

STUDIES OF THE COENZYME BINDING SITE
AND ESSENTIAL SULFHYDRYL GROUP OF
YEAST 6-PHOSPHOGLUCONATE DEHYDROGENASE

by

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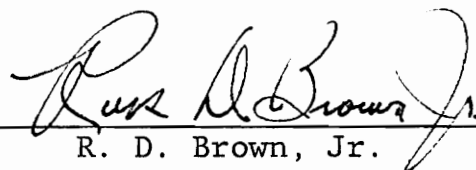
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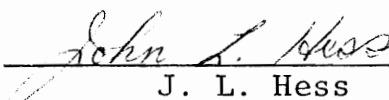
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LIST OF ABBREVIATIONS

Abbreviation

AADP - 3-aminopyridine adenine dinucleotide phosphate.

INTRODUCTION

The hexose monophosphate shunt, alternatively designated the phosphogluconate pathway, provides for most cells a means of glucose degradation in addition to the glycolysis-tricarboxylic acid cycle route. This pathway is most prominent in tissues having a high level of reductive synthesis of compounds such as fatty acids and steroids and serves the more universal function of generating pentoses for use in nucleic acid synthesis. In order to carry out reductive synthesis, large quantities of reduced nicotinamide adenine dinucleotide phosphate (NADPH) are required and the phosphogluconate pathway serves as a prime source of these reducing equivalents. These equivalents are generated at two points: first in the oxidation of glucose-6-phosphate, as catalyzed by glucose-6-phosphate dehydrogenase, to form 6-phosphoglucono- δ -lactone, and second in the oxidation of 6-phosphogluconate (with accompanying decarboxylation), as catalyzed by 6-phosphogluconate dehydrogenase, resulting in the formation of ribulose-5-phosphate. Glucose-6-phosphate dehydrogenase, as a "branch-point" enzyme, has been extensively studied while 6-phosphogluconate dehydrogenase has received little attention by comparison. Considering the importance of this enzyme in the generation of NADPH for reductive synthesis, further investigation of its properties is desirable.

Pyridine nucleotide dependent dehydrogenases catalyze the oxidation and reduction of a wide variety of substrates by means of the stereospecific transfer of a hydride ion to or from the reversibly bound coenzyme. One would thus expect to find both the substrate and the coenzyme selectively bound to specific sites on these enzymes so that a proper orientation of these molecules can be achieved to facilitate the hydride transfer. Although the properties of the substrate binding site of dehydrogenases might vary considerably due to differences in the structures of substrates, a similarity in the properties of the coenzyme binding sites of dehydrogenases would be expected. Considering the structural complexity of the pyridine nucleotide molecule, one might expect that its binding to these enzymes involves varied types of enzyme-coenzyme interactions. For example, it is possible that the enzyme-coenzyme binding results from selective interactions of regions of the coenzyme binding site with distinct portions of the coenzyme molecule. The existence of these regions could be investigated by a study of the binding of a series of compounds resembling portions of the coenzyme molecule, which would be capable of forming binary complexes with the coenzyme binding site, resulting in exclusion of the coenzyme molecule. This would be manifested as coenzyme-competitive inhibition by these compounds and the relative efficiency of binding of these coenzyme analogs could be used to identify these proposed binding regions.

In addition to providing information concerning the interactions of importance to coenzyme binding, information from this type of study could be used in the development of site-labelling reagents.

While these binding studies generate information concerning general concepts of binding, it is also of interest to investigate aspects of binding and activity on a more detailed level. For example, the nature of the functional groups important to enzyme activity is of interest. To investigate whether or not a particular functional group is important to enzyme activity, one incubates the enzyme in the presence of a reagent that selectively modifies the group of interest. A loss of enzyme activity in the presence of the reagent indicates the necessity of the free form of this group for enzyme activity. Activity loss may result from the inability of the altered residue to exert the particular influence on tertiary or quaternary structure, ligand binding, or direct catalysis, that made the unaltered residue essential to enzyme activity or the modification of the residue may engender conformational change to an inactive form of the enzyme. Dehydrogenases are generally characterized as depending on a free sulfhydryl group as evidenced by their inactivation in the presence of selective reagents which covalently modify sulfhydryl groups. Additional information about the essential sulfhydryl group can be obtained by varying the characteristics of a particular sulfhydryl reagent

such that it could interact with the surrounding environment of the essential sulfhydryl group. If the essential sulfhydryl group were to be located in a nonpolar region, for example, the reaction of a hydrophobic reagent with this group would be facilitated. If such a region were present, it may interact with the essential sulfhydryl group and also be of importance to the involvement of this group in enzyme activity.

In the present study of yeast 6-phosphogluconate dehydrogenase, the above types of studies will be applied to gain additional information concerning this enzyme. Studies of the binding of coenzyme analogs will be used to investigate interactions of importance in the binding of NADP to the enzyme. The enzyme will also be tested for chainlength effects on inactivation by N-alkylmaleimides, which can be used to identify a nonpolar region near the essential sulfhydryl group of a dehydrogenase.

LITERATURE REVIEW

Properties of Yeast 6-Phosphogluconate Dehydrogenase-

The first allusion to the existence of 6-phosphogluconate dehydrogenase was made by Warburg et al. (1,2) in their studies of glucose-6-phosphate dehydrogenase where they reported that the oxidation of glucose-6-phosphate continued beyond the initial product, 6-phosphogluconate, in yeast extracts. A requirement for NADP in this further reaction was also noted. A clarification of the exact reaction being observed was subsequently undertaken and it was demonstrated both by Warburg and Christian (3) and Dickens (4) that the reaction involved an oxidative decarboxylation of 6-phosphogluconate to a pentose phosphate which could be further metabolized. A more definite identification of the pentose product as ribulose-5-phosphate was provided by Horecker and Smyrniotis (5,6) with a partially purified preparation of yeast 6-phosphogluconate dehydrogenase containing some phosphoribose isomerase activity.

Yeast 6-phosphogluconate dehydrogenase was finally obtained in a purified form by Pontremoli et al. (7) in 1961. This preparation was demonstrated to be free of ribose-5-phosphate isomerase and glucose-6-phosphate dehydrogenase activity which, as contaminants, had complicated product identification and kinetics of previous preparations. After an initial ammonium sulfate precipitation step, the enzyme

solution was subjected to a combined charcoal treatment and heat denaturation step (48° incubation for one hour). This process was found to remove about 65 percent of the protein, 50 to 70 percent of glucose-6-phosphate dehydrogenase activity, all ribose-5-phosphatase activity, and resulted in the loss of only 30 percent of 6-phosphogluconate dehydrogenase activity. The remainder of glucose-6-phosphate dehydrogenase activity could then be removed by calcium phosphate gel adsorption. Crystallization was effected by adjustment of ammonium sulfate concentration to 50 percent saturation and allowing the solution to stand overnight at 4°. A second recrystallization resulted in a 144-fold purification of yeast 6-phosphogluconate dehydrogenase which was free of glucose-6-phosphate dehydrogenase and ribose-5-phosphate isomerase activity. With this purified preparation, accurate determination of some properties of the enzyme was possible. The Michaelis constants for NADP and 6-phosphogluconate were determined to be 26 and 160 μM , respectively, and the enzyme was shown to follow a random order of addition of substrates. The reaction product was positively identified as being ribulose-5-phosphate by paper chromatography of the dephosphorylated ribulose, isolation of the phosphate ester by ion exchange chromatography, and by a transketolase assay. At any time during the reaction, a 1:1 correlation between ribulose-5-phosphate and NADPH was demonstrated as well. This crystalline yeast 6-phosphoglu-

conate dehydrogenase was found to be capable of catalyzing the reverse reaction, oxidation of NADPH in the presence of ribulose-5-phosphate and carbon dioxide, at a rate comparable to the crude preparation. Finally, the effects of metals were determined and it was found that the enzyme is activated somewhat by Mg^{++} , Na^+ , and K^+ . No metal ion is required, however, as evidenced by no loss of enzyme activity after overnight dialysis against 0.1 mM ethylenediaminetetraacetate.

In 1967, Rippa et al. (8) reported a slightly altered purification procedure involving a protamine precipitation and elution of the enzyme from a phosphocellulose column. The crystalline enzyme from this preparation was shown to be homogenous using disc gel and starch gel electrophoresis. A molecular weight determination of this preparation using sucrose density gradient centrifugation yielded a value of 101,000. An amino acid analysis was also performed and the results are presented in Table I. An absorption spectrum of the enzyme in 10 mM sodium phosphate buffer, pH 7.0, showed only one peak at 280 nm and it was found that a solution of 1.0 g of pure enzyme per ml had an absorbance of 1.270 at 280 nm. No activity could be detected when NAD was substituted for NADP in assays with this preparation. The Michaelis constants were evaluated and found to be 20 μM for NADP and 52 μM for 6-phosphogluconate.

TABLE I

Amino Acid Composition of Yeast
6-Phosphogluconate Dehydrogenase

Amino Acid Residue	Moles/101,000 g Protein
Alanine	77
Arginine	13
Aspartic Acid	106
Half Cystine	8
Glutamic Acid	106
Glycine	118
Histidine	13
Isoleucine	58
Leucine	76
Lysine	82
Methionine	13
Phenylalanine	35
Proline	41
Serine	42
Threonine	34
Tryptophan	10
Tyrosine	33
Valine	52

The homogeneous 1967 preparation of yeast 6-phosphogluconate dehydrogenase was used in a later study (9) to determine further properties of the enzyme with regard to subunit content and number of coenzyme binding sites. In the subunit studies, the enzyme was subjected to maleylation and sodium dodecyl sulfate treatments in order to dissociate the enzyme into monomers. After an exhaustive dialysis against 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM β -mercaptoethanol and 0.1 mM ethylenediaminetetraacetate, the molecular weight of these treated enzyme samples was determined. This determination involved a comparison of the mobilities of these treated enzyme samples to those of standards on disc gel electrophoresis, which yielded a molecular weight of approximately 50,000 per subunit. Certain evidence for the identity of the subunits was also obtained. A hydrazinolysis revealed the presence of two alanine residues, i.e. one from the carboxy-terminal end of each subunit. Carboxypeptidase A was employed to release amino acids from the carboxy-terminal end in the order alanine, threonine, leucine, phenylalanine, and isoleucine in stoichiometric ratios consistent with the presence of two subunits. Finally, each subunit was shown to bind one molecule of the coenzyme using studies with tritium-labelled NADPH. In these experiments a Sephadex G-50 column was equilibrated with 0.02 M sodium phosphate buffer, pH 7.5, containing labelled NADPH at varying concentrations.

A sample of enzyme was prepared by adding an amount of labelled NADPH to the enzyme solution such that the concentration of labelled NADPH in the sample was identical to that of the equilibrating solution, and this sample was applied to the column. Studies of the elution patterns revealed that two moles of NADPH were bound per mole of enzyme with a dissociation constant of 0.48 μ M.

With the availability of purified preparations of the yeast 6-phosphogluconate dehydrogenase investigations as to the identity of the functional groups involved in enzyme activity were undertaken. Residues that have been identified as being essential for activity of this enzyme include lysine (10), histidine (11-13), tyrosine (14), and cysteine (15,16). The requirement for the cysteine residue is of particular interest due to the fact that many other dehydrogenases have also been shown to possess cysteine residues that are essential for enzyme activity, resulting in the designation of dehydrogenases as "sulfhydryl enzymes." The general approach to the study of essential sulfhydryl groups involves the use of various reagents capable of forming a covalent bond with this group, resulting in a time-dependent inactivation of these enzymes. One such reagent is iodoacetate; a 2.5 mM solution of this compound was capable of causing an 80 percent inactivation of yeast 6-phosphogluconate dehydrogenase in 40 minutes at pH 8.6 (15). The addition of 1.2 mM NADP to an incubation of the enzyme with iodoacetate afforded no

protection for the enzyme. However, the inclusion of 4.0 mM 6-phosphogluconate provided 38 percent protection of the enzyme after 40 minutes. The number of equivalents of iodoacetate bound per mole of enzyme was next determined using [^{14}C]-labelled iodoacetate and a molecular weight of 100,000 for yeast 6-phosphogluconate dehydrogenase. The average value obtained was 1.8 mole of iodoacetate bound per mole of enzyme. Another experiment was necessary, though, to prove that the carboxymethylated residue was cysteine since lysyl and histidyl residues are also carboxymethylated by iodoacetate at pH 8.6. Thus yeast 6-phosphogluconate dehydrogenase was incubated with [^{14}C]-iodoacetate until no enzyme activity remained. This modified enzyme was subjected to acid hydrolysis and the amino acids were separated by paper electrophoresis. A spot containing 80 percent of the radioactivity was found to migrate to the same location as a known sample of carboxymethylcysteine, thus providing confirmation of the identity of the altered residue as cysteine.

Other "sulfhydryl reagents" were also used in a later study of yeast 6-phosphogluconate dehydrogenase by Rippa et al. (16). Incubation of the enzyme with p-hydroxymercuribenzoate resulted in complete inactivation when the concentration of this reagent was three times that of the enzyme, thus implicating three sulfhydryl groups as being necessary for activity. Chlorodinitrobenzene was also tested and found to inactivate the enzyme 70 percent after 60 minutes

of incubation. Virtually complete protection of the enzyme from inactivation by chlorodinitrobenzene was obtained with 1.0 mM 6-phosphogluconate while 0.3 mM NADP afforded no protection. Partial protection was obtained by including 0.15 M inorganic phosphate, which was identified as a competitive inhibitor, with respect to 6-phosphogluconate, of the enzyme. In a study very similar to the labelled iodoacetate study described above, the investigators found that the binding of 1.6 moles of [^{14}C]-labelled chlorodinitrobenzene per mole of enzyme was sufficient to inactivate the enzyme completely, and the altered residue was positively identified as cysteine using paper chromatography. At this time the authors concluded that their data demonstrated that there was only one essential sulfhydryl group per enzyme molecule. With the elucidation of the subunit structure of the enzyme (9), however, the data concerning the essential cysteine residues were reevaluated and were interpreted as indicating the presence of two essential sulfhydryl groups per enzyme molecule, or one per subunit.

In 1970, Rippa et al. (13) presented a limited amount of information concerning the binding of NADP to the coenzyme binding site of yeast 6-phosphogluconate dehydrogenase. Certain compounds resembling portions of the coenzyme molecule were tested as coenzyme-competitive inhibitors of the enzyme. It was found that 2'-adenosine monophosphate was a potent competitive inhibitor with an inhibitor dissociation constant

(K_I) of 440 μ M. Other compounds that were tested but found not to inhibit the enzyme at 10 mM concentrations included 3'-adenosine monophosphate, 5'-adenosine monophosphate, and NAD. The strong binding of the 2'-adenosine monophosphate and the apparent lack of binding of the other adenosine derivatives and NAD, are interpreted as indicating the importance of this 2'-phosphate to coenzyme binding and the presence of a positively charged region at the NADP binding site. The dye, Rose Bengal, was also found to be a competitive inhibitor with respect to NADP and is thus believed to bind to the NADP binding site.

Investigations into other characteristics of yeast 6-phosphogluconate dehydrogenase are continuing to provide information about the enzyme. It was found that the addition of either substrate or coenzyme to the enzyme does not induce changes in protein conformation detectable by optical rotary dispersion measurements (17). The most recent work with this enzyme has been directed toward determination of the mechanism of the enzyme reaction with respect to the role of the coenzyme (18,19). The basic conclusion from these studies is that the coenzyme plays a redox role in the dehydrogenation step, but also that its presence in its binding site on the enzyme stimulates decarboxylation and the ketonization involved in formation of the product, ribulose-5-phosphate. Studies are still in progress to determine the nature of this stimulation.

Studies of 6-Phosphogluconate Dehydrogenase from Other

Sources - Studies of 6-phosphogluconate dehydrogenase from sources other than yeast have been performed. A list of properties of the enzyme from yeast and from other sources is presented in Table II. The work of Dyson and D'Orazio (26) with sheep liver 6-phosphogluconate dehydrogenase involves some inhibitor studies of interest. Various adenosine derivatives were tested as inhibitors of the sheep liver enzyme and, as in the case of yeast 6-phosphogluconate dehydrogenase, adenosine 2'-monophosphate was found to be a potent coenzyme-competitive inhibitor with a K_I value of 355 μM . Other adenosine derivatives examined as inhibitors included adenosine 3'-monophosphate, adenosine 5'-diphosphate, adenosine 5'-triphosphate, and adenosine. Only adenosine 5'-diphosphate inhibited the sheep liver enzyme at a level comparable to adenosine 2'-monophosphate, with a K_I value of 559 μM . The K_I values of the adenosine 3'-monophosphate and adenosine 5'-monophosphate are tenfold larger at 6.14 and 5.70 mM, respectively, and adenosine 5'-triphosphate has a K_I value of 1.34 mM. Adenosine is poorly bound with a K_I value of 15.4 mM and all inhibitors described were competitive with respect to NADP. Thus the presence of a phosphate at the 2'-position once again appears to improve the binding of an adenosine derivative to the NADP binding site of 6-phosphogluconate dehydrogenase.

TABLE II

Properties of 6-Phosphogluconate Dehydrogenase from Various Sources

Source	Molecular Weight	Subunit Weight	pH Optimum	K_m , 6-PG ^a (x10 ⁵)	K_m , NADP (x10 ⁵)	Reference
Yeast	101,000	50,000	7.3-7.5	5.4	2.0	(9-11)
Sheep Liver	129,000	65,000	7.7	1.6	0.68	(20,21)
<u>Strep. Faecalis</u>	108,000	52,000	7.5-8.0	2.4	1.5	(22)
Human Erythrocyte	105,000	52,000	8.3	2.0	3.0	(23,24)
<u>Neurospora</u>	110,000 to 120,000	57,000	7.8-9.0	1.0	3.0	(25)

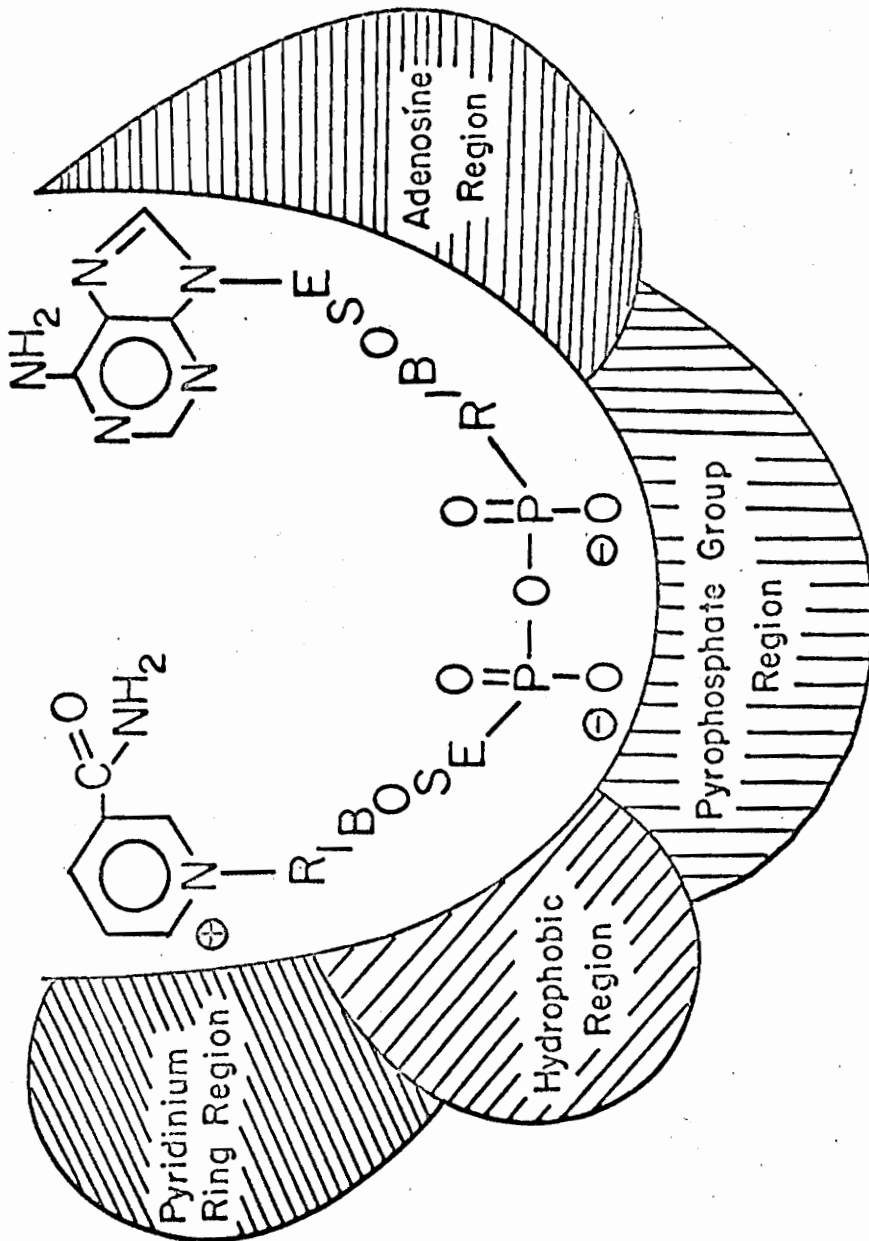
^a6-PG, 6-phosphogluconate

Coenzyme Binding Site Mapping Using Inhibitor Studies-

As mentioned previously, the pyridine nucleotide dependent dehydrogenases catalyze the oxidation and reduction of many different substrates. This occurs through a stereospecific transfer of a hydride ion to or from reversibly bound NAD(H) or NADP(H). It is thus reasonable to propose the existence of substrate- and coenzyme-specific sites on the enzyme designed to selectively bind substrate and coenzyme in an arrangement that would orient these molecules properly for the hydride transfer. As the structures of substrates vary widely among dehydrogenases, one would expect considerable variation in the properties of the binding sites of these substrates, but a similarity in coenzyme binding sites might also be expected since the same type of molecule is employed as coenzyme in all cases. In the past, the nature of this coenzyme binding has received consideration on a more detailed level. Given the structural complexity of the pyridine nucleotide coenzyme molecule, it is reasonable to consider the mode of its binding somewhat comparable to that of the hexasaccharide substrate of lysozyme in which binding regions designed to interact with each of the six sugar residues have been identified (27,28). These regions were identified through the use of substrate, partial substrates, and substrate-competitive inhibitors resembling portions of the hexasaccharide molecule. Using similar logic, a molecule

of the complexity of the pyridine nucleotide molecule might be expected to bind to an enzyme in a manner similar to that depicted in Figure 1. The so-called adenosine, pyrophosphate, and nicotinamide regions are suggested to selectively interact with distinct portions of the coenzyme molecule, and the existence of the hydrophobic region has been demonstrated experimentally. Indications of the existence of these regions have been derived from inhibitor binding studies somewhat similar to the studies which further elucidated the binding of the substrate of lysozyme (27,28). These studies involve the use of compounds which are structurally similar to the coenzyme and can thus interact with the coenzyme binding site to form binary complexes resulting in exclusion of the true coenzyme from the binding site. This process is manifested as coenzyme-competitive inhibition by these analogs and from inhibitor dissociation constants, K_I , the degree of binding of a particular inhibitor to the pyridine nucleotide binding site can be determined. By systematically increasing the chemical resemblance of an inhibitor to the actual coenzyme, and thereby causing inhibitor interaction with greater portions of the binding site, one would expect to observe increasing affinities of the enzyme for the coenzyme-competitive inhibitors. This type of logic has been used to obtain information concerning the existence and relative importance of the proposed binding regions of Figure 1.

Figure 1. A schematic representation of the different regions of the pyridine nucleotide binding regions of enzymes (29).



An example of this type of approach to coenzyme binding site study is provided by the work of Kim et al. (30) with rabbit muscle glycerophosphate dehydrogenase. The NAD binding site of this enzyme was studied using adenosine and the adenosine derivatives adenosine 5'-monophosphate, adenosine 5'-diphosphate, and adenosine diphosphoribose. Progressing through this series of inhibitors, one notes their increasing resemblance to the true coenzyme, NAD (see Figure 1), and the K_I values for these compounds were found to decrease in this order (see Table III). Referring to Figure 1, one observes that these data are consistent with the binding site model; as the resemblance to the true coenzyme molecule increases, the inhibitor is able to interact with more of the proposed binding regions and thus its K_I value is lower. This type of study has also been applied to other dehydrogenases (see Table III) and a similar pattern has been observed in beef muscle lactic dehydrogenases (31), and rat liver mitochondrial malic dehydrogenase (32) in which adenosine, pyrophosphate, and ribose-binding regions appear to exist. In the case of yeast alcohol dehydrogenase (33,34), however, it was found that adenosine 5'-monophosphate and adenosine 5'-diphosphate are not bound as well as adenosine, thus suggesting that there is no pyrophosphate binding region in this enzyme. Adenosine diphosphoribose was bound better than adenosine in all cases, but the extent of this superior binding varied widely. Thus it appears that coenzyme binding to the dehy-

TABLE III

Dissociation Constants for Competitive Inhibition of Dehydrogenases

Enzyme	K _I , mM				Reference
	Adenosine	Adenosine 5'- Monophosphate	Adenosine 5'- Diphosphate	Adenosine Di- Phosphoribose	
Rabbit Muscle Glycerophosphate Dehydrogenase	4.30	2.08	0.709	0.160	(30)
Beef Muscle Lactic Dehydro- genase	7.07	0.551	0.770	0.198	(31)
Rat Liver Mito- chondrial Malic Dehydrogenase	4.60	0.558	0.633	0.0340	(32)
Yeast Alcohol Dehydrogenase	6.00	8.90	8.45	2.65	(33, 34)

drogenases follows no orderly pattern and suggests that the mode of binding of the coenzyme, while superficially similar, is unique to each enzyme on a more detailed level.

A related type of study has been employed to investigate the existence of binding regions for the nicotinamide moiety of the pyridine nucleotide molecule. Such studies have provided the basis for proposing the existence of the nicotinamide and hydrophobic regions shown in Figure 1. The compounds employed in this study are the N¹-alkyl derivatives of nicotinamide (N¹-methyl- to N¹-dodecyl, inclusive), which have been synthesized (35) and used as structural analogs of the pyridinium portion of the coenzyme molecule. These compounds are tested as coenzyme-competitive inhibitors of the NAD(P)-dependent enzyme in question. Inhibition of the enzyme by the short-chain derivatives suggests the formation of a binary complex between these compounds and a region in the coenzyme binding site designed for interaction with the nicotinamide moiety (see Figure 1). Multiple inhibition analysis (33) using combinations of adenosine derivatives and N¹-alkylnicotinamide chlorides, has confirmed that these pyridinium derivatives bind at a region of the coenzyme binding site different from that of the adenosine derivatives. Any enhancement of inhibition in the case of the longer-chain derivatives, when compared to short-chain derivatives, could indicate increasing interactions between long-chain derivatives and a region on the enzyme capable of interacting with

the hydrophobic side chains of these compounds. If such a nonpolar region were present, one would expect that the N¹-alkylnicotinamide chlorides would be better bound to the region as the alkyl side chain increased in length and thereby became capable of greater interaction with this hydrophobic region. This phenomenon has indeed been observed, and a nonpolar region next to the nicotinamide binding region was identified in the case of yeast alcohol dehydrogenase (35), rabbit muscle glycerophosphate dehydrogenase (30), bovine liver glutamic dehydrogenase (36), and rat liver glutamic dehydrogenase (37). Such a region is believed to play a multiple role in coenzyme binding to the enzyme and in the subsequent catalytic process. This region may be responsible for hydrophobic interactions with the terminal ribose moiety in adenosine diphosphoribose that leads to its improved binding when compared to adenosine derivatives representing smaller portions of the coenzyme molecule (see Figure 1, Table III). Of perhaps greater importance is the possible interaction of this nonpolar region with the uncharged 1,4-dihyronicotinamide ring of NAD(P)H. This type of interaction may function not only in the binding of NAD(P)H, but also in the actual hydride transfer process for which it would provide an ideal (nonpolar) environment. A hydrophobic region would be effective in exclusion of solvent molecules and would thereby reduce the possibility of exchange of the transferred hydride ion with the solvent. The transition state for the oxidation-

reduction reaction with the nicotinamide ring is also relatively nonpolar and could be stabilized by this region. Thus, the presence of this hydrophobic region would appear to be very advantageous in the hydride transfer reaction of dehydrogenases and thus might be expected to be an essential component of all forms of this type of enzyme. However, such a region has not been demonstrated in bovine lactate dehydrogenase H₄ and M₄ isozymes, rat liver mitochondrial malic dehydrogenase, and yeast glucose-6-phosphate dehydrogenase¹ due to their failure to selectively bind the N¹-alkylnicotinamide derivatives and thereby exclude the binding of the coenzyme. This may indicate the lack of a pyridinium ring binding region in the case of these enzymes, which perhaps rely on conformational changes induced by the binding of other portions of the coenzyme molecule to orient the nicotinamide ring for stereospecific hydride transfer. Though not demonstrable by the N¹-alkylnicotinamide type of study, a hydrophobic region may yet exist in these enzymes that do not bind these pyridinium derivatives and may still represent a point of enzyme interaction with the coenzyme. It is of interest to note that, while these enzymes do not selectively bind the N¹-alkylnicotinamide chlorides, they are inactivated by denaturation in the presence of the micellar forms of the longer-chain derivatives.²

¹Anderson, B. M., unpublished results

²Anderson, B. M., unpublished results

Studies of the Essential Sulfhydryl Group of Dehydrogenases - The representation of the pyridine nucleotide binding site in Figure 1 is an adequate representation of a general concept of coenzyme binding, however, it is also of interest to investigate aspects of binding and activity on a more detailed level. For example, one might question the nature of the amino acid residues required for activity and the specific functions of such residues. One means of investigation of this question is to alter specific residues and to monitor the effects of these alterations on enzyme activity. In this respect, covalent modification of the sulfhydryl group of cysteine residues can be effected through the use of certain reagents and the incubation of an enzyme requiring one or more sulfhydryl group(s) for activity with these reagents would be expected to result in an inactivation of the enzyme.

Grazi et al. (15) and Rippa et al. (16) reported that incubation of yeast 6-phosphogluconate dehydrogenase in the presence of reagents capable of forming a covalent bond with sulfhydryl groups resulted in an inactivation of the enzyme. This phenomenon has been observed in the case of many dehydrogenases, resulting in their designation as "sulfhydryl enzymes." Considering the necessity of this particular moiety for activity of these enzymes, more information concerning the role and environment of essential sulfhydryl groups was needed. To this end, indications of the importance of non-

polar interactions in the binding and/or catalysis of yeast alcohol dehydrogenase led Heitz et al. (38) to investigate nonpolar effects on reactions of the essential sulfhydryl group of this enzyme. The general approach used in this study was to synthesize derivatives of a compound capable of covalently modifying sulfhydryl groups and whose chemical characteristics would resemble the proposed environment of the sulfhydryl group, which would facilitate the reaction between the reagent and the sulfhydryl group and thereby enhance the rate of inactivation. The particular group of sulfhydryl-modifying compounds employed in this study was the N-alkylmaleimides (N-methyl to N-decyl, inclusive). If the sulfhydryl group were to be located in a nonpolar region, the longer-chain N-alkylmaleimides would be expected to better interact with this region, facilitate the reaction between the sulfhydryl group and the maleimide, and display an enhanced rate of enzyme inactivation. To detect the presence of this nonpolar region, the enzyme was incubated with varying concentrations of a particular N-alkylmaleimide and the decrease in enzyme activity with time was monitored. A plot of the logarithm of residual activity versus time yielded a linear relationship and enabled the calculation of a pseudo-first order rate constant, k_1 , of inactivation. When the pseudo-first order rate constants were plotted versus N-alkylmaleimide concentration, a linear relationship was also observed and a second order rate constant, k_2 , was calculated

from the slope of this line. Increasing k_2 values across a series of N-alkylmaleimides with increasing chain length were observed in the case of yeast alcohol dehydrogenase, indicating the existence of enhanced interactions between the environment of the sulfhydryl group and the longer-chain N-alkylmaleimides. Using the logic outlined above, the sulfhydryl group was judged to be adjacent to a hydrophobic region with which the N-alkylmaleimides could have greater interactions as the length of the N-alkyl side chain increased. This type of study was extended to other dehydrogenases and similar chainlength effects were observed in the case of rabbit muscle glycerophosphate dehydrogenase (39), beef heart lactic dehydrogenase₁ (H₄ isozyme) and beef muscle lactic dehydrogenase₅ (M₄ isozyme) (31). Other enzymes possessing essential sulfhydryl groups and demonstrating chainlength effects include papain (40), ficin (41), and hog kidney D-amino acid oxidase (42). Evidence for the validity of this type of study in identification of hydrophobic regions near sulfhydryl groups comes from X-ray studies of papain (43). These studies have revealed the sole, and essential, sulfhydryl group of this enzyme to be located in a nonpolar cleft region and maleimide inactivation of papain occurred with a marked chainlength effect (40). Thus, in a number of dehydrogenases (and other enzymes) the essential sulfhydryl group appears to be proximal to a nonpolar region on the enzyme. The exact role of this region is unclear, but it may be involved with the

essential sulfhydryl group in generation of proper tertiary or quaternary structure for activity, in the binding of ligands by the enzyme, or in direct participation in catalysis. The existence of a nonpolar region near the essential sulfhydryl group is not a general feature of dehydrogenases, however. While glucose-6-phosphate dehydrogenase can be inactivated by N-alkylmaleimides, no chainlength effect is observed (38) indicating that this enzyme lacks a nonpolar region near the essential sulfhydryl group.

A more recently developed type of study of the essential sulfhydryl group of dehydrogenases makes use of data obtained from site-mapping studies. This information has been used in the design of site-labelling reagents for dehydrogenases. These are nonfunctional coenzyme analogs containing a substituent group that can be activated such that it would react with a sulfhydryl group on the enzyme. The close resemblance of this reagent to the true coenzyme would give rise to selective modification of a sulfhydryl group near the point of binding of the region of the analog to which the activated substituent is attached. To this end, the compound 3-aminopyridine adenine dinucleotide was prepared (44) and shown to be a coenzyme-competitive inhibitor of seven pyridine nucleotide-dependent enzymes. This compound can be activated through diazotization and can then be used to selectively inactivate yeast alcohol dehydrogenase through a reaction with four cysteine residues on the enzyme as evidenced

by the presence of S-3-pyridyl cysteine in the acid hydrolyzate of the modified enzyme. The usefulness of 3-aminopyridine adenine dinucleotide in the selective inactivation studies led to the preparation of 3-aminopyridine adenine dinucleotide phosphate (AADP). This compound has been shown to be a competitive inhibitor of both yeast glucose-6-phosphate dehydrogenase and yeast 6-phosphogluconate dehydrogenase. Diazotization of this compound has also been performed and the diazotized form was shown to be incapable of selective inactivation of these two NADP-dependent enzymes (45). Therefore, as in the mode of coenzyme binding, the environment and role of the essential sulfhydryl group follows no uniform pattern among dehydrogenases and further investigations of the essential sulfhydryl groups of this type of enzyme are needed.

EXPERIMENTAL PROCEDURES

Materials - Yeast 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase (decarboxylating), Type V, EC 1.1.1.44) was obtained as a crystalline suspension in 3.1 M $(\text{NH}_4)_2\text{SO}_4$ -0.2 M glycylglycine solution, pH 7.6, from Sigma Chemical Company. Stock solutions of the enzyme were prepared by dilution of the enzyme tenfold by adding 0.20 ml of the enzyme suspension to 1.8 ml of 0.033 M glycylglycine buffer, pH 7.5, and stored frozen.

Crystallized and lyophilized yeast alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) was obtained from Sigma Chemical Company. Stock solutions were prepared as 7.8 μg per ml solutions in 0.05 M potassium phosphate buffer, pH 7.5, and stored frozen.

Adenosine, adenosine 2'-monophosphate, adenosine 3'-monophosphate (crystalline), adenosine 5'-monophosphate (type II, sodium salt, crystalline), adenosine 3':5'-cyclic monophosphate (crystalline), adenosine 2',5'-diphosphate, adenosine 5'-diphosphate (grade I), adenosine diphosphoribose, 2'-monophospho-adenosine diphosphoribose, nicotinamide mononucleotide, NAD (sodium salt), NADP (sodium salt), 6-phosphogluconate (trisodium salt), hen egg ovalbumin, and cytochrome C were all purchased from Sigma Chemical Company. AADP was prepared according to Anderson et al. (45). The N^1 -alkylnicotinamide derivatives, prepared according to Anderson et al. (35), were

available in the laboratory. With the exception of N-ethylmaleimide, which was purchased from Eastman Organic Chemicals, the N-alkylmaleimides (N-methyl and N-butyl to N-decyl, inclusive), prepared according to Heitz et al. (38), were available in the laboratory. Sephadex G-150 was purchased from Pharmacia. All other chemicals (buffer salts and disc gel reagents) were reagent grade.

Methods

Determination of Enzyme Purity - The purity of the Sigma preparation of yeast 6-phosphogluconate dehydrogenase (Sigma Lot 111C-8641-1) was determined using three experiments with polyacrylamide gel electrophoresis. Prior to the first experiment, 1.0 ml of the Sigma product was dialyzed overnight at 4° against 3.0 liters of 0.1 M sodium phosphate buffer, pH 7.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis as described by Shapiro et al. (46) was performed on duplicate 25- and 50- μ g aliquots of the dialyzed enzyme. The running buffer was 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1 percent sodium dodecyl sulfate. The gels were run at 25° for three hours using 3 mA per tube. They were then stained with Coomassie blue and destained using 10 percent acetic acid. The gel electrophoresis apparatus used in all experiments was manufactured by Canalco.

In the second experiment, a 1.0 ml sample of the Sigma enzyme product was dialyzed overnight at 4° against 1.0 liter

of sodium phosphate buffer, pH 7.0 with three changes of buffer. Polyacrylamide gel electrophoresis as described by Davis (47) was employed to test the homogeneity of this preparation. In this system, the stacking pH is 8.9 and the running pH is 9.5. Duplicate aliquots containing 25 and 50 μg of the dialyzed enzyme were applied to the gels which were run for three hours at 4° with a current of 3 mA per tube. These gels were stained with Coomassie blue and destained electrophoretically in seven percent acetic acid.

For the third gel system, a 1.0 ml sample of the yeast 6-phosphogluconate dehydrogenase was dialyzed as in the second experiment, except that the buffer used was 0.05M Veronal buffer, pH 7.0. Polyacrylamide gel electrophoresis was run according to the method of Williams and Reisfeld (48) on duplicate 25- and 50- μg samples of the dialyzed enzyme. In this system, the stacking pH is 7.0 and the running pH is 8.0. These samples were run, stained, and destained exactly as in the second experiment.

Determination of Molecular Weight of Yeast 6-Phosphogluconate Dehydrogenase by Gel Filtration on Sephadex G-150- Sephadex G-150 was placed in 0.10 M sodium phosphate buffer, pH 7.0, and allowed to swell for three days at 25°. A 1.5 by 90 cm column was then packed with the swelled Sephadex G-150 and washed with additional quantities of the above buffer at 4° for one day. To this column was added 2.0 ml of a standard protein solution containing 0.75 mg yeast alcohol

dehydrogenase, 0.75 mg hen egg ovalbumin, 0.75 mg cytochrome C, 0.75 mg catalase, 1.0 mg Dextran Blue-2000, and 0.2 g sucrose. The column was eluted with the above buffer and 1.5 ml fractions were collected. The fractions were analyzed for protein content by monitoring absorbance at 280 nm and for Dextran Blue by monitoring absorbance at 660 nm. From the peak absorbance at 660 nm, the void volume (V_0) was determined to be 60.5 ml (catalase eluted with the void volume). The total volume (V_t) of the gel bed, the height of which was 84.5 cm, was determined to be 149.3 ml using the formula $V_t = \pi r^2 h$, where r is the radius of the column and h is the bed height. Values of the diffusion coefficient, K_{av} , of yeast alcohol dehydrogenase, hen egg ovalbumin, and cytochrome C were determined using the formula $K_{av} = V_e - V_0 / V_t - V_0$, where V_e is the elution volume, determined by multiplying the fraction number corresponding to the maximum 280 nm absorption by the fraction volume. These values of the partition coefficient were plotted versus the logarithm of the molecular weight of the corresponding proteins and a linear relationship was obtained. This was used as a standard curve for molecular weight determination. To determine the molecular weight of yeast 6-phosphogluconate dehydrogenase, a 2-ml sample of the crystalline suspension of the enzyme was dialyzed at 4° against 4 x 1000 ml 0.1 M sodium phosphate buffer, pH 7.0. The dialyzed sample was then applied to the column, eluted with equilibration

buffer, and 1.5 ml fractions were collected. These fractions were analyzed for protein content by monitoring their absorbance at 280 nm. The peak protein absorbance was used to determine a K_{av} of 0.247. From this value a molecular weight for this protein was established.

Kinetic Studies of Yeast 6-Phosphogluconate Dehydrogenase - The inhibitor dissociation constants, K_I , were determined using one or both of two different types of kinetic measurements. In the first type of experiments, initial velocities were studied as a function of varying inhibitor concentration at a constant substrate concentration (NADP, either 15.9 μ M or 79.3 μ M). These data were plotted according to Dixon (49). In a second type of experiment, initial velocities were studied as a function of varying substrate concentration, NADP varying from 15.9 μ M to 79.3 μ M at three concentrations of inhibitor. The inhibitor concentrations used were zero and approximately one and two times the K_I concentration determined from the Dixon plots. These data were plotted according to Lineweaver and Burk (50).

For these kinetic studies at 25 $^{\circ}$, the 3 ml reaction mixtures contained 0.06 M glycylglycine buffer, pH 7.5, NADP (15.9 to 79.3 μ M, for appropriate experiment), 0.958 mM 6-phosphogluconate, and 1.8 μ g of yeast 6-phosphogluconate dehydrogenase in the presence or absence of inhibitor. In all cases the reactions were initiated by addition of the enzyme and initial velocities were obtained by monitoring the in-

crease in the initial absorbance at 340 nm using a Beckman Acta MVI double beam recording spectrophotometer. All pH measurements were made using a Radiometer PHM-52 pH meter equipped with a Radiometer GK-2321-C combination electrode.

Kinetic Studies of Yeast Alcohol Dehydrogenase - Initial velocities of the reaction catalyzed by yeast alcohol dehydrogenase were studied as a function of varying inhibitor concentration at a constant substrate concentration. The concentration of ethanol was saturating and the concentration of NAD was 43.6 μM , well below the K_m value of 228 μM . The compounds tested as inhibitors were adenosine 2'-monophosphate and adenosine 2',5'-diphosphate at concentrations up to roughly two times the K_I values reported in the literature (33,34) for two adenosine derivatives not phosphorylated at the 2' position, 5'-adenosine monophosphate and adenosine 5'-diphosphate. The spectrophotometric assay conducted at 25 $^{\circ}$, employed 3 ml reaction mixtures containing 0.012 M sodium pyrophosphate buffer, pH 8.2, 0.1 M ethanol, 43.6 μM NAD, inhibitor (if any) and 0.39 μg of enzyme. The reaction was initiated by the addition of the enzyme and the formation of NADH was monitored using the increase in the initial absorbance at 340 nm.

Time-Dependent Maleimide Inactivation Studies - The time-dependent inactivation of yeast 6-phosphogluconate dehydrogenase by a series of N-alkylmaleimide derivatives was studied by incubating the enzyme in the presence of various

N-alkylmaleimide derivatives at 25°. Incubation mixtures included 18 µg of enzyme, 14.0 mM sodium phosphate buffer, pH 7.0, 2.0 percent ethanol, and varying concentrations of the N-alkylmaleimide derivative under investigation, in a final volume of 1.0 ml. The 2.0 percent ethanol was included to solubilize the longer-chain N-alkylmaleimides. Ethanol at this concentration was shown not to inactivate the enzyme up to a period of one hour. The time of addition of enzyme was considered time zero and, at various times thereafter, a 0.10 ml aliquot of the incubation mixture was added to 2.90 ml standard assay mixtures containing 5 mM sodium phosphate buffer, pH 7.0, 79.3 µM NADP, and 0.958 mM 6-phosphogluconate. Velocities were measured by monitoring the increase in the initial absorbance at 340 nm and corresponded to the residual enzyme activity at the time of incubation. Measurements were performed on a Beckman Acta MVI recording spectrophotometer. The logarithms of the activities were plotted against time and the resulting linear relationship was employed to determine pseudo first-order rate constants of the inactivation reactions.

Protection Against N-Ethylmaleimide Inactivation of Yeast 6-Phosphogluconate Dehydrogenase - The protection experiments performed were similar to the time-dependent inactivation studies, except that various protecting compounds (which included 2'-adenosine monophosphate, 2',5'-adenosine diphosphate, NADP, NADPH, AADP, and 6-phosphogluconate) were

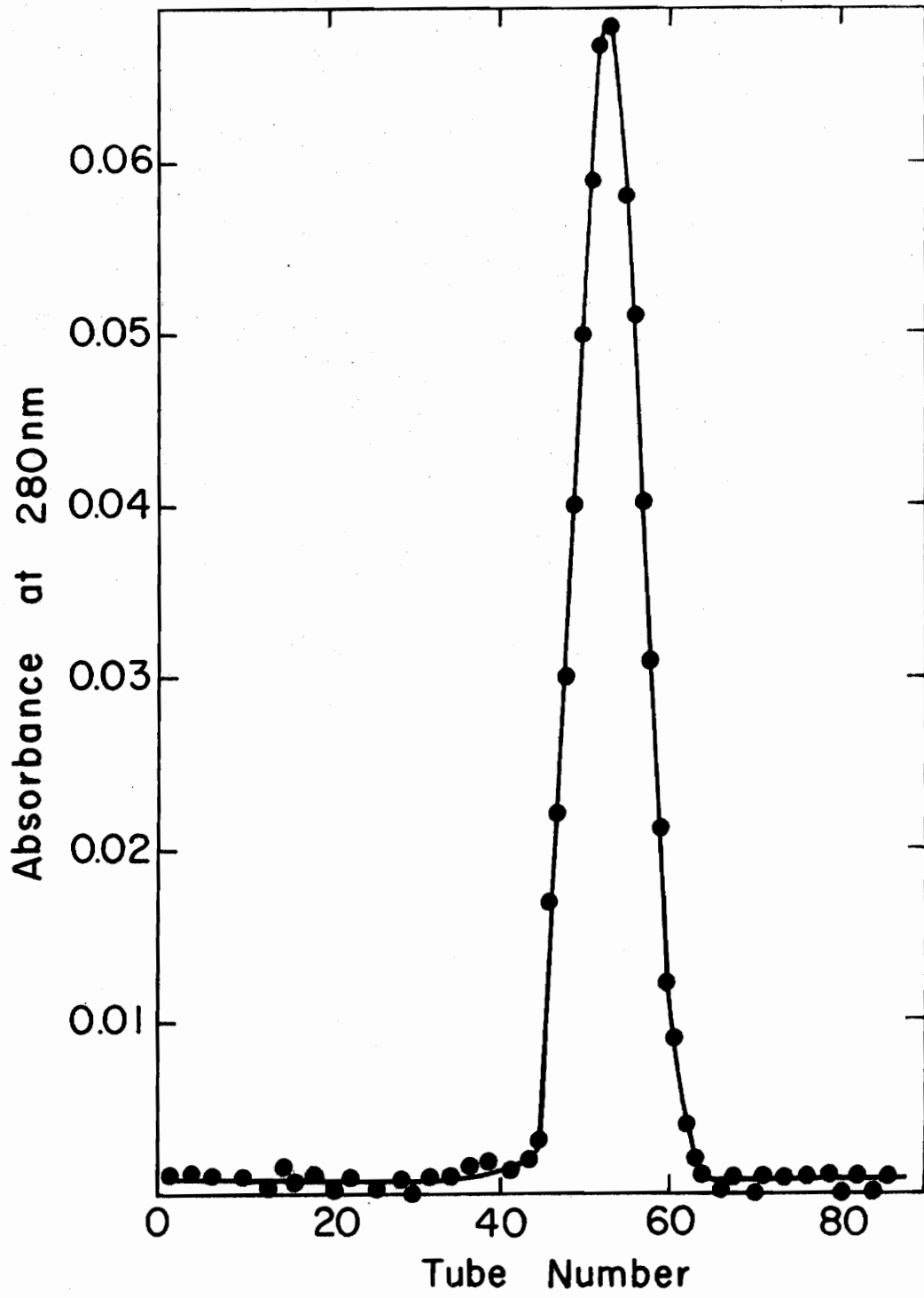
included in the incubation mixtures. These incubation mixtures included 2.28 mM N-ethylmaleimide, 18 μ g of enzyme, 21.0 mM sodium phosphate buffer, pH 7.0, 2.0 percent ethanol, and the protecting compound(s). The concentrations of the protecting compounds ranged from one to three times the K_I or K_m value of the compounds. The time of addition of enzyme was considered time zero and, at various times, a 0.10 ml aliquot of this mixture was added to 2.90 ml of the previously described assay mixture. Logarithms of residual activities were plotted against time to enable calculation of pseudo first-order rate constants. Protection by any given compound was expressed as percent decrease in the pseudo first-order rate constant of the maleimide inactivation produced by the presence of the protecting compound.

RESULTS

Purity and Molecular Weight of Yeast 6-Phosphogluconate Dehydrogenase - In order to strengthen the reliability of the kinetic parameters of the yeast 6-phosphogluconate dehydrogenase used in these studies, the purity of the preparation was determined. The Sigma product, even at 50 μ g of enzyme, was characterized as a single band on SDS polyacrylamide gels, pH 7.2, and on 10 percent polyacrylamide gels run at both pH 8.0 and pH 9.5. In addition, the enzyme eluted as a single, sharp peak from a Sephadex G-150 column (Figure 2). From the value of the partition coefficient, K_{av} , of yeast 6-phosphogluconate dehydrogenase on this column, the molecular weight of the enzyme was determined to be 87,000.

Inhibition of Yeast 6-Phosphogluconate Dehydrogenase by Adenosine Derivatives - The inhibition of yeast 6-phosphogluconate dehydrogenase by ten adenosine derivatives was studied according to the procedure described under "Methods." The ten adenosine derivatives employed included adenosine, adenosine 2'-monophosphate, adenosine 3'-monophosphate, adenosine 3':5'-cyclic-monophosphate, adenosine 5'-monophosphate, adenosine 2',5'-diphosphate, adenosine 5'-diphosphate, adenosine diphosphoribose, 2'-monophospho-adenosine diphosphoribose, and AADP. All adenosine derivatives studied were found to be coenzyme-competitive inhibitors of this enzyme. The studies of the

Figure 2. Elution pattern of yeast 6-phosphogluconate dehydrogenase from Sephadex G-150 column. A 2 mg sample of the crystalline suspension of the enzyme was dialyzed at 4° against 4 x 1000 ml 0.1 M sodium phosphate buffer, pH 7.0. The dialyzed sample was applied to a Sephadex G-150 column (1.5 by 90 cm) and eluted with this same buffer. Fractions (1.5 ml) were collected and analyzed for protein by monitoring absorbance at 280 nm.



adenosine 2',5'-diphosphate inhibition of the enzyme exemplifies the type of results obtained. The inhibition was first measured as a function of constant coenzyme and variable inhibitor concentrations and the results are presented in Figure 3. In this experiment, data were generated by measuring initial velocities at five different inhibitor concentrations with the concentration of NADP at 79.3 μM . Data for the second line were generated in a similar manner with the concentration of NADP at 15.9 μM . The K_I value obtained from this study is 64.0 μM . Secondly, the inhibition of yeast 6-phosphogluconate dehydrogenase was measured using varied NADP concentrations at constant levels of inhibitor and the results are presented in Figure 4. One line represents the initial velocities at five different NADP concentrations with no inhibitor present, and the second and third lines represent these same reactions measured in the presence of inhibitor at roughly one and two times the K_I value concentration. The K_I value determined in this study is 62.0 μM and the inhibition is shown to be competitive. All K_I values were calculated from visual-best-fit lines. Inhibitor dissociation constants were obtained in this manner for the ten adenosine derivatives studied and the results are presented in Table IV.

Inhibition of Yeast Alcohol Dehydrogenase by Adenosine Derivatives - The lack of inhibition of yeast alcohol dehydrogenase by adenosine 2'-monophosphate and adenosine 2',5'-diphosphate was demonstrated using the procedure described

Figure 3. Dixon plot of adenosine 2',5'-diphosphate inhibition of yeast 6-phosphogluconate dehydrogenase. Reaction mixtures contained 0.06 M glycylglycine buffer, pH 7.5, 0.958 mM 6-phosphogluconate, 1.8 μ g of enzyme, and NADP and inhibitor as indicated in a total volume of 3 ml.

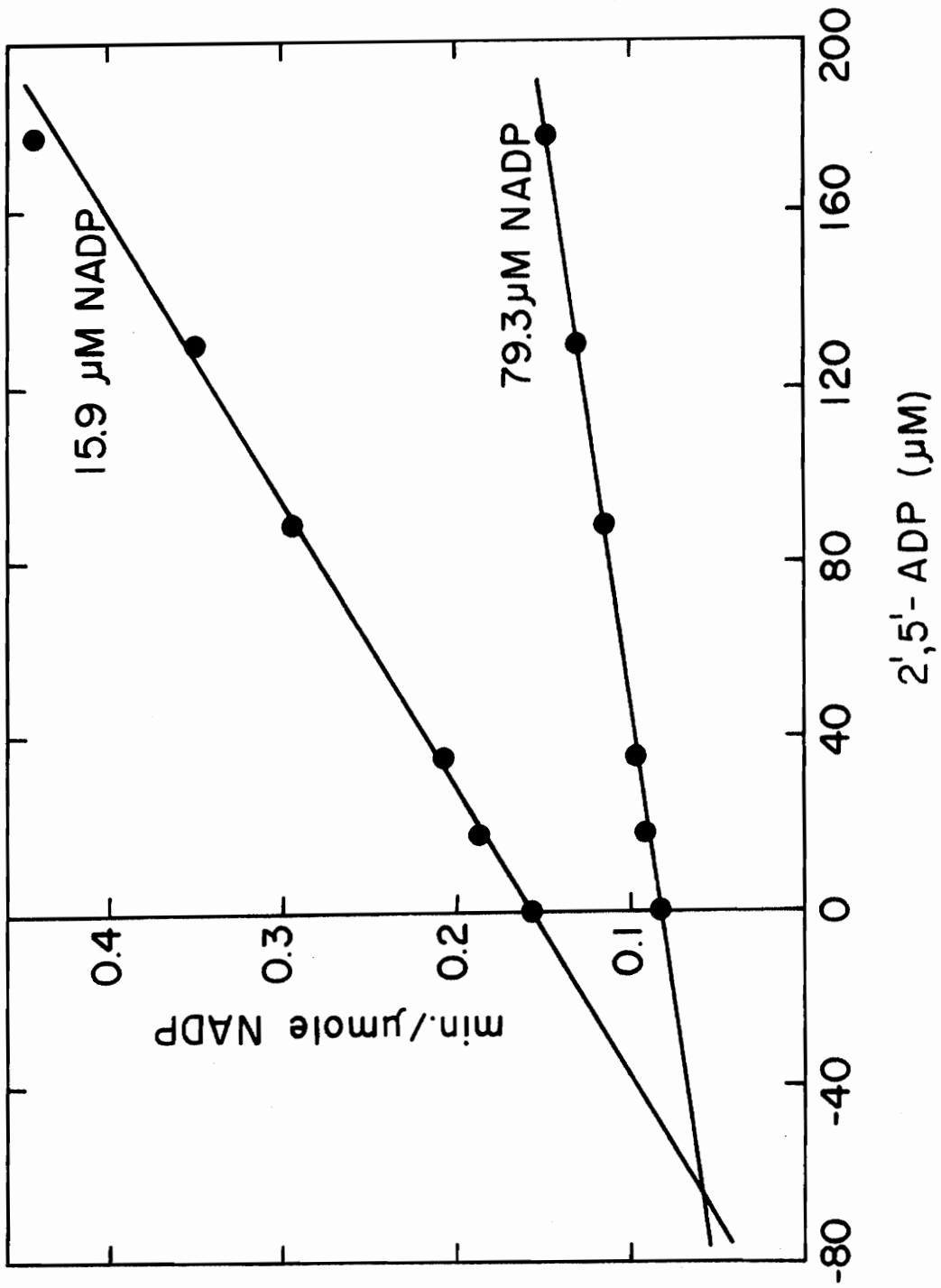


Figure 4. Lineweaver-Burk plot of adenosine 2',5'-diphosphate inhibition of yeast 6-phosphogluconate dehydrogenase. Reaction mixtures contained 0.06 M glycylglycine buffer, pH 7.5, 0.958 mM 6-phosphogluconate, NADP, 1.8 μ g of enzyme, and inhibitor as indicated in a total volume of 3 ml. NADP concentration was varied from 15.9 μ M to 79.3 μ M.

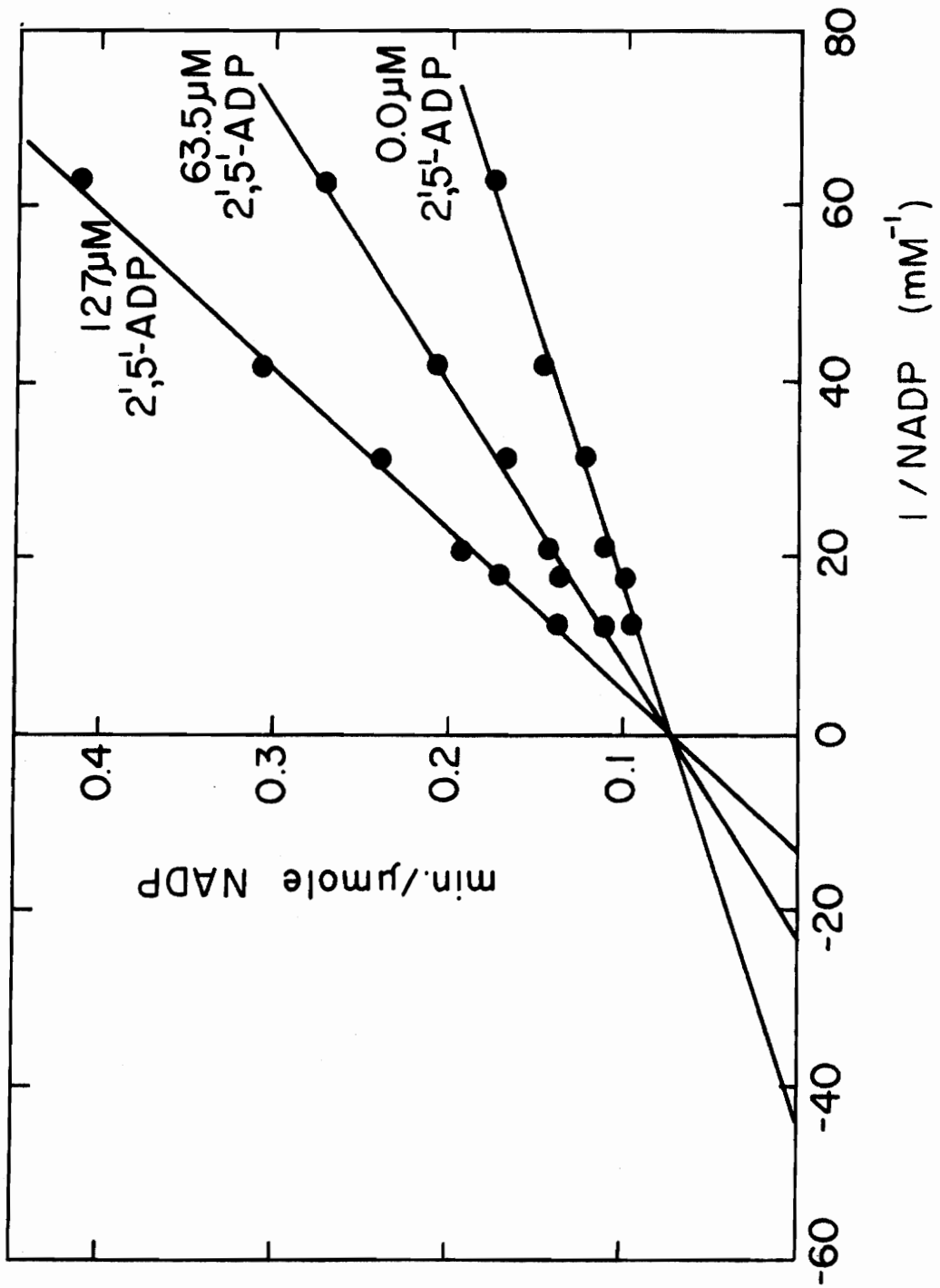


TABLE IV

Dissociation Constants for Competitive Inhibition
of Yeast 6-Phosphogluconate Dehydrogenase

Compound	$K_I, \mu\text{M}$	
	K_I, DIXON^a	$K_I, \text{LINEWEAVER-BURK}^b$
Adenosine 2'-Monophosphate	998 \pm 123	1070 \pm 120
Adenosine 2',5'-Diphosphate	58.1 \pm 6.1	55.3 \pm 6.8
2'-Phospho-adenosine Diphosphoribose	11.4 \pm 0.9	8.30 \pm 0.88
3-Aminopyridine Adenine Dinucleotide Phosphate (AADP)	38.8 \pm 1.3	74.8 \pm 5.1
Adenosine	18,600 \pm 1,500	-
Adenosine 5'-Monophosphate	9,250 \pm 100	9,300 \pm 400
Adenosine 5'-Diphosphate	3,600 \pm 120	3,870 \pm 730
Adenosine Diphosphoribose	11,400 \pm 1,200	16,500 \pm 1,500 ^a
Adenosine 3'-Monophosphate	8,000 \pm 350	5,630 \pm 820
Adenosine 3':5'-Cyclic Monophosphate	15,300 \pm 2,400	-

^aMean value \pm mean deviation, two trials

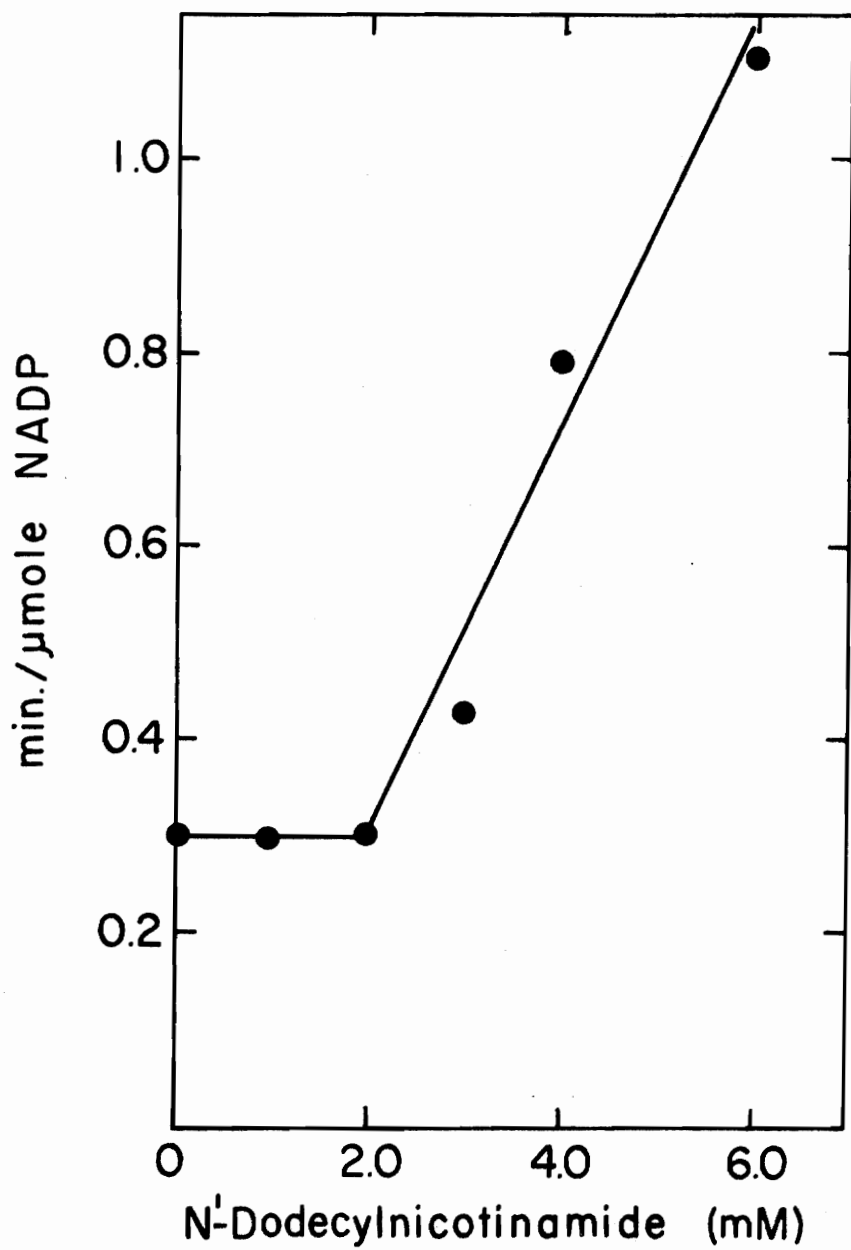
^bMean value \pm standard deviation, four trials, unless otherwise noted

under "Methods." No inhibition of yeast alcohol dehydrogenase was observed at concentrations of 22.1 mM and 19.7 mM, respectively. These concentrations are greater than twice the K_I values of two other adenosine derivatives: adenosine 5'-monophosphate ($K_I = 8.9$ mM (33,34)) and adenosine 5'-diphosphate ($K_I = 8.45$ mM (33,34)).

Inhibition of Yeast 6-Phosphogluconate Dehydrogenase by Nicotinamide Derivatives - Under conditions of saturating concentrations of 6-phosphogluconate and 15.9 μ M NADP the N^1 -methylnicotinamide chloride (80 mM), the N^1 -octylnicotinamide chloride (8.0 mM), or 3.0 mM nicotinamide mononucleotide alone did not cause inhibition of yeast 6-phosphogluconate dehydrogenase. The possibility of a synergistic effect on inhibition was examined by including sufficient 2'-adenosine monophosphate or 2',5'-adenosine diphosphate to inhibit the enzyme approximately 25 percent in the reaction mixture with the above concentrations of N^1 -alkylnicotinamides and nicotinamide mononucleotide. The combinations, however, showed no additional inhibition of the enzyme. The N^1 -dodecylnicotinamide chloride was tested and a decrease in enzyme activity was observed (Figure 5) when the concentration of this compound was between 3 and 6 mM and no activity could be detected when the concentration was greater than 6 mM.

Inactivation of Yeast 6-Phosphogluconate Dehydrogenase by N-Alkylmaleimides - When yeast 6-phosphogluconate dehydrogenase was incubated in the presence of N-alkylmaleimides,

Figure 5. Effect of micelles of N¹-dodecylnicotinamide chloride on activity of yeast 6-phosphogluconate dehydrogenase. Reaction mixtures contained 0.06 M glycylglycine buffer, pH 7.5, 0.958 mM 6-phosphogluconate, 15.9 μM NADP, 1.8 μg of enzyme, and N¹-dodecylnicotinamide chloride as indicated in a total volume of 3 ml.



enzyme activity was observed to decrease with increasing time of incubation. A plot of residual activity versus time yielded a linear relationship as exemplified by the inactivation of the enzyme by N-methylmaleimide described in Figure 6. From this linear relationship the pseudo first-order rate constants of inactivation can be calculated. Pseudo first-order rate constants were obtained for up to five different concentrations of each N-alkylmaleimide studied and were plotted against maleimide concentration. The linear relationship between these two parameters is shown in Figure 7. A second-order rate constant for inactivation of yeast 6-phosphogluconate dehydrogenase by N-methylmaleimide was calculated from the slope of the line in Figure 7. Second-order rate constants for inactivation of the enzyme were obtained in this manner for the six N-alkylmaleimides listed in Table V. These values are shown not to vary appreciably with increasing chain length of the maleimide derivatives. The longer-chain maleimides were not sufficiently soluble in 2.0 percent ethanol to perform inactivation studies.

Protection Against N-Ethylmaleimide Inactivation of Yeast 6-Phosphogluconate Dehydrogenase - The results of the protection experiments are shown in Table VI, where protection is expressed as percent decrease in the pseudo first-order rate constant of the maleimide inactivation produced by the presence of the protecting compound. It is observed that protection is obtained only in the presence of 6-phosphoglu-

Figure 6. Inactivation of yeast 6-phosphogluconate dehydrogenase by N-methylmaleimide. Incubation mixtures included 14.0 mM sodium phosphate buffer, pH 7.0, 2.0 percent ethanol, and 18 μ g of enzyme in a total volume of 1 ml. The time of addition of the enzyme was considered time zero and at various times thereafter 0.1 ml aliquots of the incubation mixture were transferred to 2.9 ml of a standard assay mixture containing 5.0 mM sodium phosphate buffer, pH 7.0, 79.3 μ M NADP, and 0.958 mM 6-phosphogluconate.

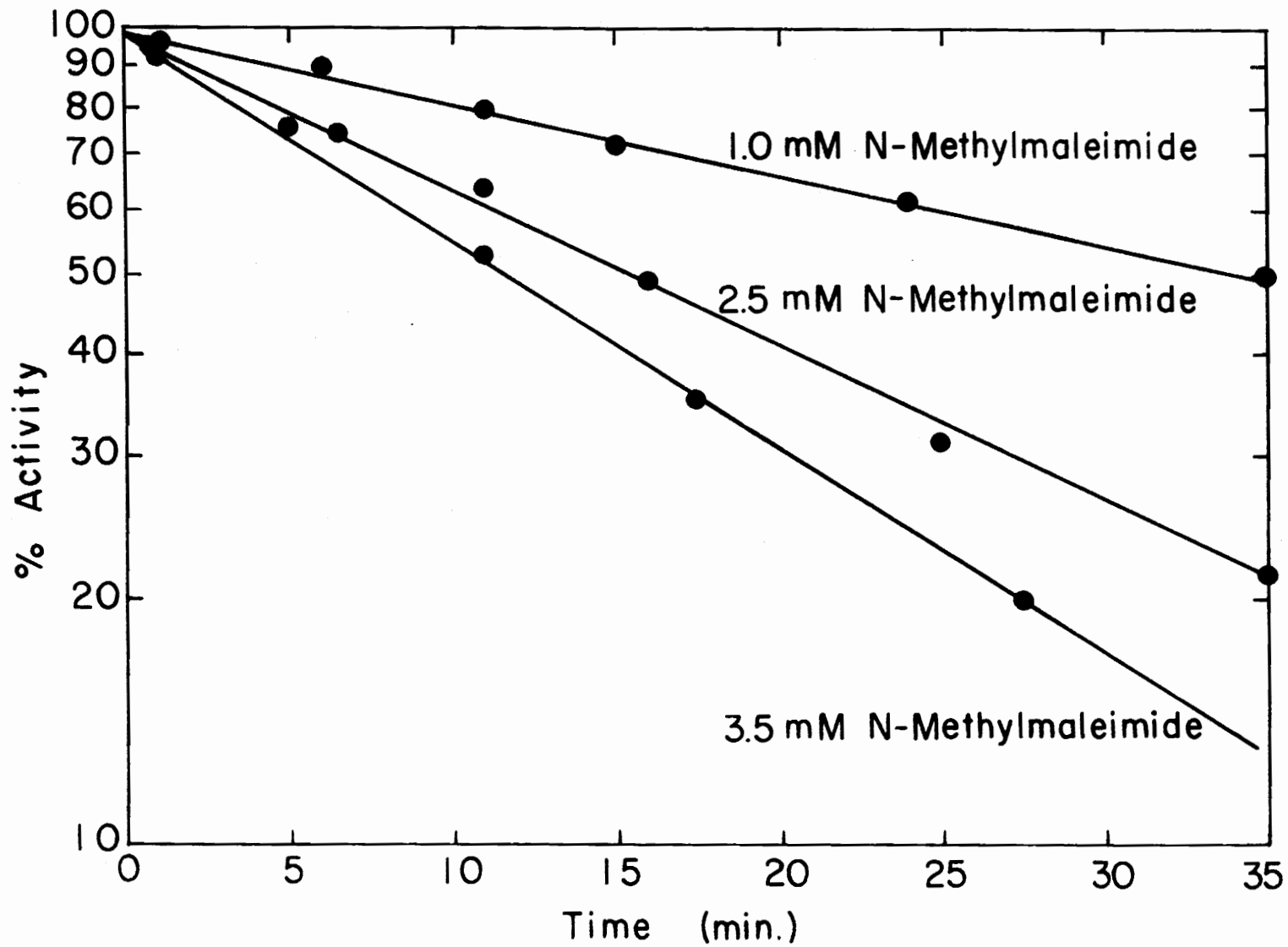


Figure 7. The effect of N-methylmaleimide concentration on the first-order rate constant of inactivation of yeast 6-phosphogluconate dehydrogenase. Incubation mixtures contained 14.0 mM sodium phosphate buffer, pH 7.0, 2.0 percent ethanol, 18 μ g of enzyme, and N-methylmaleimide as indicated in a total volume of 1 ml.

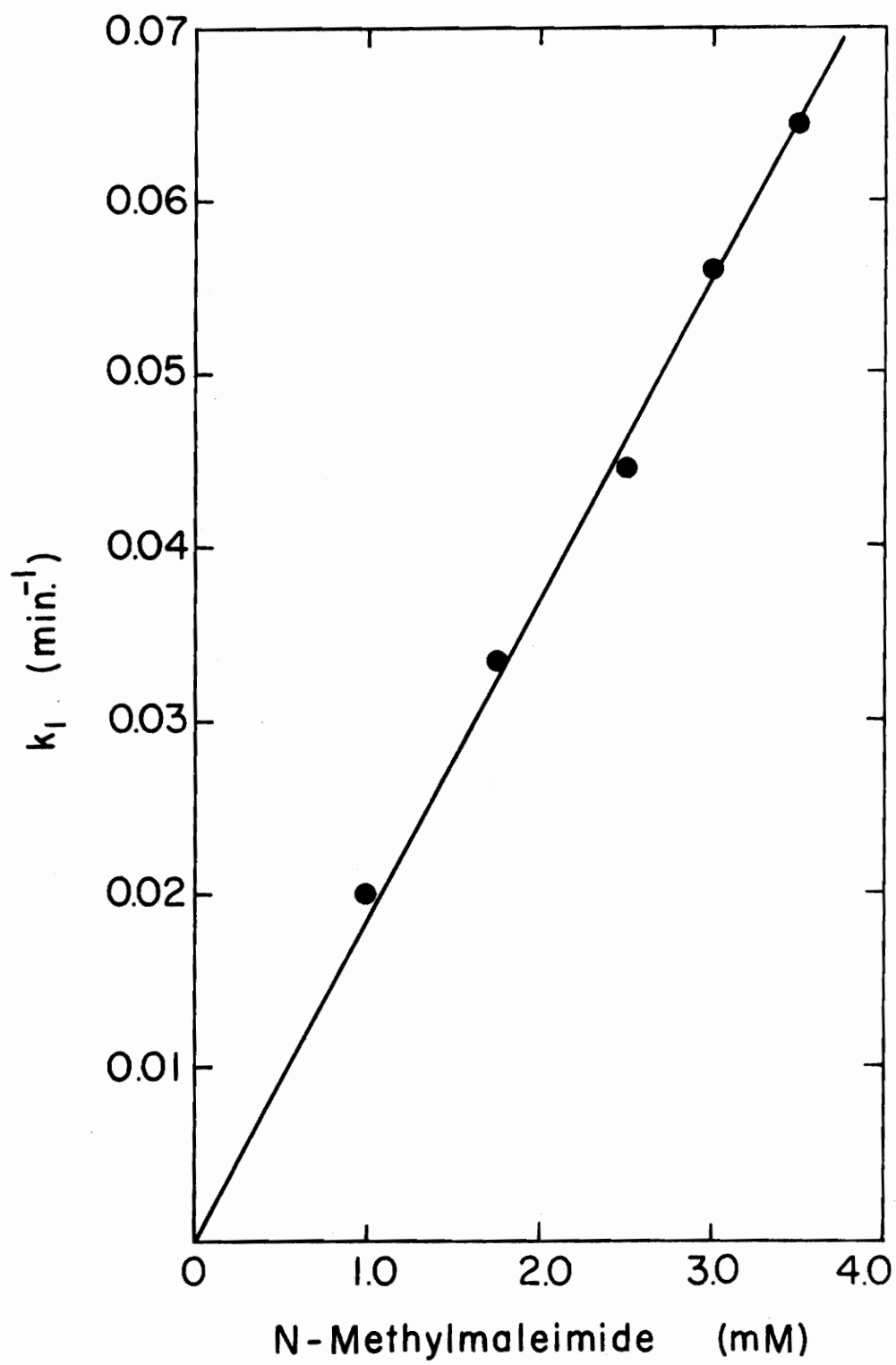


TABLE V

Irreversible Inactivation of Yeast 6-Phospho-
gluconate Dehydrogenase by N-Alkylmaleimides

Alkyl Derivative	k_2 (1/mole ⁻¹ min ⁻¹)
Methyl	18.2
Ethyl	18.5
Butyl	16.2
Pentyl	15.8
Hexyl	10.4
Phenyl	11.2

TABLE VI

Protection Against N-Ethylmaleimide Inactivation
of Yeast 6-Phosphogluconate Dehydrogenase

<u>Protecting Compounds</u>					
Compound #1	Concentration #1 (μM)	Compound #2	Concentration #2 (μM)	k_1 ($\text{min}^{-1} \times 10^2$)	Percent Protection
None	-	None	-	7.10	0.0
Adenosine 2' 5'-Diphosphate	162	None	-	7.10	0.0
NADP	60	None	-	7.10	0.0
6-Phosphogluconate	52	None	-	5.54	22.0
NADPH	1.41	None	-	7.10	0.0
6-Phosphogluconate	52	NADPH	0.47	2.80	60.6
6-Phosphogluconate	154	None	-	2.20	70.4
AADP	165	None	-	7.10	0.0
6-Phosphogluconate	52	AADP	55	2.02	71.5

^aAll values represent the results of one trial

conate ($K_m = 52 \mu\text{M}$ (8)) and this protection is greatly enhanced in the presence of NADPH ($K_m = 0.47 \mu\text{M}$ (13)) and AADP ($K_I = 55 \mu\text{M}$). However, NADP and NADPH at up to three times the K_m concentration and adenosine 2',5'-diphosphate and AADP at up to three times the K_I concentration, showed no protection.

DISCUSSION

Purity and Molecular Weight of Yeast 6-Phosphogluconate

Dehydrogenase - The Sigma preparation of yeast 6-phosphogluconate dehydrogenase appears to be homogeneous as indicated by a number of criteria. First, the enzyme was characterized as a single band on SDS polyacrylamide gel electrophoresis at pH 7.2. Similar results were obtained on 10 percent polyacrylamide gels run at both pH 8.0 and at pH 9.5. Finally, the enzyme eluted as a single, sharp peak from a Sephadex G-150 column. These results indicate that the kinetic data obtained in these studies of yeast 6-phosphogluconate dehydrogenase reflect the properties of the pure enzyme only; no contaminants that could influence the enzyme activity are present. The molecular weight of yeast 6-phosphogluconate dehydrogenase, as determined by a gel filtration experiment, was calculated to be 105,000. This is in reasonable agreement with the literature (12) value of 101,000, obtained from sucrose density gradient centrifugation.

Inhibition of Yeast 6-Phosphogluconate Dehydrogenase

by Adenosine Derivatives - The purpose of this study was to investigate the mode of binding of the pyridine nucleotide coenzyme to the NADP-dependent enzyme, yeast 6-phosphogluconate dehydrogenase. In the past, a useful approach to this type of investigation has been developed and has yielded much information concerning the enzyme-coenzyme interactions in-

volved in coenzyme binding to certain NAD-dependent dehydrogenases. Information derived from this type of study has led to the simplified representation of coenzyme binding to these enzymes, which is depicted in Figure 1. This schematically indicates the existence of binding-site regions designed for selective interaction with portions of the coenzyme molecule. The existence and relative importance of certain of these regions to coenzyme binding has been revealed through a comparison of the relative efficiencies of binding of adenosine and its derivatives, adenosine 5'-monophosphate, adenosine 5'-diphosphate, and adenosine diphosphoribose to NAD-requiring enzymes. Upon inspection of Figure 1, it becomes apparent that these compounds represent increasing portions of the NAD molecule and, as such, would be expected to selectively interact with increasing portions of the coenzyme binding site, resulting in the exclusion of NAD from the binding site. This is manifested as coenzyme-competitive inhibition of the enzyme and these regions are identified by the relative efficiency of binding of adenosine derivatives as reflected by their K_I values. Comparisons of the binding of these adenosine derivatives was presented in Table II and it is evident from these data that the binding of adenosine and its derivatives to different dehydrogenases does not follow a uniform pattern. The notable exception to the predicted pattern of steadily decreasing K_I values across this series of adenosine derivatives is yeast alcohol dehydrogenase in which adenosine 5'-monophos-

phate and adenosine 5'-diphosphate are more poorly bound than is adenosine, thus suggesting the nonexistence of a pyrophosphate binding region in this enzyme. In the other enzymes listed, adenosine, pyrophosphate, and ribose-binding regions appear to be present, but are shown to vary widely in importance to binding. Thus, coenzyme binding to NAD-dependent enzymes appears to result from selective interactions of regions of the coenzyme binding site with various portions of the coenzyme molecule. Since there appear to be regions designed for selective enzyme-coenzyme interactions in NAD-dependent enzymes, one might question whether or not these same types of regions would exist in NADP-dependent enzymes as well, given the overall resemblance of the NAD and NADP molecules. In addition, since the pyridine nucleotide-dependent enzymes are often very specific for one particular coenzyme (e.g. yeast 6-phosphogluconate dehydrogenase, for NADP (12)), one might expect the characteristics of the coenzyme binding site of NADP-dependent enzymes to differ somewhat from those of NAD-dependent enzymes, perhaps possessing some extra point of interaction that ensures the specific binding of NADP. The obvious point of difference between the NADP and NAD is the presence or absence, respectively, of the 2'-phosphate moiety, which could easily provide another point of selective enzyme-coenzyme interaction in addition to those regions proposed in Figure 1. This extra point of interaction could at least partially explain the specificity of these NADP-

requiring enzymes. By analogy with the studies of NAD-dependent enzymes, one means of testing for the existence of the proposed region of interaction with the 2'-phosphate would be to study the binding of compounds representing portions of the NADP molecule, which would possess the 2'-phosphate moiety, to the NADP-requiring enzyme. The data obtained in these studies would be compared to that obtained from the binding of similar compounds lacking the 2'-phosphate, and enhanced binding of the derivatives possessing the 2'-phosphate would be interpreted as evidence of a selective interaction of these compounds with a region suggested to interact with the 2'-phosphate of NADP.

To this end, various adenosine derivatives were studied as coenzyme-competitive inhibitors of yeast 6-phosphogluconate dehydrogenase and the results are listed in Table IV. The first four adenosine derivatives listed represent portions of the NADP molecule and, as such, possess a 2'-phosphate moiety. Very efficient binding of these compounds was observed, with K_I values ranging from approximately 100 to approximately 1.0 μM , generally decreasing with increasing resemblance of the inhibitor to the true coenzyme, NADP. The binding of the remaining adenosine derivatives, which lack the 2'-phosphate moiety, is seen to be distinctly inferior. From the data presented in Table IV several conclusions concerning the mode of coenzyme binding to the NADP-dependent enzyme, yeast 6-phosphogluconate dehydrogenase, can be drawn.

The first conclusion that one could make from these data is the existence of a region in the coenzyme binding site of yeast 6-phosphogluconate dehydrogenase which is designed for selective interaction with the 2'-phosphate moiety of NADP. The presence of this region is best illustrated by a comparison of the binding of the coenzyme analogs phosphorylated in the 2'-position (adenosine 2'-monophosphate, adenosine 2',5'-diphosphate, 2'-phospho-adenosine diphosphoribose, and 3-aminopyridine adenine dinucleotide phosphate) with their counterparts not phosphorylated in this position (adenosine, adenosine 5'-monophosphate, adenosine diphosphoribose, and NAD, respectively). In each case, the K_I value of the 2'-phosphorylated adenosine derivative is much smaller, ranging from ca. 100 times lower (for adenosine 2'-monophosphate versus adenosine) to ca. 1000 times lower (for 2'-phospho-adenosine diphosphoribose versus adenosine diphosphoribose). In the comparison of the nonfunctional coenzyme analog 3-aminopyridine adenine dinucleotide phosphate to the catalytically inactive pyridine nucleotide, NAD, the specificity of binding appears to be absolute; NAD does not inhibit the yeast 6-phosphogluconate dehydrogenase at a concentration of 70 mM. Thus, the enzyme is seen to exhibit highly preferential binding of coenzyme analogs which are phosphorylated at the 2'-position. This suggests the probable existence of a region in the coenzyme binding site which selectively interacts with the 2'-

phosphate moiety, which would at least partially explain the absolute specificity of yeast 6-phosphogluconate dehydrogenase for NADP.

The binding studies of the adenosine derivatives phosphorylated at the 2'-position follow a pattern similar to certain NAD-dependent dehydrogenases and allow the formation of a second conclusion. From the results of these studies, it appears that yeast 6-phosphogluconate dehydrogenase binds NADP through selective interactions with portions of the coenzyme molecule similar to the mode of coenzyme binding of several NAD-dependent dehydrogenases. In addition to the aforementioned 2'-phosphate binding region, regions for the selective interaction with the adenosine, pyrophosphate, and perhaps ribose, portions of the coenzyme molecule appear to exist as evidenced by adenosine inhibition and by decreasing K_I values for adenosine 2'-monophosphate, adenosine 2',5'-diphosphate, and 2'-phospho-adenosine diphosphoribose. The NADP analog, 3-aminopyridine adenine dinucleotide phosphate is less strongly bound than is 2'-phospho-adenosine diphosphoribose, which may suggest the nonexistence of a nicotinamide binding region. However, this observation may also reflect the chemical difference between the nicotinamide moiety of NADP and the 3-aminopyridine ring of 3-aminopyridine adenine dinucleotide phosphate. The change of the substituent from an amide group to an amino group and/or the resulting redistribution of electron density in the pyridinium ring

could possibly cause a decrease in enzyme-pyridinium moiety interactions resulting in slightly less efficient binding of the coenzyme analog. The binding studies of adenosine 3'-monophosphate and adenosine 3':5'-cyclic monophosphate are included to gain additional information about the proposed binding regions of the coenzyme binding site. From their K_I values, adenosine 3'-monophosphate is observed to be somewhat better bound than is adenosine 5'-monophosphate. This observation may be a result of the similarity in spatial location of the negatively-charged phosphate group in adenosine 2'-monophosphate and adenosine 3'-monophosphate, which may allow slight interactions between the latter compound and the 2'-phosphate binding region. Some interaction of a 3'-phosphate group with the pyrophosphate region is also conceivable. Cyclization of the adenosine 3'-monophosphate to form adenosine 3':5'-cyclic monophosphate results in poor binding, which is probably due to the loss of a negative charge and a resulting diminished interaction between the 3'-phosphate and the 2'-phosphate and pyrophosphate-binding regions.

A consideration of the adenosine-derivative binding data from a slightly different aspect leads to another conclusion: generally, as resemblance of the adenosine derivative to the true coenzyme is increased, the interaction of the 2'-phosphate with the binding site becomes of greater importance to efficient binding. In the case of the 2'-phosphorylated coenzyme analogs, the efficiency of binding of adenosine deri-

vatives generally increases as the derivative corresponds to more of the NADP molecule and can thereby interact with more of the binding regions of the coenzyme binding site. One might expect a similar pattern for the adenosine derivatives lacking the 2'-phosphate (adenosine, adenosine 5'-monophosphate, adenosine 5'-diphosphate, adenosine diphosphoribose) and NAD, which also correspond to progressively greater portions of the NADP molecule. This is shown to be an erroneous assumption, however, for while the binding of these compounds improves across the series adenosine, adenosine 5'-monophosphate, and adenosine 5'-diphosphate (which presumably interact with adenosine and pyrophosphate regions), it becomes much less efficient in the case of adenosine diphosphoribose, and NAD does not inhibit at 70 mM concentrations. This suggests that the interaction with the 2'-phosphate is a necessary prerequisite for efficient binding of adenosine derivatives larger than adenosine 5'-diphosphate. One possible explanation for this observation would be that the selective interaction of the NADP binding site with the 2'-phosphate induces a conformational change in the coenzyme binding site that permits selective enzyme-coenzyme interactions with the nicotinamide-ribose portions of the NADP molecule. If the adenosine derivative lacks a 2'-phosphate and thus cannot generate the proposed conformational change, the addition of a ribose or nicotinamide-ribose moiety could hinder the ligand-coenzyme interaction of an adenosine 5'-diphosphate moi-

ety through unfavorable steric interactions that would prohibit close approximation of the ligand to the appropriate binding regions. Another possible explanation is that the binding of adenosine derivatives phosphorylated at positions other than 2'- occurs through multiple types of enzyme-ligand interactions. While concomitant interactions with the adenosine and pyrophosphate regions could partially account for the binding of these adenosine derivatives, it is conceivable that there could also be binding through interactions between the negatively charged phosphate (or pyrophosphate) groups on the ligand and any region designed to interact with negatively charged phosphate, such as the 2'-phosphate region. The loss of a negatively charged, free phosphate or pyrophosphate group with the addition of a ribose moiety could greatly detract from such interactions and thereby reduce the efficiency of binding of adenosine diphosphoribose. A further addition of a nicotinamide moiety to adenosine diphosphoribose, to form NAD, could induce additional steric problems that virtually preclude its interaction with the coenzyme binding site.

Inhibition of Yeast Alcohol Dehydrogenase by Adenosine Derivatives - While yeast 6-phosphogluconate dehydrogenase, an NADP-dependent enzyme, possesses a binding region for selective interaction with the 2'-phosphate of NADP, an NAD-dependent enzyme such as yeast alcohol dehydrogenase, would be expected to lack such a region. If a 2'-phosphate region were present in yeast alcohol dehydrogenase, one would expect

to obtain enhanced binding of adenosine derivatives phosphorylated at the 2'-position when compared to similar derivatives not phosphorylated at this position, as was observed for yeast 6-phosphogluconate dehydrogenase. When adenosine 2'-monophosphate and adenosine 2',5'-diphosphate were tested as inhibitors of yeast alcohol dehydrogenase, they were observed not to inhibit the enzyme at concentrations greater than twice the K_I values of adenosine 5'-monophosphate and adenosine 5'-diphosphate. From this observation one can conclude that there is no region in yeast alcohol dehydrogenase designed to selectively interact with a 2'-phosphate and that the enhancement of binding of compounds containing the 2'-phosphate with yeast 6-phosphogluconate dehydrogenase is related to selective interactions associated with the coenzyme specificity of this NADP-dependent enzyme.

Inhibition of Yeast 6-Phosphogluconate Dehydrogenase by Nicotinamide Derivatives - An examination of Figure 1 indicates the existence of two regions not defined in the binding studies of adenosine derivatives, the pyridinium ring and hydrophobic regions. These regions were identified in a number of dehydrogenases using binding studies of structural analogs of the pyridinium moiety of the pyridine nucleotides, the N^1 -alkylnicotinamide chlorides (N^1 -methyl to N^1 -dodecyl, inclusive). These compounds were observed to be coenzyme-competitive inhibitors by means of the formation of binary complexes at the pyridine nucleotide binding site. Multiple

inhibition analysis (33) using combinations of N¹-alkylnicotinamides and adenosine derivatives indicated that these compounds bind at different regions of the coenzyme binding site, thus suggesting the existence of a region which selectively interacts with the positively charged nicotinamide moiety of NAD. The hydrophobic region proximal to the nicotinamide region of some dehydrogenases was defined using comparative binding studies of N¹-alkylnicotinamide chlorides with varying side chain lengths. Steadily decreasing K_I values with increasing length of the N¹-alkyl side chain was judged to be a consequence of interactions of these side chains with a nonpolar region proximal to the nicotinamide binding region of the coenzyme binding site. This hydrophobic region is believed to serve a multiple purpose. It may serve as a point of interaction with the (nicotinamide-) ribose moiety in NAD binding, which would at least partially explain the enhanced binding of adenosine diphosphoribose compared to that of adenosine derivatives representing smaller portions of the coenzyme molecule (see Table IV). Another function of perhaps greater importance is that this region may provide a point of interaction with the uncharged 1,4-dihyronicotinamide portion of NADH and may serve to provide a nonpolar environment in which direct hydride transfer can take place. In addition, this region may have a role in stabilization of the relatively nonpolar transition state of the oxidation-reduction reaction. A positive chainlength effect in the binding of N-alkylnico-

tinamides to some dehydrogenases (30,35-37) indicated the presence of the hydrophobic region in these enzymes. However, such a region cannot be demonstrated by this approach in certain other dehydrogenases¹ due to their failure to form binary complexes with these compounds. From the data obtained with yeast 6-phosphogluconate dehydrogenase, it too was found to be incapable of binding the N¹-alkylnicotinamide chlorides alone. In consideration of data from adenosine binding studies, it was suggested that a possible reason for this observation was that the coenzyme binding process requires interaction with a 2'-phosphate on an adenosine derivative to assume the proper conformation that would allow interactions between the coenzyme binding site and the nicotinamide-ribose portions of the coenzyme molecule. Thus, sufficient concentrations of adenosine 2'-monophosphate or adenosine 2',5'-monophosphate were included to cause partial inhibition of the enzyme and N¹-methylnicotinamide chloride was added to investigate a possible synergistic effect on inhibition. This procedure resulted in no enhancement of inhibition, thus one cannot detect the existence of either a nicotinamide binding region or a hydrophobic region in the coenzyme binding site of yeast 6-phosphogluconate dehydrogenase using this type of study. This does not provide confirmation of the nonexistence of these regions since it is possible that 2'-phosphorylated coenzyme analogs structurally related to a larger portion of

¹Anderson, B. M., unpublished results

the coenzyme molecule could be required to promote an enzyme conformational change resulting in the accessibility of a pyridinium ring region and/or a hydrophobic region to the ligand. While it is conceivable that yeast 6-phosphogluconate dehydrogenase could rely on forces other than a pyridinium ring region (such as conformational changes induced by coenzyme binding) to properly orient the nicotinamide ring for the stereospecific hydride transfer process, it is difficult to dismiss the existence of the hydrophobic region given its great potential importance to binding and catalysis. However, if this nonpolar region is present, other means will have to be devised to determine its existence.

It was found that yeast 6-phosphogluconate dehydrogenase could be inhibited by N¹-dodecylnicotinamide chloride at concentrations greater than 2 mM and no activity is observed at concentrations above 6 mM. A similar phenomenon has been observed in the past for several other dehydrogenases¹ and has been attributed to protein denaturation in the presence of micelles of the N¹-dodecylnicotinamide chloride (critical micelle concentration = 5.8 mM at ionic strength of 0.1 M (51)). Another possible explanation is that this observation is that this observation results from selective interactions between these enzymes and the micelles at the coenzyme binding site which result in enzyme inhibition. A third possibility is suggested by results obtained by Anderson and Anderson

¹Anderson, B. M., unpublished results

(51). In these studies, changes in the fluorescence properties of NADH in the presence of micelles of N¹-dodecylnicotinamide chloride were reported, indicating that interactions occur between the coenzyme and the micelles. While the concentrations of N¹-dodecylnicotinamide chlorides used in the current study are somewhat lower than the expected critical micelle concentration, loss of activity may reflect interactions of the coenzyme with submicellar molecular aggregates of N¹-dodecylnicotinamide chloride.

Inactivation of Yeast 6-Phosphogluconate Dehydrogenase by N-Alkylmaleimides - In this study, yeast 6-phosphogluconate dehydrogenase was incubated in the presence of various N-alkylmaleimides. This resulted in a time-dependent inactivation of the enzyme, confirming earlier studies (15,16) which indicated that a sulfhydryl group is necessary for activity of this enzyme. The N-alkylmaleimides tested in this study are listed in Table V along with the second-order rate constants of inactivation, k_2 , for these compounds. From the k_2 values of these reactions, it is evident that the inactivation of yeast 6-phosphogluconate dehydrogenase by these compounds proceeds without a positive chainlength effect and therefore the possession of a nonpolar side chain does not enhance the N-alkylmaleimide inactivation of this enzyme. By contrast, a positive chainlength effect has been observed for N-alkylmaleimide inactivation of several other enzymes (31,38-42) and has been suggested to result from enhanced, selective interactions of

the longer N-alkylmaleimide side chains with a nonpolar region near the essential sulfhydryl group. These selectively bound N-alkylmaleimides are believed to then react with the sulfhydryl group to irreversibly inactivate the enzyme. As such effects are not observed with yeast 6-phosphogluconate dehydrogenase, the essential sulfhydryl group of this enzyme is presumably not in a hydrophobic environment, but rather in a more polar, perhaps well-solvated location. This explanation is supported by the fact that the simple sulfhydryl compounds cysteine and glutathione, in which such nonpolar interactions are impossible, also exhibit no chainlength effect in reactions with N-alkylmaleimides (38). Thus, if there is no selective interaction between yeast 6-phosphogluconate dehydrogenase and the N-alkylmaleimides, one would expect that the reaction between the essential sulfhydryl group of this enzyme and the N-alkylmaleimides relies mostly on random collisions of enzyme and reagent. The second-order plots of yeast 6-phosphogluconate dehydrogenase inactivation by N-alkylmaleimides indicate that this is true; within the range of assay, the first-order rate constant displays a linear increase with increasing maleimide concentration. This is in contrast to D-amino acid oxidase (42) and papain (40) which display saturation kinetics in these same plots and which also exhibit a positive chainlength effect with N-alkylmaleimide inactivation.

Protection Against N-Ethylmaleimide Inactivation of Yeast 6-Phosphogluconate Dehydrogenase - Yeast 6-phosphogluconate dehydrogenase can be protected from N-ethylmaleimide inactivation by including certain compounds in the incubation of the enzyme with N-ethylmaleimide. The compounds employed are listed in Table VI and all are known to be capable of selectively binding to the enzyme; 6-phosphogluconate at the substrate binding site and adenosine 2',5'-diphosphate, NADP, NADPH, and AADP at the coenzyme binding site. When the enzyme is incubated with N-ethylmaleimide and the substrate at its K_m concentration, the enzyme is protected 22 percent from inactivation and this protection increases to 71.5 percent at three times the K_m value of this compound. These results are similar to those reported by Grazi et al. (15) and Rippa et al. (16) who observed protection of yeast 6-phosphogluconate dehydrogenase from inactivation by iodoacetate and chlorodinitrobenzene by 6-phosphogluconate. These results indicate that the binding of 6-phosphogluconate to the enzyme gives rise to protection of the essential sulfhydryl group, perhaps due to the presence of the sulfhydryl group at the substrate binding site or may result from a conformational change brought about by substrate binding. With the addition of NADPH or AADP, however, the protection by 6-phosphogluconate is greatly enhanced, increasing the degree of protection approximately threefold in each case. This indicates that the ternary complex between enzyme, substrate, and coenzyme

somehow very effectively shields the sulfhydryl group from reaction with N-ethylmaleimide. While 6-phosphogluconate alone can afford protection for the enzyme, there is a large synergistic effect in protection brought about by the binding of the coenzyme or coenzyme analog. This suggests that interaction of the enzyme with the coenzyme or coenzyme analog, gives rise to a change in the enzyme conformation that enhances the protective effect of the substrate. The presence of the substrate is necessary for protection, though, as evidenced by lack of protection by adenosine 2',5'-diphosphate, NADP, NADPH, or AADP alone.

The synergistic effect on protection of the enzyme is an interesting observation, and suggests an area for future study. This would involved a further investigation of this synergistic effect by applying information from a combination of coenzyme binding site studies to maleimide inactivation studies. Specifically, the enzyme could be incubated with adenosine derivatives representing portions of the coenzyme molecule and N-ethylmaleimide and the synergistic effect on protection by each compound determined. A comparison of protective effects would provide information as to the specific interactions required to generate this synergistic effect and hopefully provide information about conformational changes induced by ternary complex formation.

SUMMARY

Studies of the binding of coenzyme analogs to yeast 6-phosphogluconate dehydrogenase indicate that NADP binding to the enzyme results from selective interactions between regions of the coenzyme binding site and portions of the NADP molecule. These studies suggested the existence of coenzyme binding site regions which selectively interact with the adenosine, 2'-phosphate, and pyrophosphate moieties of NADP. The importance of the 2'-phosphate to coenzyme binding was indicated by enhanced binding of adenosine derivatives possessing this moiety when compared to adenosine derivatives not phosphorylated at this position. The better binding of the 2'-phosphorylated derivatives became more pronounced with increasing resemblance of the derivative to the NADP molecule, and NAD was not inhibitory up to 70 mM. These results substantiate the concept that interaction of the enzyme with the 2'-phosphate is a key factor in the specificity of yeast 6-phosphogluconate dehydrogenase for NADP.

Structural analogs of the pyridinium portion of the NADP molecule, N¹-alkylnicotinamide chlorides, did not inhibit yeast 6-phosphogluconate dehydrogenase at concentrations normally required for selective interactions with dehydrogenases; however, enzyme activity was decreased at micellar concentrations of N¹-dodecylnicotinamide chloride.

Investigations of the role and environment of the essential sulfhydryl group of this enzyme were also performed.

N-alkylmaleimides (N-methyl - N-hexyl, inclusive) were shown to inactivate the enzyme, but without a chainlength effect. In the presence of 6-phosphogluconate, the enzyme was protected from N-ethylmaleimide inactivation and this protection was enhanced by the addition of NADPH or AADP.

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STUDIES OF THE COENZYME BINDING SITE AND ESSENTIAL SULFHYDRYL
GROUP OF YEAST 6-PHOSPHOGLUCONATE DEHYDROGENASE

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(ABSTRACT)

Studies of the binding of coenzyme analogs to yeast 6-phosphogluconate dehydrogenase indicate that NADP binding to the enzyme results from selective interactions between regions of the coenzyme binding site and portions of the NADP molecule. These studies suggested the existence of coenzyme binding site regions which selectively interact with the adenosine, 2'-phosphate, and pyrophosphate moieties of NADP. The importance of the 2'-phosphate to coenzyme binding was indicated by enhanced binding of adenosine derivatives possessing this group. The better binding of the 2'-phosphorylated derivatives became more pronounced with increasing resemblance to the NADP molecule. NAD was not inhibitory up to 70 mM. These results substantiate the concept that interaction of the enzyme with the 2'-phosphate is a key factor in the specificity for NADP.

Structural analogs of the pyridinium portion of the NADP molecule, N¹-alkylnicotinamide chlorides, did not inhibit yeast 6-phosphogluconate dehydrogenase at concentrations normally required for selective interactions with dehydrogenases; however, enzyme activity was decreased at micellar concentrations of N¹-dodecylnicotinamide chloride.

Investigations of the role and environment of the essential sulfhydryl group of this enzyme were also performed. N-Alkylmaleimides (N-methyl-N-hexyl, inclusive) were shown to inactivate the enzyme, but without a chainlength effect. In the presence of 6-phosphogluconate, the enzyme was protected from N-ethylmaleimide inactivation and this protection was enhanced by the addition of NADPH or 3-aminopyridine adenine dinucleotide phosphate.