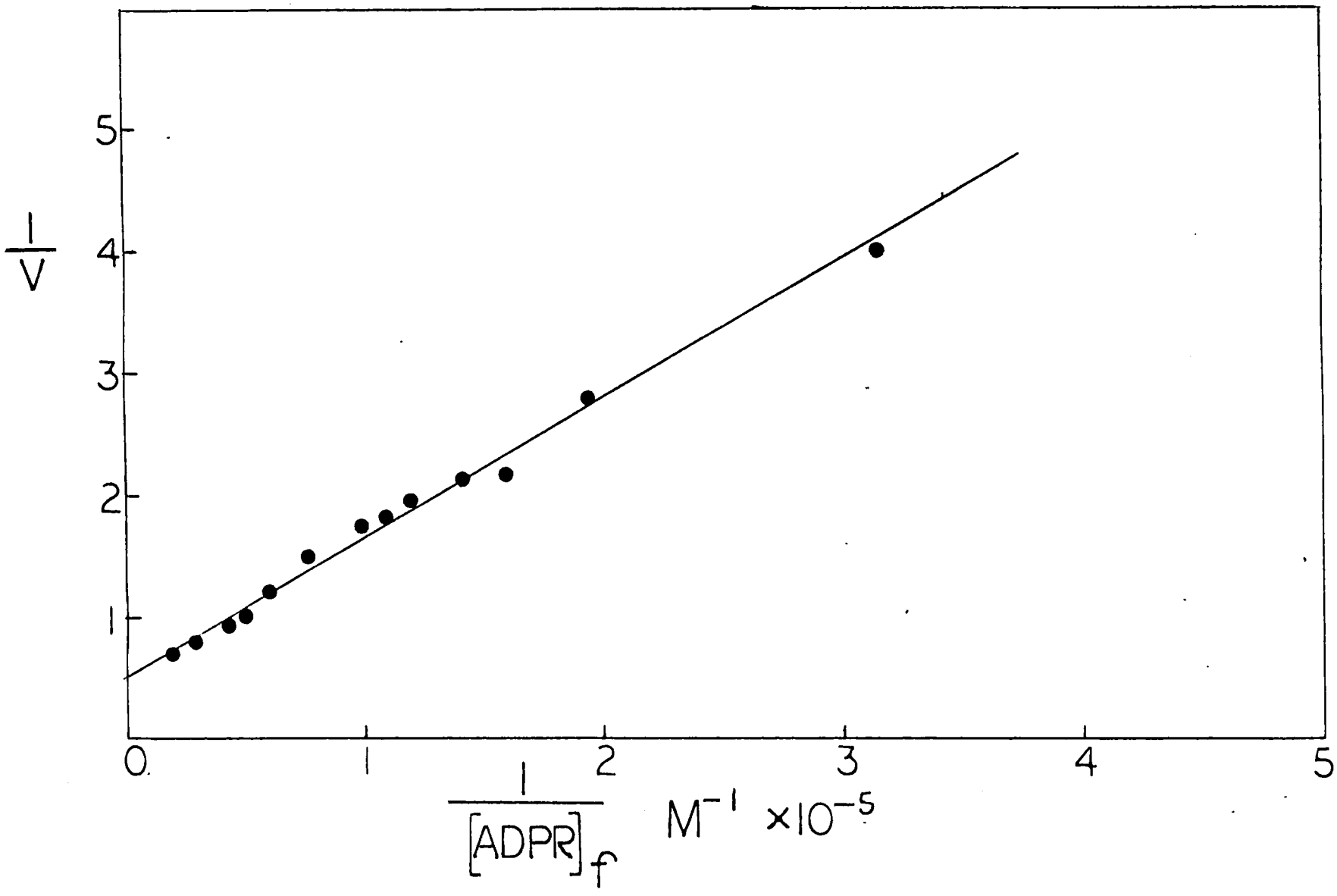
A plot of $\bar{\upsilon}/[A]_f$ as the ordinate against $\bar{\upsilon}$ as the abscissa gives n on the intercept of the absicca axis and $K_a n$ on the intercept of the ordinate axis. Data plotted according to Equation 7 for the titration of α-glycerophosphate dehydrogenase with adenosine diphosphoribose both in the presence and absence of substrate are shown in Figures 18 and 19, respectively. Dissociation constants for the binding of adenosine diphosphoribose in the absence of substrate were found to be 2.2×10^{-4}M and 4.5×10^{-5}M with extrapolation to 1.8 sites per enzyme dimer. In the presence of substrate, a linear Scatchard plot was obtained with a dissociation constant equaling 1.0×10^{-3}M. Through extrapolation 2.0 sites per enzyme dimer were found. Data obtained from the titration of α-glycerophosphate dehydrogenase with adenosine diphosphoribose was also plotted, Figures 20 and 21, according to the method of Klotz (19) shown in Equation 8.

$$\frac{1}{[S]_b} = \frac{Ks}{n\,[E]_t} \quad \frac{1}{[S]_f} + \frac{1}{n\,[E]_t} \; ; \text{ Eq. 8}$$


V/[ADPR]f
x10⁻⁴M⁻¹
4
3
2
1
0
0.5
1.0
1.5
2.0
V


10
5
$\frac{V}{[ADPR]_f}$ μM^{-1}
0.5
1.0
1.5
2.0
V


12
10
8
6
4
2
1/v
0
0.1
0.2
0.3
0.4
1/[ADPR]f ×10^6 M^-1


1/V
5
4
3
2
1
0
1
2
3
4
5
1/[ADPR]f
M⁻¹ ×10⁻⁵

The intercept on the ordinate axis gives 1/n and the slope K_d/n where K_d is the dissociation constant for the binding of the ligand to enzyme. Dissociation constants in the absence of substrate were found to be 1.8×10^{-4}M and 1.3×10^{-5}M and in the presence of substrate 1.5×10^{-3}M. Total number of sites per enzyme dimer, n, was found to be 2.0 both in the presence and absence of substrate.

The type of cooperativity between ∝-glycerophosphate dehydrogenase and adenosine diphosphoribose was also determined using the Hill plot (18). The Hill equation relates the saturation function, $\bar{Y}$, to substrate concentration, S, through Equation 9 where n_H, the Hill coefficient, is the slope of the Hill plot measured where $\log \bar{Y}/(1-\bar{Y}) = 0$ or $\bar{Y} = 0.5\ (1-\bar{Y})$.

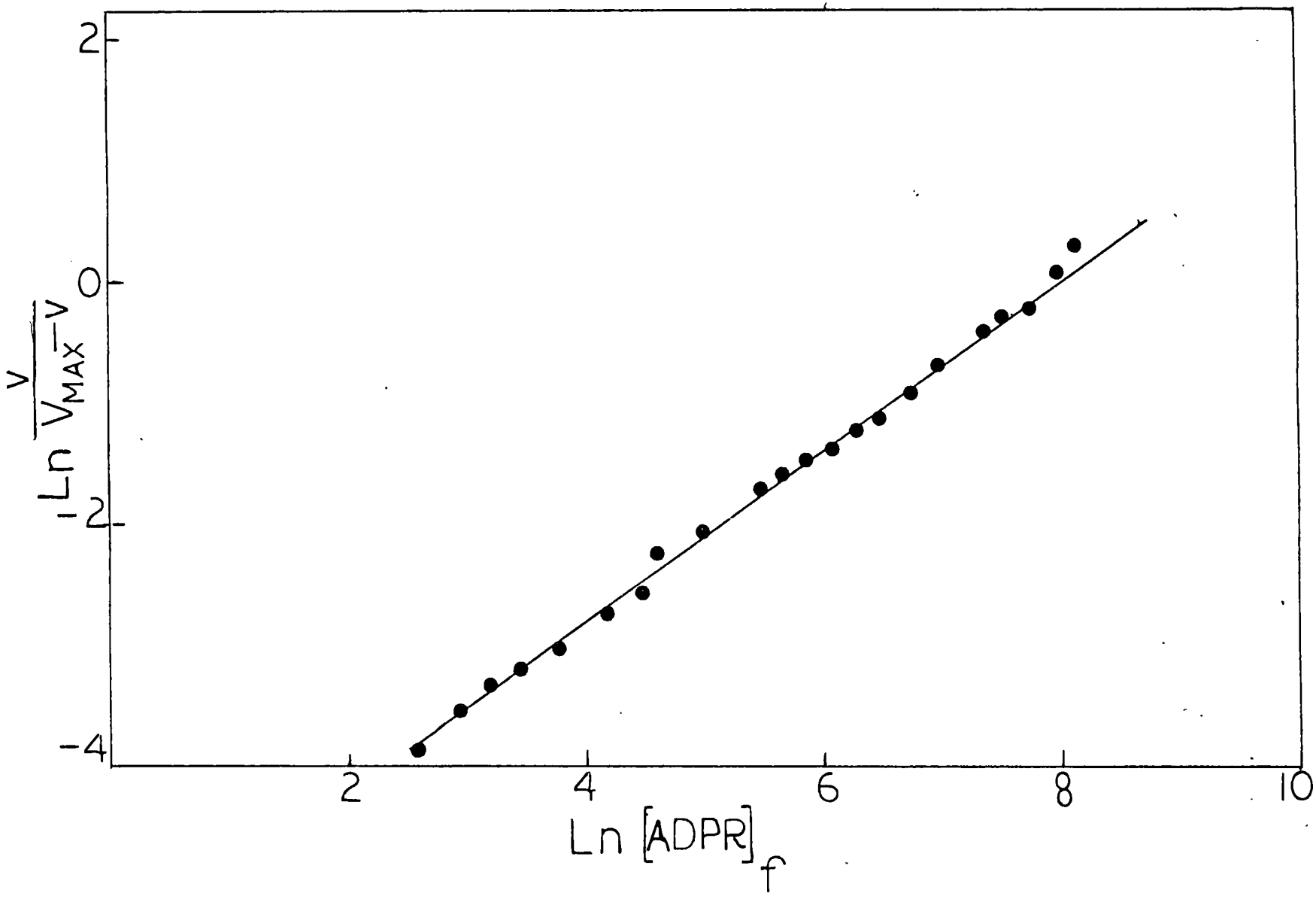
$$n_H = \frac{d \log \left[\bar{Y}/\ (1-\bar{Y})\right]}{d \log S} \quad ; \quad \text{Eq. 9}$$

The linear form of the Hill equation, shown in Equation 10, was used to plot the titration date of ∝-glycerophosphate dehydrogenase with adenosine diphosphoribose where $\log \bar{Y}/\ (1-\bar{Y})$ is equal to $\log \frac{[S]_b}{\text{Enzyme}_{\text{free}}}$ and K^1 a constant comprising the interaction constants and the intrinsic dissociation constant.

$$\text{Log} \frac{\bar{Y}}{(1-\bar{Y})} = n \log [S]_{free} - \text{Log } K^1 ; \text{ Eq. 10}$$

Figures 22 and 23 show the Hill plot for ∝-glycerophosphate dehydrogenase titrated with ADP-ribose in the absence and presence of substrate, respectively. Hill coefficients in the absence and presence of ∝-glycerophosphate were found to be 0.69 and 1.00, respectively.

Chicken muscle L-∝-glycerophosphate dehydrogenase was also titrated with 3-aminopyridine adenine dinucleotide, adenosine monophosphate and disodium n-dodecyl phosphate both in the absence and presence of ∝-glycerophosphate. Table 5 gives the dissociation constants, calculated from the Scatchard and Klotz Plots, and the Hill coefficient for these ligands in the absence of substrate and Table 6 for the same in the presence of substrate. Rabbit muscle L-∝-glycerophosphate was also titrated by this method using 3-aminopyridine adenine dinucleotide and the results are summarized in Table 7.


2
0
-2
-4
2
4
6
8
10
Ln [ADPR]f
Ln V/(VMAX−V)

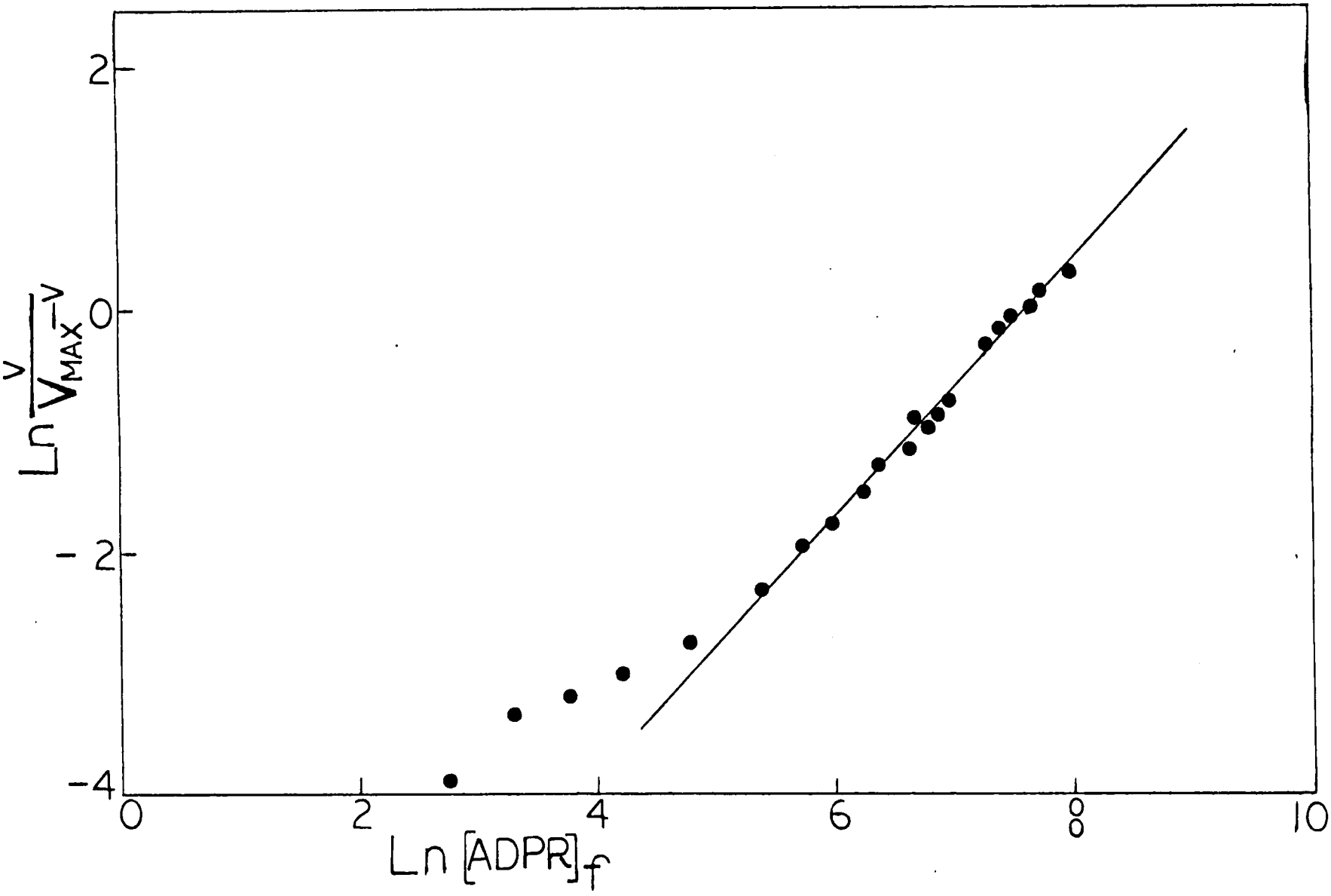

2
0
-2
-4
0
2
4
6
8
10
Ln $\frac{V}{V_{MAX}-V}$
Ln [ADPR]$_f$

TABLE 5

Coenzyme Binding Studies on Chicken Muscle L- ∝-Glycerophosphate Dehydrogenase in the Absence of ∝-Glycerophosphate.

Ligand Titrated With	Scatchard Plot Kd (M)	Klotz Plot Kd (M)	Hill Coefficient
3-Aminopyridine adenine dinucleotide	2.2×10^{-4} 1.4×10^{-5}	1.4×10^{-4} 2.4×10^{-5}	0.75
Adenosine diphospho-ribose	2.2×10^{-4} 4.5×10^{-5}	1.8×10^{-4} 1.3×10^{-5}	0.69
Adenosine monophosphate	1.4×10^{-3}	1.2×10^{-3}	0.80
Disodium n-dodecyl phosphate	4.1×10^{-3}	4.0×10^{-3}	1.0

TABLE 6

Coenzyme Binding Studies on Chicken Muscle L-α-Glycerophosphate Dehydrogenase in the Presence of α-Glycerophosphate.

Ligand Titrated With	Scatchard Plot Kd (M)	Klotz Plot Kd (M)	Hill Coefficient
3-Aminopyridine adenine dinucleotide	1.3×10^{-4}	9.0×10^{-5}	0.93
Adenosine diphosphoribose	1.0×10^{-3}	1.5×10^{-3}	1.00
Adenosine monophosphate	5.0×10^{-4} 4.2×10^{-3}	5.3×10^{-4} 2.2×10^{-3}	0.65
Disodium n-dodecyl phosphate	6.5×10^{-3}	6.8×10^{-3}	0.98

TABLE 7

3-Aminopyridine Adenine Dinucleotide Binding Studies on Rabbit Muscle L- α-Glycerophosphate Dehydrogenase Both in the Absence and Presence of α-Glycerophosphate.

α-Glycerophosphate	Scatchard Plot Kd (M)	Hill Coefficient
Absent	2.71×10^{-4} 4.88×10^{-5}	0.86
Present	1.75×10^{-4}	1.00

DISCUSSION

The binding of disodium monoalkyl phosphates, n-butyl phosphate through n-dodecyl phosphate to rabbit and chicken muscle L-α-glycerophosphate dehydrogenase was found to be competitive with respect to the coenzyme, NAD, in each case. The inhibitor dissociation constants decreased with increasing alkyl chain length in both the rabbit and chicken muscle forms as shown by the data given in Table 2. It is felt that the binding of these compounds arises from a combination of interactions involving the pyrophosphate and hydrophobic regions in the NAD coenzyme binding site. These regions along with an adenosine and "pyridinium ring" region have been previously shown to exist in rabbit muscle L-α-glycerophosphate dehydrogenase (11). Although similar studies have not been carried out on the chicken muscle form of the enzyme, it is felt that due to the mechanistic similarity of all dehydrogenases, to a first approximation, the chicken muscle form would also contain these same four regions for the binding of one molecule of NAD in the coenzyme binding site.

The increased inhibition with increased chain length of the alkyl groups is shown by the linear relationship between the logarithm of $1/K_I$ and the number of carbons in the alkyl groups, shown in Figure 9. Equation 1 was used to calculate the change in free energy of binding per methylene group. A free energy change

per methylene group of 0.40 ± .04 kcal per mole and 0.17 ± .04 kcal per mole was obtained for the rabbit and chicken muscle forms, respectively. Interactions between the nonpolar groups on substrates or inhibitors with the nonpolar residues in the enzyme can contribute a significant fraction of the total binding energy in the binding of these compounds. Enhancement of the binding of the larger disodium monoalkyl phosphates indicates the presence of a hydrophibic region, in the coenzyme binding site of the enzyme, located close to the pyrophosphate binding region. A value of 0.40 ± .04 per mole per methylene group for the rabbit muscle enzyme lies within the range, 0.36 - 0.95 kcal per mole, suggested for interactions through dispersion forces (13). Dispersion interactions are the primary cause of attractive forces between neutral molecules. These interactions are due to instantaneous dipoles and the polarization they produce. This value, 0.40 ± .04 kcal per mole per methylene group, obtained for the disodium monoalkyl phosphates is very close to 0.383 kcal/mole obtained by Kim and Anderson (14) in a study of carboxylic acid inhibition of rabbit muscle L-α-glycerophosphate dehydrogenase. The change in free energy of 0.40 ± .04 and 0.383 kcal per mole observed with disodium monoalkyl phosphates and carboxylic acids, respectively, is somewhat lower than 0.47 and 0.59 kcal per mole obatined by Anderson and coworker (11, 12), with N'-alkylnicotinamide chlorides and n-alkylammonium chlorides, respectively. Therefore, it would appear that the alkyl groups of the monoalkyl phosphates cannot

orient themselves in the hydrophobic region as effectively as do those of other compounds. A possibility of this decrease in ability to bind in the hydrophobic region could be due to the looseness of fit of the phosphate moiety in the pyrophosphate binding region.

A free energy change per methylene group of 0.17 $\pm$ .04 kcal per mole has been found for chicken muscle L-α-glycerophosphate dehydrogenase. This value lies below the range suggested for interactions through dispersion forces only. Therefore, a more complicated nature of the interaction of the alkyl groups of disodium monoalkyl phosphates with the enzyme was indicated. It is felt that the relatively low change in free energy per methylene group observed in the binding of disodium monoalkyl phosphates reflects the negative influence of steric effects on a normal process of nonpolar interactions.

Inhibition of chicken muscle L-α-glycerophosphate dehydrogenase was also studied with compounds structually related to the adenosine portion of the NAD molecule. The inhibitor dissociation constants for the three adenosine derivatives, adenosine monophosphate, adenosine diphosphate and adenosine diphosphoribose, are listed in Table 3. From these inhibitor dissociation constants, it can be seen that adenosine monophosphate is least strongly bound to the enzyme compared to the other adenosine derivatives studied. The small stepwise increase observed in the binding of adenosine diphosphate over

adenosine monophosphate may suggest a favorable interaction of the pyrophosphate binding region, in the coenzyme binding site, with the additional negatively charged phosphate group. Adenosine diphosphoribose, which represents an even greater portion of the NAD molecule, was more effectively bound to the enzyme than either adenosine monophosphate or adenosine diphosphate. The coenzyme-competitive inhibition studies with adenosine derivatives suggests the apparent recognition by the enzyme of several parts of the coenzyme molecule. From these studies the adenosine moiety, the pyrophosphate group and a hydrophobic region would be expected to contribute to the over-all binding of the coenzyme. Further studies using nicotinamide derivatives as coenzyme competitive inhibitors would be necessary before it can be shown conclusively that a "pyridinium ring" region exists in the coenzyme binding site. However, based on the similarity of the chicken muscle and rabbit muscle forms of the enzyme a "pyridinium ring" region is certainly implicated. Multiple inhibition analyses using inhibitor pairs disodium n-heptyl phosphate and adenosine monophosphate, adenosine diphosphate, adenosine diphosphoribose, and disodium n-undecyl phosphate were performed and the results observed are given in Table 4. The components of inhibitor pairs composed of disodium n-heptyl phosphate with either adenosine diphosphate or adenosine diphosphoribose mutually exclude one another from binding to the enzyme. This is exemplified by the parallel line relationship observed with

the inhibitor pair disodium n-heptyl phosphate and adenosine diphosphoribose shown in Figure 11. Such mutual exclusion would be expected for any combination of inhibitors that interact with the same regions in the NAD binding site. The results were as expected since it was proposed that the phosphate moiety of the monoalkyl phosphate would bind in the pyrophosphate binding region of the coenzyme binding site. Each of the inhibitors used with disodium n-heptyl phosphate contained a pyrophosphate group which should exclude the phosphate moiety on the monoalkyl phosphate if it were binding in the pyrophosphate region of the coenzyme binding site. Mutual exclusion was also observed with the inhibitor pair composed of disodium n-heptyl phosphate and disodium n-undecyl phosphate indicating that only one alkyl phosphate may be bound in the pyrophosphate binding region. Multiple inhibition studies with the inhibitor pair composed of disodium n-heptyl phosphate and adenosine monophosphate, shown in Figure 12, indicated the simultaneous binding of both inhibitors. The interaction constant, calculated from Figure 12, was 0.98 which indicates little or no interaction between these inhibitors. An interaction constant of 1.0 signifies the absence of interactions between inhibitors in a binding process (29). The pyrophosphate binding region, in the coenzyme binding site, is capable of simultaneously binding both the phosphate moiety from adenosine monophosphate and the phosphate moiety from the monoalkyl phosphate. Thus, from the multiple inhibition studies composed of

the inhibitor pairs, given in Table 4, it was concluded that the phosphate moiety of the monoalkyl phosphate binds to the pyrophosphate binding region, in the coenzyme binding site, and that only one molecule of monoalkyl phosphate can be bound.

The present studies on chicken muscle α-glycerophosphate dehydrogenase suggest, through the coenzyme-competitive inhibition studies using adenosine derivatives, the presence of the same four regions for the binding of one molecule of NAD coenzyme binding site as found in the rabbit muscle form of the enzyme. However, the presence of the "pyridinium ring" region can only be assumed because of the similarity of these two forms of the enzyme.

The phosphate moiety of the disodium monoalkyl phosphate has been shown, in the chicken muscle form of the enzyme, to bind to the pyrophosphate region in the coenzyme binding site. Again, due to the similarity of NAD dependent dehydrogenases, it is assumed that the phosphate moiety of the monoalkyl phosphate also binds to the pyrophosphate binding region in the coenzyme binding site of rabbit muscle α-glycerophosphate dehydrogenase. The positive chain length effect of the monoalkyl phosphates was demonstrated in both forms of the enzyme. In the rabbit muscle form of α-glycerophosphate dehydrogenase, the value of $0.40 \pm .04$ kcal per mole per methylene group clearly lies in the range expected for interactions through dispersion forces and, therefore, clearly demonstrates the presence of a hydrophobic region next to the pyrophosphate region in the coenzyme

binding site. However, in chicken muscle α-glycerophosphate dehydrogenase, a value of $0.17 \pm .04$ kcal per mole per methylene group is not in the range expected for interaction through dispersion forces. Thus, a hydrophobic region was not clearly demonstrated as in the case of the rabbit muscle form; however, the positive chain length effect observed, although a relatively minor effect, does suggest nonpolar interactions at the substrate-binding site.

Coenzyme-competitive inhibitors, 3-aminopyridine adenine dinculeotide, adenosine diphosphoribose, adenosine monophosphate and disodium n-dodecyl phosphate were used to study the coenzyme binding to chicken muscle L-α-glycerophosphate dehydrogenase. The binding of these competitive inhibitors to the enzyme was followed using the technique of protein fluorescence quenching. Fluorescence quenching is believed to be linerly proportional to ligand site occupancy both in the presence and absence of substrate based on the presence of an iosemissive point in the fluorescence emission spectra. Isoemissive points were found both in the presence and absence of substrate, shown in Figures 13 and 14, indicating that relatively simple change in the fluor environment with ligand binding was being observed.

Binding data obtained with the coenzyme-competitive inhibitors was plotted according to the methods of Lineweaver-Burke(17), Klotz (19), Scatchard (20) and Hill (18). The Scatchard plot,

based on Equation 6, is the most sensitive method of plotting binding data since this method of analyzing data places less importance on the values of $\bar{\upsilon}$ obtained at very low A values and gives more even relative weight to the different points than the Lineweaver-Burke and Klotz plots.

The Hill coefficient, n_H, determined from the Hill plot, based on Equation 10, is often confused with the number of ligand-binding sites. The number of ligand-binding sites are related to the Hill coefficient only by the restriction that the Hill coefficient cannot exceed the number of sites. The Hill plot is also sensitive to the effect of systematic and random error (33). Systematic error can create severe problems due to the way in which $\bar{Y}$ is calculated. $\bar{Y}$ is found by dividing the concentration of bound ligand by the total concentration of binding sites and thus the result depends heavily on the accurate knowledge of protein concentration and number of binding sites (33). Errors can be diagnosed by the requirement that a correct Hill plot must approach an asymptote of unit slope at high values of log $\bar{Y}/1-\bar{Y}$. Random experimental error can also cause severe problems since the transformation of $\bar{Y}$ to log $\bar{Y}/1-\bar{Y}$ results in considerable scale expansion at both ends, but very little distortion in the middle range (33). Therefore, although the Hill plot is valuable for determining the type of cooperativity, the values obtained for the Hill coefficient

should be interpreted carefully since triplicate analysis and firm statistical analysis were not performed due to the large concentrations of enzyme required.

Equilibrium dialysis was attempted in order to determine dissociation constants and Hill coefficient using another method with several coenzyme-competitive inhibitors, but results were not conclusive since concentrations of enzyme used were not high enough.

Coenzyme binding of 3-aminopyridine adenine dinucleotide and adenosine diphosphoribose, respectively, was found to be anticooperative in chicken muscle α-glycerophosphate dehydrogenase in the absence of α-glycerophosphate. Anticooperativity, also called negative cooperativity, results from the binding of one ligand to either of the two identical sites causing a decrease in the intrinsic affinities of the vacant site for another ligand molecule. The binding of 3-aminopyridine adenine dinucleotide and adenosine diphosphoribose was determined to be anticooperative based on the double-reciprocal, Klotz, Scatchard and Hill plots. Characteristics of anticooperativity are a concave double reciprocal and Klotz plot, shown in Figures 16 and 20, respectively, for the binding of adenosine diphosphoribose to α-glycerophosphate dehydrogenase in the absence of substrate. Also characteristic of anticooperativity is a Scatchard plot that curves upward, shown in Figure 18, and a Hill plot with a Hill coefficient less than one, shown in Figure 22 for the same system as above. Similar results were found for the binding of

3-aminopyridine adenine dinucleotide to the enzyme in the absence of substrate. The binding of adenosine monophosphate and disodium n-dodecyl phosphate to α-glycerophosphate dehydrogenase in the absence of substrate was found to be noncooperative in both cases, as shown in Table 5. In the noncooperative system, the binding of the first molecule of ligand to the protein molecule exerts no influence on the binding of the second ligand molecule to the protein molecule. The change in cooperativity in the binding of these coenzyme-competitive inhibitors is thought to involve an interaction in the coenzyme binding site with the ribose on the adenosine derivative. However, the binding of disodium n-dodecyl phosphate which involves both the pyrophosphate and hydrophobic binding regions in the coenzyme binding site results in noncooperativity in the absence of substrate in the chicken muscle form of the enzyme. It is felt that the binding of the monoalkyl phosphate is either sterically hindered, as implied previously in this paper or the binding is simply too "loose" to cause tertiary changes in the enzyme structure. Therefore, for tertiary conformational changes to occur (i.e. negative cooperativity) both the adenosine and ribose binding region in the coenzyme binding site must be bound with ligand. It is felt that the conformational change is due to the steric requirements of a ligand binding over the adenosine, pyrophosphate and ribose binding regions rather than simply binding two seperate ligands to the adenosine and ribose binding regions, respectively.

Binding studies using 3-aminopyridine adenine dinucleotide, adenosine diphosphoribose and disodium n-dodecyl phosphate were all found to be noncooperative in the binding to α-glycerophosphate dehydrogenase in the presence of substrate. Characteristics of noncooperativity are linear double-reciprocal, Klotz and Scatchard plots and Hill coefficients of approximately one. As shown in Table 6, one dissociation constant for each inhibitor was obtained indicating that the system obeys Michaelis-Menten kinetics. Hill coefficients for 3-aminopyridine adenine dinucleotide, adenosine diphosphoribose and disodium n-dodecyl phosphate were one within experimental error.

In the presence of α-glycerophosphate, the binding of adenosine monophosphate to chicken α-glycerophosphate dehydrogenase was determined to be noncooperative. The finding of two dissociation constants, 5.2×10^{-4}M and 3.2×10^{-4}M and a Hill coefficient of 0.64, shown in Table 6, indicated negative cooperativity. Again, any explanation for the apparent change in cooperativity of adenosine monophosphate from the other adenosine derivatives must involve the ribose binding region in the coenzyme binding site. The binding of coenzyme-competitive inhibitors that occupy a smaller region in the coenzyme binding site than adenosine diphosphoribose cannot provide the steric requirements necessary for the normal cooperativity seen with NAD and its analog 3-aminopyridine adenine

dinucleotide. Further studies would be necessary to determine the exact mechanism of the change in cooperativity upon removing the ribose from the adenosine derivative.

Rabbit muscle α-glycerophosphate was also studied with 3-aminopyridine adenine dinucleotide both in the presence and absence of substrate. The results were the same as those for the chicken muscle form of the enzyme. Table 7 shows that in the absence of substrate negative cooperativity was observed and noncooperativity was observed in the presence of substrate.

The change from negative cooperativity in the absence of substrate to noncooperativity in the presence of α-glycerophosphate for 3-aminopyridine adenine dinucleotide and adenosine diphosphoribose can be explained by two alternative microscopic explanations which both predict similar changes in the binding of coenzyme-competitive inhibitor to the enzyme. The first explanation supposes that the binding sites on each subunit are identical and the apparent negative cooperativity in ligand binding results from interactions induced between binding sites. The alternative explanation supposes that, despite the fact that both subunits have identical amino acid sequences, the shape of the dimeric molecule imposes an asymmetry in the binding site such that the ligand-free binding sites are nonidentical. The induced model would imply that α-glycerophosphate decreases the communication between binding sites where the asymmetry model would require

α-glycerophosphate to cause a conformational change in the tertiary structure making both binding sites on each subunit identical. Information obtained in the binding studies is, at the present, not enough to clearly distinguish between these microscopic explanations.

Models for cooperative ligand binding to proteins have been put forth by Monod, Wyman, and Changeux (16) and Atkinson (21). The Monod, Wyman and Changeux model for allosteric interactions assumes that protein can exist in one of either two forms in which all of the subunits are in the same form. In Atkinson's model for allosteric interactions, the binding of one ligand can either increase or decrease the affinity of ligand binding at a second site. Further, Atkinson's model assumes progressive changes in ligand site interactions. Paulus and KeKiel (23) have shown through theoretical derivations of the Hill coefficient, based on parameters customarily employed in Hill plots and under situations normally encountered with enzymes, that negative cooperativity cannot be accounted for by the model of Monod, Wyman and Changeux and can only be accounted for by the model of Atkinson. Consequently, the binding of coenzyme-competitive inhibitor to α-glycerophosphate dehydrogenase should be described in terms of Atkinson's model in which progressive changes in ligand site interactions occur rather than the "all or none" change proposed by Monod et al.

A stepwise increase in the binding of 3-aminopyridine adenine dinucleotide over adenosine diphosphoribose was found in the coenzyme-

competitive binding studies on chicken muscle α-glycerophosphate dehydrogenase in the presence of substrate, as shown in Table 6. Another stepwise increase in binding of adenosine diphosphoribose over adenosine monophosphate was also observed with adenosine phosphate being the least effectively bound. These results support kinetic evidence, shown in Table 3, that the coenzyme binding site of chicken muscle α-glycerophosphate dehydrogenase, like that of the rabbit muscle form, also possesses four binding regions for the binding of one molecule of NAD. These regions consists of an adenosine, pyrophosphate, ribose and "pyridinium ring" region. The hydrophobic binding regions in the rabbit muscle form of the enzyme has been called the ribose binding region in the chicken muscle form of the enzyme since the positive alkyl chain length effect of the monoalkyl phosphates does not clearly indicate hydrophobic interactions. Further, it has been shown that the ribose binding region is necessary for the normal type of cooperativity seen in NAD and its analog 3-aminopyridine adenine dinucleotide. The exact nature of the ribose binding region and its function in the binding of NAD cannot be determined based on existing evidence and awaits further study.

A stepwise increase in the binding of 3-aminopyridine adenine dinucleotide over adenosine diphosphoribose was not seen in the binding of these inhibitors to chicken muscle α-glycerophosphate dehydrogenase in the absence of substrate. However, both of the above

adenosine derivatives were found to bind approximately ten times better than adenosine monophosphate. Reason for the apparent deviation from the stepwise binding seen previously in both kinetic and binding studies in the presence of substrate, must lie in the absence of substrate, α-glycerophosphate.

In summary, disodium monoalkyl phosphates, n-butyl phosphate through n-dodecyl phosphate were shown to inhibit the L-α-glycerophosphate dehydrogenase catalyzed reaction competitively with respect to NAD. A positive chainlength effect was observed in the binding of these compounds to the enzyme, suggesting the presence of a hydrophobic region in or near the coenzyme binding site of the enzyme. Multiple inhibition analyses demonstrated the binding of the disodium monoalkyl phosphates to the pyrophosphate binding region in the coenzyme binding site. These results suggested the presence of a hydrophobic binding region in or nearby the ribose binding region in the coenzyme binding site of the enzyme.

Fluorescence quenching was used to study the properties of the binding of coenzyme-competitive inhibitors to L-α-glycerophosphate dehydrogenase. Anticooperativity was found in the binding of 3-aminopyridine adenine dinucleotide and adenosine diphosphoribose in the absence of substrate and noncooperativity was found with the same in the presence of substrate. Adenosine monophosphate was found to bind noncooperatively in the presence of substrate and anticooperatively in the absence of substrate. Disodium n-heptyl phosphate was

found to bind noncooperatively both in the presence and absence of substrate. Results indicate that the binding of coenzyme-competitive inhibitors that occupy an area in the coenzyme binding site smaller than adenosine diphosphoribose cannot provide the steric requirements necessary for the normal type of cooperativity seen with the analog of NAD, 3-aminopyridine adenine dinucleotide. The change from anticooperativity in the absence of substrate to noncooperativity in the presence of substrate results from decreased interactions between the NAD binding region on each subunit through formation of the ternary complex.

Future work would involve coenzyme-competitive inhibition studies using N-alkyl nicotinamide chlorides and alkyl ammonium chlorides. Results obtained from these studies would determine the presence or absence of a "pyridinium ring" region and a hydrophobic region between the "pyridinium ring" region and the pyrophosphate binding region. The increased inhibition with increased chain length of the alkyl groups could be used to calculate the free energy change per methylene group. Comparison of the free energy values per methylene group, obtained for the chicken and rabbit muscle forms of the enzyme, would provide more data on the differences and similarities of the hydrophobic region in these two forms of the enzyme.

Further work is necessary to determine if the hydrophobic binding region is actually the area responsible for binding the

ribose of the coenzyme or is only a region closely associated with the ribose binding region. The synthesis of nicotinamide riboside for use as a coenzyme-competitive inhibitor and in multiple inhibition analyses with disodium monoalkyl phosphates, aliphatic carboxylic acids and adenosine diphosphoribose would determine if the hydrophobic binding region was actually responsible for binding the ribose on the coenzyme or only closely associated with the region binding the ribose. Nicotinamide riboside could be obtained by incubating nicotinamide mononucleotide with a phosphatase enzyme and separating the reaction products by gel filtration. If the hydrophobic binding region is actually responsible for the binding of the ribose in the coenzyme, mutual exclusion should be obtained between the inhibitor pairs nicontinamide riboside and disodium monoalkyl phosphate and aliphatic carboxylic acid, respectively. However, an interaction constant of one would indicate the simultaneous binding of the same inhibitors indicating that the hydrophobic binding region is not directly responsible for the binding of the ribose in the coenzyme.

Future work on coenzyme binding to chicken muscle α-glycerophosphate dehydrogenase would involve binding studies using adenosine, adenosine diphosphate, nicotinamide riboside and n-alkyl nicotinamide chlorides. Data obtained with these coenzyme-competitive inhibitors along with data already presented in this paper could determine the

region or area in the coenzyme binding site needed to be occupied to obtain the normal type of cooperativity seen with 3-aminopyridine adenine dinucleotide, an NAD analog.

Binding studies should also be performed using saturating coenzyme-competitive inhibitor and titrating with substrate, α-glycerophosphate dehydrogenase. This data would provide more information for distinquishing between the induced and preexisting asymmetry model.

Finally, binding studies should also be repeated using a direct equilibrium binding technique; such as, ultrafiltration dialysis to assure against artifacts in binding due to the technique of protein fluorescence quenching.

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