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INHIBITION OF L-α-GLYCEROPHOSPHATE DEHYDROGENASE BY ALKYLAMMONIUM CHLORIDES*

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Abstract—Eight *n*-alkylammonium chlorides were demonstrated to inhibit the L-α-glycerophosphate dehydrogenase-catalyzed oxidation of L-α-glycerophosphate. The inhibition obtained was shown to be competitive with respect to the coenzyme, NAD. Interactions of these inhibitors with a "pyridinium ring" region at the NAD binding site of the enzyme was suggested by the competitive nature of the inhibition obtained and by the simultaneous binding of *n*-decylammonium chloride and adenylic acid to the enzyme. The binding of *n*-alkylammonium chlorides to L-α-glycerophosphate dehydrogenase increases with increasing chain length of the inhibitor, indicating non-polar interactions with a hydrophobic region of the enzyme to be of importance in these binding processes.

THE AMINO group, which can be used to confer a specific biological activity upon an organic molecule, can also result in the formation of a potent inhibitor of several enzyme systems. There are many enzyme-catalyzed reactions in which a specific interaction between a protonated amino group and the protein involved contributes significantly to the essential binding of coenzymes or substrates. In the binding of substrates and inhibitors to cholinesterases,[1, 2] trypsin[3, 4] and monoamine oxidase,[5] the importance of a protonated amino group is immediately suggested by the substrate specificity of these enzymes. The effective inhibition of yeast alcohol dehydrogenase[6] by *n*-alkylammonium chlorides, which was demonstrated to be competitive with respect to NAD, was not anticipated on the basis of structural analogy to the oxidized coenzyme. The efficiency of binding of *n*-alkylammonium chlorides to yeast alcohol dehydrogenase does not reside in interactions of the protonated amino group alone, but includes non-polar interactions of the alkyl groups with a hydrophobic region of this enzyme. The NAD binding site of yeast alcohol dehydrogenase contains a "pyridinium ring" region that interacts with the positively charged ring nitrogen of the oxidized coenzyme[7] and a hydrophobic region close by, which may, in the binding of NAD, interact with the ribose attached to the nicotinamide moiety of the oxidized coenzyme. As a result of the presence of these two regions, *n*-alkylammonium chlorides are bound very effectively to this enzyme. Such a combination of regions is not a feature common to all dehydrogenases,[8] as witnessed by the lack of inhibition of many dehydrogenases by N^1-alkylnicotinamide chlorides, which are also bound through

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the same combination of interactions. Recently, rabbit muscle L-α-glycerophosphate dehydrogenase was demonstrated to be effectively inhibited by N^1-alkylnicotinamide chlorides.[9] This inhibition was shown to be coenzyme-competitive and to involve interactions of the inhibitors with a pyridinium ring region and a closely associated hydrophobic region on the enzyme. The present study involves the investigation of selective binding of *n*-alkylammonium chlorides to this enzyme.

MATERIALS AND METHODS

Materials. Crystalline rabbit muscle L-α-glycerophosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8), obtained in ammonium sulfate suspension from the Sigma Chemical Company, St. Louis, Mo., was diluted in 0·05 M Tris-HCl buffer, pH 7·85, containing 0·1% crystalline bovine serum albumin. N^1-decylnicotinamide chloride and N^1-butylnicotinamide chloride were prepared as described previously.[7] NAD, adenylic acid and L-α-glycerophosphate were also purchased from the Sigma Chemical Company. *n*-Alkylamines were obtained from Eastman Organic Chemicals, Rochester, N.Y.

Methods. The L-α-glycerophosphate dehydrogenase-catalyzed oxidation of L-α-glycerophosphate was studied at 25° in 3·0 ml of reaction mixtures containing 0·05 M Tris-HCl buffer, pH 7·85. The concentrations of the components of these reaction mixtures, such as NAD, inhibitors and enzyme, will be included in the specific descriptions of individual experiments. Reactions were initiated by the addition of enzyme and initial velocities were obtained by measuring the linear increase in fluorescence intensity for 1 min at 460 mμ with excitation at 368 mμ. Multiple inhibition studies, using two different competitive inhibitors,[10] were carried out as described previously.[11]

Spectrophotofluorometric measurements were carried out at 25° in a temperature controlled cell compartment of an Aminco-Bowman spectrophotofluorometer with a xenon mercury lamp, Pacific photometric recording photometer model 15 fitted with an EMI 9502 photocell, and a Mosely Autograf model 135A X-Y recorder. Measurements of pH were made at 25° with a Radiometer pH meter, type PHM 4b, with a G-200-B glass electrode.

RESULTS

Eight *n*-alkylammonium chlorides were studied as inhibitors of rabbit muscle L-α-glycerophosphate dehydrogenase. All of the *n*-alkylammonium chlorides studied (the *n*-butyl to the *n*-decyl derivative inclusive) and the *n*-dodecyl derivative effectively inhibited the oxidation of L-α-glycerophosphate catalyzed by this enzyme. The inhibition obtained with these compounds, when studied as a function of varying NAD concentration, was observed to be competitive with respect to NAD in each case. Initial velocity measurements made in the presence of *n*-hexylammonium chloride, plotted according to Lineweaver and Burk,[12] are shown in Fig. 1. The competitive nature of the inhibition caused by *n*-hexylammonium chloride is representative of the relationship observed in the inhibition by all of the *n*-alkylammonium chlorides studied. Inhibition by these compounds was also studied under conditions of constant NAD and L-α-glycerophosphate concentrations and varying inhibitor concentrations. The inhibition obtained at six different concentrations of *n*-octylammonium chloride,

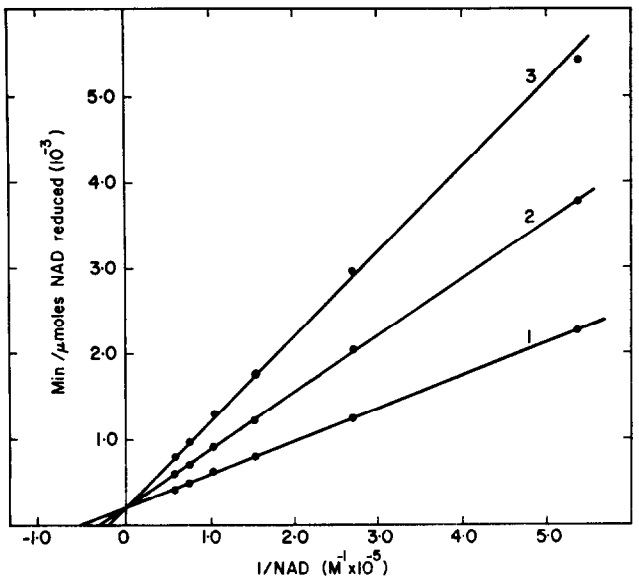

FIG. 1. Competitive inhibition of L-α-glycerophosphate dehydrogenase by *n*-hexylammonium chloride. NAD concentrations were varied from $1{\cdot}84 \times 10^{-6}$ M to $1{\cdot}66 \times 10^{-5}$ M. Reaction mixtures contained 0·05 M Tris buffer (pH 7·85), $4{\cdot}33 \times 10^{-3}$ M L-α-glycerophosphate, 0·2 μg L-α-glycerophosphate dehydrogenase, NAD and inhibitor as indicated in a total volume of 3 ml. Line 1, no inhibitor; line 2, $1{\cdot}0 \times 10^{-2}$ M *n*-hexylammonium chloride; line 3, $2{\cdot}0 \times 10^{-2}$ M *n*-hexylammonium chloride.

plotted according to Dixon,[13] is shown in Fig. 2. A similar relationship was obtained for the other seven *n*-alkylammonium chlorides when studied in this manner. Inhibitor dissociation constants for each of the *n*-alkylammonium chlorides, calculated from the two independent types of experiments described above, are listed in Table 1. The

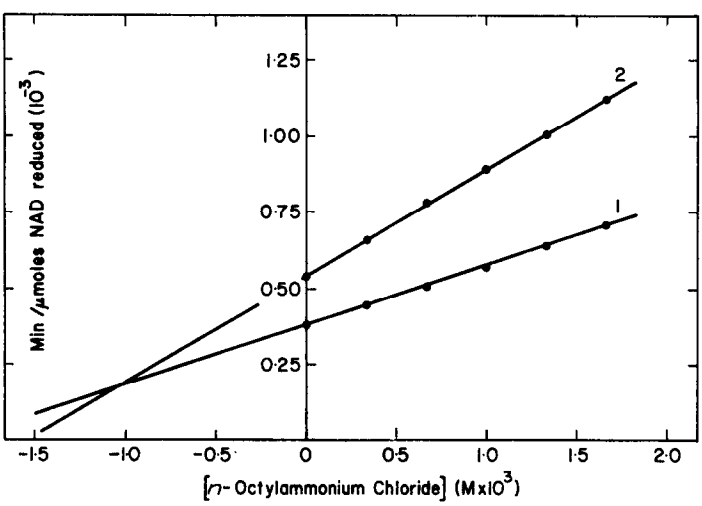

FIG. 2. Inhibition of L-α-glycerophosphate dehydrogenase as a function of *n*-octylammonium chloride concentration. Reaction mixtures contained 0·05 M Tris buffer (pH 7·85), $4{\cdot}33 \times 10^{-3}$ M L-α-glycerophosphate, 0·2 μg of enzyme, NAD and inhibitor as indicated in a total volume of 3 ml. Line 1, $1{\cdot}84 \times 10^{-5}$ M NAD; line 2, $1{\cdot}1 \times 10^{-5}$ M NAD.

inhibitor constants obtained decrease with increasing chain length of the inhibitor. This chain length effect on the inhibition of L-α-glycerophosphate dehydrogenase by *n*-alkylammonium chlorides is illustrated in Fig. 3.

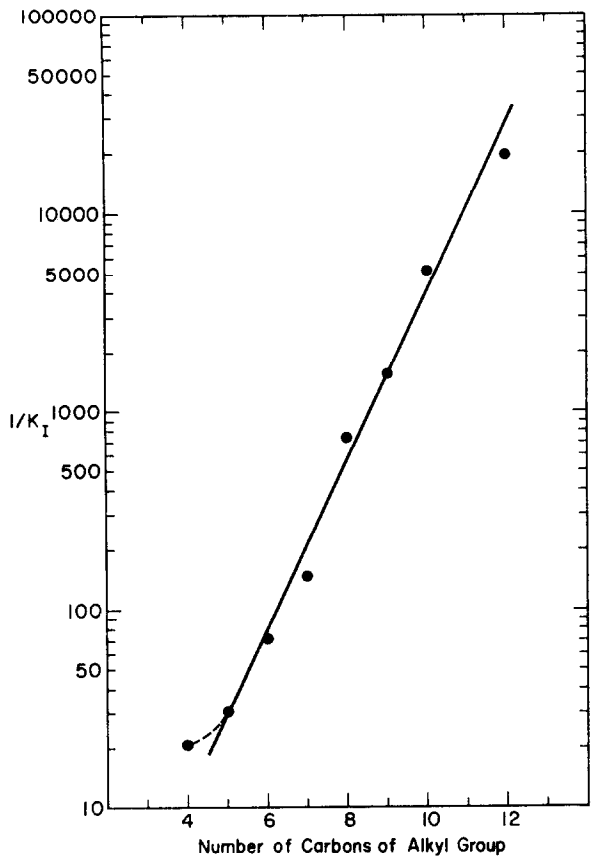

FIG. 3. The relationship of the logarithm of the reciprocals of the inhibitor constants to the chain length of the alkyl substituents of the inhibitors. K_I values employed represent averages of those listed in Table 1.

Since the *n*-alkylammonium chlorides were demonstrated to be coenzyme-competitive inhibitors of L-α-glycerophosphate dehydrogenase, their mode of binding was compared to that of other coenzyme-competitive inhibitors of this enzyme. Multiple inhibition analysis described previously by Yonetani and Theorell[10] was used for this purpose. In the study of any given inhibitor pair, initial velocities were measured at five different concentrations of one inhibitor with the second inhibitor present at a constant concentration. This series of five reactions was then repeated so that a total of five different concentrations of the second inhibitor were employed. The data obtained were plotted as the ratio of the initial velocity in the absence of inhibitor (v_0) to the initial velocity in the presence of inhibitor (v_i) vs. the concentration of this inhibitor. The multiple inhibition observed with the inhibitor pair, *n*-decylammonium chloride—N^1-decylnicotinamide chloride, plotted in this manner

TABLE 1. INHIBITOR CONSTANTS OF THE *n*-ALKYLAMMONIUM CHLORIDES*

Alkyl substituent of inhibitor	K_I(M) Lineweaver–Burk plot	K_I(M) Dixon plot
Butyl	$4{\cdot}75 \times 10^{-2}$	$4{\cdot}80 \times 10^{-2}$
Pentyl	$3{\cdot}36 \times 10^{-2}$	$3{\cdot}10 \times 10^{-2}$
Hexyl	$1{\cdot}42 \times 10^{-2}$	$1{\cdot}50 \times 10^{-2}$
Heptyl	$6{\cdot}94 \times 10^{-3}$	$7{\cdot}60 \times 10^{-3}$
Octyl	$1{\cdot}30 \times 10^{-3}$	$1{\cdot}04 \times 10^{-3}$
Nonyl	$5{\cdot}86 \times 10^{-4}$	$6{\cdot}80 \times 10^{-4}$
Decyl	$1{\cdot}88 \times 10^{-4}$	$1{\cdot}67 \times 10^{-4}$
Dodecyl	$5{\cdot}33 \times 10^{-5}$	$5{\cdot}00 \times 10^{-5}$

* Inhibitor dissociation constants were calculated from kinetic studies carried out under conditions described in Figs. 1 and 2. Experiments that employed varying substrate concentrations (Lineweaver–Burk plots[12]) were performed at two inhibitor concentrations, one above and one below the value of the inhibitor dissociation constant. Experiments employing constant substrate concentrations (Dixon plots[13]), in all cases utilized inhibitor concentration ranges extending to approximately 70 per cent inhibition.

resulted in a series of parallel lines (Fig. 4). A parallel line relationship was also obtained in the multiple inhibition by the inhibitor pair composed of *n*-decylammonium chloride and N^1-butylnicotinamide chloride (Fig. 5). Multiple inhibition by the inhibitor pair, *n*-decylammonium chloride—adenylic acid, resulted in a converging

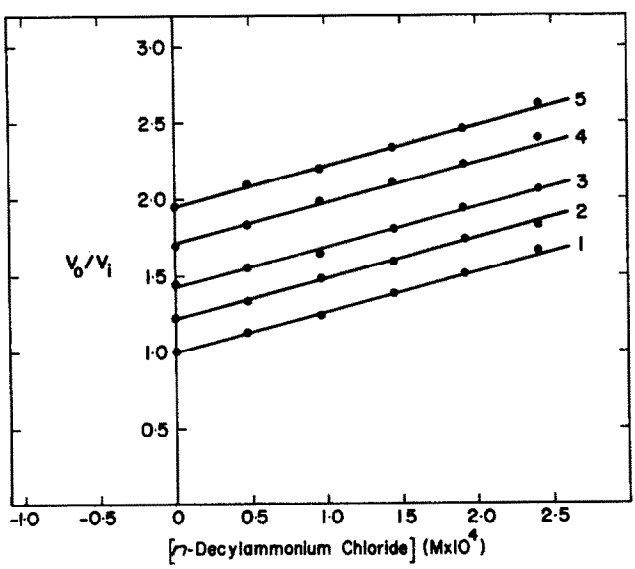

FIG. 4. Multiple inhibition of L-α-glycerophosphate dehydrogenase by *n*-decylammonium chloride and N^1-decylnicotinamide chloride. Reaction mixtures contained 0·05 M Tris buffer (pH 7·85), $1{\cdot}77 \times 10^{-5}$ M NAD and $4{\cdot}33 \times 10^{-3}$ M L-α-glycerophosphate in a total volume of 3 ml. The concentration of *n*-decylammonium chloride was varied from 0 to $2{\cdot}4 \times 10^{-4}$ M. The concentration of N^1-decylnicotinamide chloride used was as follows: line 1, zero; line 2, $3{\cdot}0 \times 10^{-4}$ M; line 3, $6{\cdot}0 \times 10^{-4}$ M; line 4, $9{\cdot}0 \times 10^{-4}$ M; line 5, $1{\cdot}2 \times 10^{-3}$ M. Reactions were initiated by the addition of 0·3 μg of enzyme.

line relationship as shown in Fig. 6. The interaction constant (α) was calculated from this plot[10] to be 0·89.

DISCUSSION

The *n*-butyl to the *n*-decylammonium chloride inclusive, and *n*-dodecylammonium chloride were demonstrated to inhibit the L-α-glycerophosphate dehydrogenase-catalyzed oxidation of L-α-glycerophosphate. With each of these inhibitors, the

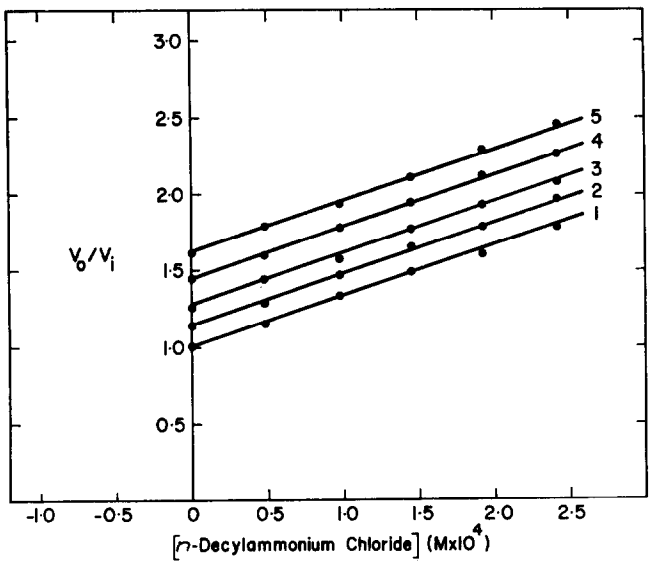

FIG. 5. Multiple inhibition of L-α-glycerophosphate dehydrogenase by *n*-decylammonium chloride and N^1-butylnicotinamide chloride. Reaction mixtures contained 0·05 M Tris buffer (pH 7·85), $1{\cdot}77 \times 10^{-5}$ M NAD and $3{\cdot}3 \times 10^{-3}$ M L-α-glycerophosphate in a total volume of 3 ml. The concentration of *n*-decylammonium chloride was varied from 0 to $2{\cdot}4 \times 10^{-4}$ M. The concentration of N^1-butylnicotinamide chloride used was as follows: line 1, zero; line 2, $6{\cdot}67 \times 10^{-3}$ M; line 3, $1{\cdot}33 \times 10^{-2}$ M; line 4, $2{\cdot}0 \times 10^{-2}$ M; line 5, $2{\cdot}67 \times 10^{-2}$ M. Reactions were initiated by the addition of 0·4 μg of enzyme.

inhibition was demonstrated to be competitive with respect to the coenzyme, NAD. The inhibition obtained was observed to increase with increasing chain length of the inhibitor (Fig. 3). This chain length effect on inhibition can be related to nonpolar interactions in the binding of inhibitors to the enzyme. The difference in free energy of binding of the various inhibitors to the enzyme can be evaluated from the linear relationship shown in Fig. 3. By using the change in the logarithm of $1/K_I(\Delta pK_I)$ per methylene group, free energy changes can be calculated from the following relation:

$$\Delta\Delta F = 2{\cdot}3\ RT\ \Delta pK_I.$$

In this manner, a free energy change per methylene group of 0·59 kcal/mole was obtained. This value lies within the range suggested for interactions through dispersion forces[14] and is very close to the value (0·471 kcal/mole) calculated from the chain length effect observed in the binding of N^1-alkylnicotinamide chlorides to the enzyme.[9] This similarity of nonpolar interactions, along with the coenzyme-competitive nature

of the inhibition by *n*-alkylammonium chlorides, suggested that the binding of these compounds to L-α-glycerophosphate dehydrogenase occurs through interactions similar to those of importance in the binding of N^1-alkylnicotinamide chlorides to this enzyme. N^1-alkylnicotinamide chlorides were demonstrated previously[9] to be coenzyme-competitive inhibitors of L-α-glycerophosphate dehydrogenase, which interacted with a pyridinium ring region and a closely associated hydrophobic region at the NAD binding site of this enzyme. Coenzyme-competitive inhibitors interacting with

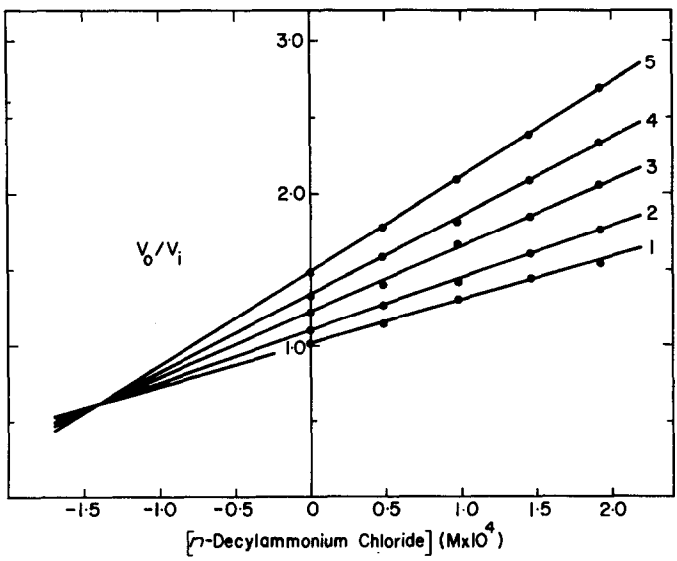

FIG. 6. Multiple inhibition of L-α-glycerophosphate dehydrogenase by *n*-decylammonium chloride and adenylic acid. Reaction mixtures contained 0·05 M Tris buffer (pH 7·85), $1{\cdot}77 \times 10^{-5}$ M NAD and $3{\cdot}3 \times 10^{-3}$ M L-α-glycerophosphate in a total volume of 3 ml. The concentration of *n*-decylammonium chloride was varied from zero to $1{\cdot}93 \times 10^{-4}$ M. The concentration of adenylic acid used was as follows: line 1, zero; line 2, $6{\cdot}7 \times 10^{-4}$ M; line 3, $1{\cdot}33 \times 10^{-3}$ M; line 4, $2{\cdot}0 \times 10^{-3}$ M; line 5, $2{\cdot}67 \times 10^{-3}$ M. The reactions were initiated by the addition of 0·4 μg of enzyme.

the same regions of the NAD binding site should mutually exclude one another from binding to the enzyme. In the same sense, inhibitors interacting at different regions of the binding site, barring unfavorable steric interactions, should be bound simultaneously to the enzyme. In this respect, the converging line relationship ($\alpha = 0{\cdot}89$) observed in the multiple inhibition study of *n*-decylammonium chloride with adenylic acid (Fig. 6) indicates simultaneous binding of these inhibitors to the enzyme. Thus, as expected, adenylic acid, which interacts with an adenosine and pyrophosphate region of the NAD binding site,[9] does not prevent the binding of *n*-decylammonium chloride. N^1-alkylnicotinamide chlorides are also bound simultaneously with adenylic acid.[9] The parallel line relationship observed in the multiple inhibition study of *n*-decylammonium chloride with N^1-decylnicotinamide chloride (Fig. 4) indicates these inhibitors to mutually exclude one another from binding to the enzyme. Since these inhibitors are thought to interact with the enzyme at both the pyridinium ring region and the hydrophobic region of the NAD binding site, mutual exclusion could arise from overlapping at either one or both of these regions. For this reason, the binding

of *n*-decylammonium chloride was investigated in the presence of N^1-butylnicotinamide chloride, since this nicotinamide derivative interacts predominantly with the pyridinium ring region and shows little, if any, nonpolar interaction with the hydrophobic region.[9] Mutual exclusion was again observed in the multiple inhibition studies of *n*-decylammonium chloride and N^1-butylnicotinamide chloride (Fig 5). Therefore, it does appear that *n*-decylammonium chloride utilizes the pyridinium ring region in binding, presumably through interactions of the positively charged nitrogen.

The functioning of *n*-alkylammonium chlorides as inhibitors of L-α-glycerophosphate dehydrogenase is therefore similar in many respects to the behavior of N^1-alkylnicotinamide chlorides with this enzyme. Both types of compounds are coenzyme-competitive inhibitors of the enzyme, both interact with the pyridinium ring region of the NAD binding site, both show similar chain length effects in binding and both are bound simultaneously with adenylic acid. The competitive nature of the binding of these compounds with respect to NAD is consistent with the "order bi bi" mechanism suggested by Black[15] in which NAD is bound to the enzyme prior to the binding of L-α-glycerophosphate. The effectiveness of binding of *n*-alkylammonium chlorides to L-α-glycerophosphate dehydrogenase, as in the case of yeast alcohol dehydrogenase,[6] results from a structural analogy to NAD that could not be anticipated without prior knowledge of the presence of a hydrophobic region at the NAD binding site of the enzyme. It is of interest that an appropriately located hydrophobic region can facilitate the binding and therefore the resulting inhibition of a series of compounds even though selective inhibition based on the structure of the compounds was not immediately obvious.

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