Inhibition of Yeast Alcohol Dehydrogenase by N^1-Benzylpyridinium Chlorides

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SUMMARY

Ten N^1-benzylpyridinium chlorides were prepared and studied as inhibitors of the yeast alcohol dehydrogenase-catalyzed oxidation of ethanol. The inhibitors, although containing a variety of functional groups substituted on the pyridinium ring, all inhibited this reaction and in each case the inhibition observed was competitive with respect to NAD^+. Interactions of these inhibitors with the "pyridinium ring" region of the NAD^+ binding site was suggested by the competitive nature of the inhibition and the simultaneous binding of all the inhibitors with adenylic acid. Varying the substituent group on the pyridinium ring did not alter significantly the binding properties of the N^1-benzyl derivatives except in those cases where charged groups were employed. In comparison to the binding of N^1-benzylnicotinamide chloride, N^1-benzyl-3-carboxylpyridinium chloride was demonstrated to be bound more poorly. N^1-Benzyl-3-aminomethylpyridinium chloride, on the other hand, was found to be a better inhibitor than N^1-benzylnicotinamide chloride.

INTRODUCTION

The study of competitive inhibitors designed through structural analogy to substrates and coenzymes has provided considerable information concerning the properties of specific binding sites of enzymes. The introduction of reactive groups into such inhibitors has in some cases produced irreversibly bound site-labeling reagents. Inhibitors of this type, in addition to their obvious importance in selectively inactivating a specific enzyme, provide protein derivatives which upon degradation can yield further information concerning segments of the primary structure involved in a given binding site. An understanding of the types of interactions that are of importance in the specific binding of various components of an enzyme system is required for the efficient designing of such inhibitors. When considering the binding of larger coenzyme molecules such as pyridine and flavin nucleotides, interactions with the enzyme concerned can involve a combination of effects related to different portions of the coenzyme molecule. Thus, in the case of the binding of NAD^+ to dehydrogenases one often finds competitive inhibition by compounds structurally related to the pyridinium ring moiety as well as the purine moiety of the NAD^+ molecule. Such is the case with yeast alcohol dehydrogenase where both adenine derivatives (1–4) and pyridinium derivatives (5, 6) serve as competitive inhibitors with respect to NAD^+ in the reaction catalyzed. In the study of pyridinium derivatives such as N^1-alkylnicotinamide chlorides of varying chain lengths, an increased binding to yeast alcohol dehydrogenase was observed with increasing chain length of the alkyl substituent (6). These and related studies with other nitrogen bases (7) have indicated the presence of a hydrophobic area at the "pyridinium ring" region of the yeast alcohol dehydrogenase NAD^+ binding site. Multiple inhibition kinetics (8) has been used to demonstrate that N^1-alkylnicotin-

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amide chlorides can be bound simultaneously with adenine derivatives (9). The importance of a positively charged nitrogen in combination with a nonpolar side chain in the binding of compounds at the "pyridinium ring" region of this NAD^+ binding site was further indicated by the demonstration that *n*-alkylammonium ions also inhibit the yeast enzyme competitively with respect to NAD^+ (10). The lower inhibitor dissociation constants observed with N^1-alkylnicotinamide chlorides compared to those of *n*-alkylammonium chlorides of the same chain length suggested a contribution to binding by the nicotinamide ring system (10). It was of interest to investigate the role of the substituent group in the 3-position of the pyridine ring in binding to this enzyme.

MATERIALS AND METHODS

Materials. Twice crystallized yeast alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) was obtained from the Worthington Biochemical Corporation. Unless otherwise noted, stock solutions of the enzyme were prepared in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.1% crystalline bovine serum albumin. NAD^+ and adenylic acid were purchased from the Sigma Chemical Company. 3-Acetylpyridine, 3-cyanomethylpyridine, 3-aminomethylpyridine, 3-pyridylcarbinol, and 3-pyridylacrylic acid were obtained from the Aldrich Chemical Company. Ethylnicotinate and 3-methylpyridine were obtained from Eastman Organic Chemicals. 3-Cyanopyridine and isonicotinamide were obtained from the Nepera Chemical Company. Nicotinamide was purchased from Matheson, Coleman and Bell.

3-Pyridylacryloamide was prepared from 3-pyridylacrylic acid according to Panizzon (11). N^1-Benzylpyridinium chlorides were synthesized by a slight modification of the method reported by Karrer and Stare (12). Benzyl chloride and the appropriate pyridine base were refluxed at a 2:1 molar ratio for varying periods of time. The solvents and time periods used for refluxing are as follows: for nicotinamide, methanol, 3 hr; for 3-acetylpyridine, methanol, 2 hr; for 3-methylpyridine, butanol, 16 hr; for 3-pyridylcarbinol, acetonitrile, 16 hr; for 3-cyanomethylpyridine, acetonitrile, 1 hr; for 3-cyanopyridine, butanol, 15 hr; for 3-aminomethylpyridine, butanol, 1 hr; for 3-pyridylacryloamide, butanol, 26 hr, and for isonicotinamide, butanol, 1.5 hr. N^1-Benzylethylnicotinate was prepared as described above by refluxing in butanol for 16 hr and was then converted to N^1-benzyl-3-carboxylpyridinium chloride by mild alkaline hydrolysis.

Various properties of the N^1-benzylpyridinium chlorides prepared are listed in Table 1. All the pyridinium derivatives were recrystallized three times from the solvent systems given in Table 1. Carbon, hydrogen, and nitrogen analyses were performed by the Galbraith Laboratories, Knoxville, Tennessee.

Methods. Yeast alcohol dehydrogenase reactions were studied at 25° in 3-ml reaction mixtures containing 0.04 M sodium pyrophosphate buffer, pH 8.0 and 0.1 M ethanol. The concentrations of other components used in the reaction mixtures will be included in the specific descriptions of individual experiments. Initial velocities were measured by following the formation of NADH at 340 mμ. Multiple inhibition studies (8) were carried out as described previously (3).

Spectrophotometric measurements were carried out at 25° in temperature-controlled cell compartments of a Gilford Model 2000 recording spectrophotometer or a Zeiss PMQII spectrophotometer. Measurements of pH were made at 25° with a Radiometer pH meter, type PHM4b, with a G-200-B glass electrode.

RESULTS

Ten N^1-benzylpyridinium chlorides containing a variety of functional groups substituted on the pyridine ring were prepared and studied as inhibitors of the yeast alcohol dehydrogenase-catalyzed oxidation of ethanol. The substituent groups were varied in order to investigate the necessity of having a carboxamido group in

TABLE 1
Properties of N^1-benzylpyridinium chlorides

Substituent group on pyridine ring	Melting point (°C)		Analysis						Recrystallization solvent	Reference
			Calculated			Found				
	Uncorrected	Literature	%C	%H	%N	%C	%H	%N		
3-CH_2CN	157–158	—	68.71	5.35	11.45	68.85	5.55	11.34	Acetonitrile	—
3-$CH{=}CHCONH_2$	228–229	—	65.57	5.50	10.20	65.37	5.49	10.05	Acetonitrile	—
4-$CONH_2$	214–215	—	62.78	5.27	11.26	61.01	5.21	10.89	Ethanol	—
3-CH_2NH_2	—[a]	—	66.52	6.44	11.94	66.45	6.55	12.05	Ethanol	—
3-CH_2OH	—[b]	—	62.24	5.99	5.94	66.50	5.90	5.74	Ethanol–ether	—
3-$CONH_2$	242–243	236	—[c]	—	—	—	—	—	Acetonitrile	(12)
3-$COCH_3$	190–191	185–186	—[c]	—	—	—	—	—	Acetonitrile	(13)
3-CH_3	218–219	220	—[c]	—	—	—	—	—	Acetonitrile	(14)
3-COOH	186–187	183–184	—[c]	—	—	—	—	—	Ethanol	(15)
3-CN	196–197	205–206	67.68	4.81	12.14	67.40[d]	4.95	12.09	Ethanol–ether	(16)

[a] Compound gradually sublimed on heating with no recognizable sublimation point.
[b] Hygroscopic properties of the compound prevent accurate measurement of the melting point.
[c] Analyses were not performed on this compound since properties for comparison were available in the literature.
[d] Analyses were performed since the observed melting point was slightly lower than the reported value.

the 3-position of the pyridinium ring to facilitate binding at the "pyridinium ring" region of the NAD^+ binding site of the yeast enzyme. Previous studies of the binding of compounds at this region dealt for the most part with straight-chain N^1-alkylnicotinamide chlorides. The decision to use N^1-benzyl derivatives was based primarily on preliminary studies indicating that with some of the substituted pyridines of interest, benzylation yielded products more readily purified. It was necessary therefore, to demonstrate that N^1-benzylnicotinamide chloride, having an additional aromatic nucleus, was not bound differently than the previously studied alkyl derivatives. The yeast alcohol dehydrogenase reaction was found to be inhibited by N^1-benzylnicotinamide chloride and the data obtained plotted according to Lineweaver and Burk (17) are shown in Fig. 1. The average inhibitor dissociation constant calculated from this plot was 1.18×10^{-2} M. The purely competitive nature of the inhibition indicated, as well as the value of the inhibitor dissociation constant were further evaluated in a second and separate experiment, the data from which, plotted according to Dixon (18) are shown in Fig. 2. The K_I of 1.21×10^{-2} M obtained from these data agrees well with that in the Lineweaver-Burk experiment. Multiple inhibition studies were carried out with N^1-benzylnicotinamide chloride in order to determine the region of the NAD^+ binding site of importance in the binding of the benzyl derivative. Multiple inhibition by the inhibitor pair, N^1-benzylnicotinamide chloride–N^1-methylnicotinamide chloride is shown in Fig. 3. A parallel line relationship was obtained yielding an interaction constant (α) of infinity indicating that these inhibitors mutually exclude one another from binding to the enzyme. In contrast to this, multiple inhibition by the inhibitor pair, N^1-benzylnicotinamide chloride-adenylic acid (Fig. 4) resulted in a converging line relationship. The interaction constant of 1.0 obtained in this case indicates simultaneous binding of these two inhibitors.

The nine N^1-benzylpyridinium chlorides containing substituent groups on the pyridine ring other than the 3-carboxamido group were next tested for inhibition of the yeast enzyme. In each case for the evaluation of the type of inhibition and the inhibitor dissociation constant two separate

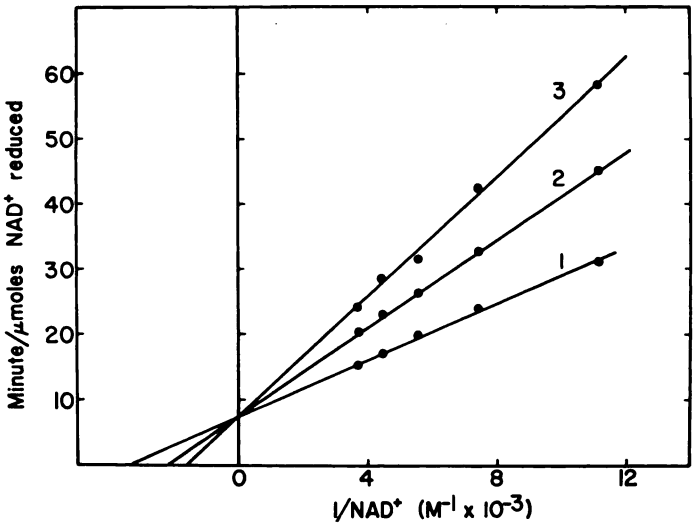

FIG. 1. *Competitive inhibition of yeast alcohol dehydrogenase by N^1-benzylnicotinamide chloride*

NAD^+ concentrations were varied from 8.97×10^{-5} M to 2.69×10^{-4} M. Curve *1*, reaction mixtures contained no inhibitor; curve *2* reaction mixtures contained 6.67×10^{-3} M N^1-benzylnicotinamide chloride; curve *3*, reaction mixtures contained 1.33×10^{-3} M N^1-benzylnicotinamide chloride. Reactions were initiated by addition of 1.3 μg of enzyme.

experiments were carried out as described above for N^1-benzylnicotinamide chloride in Figs. 1 and 2. All the N^1-benzylpyridinium chlorides studied inhibited the yeast alcohol dehydrogenase reaction, and in every case the inhibition was competitive with respect to NAD^+. Inhibitor dissociation constants determined in the two experiments carried out with each of the N^1-benzylpyridinium chlorides are listed

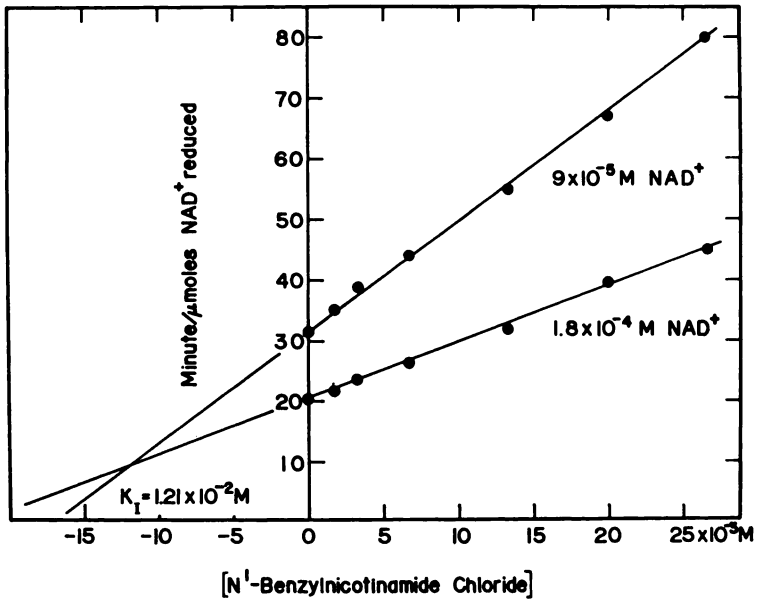

FIG. 2. *Inhibition of yeast alcohol dehydrogenase as a function of N^1-benzylnicotinamide chloride concentration.*

Reactions were initiated by the addition of 1.3 μg of enzyme.

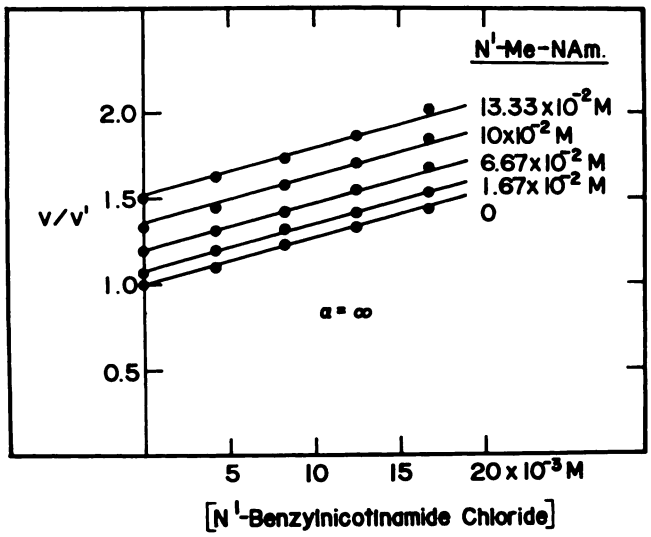

FIG. 3. *Multiple inhibition of yeast alcohol dehydrogenase by N^1-benzylnicotinamide chloride and N^1-methylnicotinamide chloride*

The data are plotted as the ratio of initial velocity in the absence of N^1-benzylnicotinamide chloride (v) over the initial velocity in the presence of N^1-benzylnicotinamide chloride (v') versus N^1-benzylnicotinamide chloride concentration. NAD^+ concentration used was 2.66×10^{-4} M. Reactions were initiated by addition of 1.3 μg of enzyme. Each line is identified by the concentration of N^1-methylnicotinamide chloride employed.

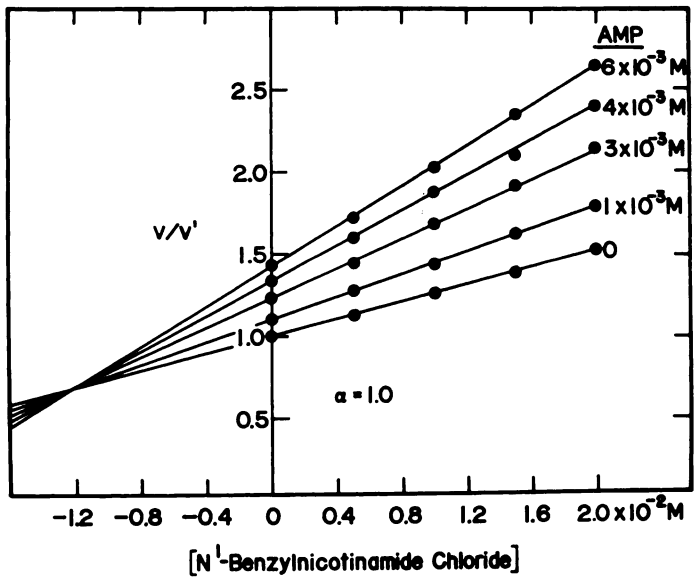

FIG. 4. *Multiple inhibition of yeast alcohol dehydrogenase by N^1-benzylnicotinamide chloride and adenylic acid (AMP)*

Data are plotted as described in Fig. 3. NAD^+ concentration used was 2.66×10^{-4} M. Reactions were initiated by addition of 1.3 μg of enzyme. Each line is identified by the concentration of adenylic acid employed.

TABLE 2
Studies of N^1-benzylpyridinium chlorides

Substituent group on pyridine ring	K_I (M)		α_{AMP}
	Lineweaver-Burk plots	Dixon plots	
3-$CONH_2$	1.18×10^{-2}	1.21×10^{-2}	1.00
3-COOH	4.38×10^{-2}	4.25×10^{-2}	1.04
3-$COCH_3$	1.24×10^{-2}	1.19×10^{-2}	1.08
3-CH_3	1.16×10^{-2}	1.37×10^{-2}	0.96
3-CN	0.86×10^{-2}	0.99×10^{-2}	1.19
3-CH_2CN	1.34×10^{-2}	1.40×10^{-2}	1.23
3-CH_2NH_2	0.34×10^{-2}	0.45×10^{-2}	0.86
3-CH_2OH	0.80×10^{-2}	0.61×10^{-2}	1.09
3-CH=CH—$CONH_2$	0.75×10^{-2}	0.92×10^{-2}	1.05
4-$CONH_2$	1.17×10^{-2}	0.83×10^{-2}	1.06

in Table 2. Most of the inhibitor dissociation constants determined were very close to those obtained for N^1-benzylnicotinamide chloride with the exception of those observed with the 3-carboxyl derivative, which were significantly higher, and those observed with the 3-aminomethyl derivative, which were significantly lower.

Multiple inhibition studies were carried out with all the N^1-benzylpyridinium chlorides using adenylic acid as the other component in the inhibitor pairs. Data from these experiments were plotted as described in Fig. 4 and in each case converging line relationships were observed. The interaction constants calculated from these plots are listed in Table 2.

The higher inhibitor dissociation constants observed in the case of N^1-benzyl-3-carboxylpyridinium chloride prompted a study of this pyridine base as the N^1-methyl derivative. N^1-Methyl-3-carboxylpyridinium chloride inhibited the yeast alcohol dehydrogenase-catalyzed oxidation of ethanol and the inhibition observed was competitive with respect to NAD^+. Inhibitor dissociation constants calculated from Lineweaver-Burk and Dixon plots were 2.68×10^{-1} M and 2.5×10^{-1} M, respectively. These values can be compared to 6.5×10^{-2} M and 7.0×10^{-2} M, the values obtained with N^1-methyl-3-carboxamidopyridinium chloride in previous studies (6).

Reversibility upon dilution of the inhibition obtained with the N^1-benzylpyridinium chlorides was studied by subjecting yeast alcohol dehydrogenase to concentrations of inhibitors ten times those necessary to produce approximately 25% inhibition. Twenty micrograms of yeast alcohol dehydrogenase was added to 5 ml of 0.1 M sodium pyrophosphate, pH 8.0 containing sufficient inhibitor to cause 25% inhibition when diluted to one-tenth. A 0.3-ml aliquot of this solution was transferred to a 2.7-ml reaction mixture containing 0.1 M sodium pyrophosphate, pH 8.0, 0.1 M ethanol, and 2.8×10^{-4} M NAD^+. A second sample was prepared by transferring 0.3 ml of a 5 ml solution containing 20 μg of yeast alcohol dehydrogenase in 0.1 M sodium pyrophosphate alone to a 2.7-ml reaction mixture containing the above-mentioned components plus the inhibitor. Enzyme and inhibitor concentrations in the two resulting reaction mixtures were therefore identical. Initial velocities of NAD^+ reduction were recorded for these two reaction mixtures and compared with the initial velocity obtained with this amount of enzyme in the absence of inhibitor. With all the ten N^1-benzyl derivatives studied, no significant additional inhibition was observed by first combining the enzyme with the 10-fold higher inhibitor concentration.

DISCUSSION

Oxidation-reduction reactions catalyzed by pyridine nucleotide-dependent dehy-

drogenases are of major importance to many metabolic pathways. The mode of binding of the pyridine nucleotide coenzymes can be an important factor in the functioning of these enzymes in their respective environments. Compounds structurally analogous to portions of the NAD^+ or $NADP^+$ molecules which occur naturally in the same environment as the dehydrogenases may very well interact with the dehydrogenases in a manner related to specific properties of the coenzyme binding sites of these enzymes. Adenosine diphosphate (ADP) for example, inhibits both yeast alcohol dehydrogenase (4) and horse liver alcohol dehydrogenase (8) competitively with respect to NAD^+. With bovine liver glutamic dehydrogenase on the other hand, low concentrations of ADP exhibit an activating effect (19, 20). Differences in the effects of other adenine derivatives with these dehydrogenases have likewise been observed (4, 8, 19).

It is of interest to note that many quaternary derivatives of nitrogen bases could affect dehydrogenase activities through a competitive-like inhibition based on structural analogy to the pyridinium ring moiety of pyridine nucleotide coenzymes. The effectiveness of such compounds could vary with the specific properties of the pyridine nucleotide binding sites of the various dehydrogenases. For example, without prior knowledge of the presence of a nonpolar region at the NAD^+ binding site of yeast alcohol dehydrogenase based on previous studies of N^1-alkylnicotinamide chlorides, one would not have expected to find coenzyme-competitive inhibition of this enzyme by *n*-alkylammonium chlorides (10).

The extension of the studies of inhibition by nicotinamide and adenine derivatives to the investigation of other dehydrogenases suggested the need for further knowledge of the role of substituent groups in the binding of pyridinium derivatives. Ten N^1-benzylpyridinium chlorides were prepared and studied as inhibitors of the yeast alcohol dehydrogenase-catalyzed oxidation of ethanol. Inhibition was obtained with all ten compounds, and in each case the inhibition observed was competitive with respect to NAD^+. The presence of the additional aromatic nucleus of N^1-benzylnicotinamide chloride as compared to the previously studied N^1-alkylnicotinamide chlorides, does not appear to alter the mode of binding of the N^1-benzyl derivative to the yeast enzyme. This is exemplified in the mutual exclusion observed in the multiple inhibition studies of N^1-benzylnicotinamide chloride with N^1-methylnicotinamide chloride (Fig. 3), and the simultaneous binding of inhibitors indicated in the multiple inhibition studies of N^1-benzylnicotinamide chloride with adenylic acid (Fig. 4). The same relationships were observed when other N^1-alkylnicotinamide chlorides were studied in combination with N^1-methylnicotinamide chloride and adenylic acid (9).

The lack of any significant deviation from unity in the interaction constants obtained in multiple inhibition studies of the remaining nine N^1-benzylpyridinium chlorides in combination with adenylic acid (Table 2) indicates that adenylic acid can be bound simultaneously with all the N^1-benzyl derivatives studied.

Some variation in inhibitor dissociation constants was observed with the different N^1-benzyl derivatives studied (Table 2); however, most of the values obtained did not differ significantly from those calculated for N^1-benzylnicotinamide chloride. With the exception of the 3-carboxyl and the 3-aminomethyl derivatives, it can be noted that the substituent group on the pyridine ring can be varied considerably without significantly changing the binding constant of the compound. If one considers possible interactions that may occur between the carboxamido group of N^1-benzylnicotinamide chloride and the enzyme, the substitution of a methyl group as in N^1-benzyl-3-methylpyridinium chloride would be expected to result in a recognizable change in binding constants. No differences were observed in the inhibitor dissociation constants obtained for the 3-carboxamido and 3-methyl derivatives (Table 2). Unless one considers properties common to both of these groups, it is diffi-

cult to visualize how the carboxamido group could contribute to the binding of the pyridinium derivatives. Also, it can be noted that having the carboxamido group in the 4-position of the pyridinium ring did not significantly alter binding properties.

In studies of NAD^+ analogs containing groups other than the carboxamido group in the 3-position of the pyridinium ring (21), it was concluded that the carboxamido group was not absolutely essential for the functioning of these compounds as coenzymes for a variety of dehydrogenases. Coenzyme activity was lost when the properties of the 3-position group differed too greatly from those of the carboxamido; however, it was noted that with this type of analog the reactivity of the 4-position of the pyridinium ring toward hydride transfer was so diminished that one could not expect reduction to occur. Studies of the N^1-benzylpyridinium chlorides would indicate that although the above-mentioned NAD^+ analogs are not functional as coenzymes, they should be bound as well as NAD^+ to yeast alcohol dehydrogenase.

N^1-Benzyl-3-carboxylpyridinium chloride was observed to be more poorly bound to yeast alcohol dehydrogenase than the carboxamido derivative, the inhibitor dissociation constants being 3–4 times higher for the carboxyl compound (Table 2). Higher inhibitor constants were also observed with N^1-methyl-3-carboxylpyridinium chloride as compared to the N^1-methyl-3-carboxamido derivative. Since more drastic changes in the substituent group failed to affect binding significantly, the poorer binding of these carboxyl derivatives is most likely related to unfavorable interactions of the additional negative charge with the enzyme. A similar unfavorable interaction was one of the possibilities suggested for the failure of nicotinic acid adenine dinucleotide to function as a coenzyme in the yeast alcohol dehydrogenase-catalyzed oxidation of ethanol (22).

The inhibitor dissociation constants obtained with N^1-benzyl-3-aminomethylpyridinium chloride were significantly lower than those obtained with other N^1-benzyl derivatives studied (Table 2). It is not immediately clear why this should be the case. The N^1-benzyl-3-aminomethylpyridinium chloride, at the pH used in the yeast alcohol dehydrogenase reactions would have two positive charges as compared to one for the other benzyl derivatives. The greater binding of the aminomethyl derivative may be due to the fact that two modes of binding are available for this compound.

The N^1-benzylpyridinium chlorides are currently being studied with a variety of dehydrogenases to further investigate the role of the pyridinium ring in the binding processes of these enzymes.

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