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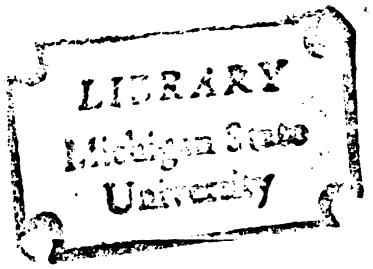

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STRUCTURE-ACTIVITY RELATIONSHIPS IN THE OXIDATION OF QUATERNARY AMMONIUM SALTS OF AMINOALCOHOLS WITH HORSE LIVER ALCOHOL DEHYDROGENASE

presented by

SHEK-HONG HERMAN LAU

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Chemistry

Gerosimos J. Karabatsos
Major professor

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STRUCTURE-ACTIVITY RELATIONSHIPS IN THE OXIDATION OF QUATERNARY AMMONIUM SALTS OF AMINOALCOHOLS WITH HORSE LIVER ALCOHOL DEHYDROGENASE

By

Shek-Hong Herman Lau

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1981

ABSTRACT

STRUCTURE-ACTIVITY RELATIONSHIPS IN THE
OXIDATION OF QUATERNARY AMMONIUM SALTS
OF AMINOALCOHOLS WITH
HORSE LIVER ALCOHOL DEHYDROGENASE

By

Shek-Hong Herman Lau

Hydrophobic, hydrophilic, and electronic interactions have long been recognized as important in binding substrate to enzyme, and in facilitating enzymatic reactions. Understanding individual as well as integral effects of these interactions on enzymatic reactions can provide valuable information about structure-activity relationships between substrate and enzyme and about the mechanism of the reaction.

Horse liver alcohol dehydrogenase (HLADH) (E.C.1.1.1.1.), is an interesting enzyme for investigation of the effect of hydrophobic interactions on the enzymatic reaction. The active site of the enzyme has a catalytic zinc atom at the bottom of a deep hydrophobic substrate binding pocket about

20 Å from the surface of the enzyme. Hansch and coworkers have discussed hydrophobic interaction in the binding of small molecules to the enzyme. Their work and that of others have led to the conclusion that binding of the substrate or the inhibitor to the enzyme is enhanced by increasing the length of the alkyl chain of the molecule (up to fifteen carbons).

To provide further data on the effective size and length of the substrate binding pocket, quaternary ammonium salts of aminoalcohols with a bulky group at one end of the molecule and a variable alkyl chain, I, were synthesized, and their reactions with HLADH and NAD^+ were studied.

$$R_3N^+-(CH_2)_n-OH\ X^-$$

I ($R = CH_3$, CH_3CH_2; $n = 2$ to 10; $X = I$ or Cl)

The initial rates and equilibrium constants for the oxidation of the quaternary ammonium salts of these aminoalcohols with HLADH and NAD^+ at six pHs at room temperature were determined. Both were found to depend on the pH and the length of the alkyl chain of the substrate. In general, the initial rates and the equilibrium constants increase at all pHs, as the alkyl chain length increases. For example, there is an 8-fold increase in initial rates going from n = 2 to the maximum at n = 7 in the N,N,N-trimethylaminoalcohol series at pH 8.0 compared to a 33-fold increase in the N,N,N-triethylaminoalcohol series. The initial rates vary with the pH and a 21-fold increase is observed going from pH 6.5 to pH 10.0 for

n = 7 in the trimethyl series and a 30-fold increase from pH 7.0 to pH 10.0 for n = 7 in the triethyl series. The ratio of the equilibrium constants for n = 2 and n = 10 in the trimethyl series at 36 hours at pH 8.0 is 1:1700 and that for the triethyl series is 1:600. This finding is consonant with the idea that hydrophobic interactions between substrate and enzyme at the binding pocket increase the binding of the substrate to the enzyme and enhance its reactivity. The pH effect on the initial rates suggests that the enzyme undergoes a conformational change as the pH changes and that the substrate binding pocket perhaps becomes more constricted at lower pHs. Consequently, the enzyme becomes more selective with respect to the size and length of the substrate.

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Professor Gerasimos J. Karabatsos for his guidance throughout this investigation.

A special thanks goes to Professor John C. Speck, Jr. without whom this would not be possible.

The author would also like to thank his wife, Amy; his parents and her parents; and members of the family for their support and patience throughout the years; and last but not least, to his daugher, Bonnie, for making the past couple of years more enjoyable.

Financial support provided by the Department of Chemistry, Michigan State University, is gratefully acknowledged.

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INTRODUCTION

Horse liver alcohol dehydrogenase (HLADH) (E.C.1.1.1.1.) is a nicotinamide adenine dinucleotide (NAD^+/NADH)-dependent enzyme that catalyzes the reversible oxidoreduction reaction of a variety of primary and secondary aliphatic and cyclic alcohols, ketones and aldehydes, and some steroids. The enzyme has been extensively studied since its discovery in the late nineteenth century. Several reviews on its structure, properties, and mechanism of reaction have been published.[1-4]

The overall reaction is represented by equation 1.

$$R_1R_2CHOH + NAD^+ \xrightleftharpoons{HLADH} R_1R_2C{=}O + NADH + H^+ \quad \text{(eq. 1)}$$

The equilibrium of the reaction overwhelmingly favors the reduction of the carbonyl compounds. A typical equilibrium constant for the oxidation of primary alcohols is about 8.0×10^{-12} M.

Although the mechanisms of the catalytic reaction has been extensively investigated by many researchers through the use of various kinetic methods,[5-9] it is not completely agreed upon. At the present time, the mechanistic sequence generally accepted by most is a modified Theorell-Chance ordered Bi-Bi mechanism.[3, 10] This is shown in Figure 1.

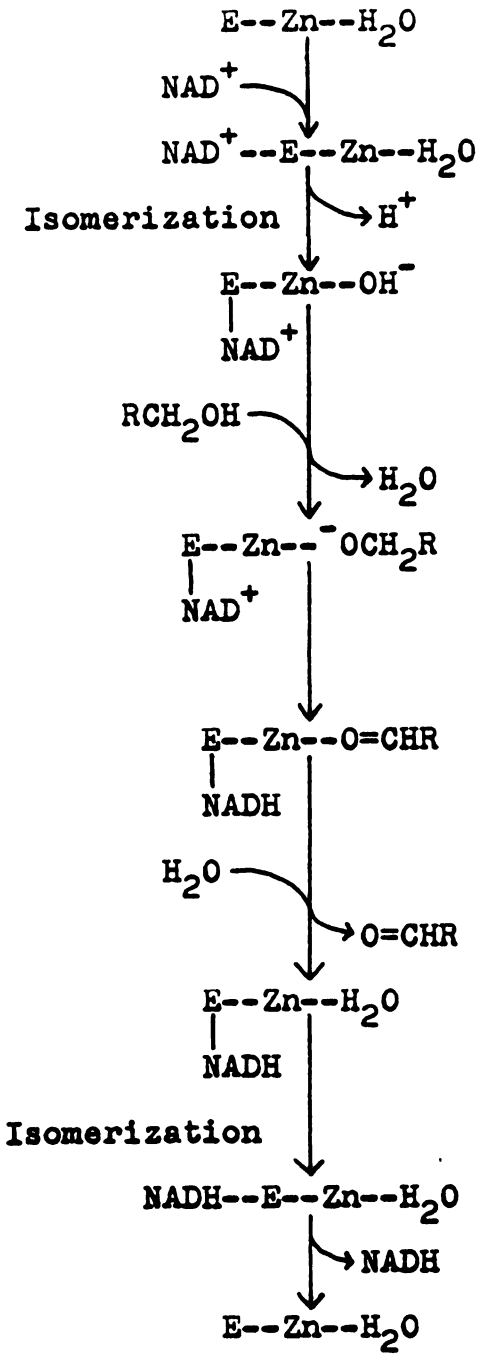


Figure 1. - Modified Theorell-Chance mechanism of HLADH

The structure of the enzyme has been thoroughly worked out. X-ray crystallography of the enzyme has recently been extended to 2.4 Å resolution.[11, 12] The active enzyme has a molecular weight of 80,000 and is a dimer of identical subunits. Each subunit is further divided into a coenzyme binding site and a substrate binding site, and firmly binds a "catalytic" and a "structural" zinc atom. The catalytic zinc atom is situated at the bottom of a predominantly hydrophobic substrate binding pocket some 20 Å from the surface of the enzyme. It is bonded to the enzyme through three bonds with the sulfhydryl and imidazole groups of Cysteine 46, Histidine 67, and Cysteine 174. The fourth position of the coordination sphere of zinc is occupied by either a water molecule or hydroxide depending on the pH of the solution.

The structure of the reduced coenzyme (NADH)[13] is shown in Figure 2.

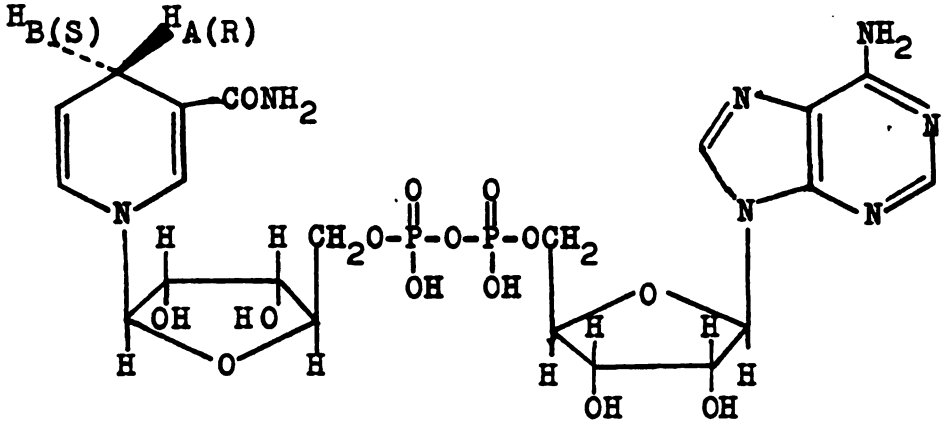


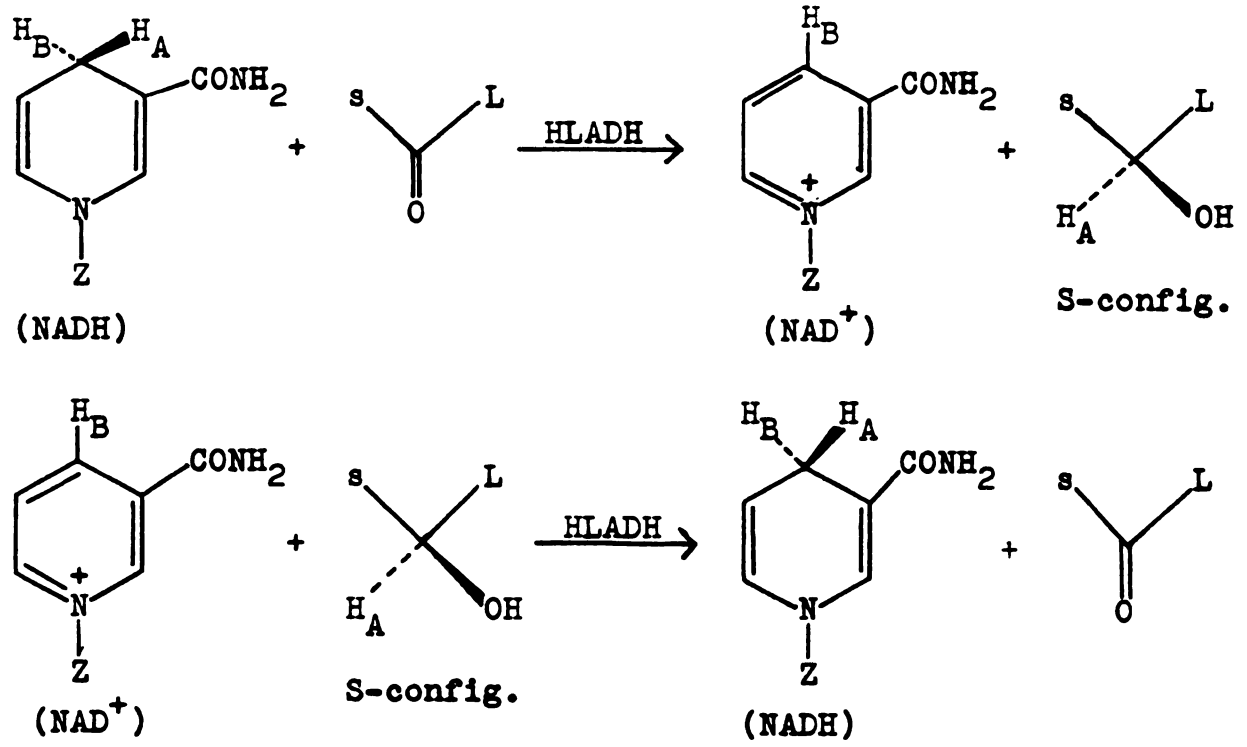
Figure 2. - Structure of Reduced Nicotinamide Adenine Dinucleotide

The stereochemistry of the hydrogens on the C-4 position of of the nicotinamide ring is labeled.

Alcohol dehydrogenases are divided into type A and type B enzymes depending on which hydrogen atom on the C-4 position of the nicotinamide ring of NADH is transferred to the substrate. The A enzymes transfer the pro-R hydrogen and the B enzymes the pro-S hydrogen. HLADH belongs to the A enzyme category. Furthermore, HLADH is not only stereospecific with respect to the coenzyme, but also with respect to the substrate. It transfers the C-4 hydrogen of the coenzyme exclusively or predominantly onto the re-face of the carbonyl group of the carbonyl compound; conversely, it oxidizes exclusively or predominantly secondary alcohols having the S-configuration. This stereospecificity with the substrate and coenzyme is illustrated in Figure 3.

At the present time, there is no definitive picture on the spatial orientation between the substrate and the coenzyme or on the role of the catalytic zinc atom in the reaction and its interaction with the substrate in the active site pocket.

It is very important to have a detailed knowledge of the spatial arrangement between the coenzyme and the substrate in the ternary enzyme complex in order to understand the reaction mechanism of alcohol dehydrogenases. Even though there have been several models proposed on this subject, none has been unambiguously confirmed or unanimously accepted.



Z = ribose-diphosphate-ribose-adenine

Figure 3. - Stereospecificity of hydrogen transfer between substrate and coenzyme

Some researchers consider steric effects the dominating factor in controlling and determining the orientation. Others propose that hydrophobic and hydrophilic interactions play an important part.

Prelog suggested a model shown in Figure 4[14] to explain the stereochemistry of alcohols obtained from the reduction of cyclic ketones with a type B enzyme from Curvularia falcata.

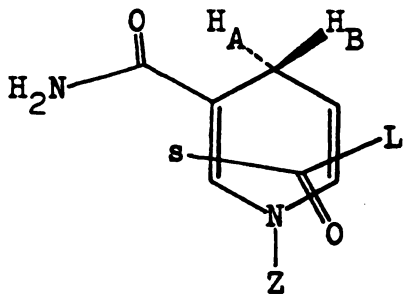


Figure 4. - Prelog's model

In his model, steric interactions would be minimized with the smaller group, 's', of the carbonyl compound placed on the side of the carbamido group of NADH, if the carbonyl group of the ketone was pointed down towards the pyridinium nitrogen. However, when this orientation was applied to the A type enzymes, it predicted products having the opposite configuration (R-configuration) from those obtained experimentally (S-configuration).[15] With this observation and the results of his group's work on some type A alcohol dehydrogenases,[16-18] Karabatsos proposed that the carbamido group played no role in determining the stereochemistry of the product. He suggested the arrangement between the substrate and coenzyme shown in Figure 5,[16] where the larger group, 'L', not the smaller group, 's', of the carbonyl compounds was put

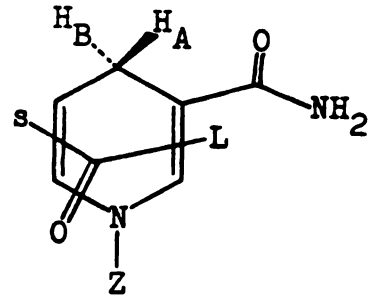


Figure 5. - Karabatsos' model

on the carbamido side of the nicotinamide ring. The model still maintained the carbonyl group pointing down towards the pyridinium nitrogen. With this spatial orientation, the product with the S-configuration was expected. In addition, his group[17, 18] had also found that hydrophobic and hydrophilic interactions might be more important than steric interactions in determining the spatial arrangement of substrate-coenzyme. Their findings are summarized in Table 1.

The results indicated that for nonpolar aliphatic alkyl substituents on the carbonyl compound, the predominant product was the S-enantiomer as predicted by the Karabatsos' model. However, when the substituent group was sufficiently hydrophilic, the product configuration could be completely reversed as evidenced from the result of the oxidation of hydroxyacetone by glycerol dehydrogenase (Gly-DH).

Table 1. - Effects of hydrophobic and hydrophilic groups on product stereospecificity

$$R_1R_2C{=}O + NADH + H^+ \xrightleftharpoons{ADH} R_1R_2CHOH + NAD^+$$

R_1	R_2	Enzyme	% R-config.	% S-config.
CH_3-	CH_3CH_2-	HLADH, Gly-DH	33-36	64-67
CH_3-	$HOCH_2$-	Gly-DH	100	0
CH_3-	$ClCH_2$-	HLADH	48	52

Both Karabatsos' and Prelog's models had the carbonyl group of the carbonyl compound pointed down towards the pyridinium nitrogen as initially suggested by Kosower.[19] However, there is no direct evidence against having the carbonyl group oriented in some other direction. Indeed, the models proposed by Graves, Clark, and Ringold,[20] and by Cerinka and Hub,[21] Figure 6, had the carbonyl group pointed away from the pyridinium nitrogen or towards the carbamido group. These alternative arrangements could change the entire spatial orientation of the substituent groups of the carbonyl compound with respect to the coenzyme.

Therefore, it is clear that unless the spatial orientation of the carbonyl group with respect to the coenzyme is determined, the arguments of the importance of steric, hydrophobic, and hydrophilic control of product stereospecificity are meaningless.

Figure 6. -

Graves model Cervinka and Hub model

It was with this in mind that Miedema,[22] May,[23] and Kroon[24] set out to synthesize some coenzyme analogs by attaching one end of the substrate molecule onto the carbamido group of NAD^+. These coenzyme analogs are shown in Figure 7.

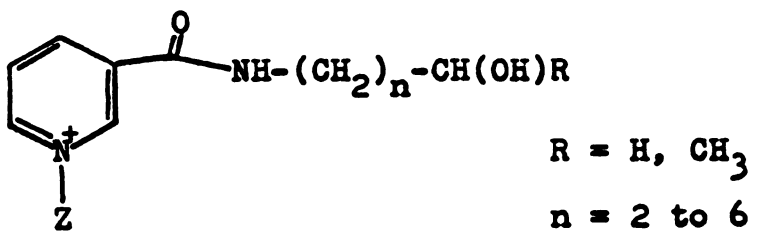
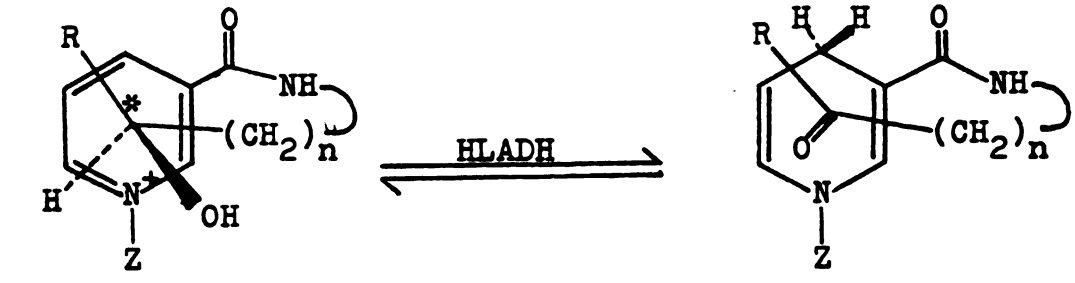
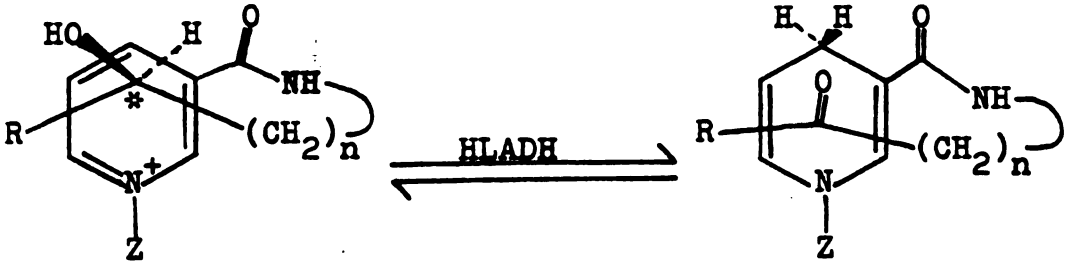


Figure 7. - Structure of NAD^+ analogs

By reducing the number of possible orientations that each face of the substrate could have with respect to the coenzyme, and by determining the configuration of the coenzyme analogs that reacted under the enzymatic conditions, it would be possible to deduce the orientation of the carbonyl group in the complex. The possible arrangements are illustrated in Figure 8. However, the results would not be definitive unless the reaction is strictly intramolecular. Experiments on the coenzyme analogs with added NAD^+ in the enzyme system indicated otherwise.[24] An increase in reactivity in the system with the coenzyme analogs and NAD^+ over that of the coenzyme analogs alone acting as both the coenzyme and the substrate led



*S-config.



*R-config.

Figure 8. - Possible spatial arrangements of the NAD^+ analogs

to the conclusion that both intra- and inter-molecular reactions could be possible. Therefore, the question of the actual orientation of the substrate and the coenzyme remained unanswered.

The role of the catalytic zinc atom in the active site is not clearly understood. From the X-ray structure determination of HLADH, the inference may be drawn that the only groups in the enzyme that might be responsible for binding and polarizing the reactive part of the substrate are the catalytic zinc atom, the zinc bound water molecule or hydroxide ion, the hydroxyl group of Serine 48, and indirectly, the imidazole group of Histidine 51 through its hydrogen bonding to Serine 48. The active site of HLADH as observed from X-ray studies is shown in Figure 9.

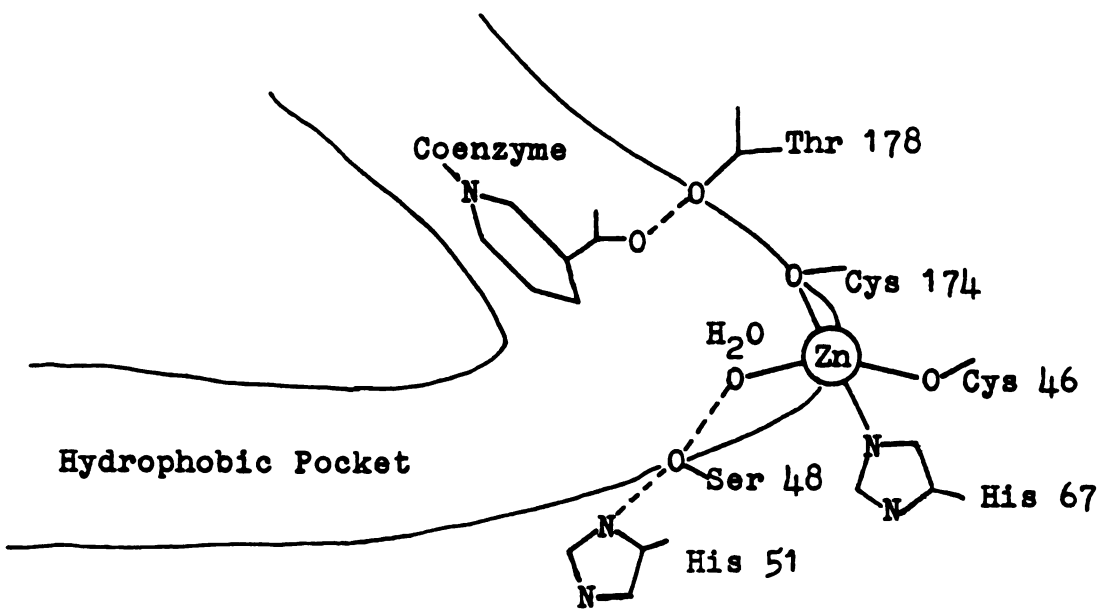
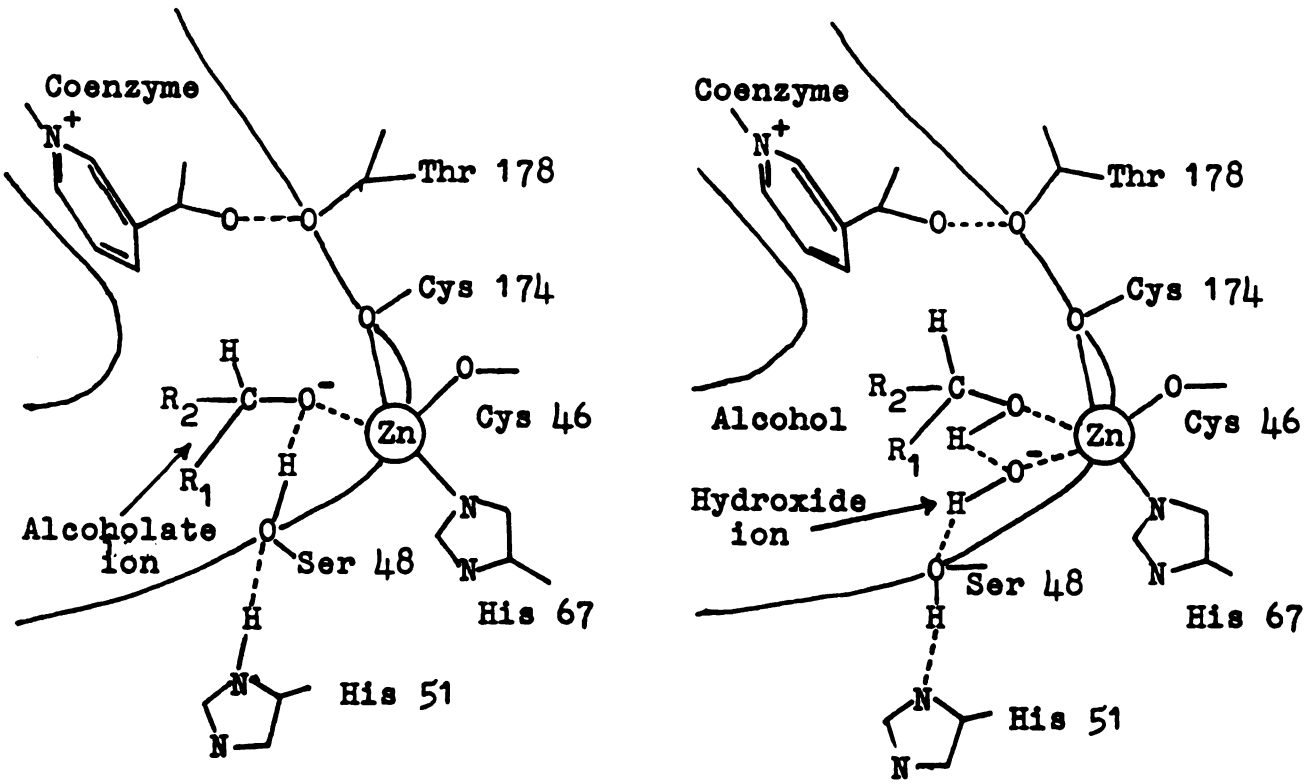


Figure 9. - Active site of HLADH as observed from X-ray studies

Two mechanisms involving the catalytic zinc atom have been suggested. The first was based on an electrophilic catalysis[3, 25a] mediated by the zinc atom. In this mechanism, alcohol was bonded directly to zinc as the negatively charged alcoholate ion replacing the hydroxide ion on zinc. The other mechanism was a general acid-base catalysis[25a, b] with the hydroxide ion remaining on the zinc atom and the neutral alcohol attached as a fifth ligand to zinc. The two mechanistic arrangements are depicted in Figure 10.



Electrophilic catalysis

Acid-base catalysis

Figure 10. - Mechanistic arrangements in the ternary complex of HLADH

The importance of the deep hydrophobic substrate binding pocket of the enzyme is well established. The hydrophobic interaction between the substrate and the enzyme is essential in facilitating substrate binding and catalytic reaction. Brooks and Shore[26] showed that while ethanol reacts readily with HLADH, methanol shows little or no activity at all. It is difficult to explain the relative reaction rates of methanol and ethanol of 1:1400. It has been proposed[27] that for alcoholic substrates the proper molecular orientation for hydride transfer at an optimum rate could be obtained by the substrate binding to two sites, a hydroxyl binding site and a hydrophobic binding site. Thus, methanol, with only one carbon atom in the molecule, apparently is not large enough to bind at both sites at the same time. On the other hand, ethanol and the higher homologs are able to bind at both sites. Consequently, the affinity for methanol by the enzyme is drastically reduced. This is reflected in the observation that 50 m$\underline{M}$ ethanol, as compared to 1 $\underline{M}$ for methanol, is required for saturation. Furthermore, increased alkyl chain length in the alcohol enhances this affinity of the enzyme-coenzyme binary complex for the substrates, since only 1.5 m$\underline{M}$ 1-propanol is required for saturation and it reacts four to five times faster than ethanol.

This thesis describes the synthesis of the quaternary ammonium halides of primary aminoalcohols shown in Figure 11 and their reactivity with HLADH at six pHs at room temperature. These compounds of variable alkyl chain length with a large

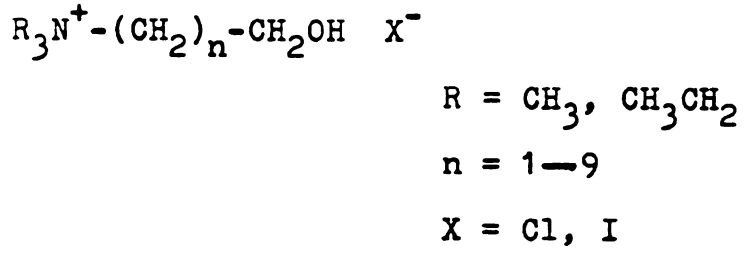


Figure 11. - Structure of quaternary ammonium halides of primary aminoalcohols

bulky group at one end and a hydroxyl group at the other could serve as probes into the effective length and size of the hydrophobic substrate binding pocket. They also could serve as a preliminary study of the viability of this kind of compound as possible substrates for the enzyme. If they are successful as substrates, the procedure could be extended to similar compounds with secondary aminoalcohols or carbonyl groups and could further be extended to the study of other enzyme systems.

In recent years, both the catalytic and the structural zinc atoms of the enzyme have been completely or selectively replaced by either cobalt[28, 29] or cadmium.[30] Bobsein and Myers[31] recently published the Cadmium-113 NMR spectra of the totally cadmium-substituted HLADH. With the compounds studied here, if the bulky group is too large to enter the cleft leading into the active site region of the enzyme, then only those molecules with an alkyl chain long enough to reach into the catalytic site would react. It would then seem probable

that by using Cadmium NMR spectroscopy with these compounds and cadmium-substituted HLADH, the two catalytic mechanisms involving zinc and the role of zinc in the catalytic reaction could be further clarified.

RESULTS AND DISCUSSION

Preparations of the quaternary ammonium salts of aminoalcohols

Of paramount importance for the studies described in this thesis was the availability of various aminoalcohols to convert to the desired quaternary ammonium salts. Since only a few of the desired N,N-dialkylaminoalcohols are commercially available, and since separation of the various mono-, di-, and tri-alkylated aminoalcohols is difficult and tedious, and since not all of the aminoalcohols are readily available (only up to six carbons long are available commercially), direct alkylation of the aminoalcohols is not practical and suitable preparative methods had to be devised. Before proceeding with a discussion of the kinetic studies, the preparation and identification of the aminoalcohols and their quaternary ammonium salts will be described.

The quaternary ammonium salts were prepared according to the general reaction routes shown in equation 2 for the iodides and in equation 3 for the chlorides. The starting point for the iodides varied depending on the availability of the starting materials (diols or N,N-dialkylaminoalcohols).

$$HO\text{-}(CH_2)_n\text{-}OH + HCl \longrightarrow HO\text{-}(CH_2)_n\text{-}Cl$$

$$HO\text{-}(CH_2)_n\text{-}Cl + R_2NH \longrightarrow R_2N\text{-}(CH_2)_n\text{-}OH \qquad \text{(eq. 2)}$$

$$R_2N\text{-}(CH_2)_n\text{-}OH + RI \longrightarrow R_3N^+\text{-}(CH_2)_n\text{-}OH\ I^-$$

$$R = CH_3,\ CH_3CH_2$$

$$n = 4 — 10$$

$$HO\text{-}(CH_2)_n\text{-}OH + HCl \longrightarrow HO\text{-}(CH_2)_n\text{-}Cl$$

$$HO\text{-}(CH_2)_n\text{-}Cl + R_3N \longrightarrow R_3N^+\text{-}(CH_2)_n\text{-}OH\ Cl^- \qquad \text{(eq. 3)}$$

$$R = CH_3$$

$$n = 6 — 10$$

With the exception of the butamethylene chlorohydrin, all of the other chlorohydrins not purchased were prepared from the corresponding diols. Butamethylene chlorohydrin was prepared from dry, gaseous hydrogen chloride and tetrahydrofuran primarily because these reagents are inexpensive and readily available.

In general, the yields of the chlorohydrins were reasonably good (about 45 to 75%) except for pentamethylene chlorohydrin (about 20%). For this reaction to be successful, it is important to remove the chlorohydrin from the reaction vessel by continuous extraction with a suitable solvent to minimize the formation of such by-products as the dichlorides. The yields of the chlorohydrins were increased considerably (from 10--20% to about 50--60%) by this method over those

obtained from other methods[32] without the continuous removal of the chlorohydrins. Those methods gave lower yields of the desired product and considerably larger amounts of the dichlorides.

The low yield of pentamethylene chlorohydrin is probably due to the instability of this compound when heated at high temperature (140--145^{0}) for an extended period of time (overnight). In fact, this compound decomposed slightly on standing for about a month at room temperature and required redistillation prior to its use.

The identity of the quaternary ammonium salts was ascertained by qualitative chemical analysis (see experimental), by proton NMR, and by mass spectroscopy with chemical ionization in methane gas (CI-MS). Figures 12 and 13 show a couple of the CI-MS spectra of the quaternary ammonium salts. None of the mass spectra obtained gave the ion peak of the parent compound. Instead, the ion peak of the dealkylhalogenated quarternary ammonium salt (N,N-dialkylaminoalcohol) and a much more intense m + 1 ion peak were observed (m is the mass of the N,N-dialkylaminoalcohol). A typical mass spectral pattern for chemical ionization with methane gas[33, 34] was observed in all cases. Some of the ion peaks of m + 1, m + 15, m + 29, and m + 41, of variable intensity, due to the interactions of H^{+}, CH_3^{+}, $C_2H_5^{+}$ ions from methane with the parent compound respectively, were observed in the spectra. The ion corresponding to the dehydration product of the m + 1 ion was also observed in the spectra indicating the presence

of a hydroxyl group. With all the iodides studied, the methyl iodide peak (m/e = 142) and that of its protonated ion (m/e = 143) appeared in every spectrum. The 143 peak was always more intense than the 142 peak. In contrast to the iodides, the chlorides gave no peak for the methyl chloride (m/e = 50) or for its protonated ion (m/e = 51). The CI-MS spectrum of N,N,N-trimethyl-10-hydroxydecyl ammonium chloride is shown in Figure 12. The dealkylhalogenated ion of mass 201 has 8% of the intensity of the m + 1 ion (m/3 = 202), whose intensity is 100%. The m + 15 ion (m/e = 216) and the m + 29 ion (m/e = 230) have relative intensities of 4% and 17% respectively. The m/e = 164 ion is the dehydration product of the m + 1 ion. Figure 13 shows the mass spectrum of N,N,N-triethyl-10-hydroxydecyl ammonium iodide. In the spectrum appear the m/e = 229 ion (4% intensity) that corresponds to the dealkylhalogenated quaternary ammonium salt, the m + 1 ion (m/e = 230, 53% intensity), and the m + 29 ion (m/e = 258, 7% intensity). In addition, the methyl iodide ion (m/e = 156, 9% intensity) and its protonated ion (m/e = 157, 100% intensity) are observed. Although no good explanation for not observing the methyl chloride ion or its protonated ion in the mass spectrum can be offered, nevertheless, the mass spectra are consistent with the structures of the quaternary ammonium salts.

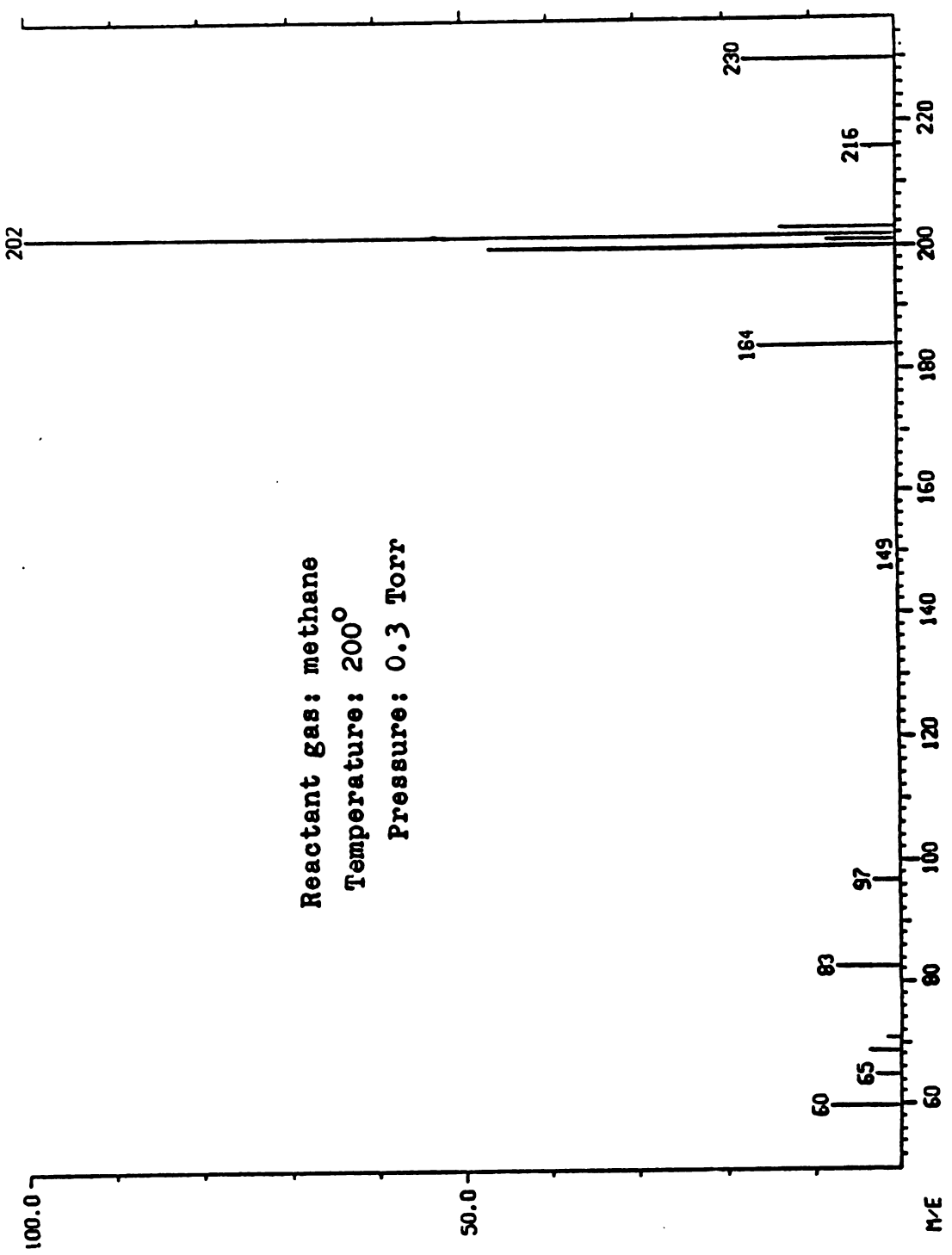


Figure 12. - Chemical ionization mass spectrum of N,N,N-trimethyl-10-hydroxydecyl ammonium chloride

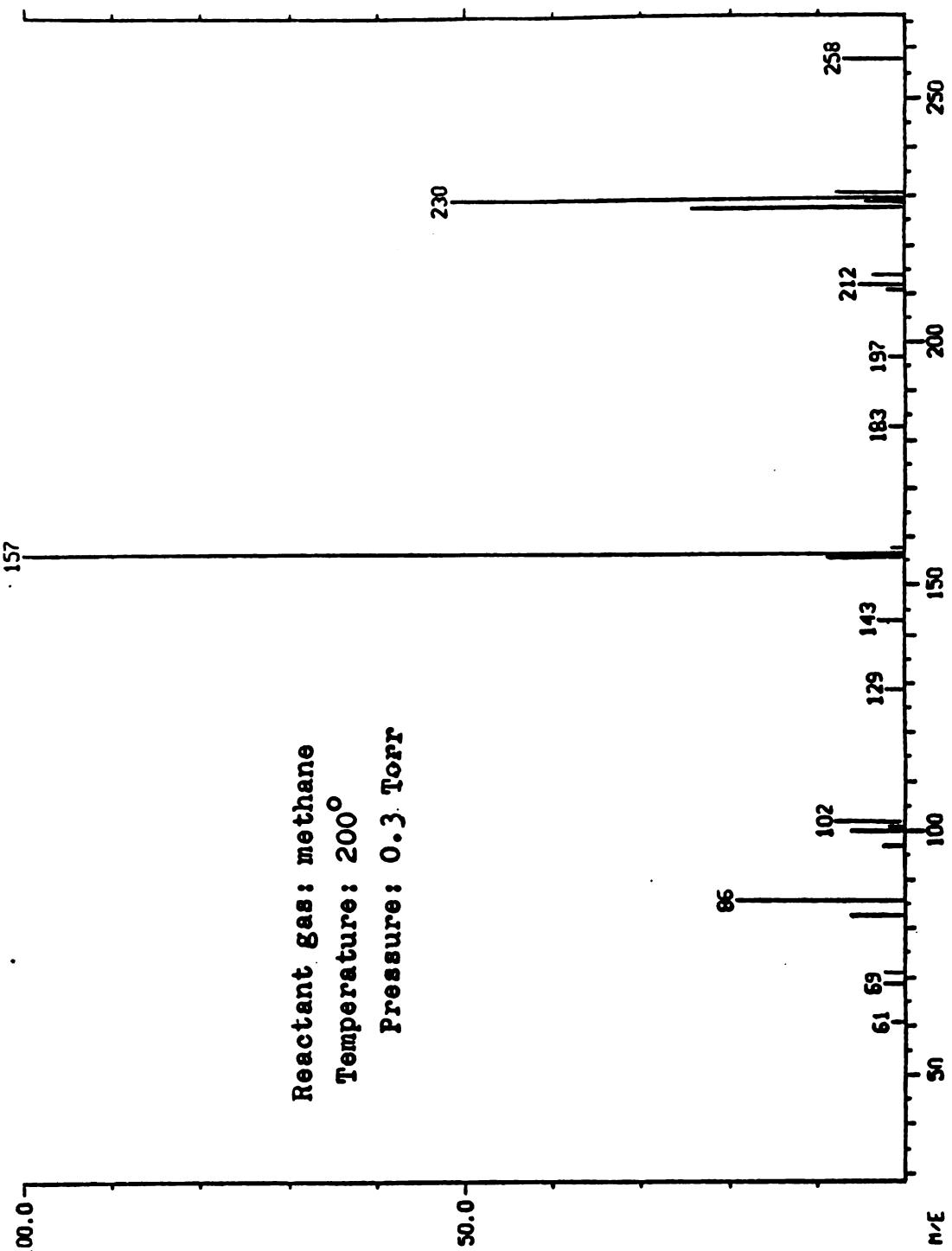


Figure 13. - Chemical ionization mass spectrum of N,N,N-triethyl-10-hydroxydecyl ammonium iodide

The proton NMR spectra of the N,N,N-trimethyl- and the N,N,N-triethylalkyl ammonium salts were taken at 250 MHz, with deuterium oxide as the solvent and spectral grade acetone as the internal standard (δ = 2.05). They are consistent with and support the structures of the salts. Some of these spectra are shown in Figures 14 to 17. In the methyl series (Figures 14 and 15), the methyl group and the methylene group of the quaternary nitrogen appear as a singlet (δ = 2.9) and as a multiplet (δ = 3.1 to 3.4 for n = 10 to n = 2), respectively. The methylene group next to the oxygen appears as a triplet (except for n = 2, which is a multiplet) at δ = 3.4 to 3.9 for n = 10 to n = 2. All other methylene groups in the carbon chain appear as multiplets in the range δ = 1.1 to 1.9. Understandably, no hydroxyl hydrogen was observed with water as the solvent. In the ethyl series (Figures 16 and 17), except for the hydrogens of the ethyl group, all other groups of hydrogens have chemical shifts and splitting patterns that are similar with those of their counterparts in the methyl series. The multiplicity and chemical shifts of the methyl (triplet at δ = 1.1) and the methylene (quartet at δ = 3.1 to 3.2) of the ethyl group are typical of such hydrogens in similar compounds.

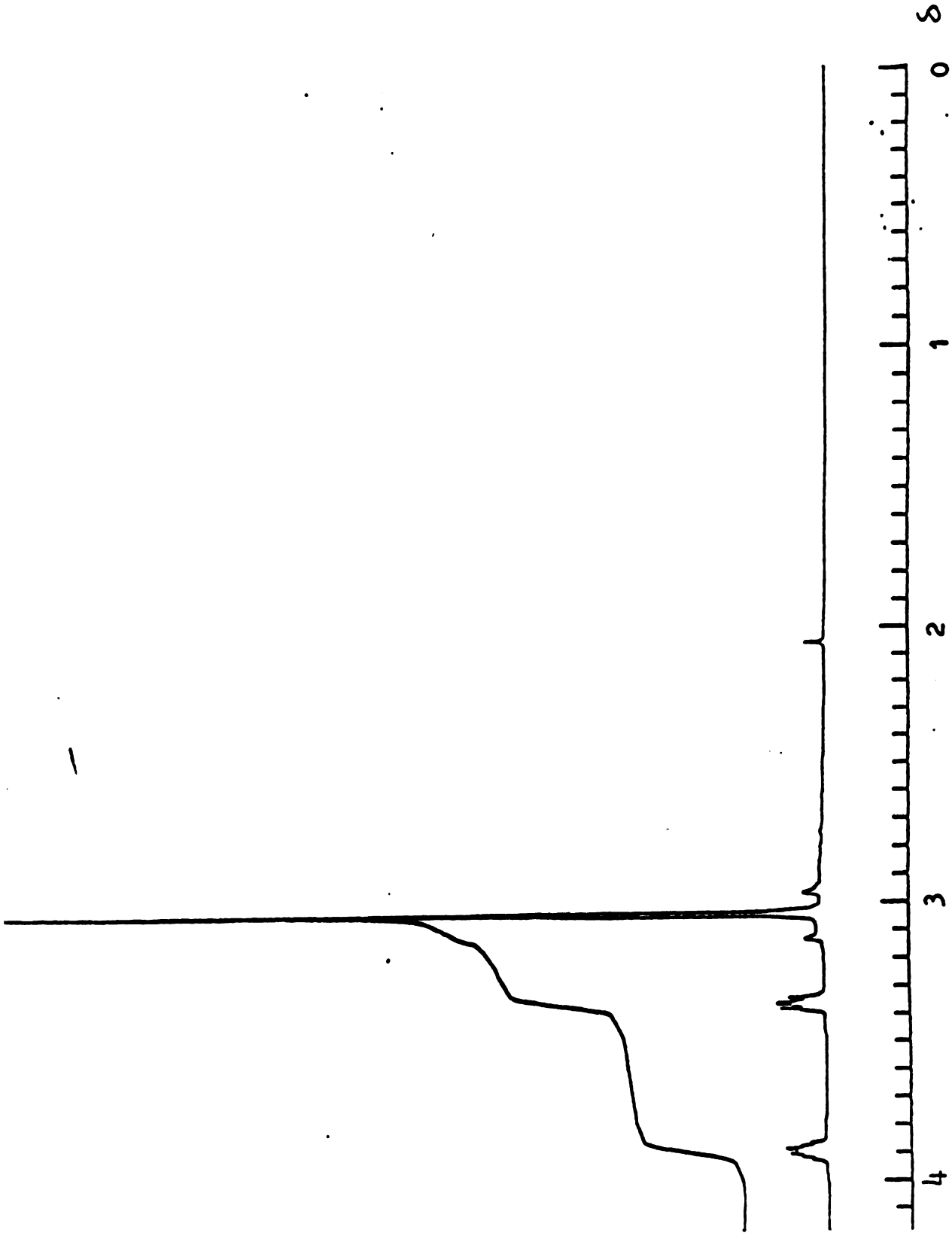


Figure 14. - 250 MHz Proton NMR spectrum of N,N,N-trimethyl-2-hydroxyethyl ammonium iodide in deuterium oxide with internal standard acetone (δ = 2.05) at room temperature

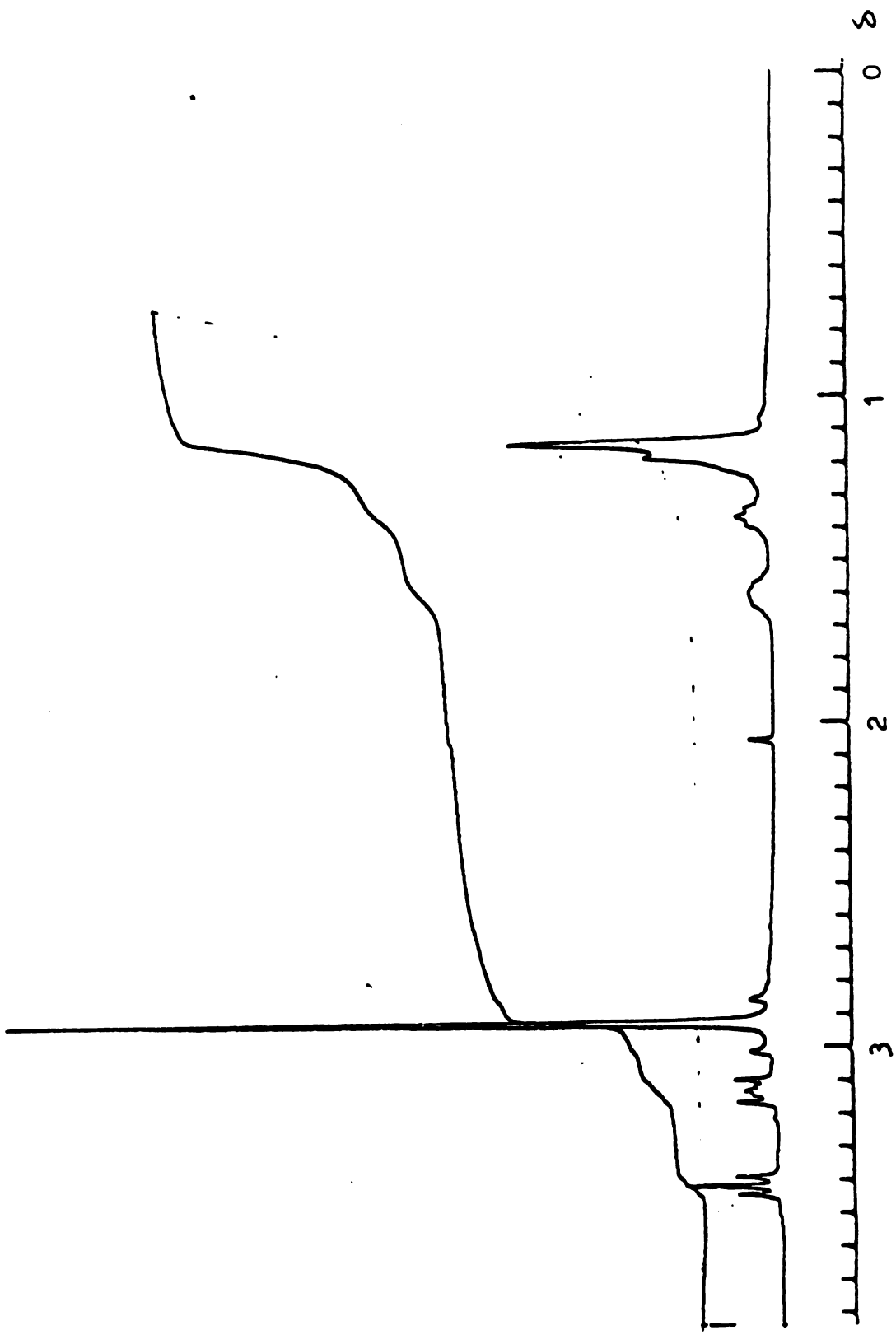


Figure 15. - 250 MHz Proton NMR spectrum of N,N,N-trimethyl-10-hydroxydecyl ammonium chloride in deuterium oxide with internal standard acetone (δ = 2,05) at room temperature

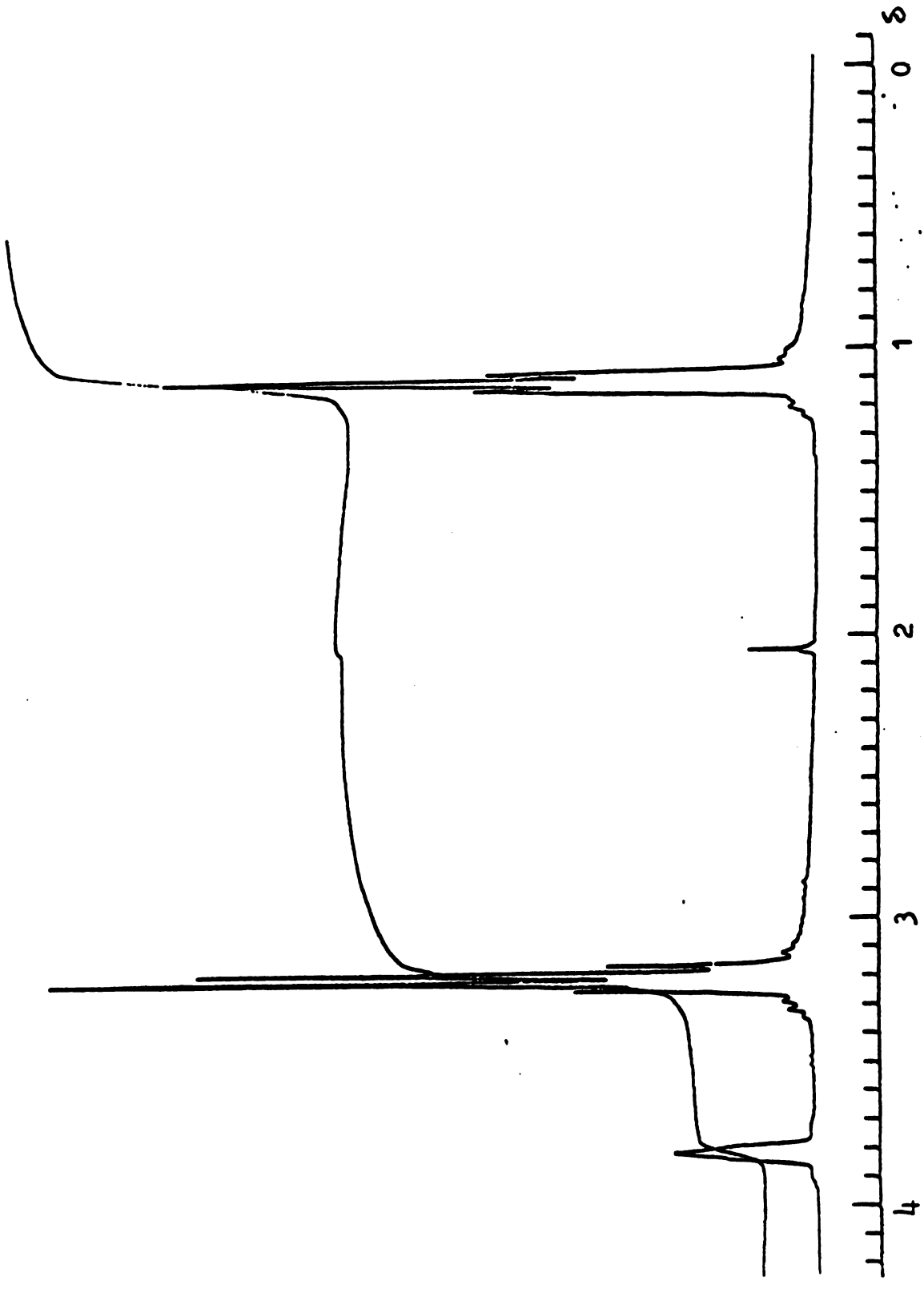


Figure 16. - 250 MHz Proton NMR spectrum of N,N,N-triethyl-2-hydroxyethyl ammonium iodide in deuterium oxide with internal standard acetone (δ = 2.05) at room temperature

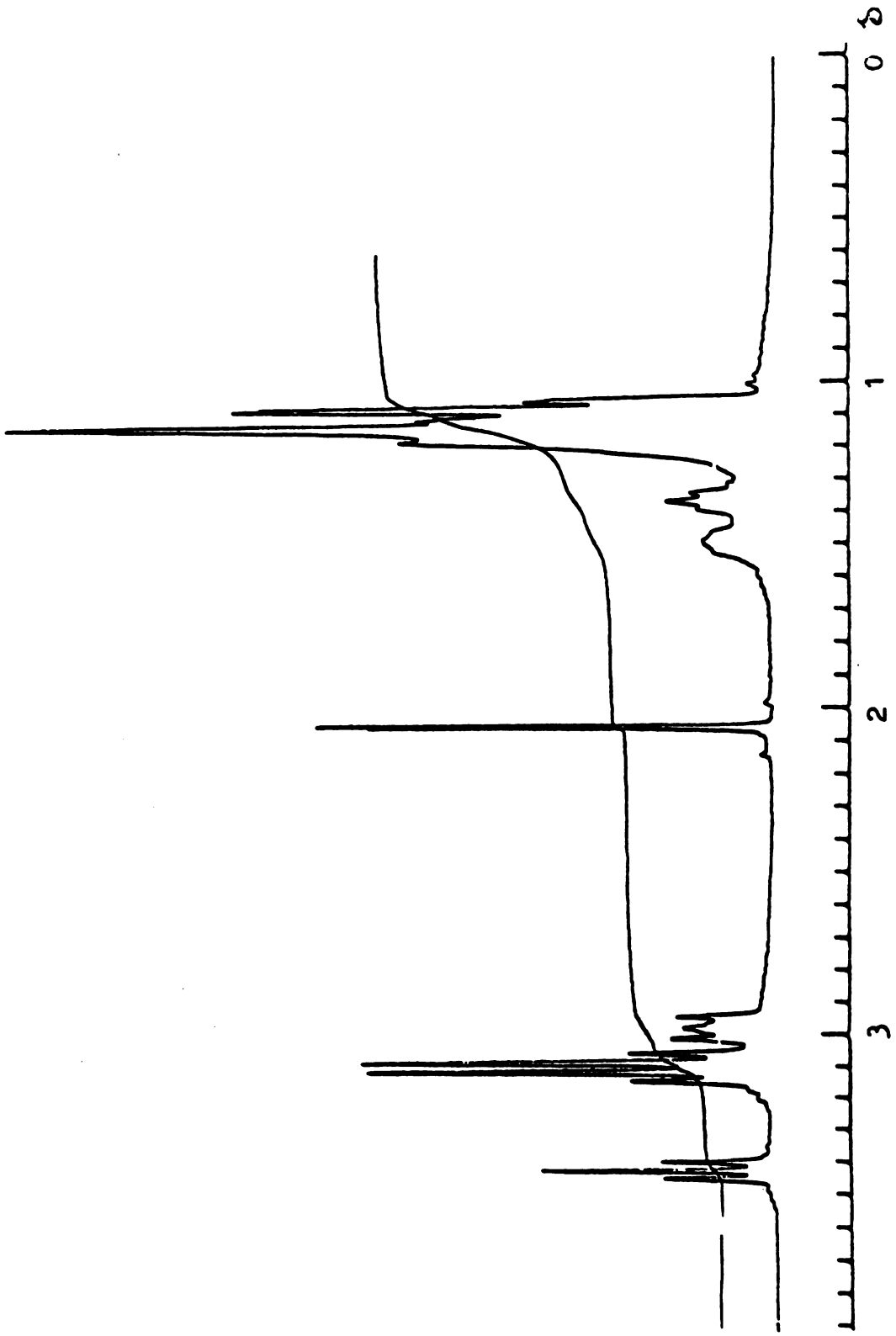


Figure 17. - 250 MHz Proton NMR spectrum of N,N,N-triethyl-10-hydroxydecyl ammonium iodide in deuterium oxide with internal standard acetone (δ = 2.05) at room temperature

Initial rate studies of the
quaternary ammonium salts of aminoalcohols

The initial rates of the aminoalcohols measured at 340 nm at the six pHs studied are shown in Figures 18 and 19, and in Tables 2 and 3. The relative rates at each pH for both the methyl and the ethyl series of aminoalcohols are given in Tables 4 and 5 respectively.

The results shown indicate that there is a general pattern in the initial velocities of the substrates. With a few exceptions, the initial rate reaches a maximum with the 7-carbon alkyl chain aminoalcohols in both series. The higher homologs in the same series taper off in reactivity while the shorter alkyl chain aminoalcohols show less reactivity or, in some cases, do not react at all at the substrate concentration used.

The effect of hydrophobic binding to enzymatic activity is vividly illustrated here. It has long been recognized that hydrophobic binding plays an important part in enzyme catalysis.[35] With HLDAH, the activity of primary aliphatic alcohols increases as the chain length increases. Kroon,[24] in his work with the aminoalcohols $CH_3CHOH(CH_2)_nNH_2$, where n = 3,4,5, and 6, as substrates for HLADH, observed a relative reactivity rate of 1.0/1.5/2.0/34 respectively. The present work also shows a similar trend in relative activity. The sudden surge in reactivity in going from the 6-carbon alkyl chain aminoalcohol to the 7-carbon chain aminoalcohol

Table 2. - Initial rates for the oxidation of $(CH_3)_3N^+(CH_2)_nOH\ X^-$ at six pHs at room temperature

		Initial rates at 340 nm, O.D. unit/min.								
pH	n =	2	3	4	5	6	7	8	9	10
6.5		---	---	---	---	0.005 ±0.002	0.034 ±0.009	0.020 ±0.003	0.036 ±0.005	0.033 ±0.006
7.0		---	---	---	---	0.008 ±0.002	0.032 ±0.004	0.019 ±0.001	0.051 $0.005	0.071 ±0.004
7.5		---	---	---	0.009 ±0.002	0.023 ±0.004	0.146 ±0.010	0.124 ±0.010	0.126 ±0.006	0.111 ±0.008
8.0		0.032 ±0.004	0.044 ±0.005	0.034 ±0.005	0.044 ±0.005	0.087 ±0.010	0.25 ±0.02	0.21 ±0.01	0.18 ±0.02	0.18 ±0.02
8.8		0.016 ±0.003	0.045 ±0.010	0.015 ±0.003	0.038 ±0.006	0.12 ±0.01	0.37 ±0.03	0.25 ±0.03	0.18 ±0.02	0.19 ±0.02
10.0		0.044 ±0.005	0.076 ±0.007	0.035 ±0.004	0.072 ±0.006	0.32 ±0.03	0.72 ±0.05	0.45 ±0.03	0.30 ±0.01	0.29 ±0.02

Table 3. - Initial rates for the oxidation of $(CH_3CH_2)_3N^+(CH_2)_nOH\ X^-$ at six pHs at room temperature

		Initial rates at 340 nm, O.D. unit/min.								
pH	n =	2	3	4	5	6	7	8	9	10
6.5		---	---	---	---	---	---	---	0.007 ±0.003	0.024 ±0.009
7.0		---	---	---	---	---	0.024 ±0.007	0.014 ±0.001	0.024 ±0.005	0.052 ±0.009
7.5		---	---	---	---	0.048 ±0.005	0.105 ±0.008	0.067 ±0.005	0.097 ±0.015	0.13 ±0.01
8.0		0.007 ±0.002	0.004 ±0.003	0.010 ±0.002	0.012 ±0.006	0.084 ±0.005	0.24 ±0.03	0.19 ±0.03	0.17 ±0.03	0.18 ±0.02
8.8		0.02 ±0.01	0.014 ±0.008	0.023 ±0.010	0.021 ±0.006	0.11 ±0.03	0.34 ±0.04	0.32 ±0.11	0.22 ±0.09	0.20 ±0.04
10.0		0.054 ±0.008	0.03 ±0.01	0.07 ±0.02	0.09 ±0.02	0.19 ±0.01	0.74 ±0.04	0.70 ±0.05	0.56 ±0.07	0.38 ±0.05

Table 4. - Relative rates for the oxidation of $(CH_3)_3N^+(CH_2)_nOH\ X^-$ at each pH with n = 10 used as standard

pH	n = 2	3	4	5	6	7	8	9	10
6.5	---	---	---	---	15	103	61	109	100
7.0	---	---	---	---	11	45	27	72	100
7.5	---	---	---	8	21	132	112	114	100
8.0	18	25	19	25	49	137	117	99	100
8.8	8	24	8	20	61	191	131	96	100
10.0	15	26	12	25	112	248	156	104	100

Table 5. - Relative rates for the oxidation of $(CH_3CH_2)_3N^+(CH_2)_nOH\ X^-$ at each pH with n = 10 used as standard

pH	n = 2	3	4	5	6	7	8	9	10
6.5	---	---	---	---	---	---	---	29	100
7.0	---	---	---	---	---	46	27	46	100
7.5	---	---	---	---	38	83	53	76	100
8.0	4	2	6	7	46	133	103	94	100
8.8	9	7	11	10	56	168	155	107	100
10.0	14	7	20	23	50	196	185	149	100

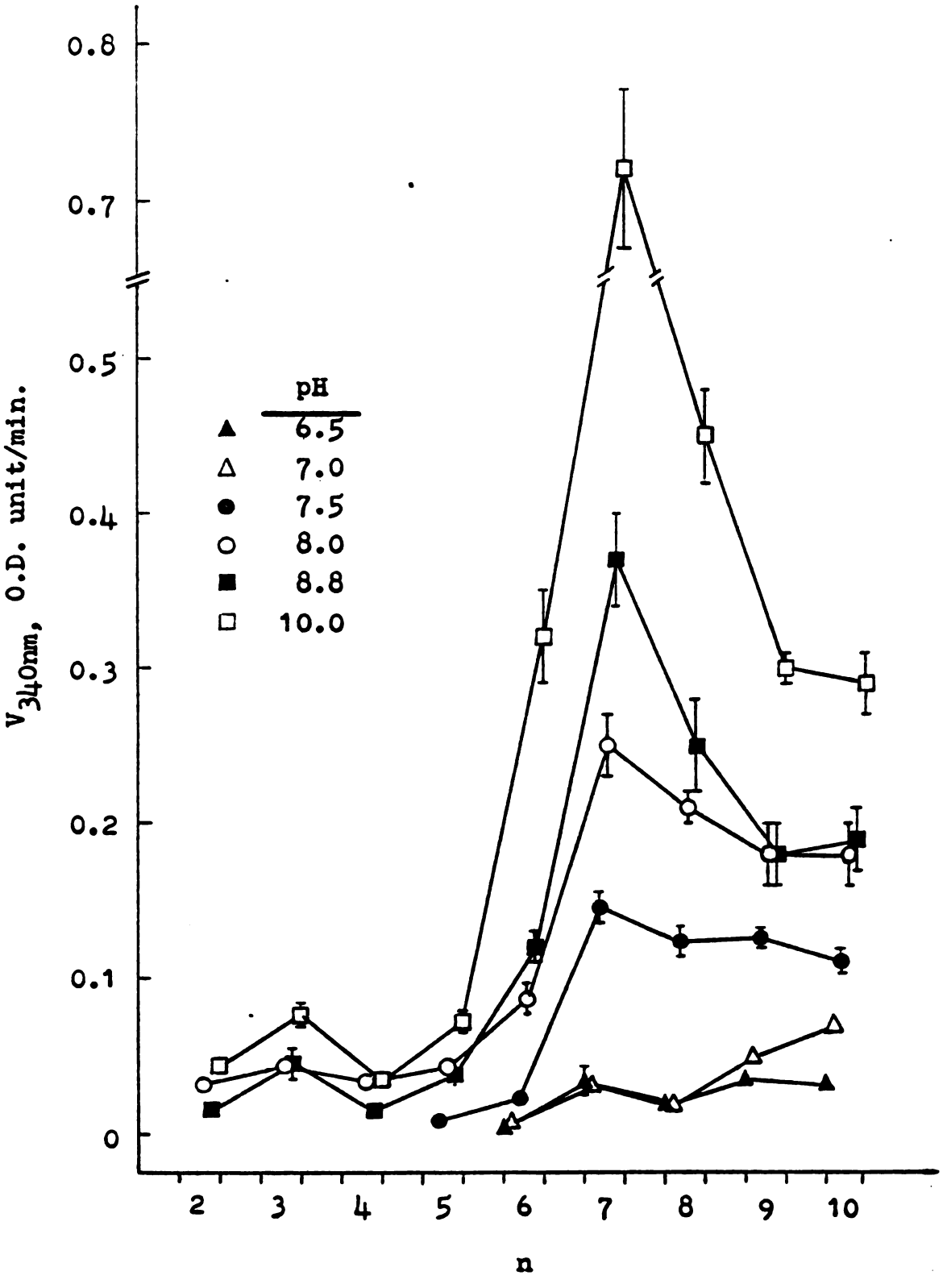


Figure 18. - Initial rates for the oxidation of $(CH_3)_c N^+(CH_2)_n OH\ X^-$ as a function of pH and n at 340 nm at room temperature

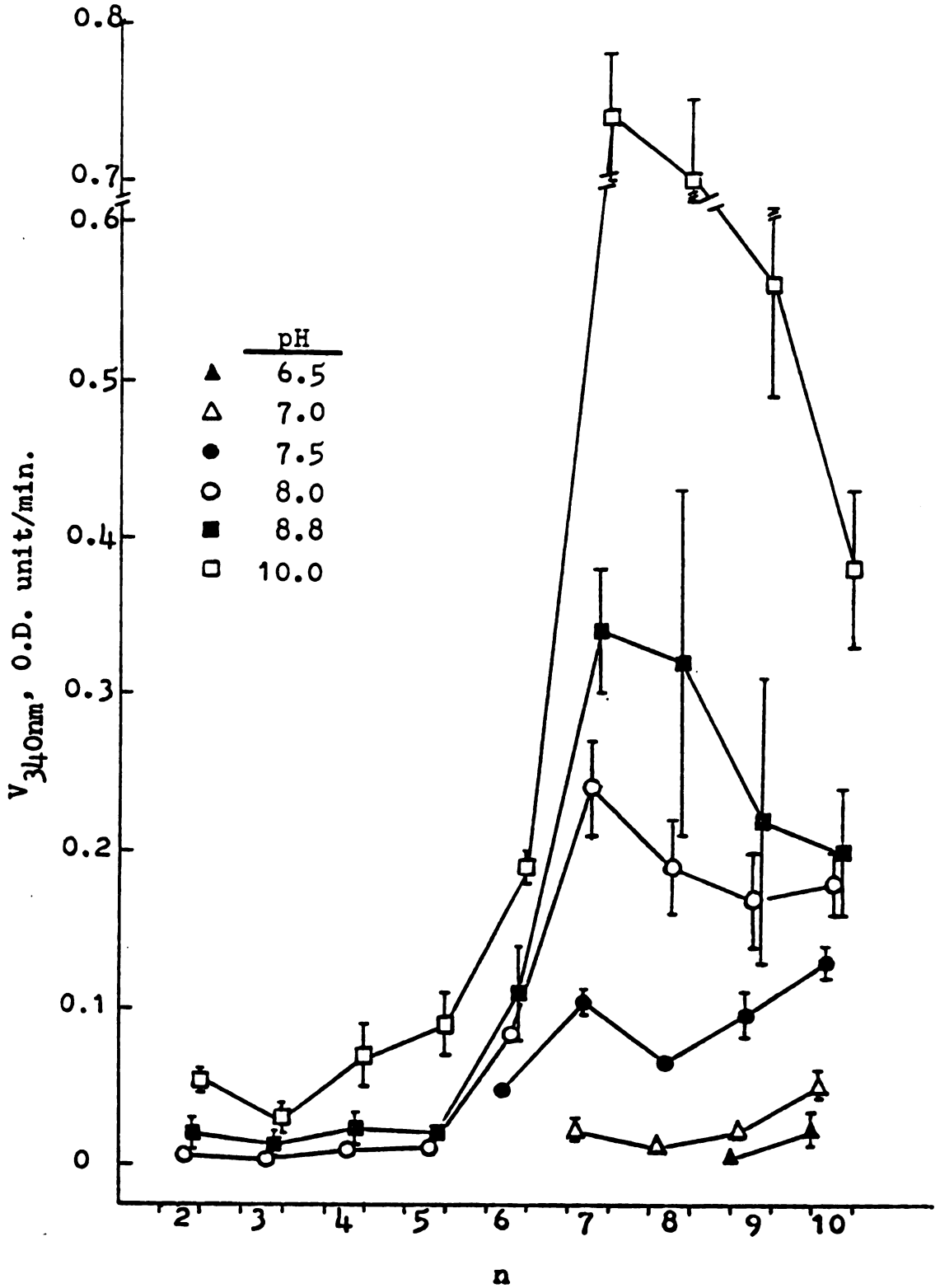


Figure 19. - Initial rates for the oxidation of $(CH_3CH_2)_3N^+(CH_2)_nOH\ X^-$ as a function of pH and n at 340 nm at room temperature

observed in this work and from 5-carbon to 6-carbon observed in Kroon's work can be explained by the best "fit" phenomenon. Since the active site of the enzyme is situated at the bottom of a deep hydrophobic substrate binding pocket, the substrate that would best fit into this pocket is expected to be kinetically favored to bind to the enzyme. It would produce the most favorable combined hydrophobic, hydrophilic, and steric interactions with the enzyme, and would require the lowest activation energy in the rate determining step of the catalytic sequence. It would seem that in the reaction of HLADH with aminoalcohols, the 7-carbon chain aminoalcohols are the most favored substrates at most of the pHs studied.

The effect of pH on the reactivity of the substrates with HLADH is shown in Figures 18 and 19. The reactivity of all substrates increases as the pH increases. At pH values of 7.5 or lower, not all substrates react. Indeed, as the pH decreases, the reactivity of the short alkyl chain aminoalcohols decreases. At the lowest pH used, 6.5, only the 9-carbon and the 10-carbon alkyl chain aminoalcohols in the ethyl series show some activity. This is perhaps an indication that the enzyme undergoes a conformational change at the hydrophobic substrate binding pocket as the pH changes. The substrate binding pocket becomes more constricted at lower pHs, and consequently, the enzyme becomes more selective with respect to the size and length of the substrate. Probably, the bulky group attached at one end of a molecule is too large to fit into the cleft at low pH and must remain outside

of it. Thus, only those substrates with an alkyl chain long enough to reach the active site will be able to react. This could be an important factor in designing NMR experiments to determine the role of zinc in the catalytic process. If the metal did play a role in the binding of the substrate at the active site, then at low pH and with a totally cadmium-substituted HLADH, only those substrates that showed activity at that pH would have any appreciable effect on the Cadmium NMR spectrum. On the other hand, if the metal did not interact with the substrate directly, none of the substrates, either active or inactive at that pH, would have much effect on the Cadmium NMR spectrum.

The quaternary ammonium salts of the aminoalcohols used in this study have thus proven to be useful probes of the hydrophobic substrate binding pocket of HLADH in terms of the depth of the cleft at the bottom of which is the active site. Their use as probes to other alcohol dehydrogenase enzyme systems whose structure is not known is something that is recommended for future studies.

The N,N,N-trimethyl and the N,N,N-triethyl derivatives of the aminoalcohols showed similar reactivities where activities were observed. The enzyme reacted with all of the substrates, in both series, at pHs 8.0 and above. It seemed desirable to test aminoalcohols with even bulkier groups attached at the amino group to see if the enzyme would show selectivity among substrates at higher pH. Attempts to prepare the propyl series of aminoalcohols were unsuccessful,

as only the 2-carbon alkyl chain compound was successfully synthesized. Further work in this area is required in order to probe the substrate binding pocket at high pHs.

Equilibrium studies of the quaternary ammonium salts of aminoalcohols with HLADH

The timed study of the equilibrium constants of the aminoalcohols at six pHs at room temperature are listed in Tables 6 and 7 for the methyl and ethyl series, respectively. These are also shown graphically as a function of pH and alkyl chain length at 36 hours in Figures 20 and 21, and as a function of time and alkyl chain length at each pH in Figures 22 to 33. The absorbance of the reduced nicotinamide adenine dinucleotide at 340 nm was recorded every twelve hours and the equilibrium constants were calculated by using equation 4 (see experimental). The equilibrium constants shown are the average of six determinations for each substrate at each pH.

Initially, the equilibrium constants were calculated after a reaction time of 30 minutes to an hour. However, it soon became apparent that this reaction time was not adequate, as many of the reactions had not reached equilibrium. The reaction time was then extended to three days. Measurements were made at twelve hour intervals up to three days, and then a final measurement was taken at the end of the seventh day.

Table 6. - Equilibrium constants for the oxidation of $(CH_3)_3N^+(CH_2)_nOH\ X^-$ with HLADH at six pHs at room temperature

pH	Time (hour)	n =	K_{eq}, M x 10^{13} 2	3	4	5	6	7	8	9	10
6.5	12		3.9 ±0.7	6.5 ±1.2	0.5 ±0.1	16 ± 2	200 ± 20	540 ± 30	560 ± 30	830 ± 30	830 ± 30
	24		1.0 ±1.0	42 ± 4	0	8.1 ±1.2	280 ± 40	630 ± 40	710 ± 20	1010 ± 40	1080 ± 90
	36		0.8 ±0.4	39 ± 8	1.1 ±0.9	15 ± 4	360 ± 40	690 ± 40	720 ± 50	1070 ± 100	1120 ± 120
	48		0.10 ±0.09	30 ±20	2.0 ±1.0	21 ± 7	420 ± 40	650 ± 40	720 ± 70	920 ±100	1210 ± 160
	60		2.0 ±1.0	7.0 ±3.0	3.0 ±0.5	7.9 ±0.8	550 ± 80	600 ± 30	790 ± 90	1050 ± 120	1140 ± 40
	72		3.0 ±2.0	0.8 ±0.4	8.0 ±3.0	25 ±13	660 ± 50	510 ± 20	810 ± 70	880 ± 50	1050 ± 90

Table 6. (cont'd)

pH	Time (hour)	n =	K_{eq}, M x 10^{13} 2	3	4	5	6	7	8	9	10
7.0	12		0.12 ±0.08	1.7 ±0.3	0	14 ± 3	200 ± 20	550 ± 20	560 ± 20	680 ± 20	730 ± 40
	24		1.2 ±0.4	9.0 ±1.0	0.8 ±0.3	18 ± 2	320 ± 40	770 ± 40	810 ± 30	1040 ± 40	1070 ± 70
	36		2.1 ±0.8	36 ±13	0.5 ±0.3	14 ± 4	380 ± 40	830 ± 40	860 ± 30	1050 ± 40	1080 ± 80
	48		3.0 ±2.0	70 ±16	0.02 ±0.02	13 ± 4	480 ± 40	940 ± 50	960 ± 50	1070 ± 60	1110 ± 90
	60		1.6 ±1.4	68 ±44	0.7 ±0.3	9.7 ±1.5	710 ±100	940 ± 30	1000 ± 70	1040 ± 70	1090 ± 80
	72		5.0 ±3.0	23 ±20	3.0 ±2.0	14 ± 4	660 ±200	930 ± 50	1020 ± 70	1160 ± 110	1150 ± 110

Table 6. (con'd)

pH	Time (hour)	n =	K_{eq}, M x 10^{13} 2	3	4	5	6	7	8	9	10
7.5	12		0.22 ±0.07	3.0 ±1.0	0.009 ±0.008	9.1 ±0.6	160 ± 20	430 ± 20	440 ± 20	490 ± 20	510 ± 20
	24		1.0 ±0.4	6.8 ±0.6	0.03 ±0.03	11 ± 1	210 ± 9	600 ± 20	620 ± 20	740 ± 40	720 ± 30
	36		3.1 ±0.9	42 ± 3	0.3 ±0.3	12 ± 2	280 ± 50	710 ± 40	720 ± 30	930 ± 50	910 ± 40
	48		6.0 ±1.0	97 ± 8	0.8 ±0.2	14 ± 2	350 ± 30	820 ± 40	890 ± 60	1110 ± 80	1070 ± 80
	60		3.0 ±1.0	180 ± 30	0.7 ±0.3	14 ± 3	430 ± 50	890 ± 30	1020 ± 50	1100 ± 60	1130 ± 60
	72		3.0 ±2.0	170 ± 30	3.0 ±0.9	20 ± 2	520 ± 50	940 ± 50	1120 ± 60	1200 ± 100	1180 ± 100

Table 6. (cont'd)

pH	Time (hour)	n = 2	3	4	5	6	7	8	9	10
		K_{eq}, M x 10^{13}								
8.0	12	0.14 ±0.03	2.5 ±0.3	0.002 ±0.002	47 ± 3	220 ± 20	380 ± 20	340 ± 20	420 ± 20	430 ± 10
	24	0.19 ±0.03	1.9 ±0.3	0	64 ± 5	220 ± 20	410 ± 30	400 ± 20	490 ± 20	520 ± 20
	36	0.3 ±0.1	3.5 ±1.6	0.4 ±0.3	73 ± 7	210 ± 40	420 ± 40	390 ± 20	480 ± 30	520 ± 20
	48	0.26 ±0.07	7.4 ±0.9	0.03 ±0.03	84 ± 6	190 ± 20	420 ± 20	390 ± 20	520 ± 20	590 ± 10
	60	0.3 ±0.1	18 ± 2	0.14 ±0.04	90 ± 4	190 ± 20	450 ± 30	400 ± 30	580 ± 20	660 ± 20
	72	0.19 ±0.10	28 ± 2	0.04 ±0.02	89 ± 6	160 ± 20	410 ± 30	370 ± 40	560 ± 50	660 ± 40

Table 6. (cont'd)

pH	Time (hour)	n = 2	3	4	5	6	7	8	9	10
		K_{eq}, $\underline{M} \times 10^{13}$								
8.8	12	0.072 ±0.006	1.2 ±0.1	0	4.5 ±0.3	27 ± 4	80 ± 2	78 ± 2	70 ± 2	77 ± 3
	24	0.10 ±0.01	1.5 ±0.2	0	3.6 ±0.4	34 ± 3	110 ± 10	110 ± 10	110 ± 10	110 ± 10
	36	0.16 ±0.09	1.8 ±0.3	0.007 ±0.007	3.5 ±1.0	42 ± 2	120 ± 20	120 ± 20	140 ± 10	130 ± 10
	48	0.18 ±0.02	2.4 ±0.5	0.005 ±0.003	3.1 ±0.3	48 ± 1	160 ± 20	150 ± 20	150 ± 10	160 ± 20
	60	0.26 ±0.04	2.7 ±0.3	0.014 ±0.005	3.3 ±0.3	53 ± 2	170 ± 20	160 ± 10	170 ± 10	180 ± 8
	72	0.28 ±0.05	2.6 ±0.2	0.006 ±0.003	2.9 ±0.3	54 ± 5	180 ± 10	160 ± 10	170 ± 10	190 ± 9

Table 6. (cont'd)

pH	Time (hour)	n = 2	3	4	5	6	7	8	9	10
		K_{eq}, M x 10^{13}								
	12	0.051 ±0.002	0.65 ±0.04	0.0002 ±0.0001	2.8 ±0.2	13 ± 3	20 ± 1	20 ± 1	20 ± 1	20 ± 1
	24	0.063 ±0.004	0.82 ±0.02	0.0028 ±0.0003	3.5 ±0.4	10 ± 1	19 ± 2	18 ± 1	18 ± 2	18 ± 2
	36	0.08 ±0.02	0.9 ±0.1	0.002 ±0.002	3.0 ±0.8	10.1 ± 0.8	21 ± 2	20 ± 2	20 ± 1	20 ± 1
10.0	48	0.06 ±0.01	0.89 ±0.06	0.004 ±0.002	2.5 ±0.2	9.9 ±0.2	20 ± 1	20 ± 1	20 ± 1	20.3 ± 0.6
	60	0.046 ±0.009	0.94 ±0.06	0.0012 ±0.0007	2.1 ±0.2	9.9 ±0.3	19 ± 2	19 ± 2	20.4 ± 0.9	20 ± 1
	72	0.04 ±0.01	0.84 ±0.09	0.008 ±0.002	1.8 ±0.2	10.1 ± 0.6	17 ± 1	18.6 ± 0.9	19 ± 1	18 ± 1

Table 7. - Equilibrium constants for the oxidation of $(CH_3CH_2)_3N^+(CH_2)_nOH\ X^-$ with HLADH at six pHs at room temperature

pH	Time (hour)	n =	K_{eq}, M x 10^{13} 2	3	4	5	6	7	8	9	10
	12		1.6 ±0.5	0.06 ±0.05	1.0 ±0.6	18 ± 5	40 ± 9	84 ±13	390 ± 50	490 ± 40	520 ± 30
	24		4 ±2	0	0.03 ±0.02	14 ± 2	23 ± 5	92 ±10	440 ± 30	580 ± 40	700 ± 30
	36		23 ± 4	0.05 ±0.05	16 ± 4	17 ± 4	23 ± 3	93 ± 3	520 ± 20	680 ± 20	870 ± 30
6.5											
	48		17 ± 4	0.02 ±0.02	7.8 ±3.0	26 ± 6	21 ± 3	76 ± 2	540 ± 40	760 ± 40	890 ± 20
	60		3.6 ±0.8	0.2 ±0.1	2.0 ±1.0	25 ± 6	16 ± 2	63 ± 5	450 ± 30	850 ± 40	1050 ± 50
	72		0	0	0	17 ± 4	8.7 ±0.4	56 ± 5	460 ± 30	680 ± 10	980 ± 30

Table 7. (cont'd)

pH	Time (hour)	K_{eq}, M x 10^{13} n = 2	3	4	5	6	7	8	9	10
7.0	12	0.39 ±0.04	0	1.0 ±0.3	17 ± 4	36 ± 4	82 ±10	300 ± 40	510 ± 10	470 ± 50
	24	2.4 ±0.4	0.4 ±0.2	5.7 ±0.5	15 ± 3	39 ± 9	120 ± 10	500 ± 30	710 ± 20	700 ± 30
	36	13 ± 2	1.0 ±0.3	13 ± 2	17 ± 2	31 ± 3	140 ± 20	650 ± 30	920 ± 40	890 ± 40
	48	22 ± 4	0.2 ±0.1	27 ± 7	17 ± 5	30 ± 2	160 ± 20	710 ± 30	1000 ± 80	970 ± 70
	60	16 ± 6	0.03 ±0.03	14 ±11	18 ±12	33 ± 4	150 ± 30	710 ± 20	1000 ± 10	1070 ± 60
	72	9.5 ±0.3	0.2 ±0.1	14 ± 6	12 ± 8	24 ± 3	130 ± 20	640 ± 40	1060 ± 60	1070 ± 40

Table 7. (cont'd)

pH	Time (hour)	K_{eq}, M x 10^{13} n = 2	3	4	5	6	7	8	9	10
	12	0.5 ±0.1	0	0.90 ±0.07	14 ± 2	24 ± 3	80 ± 3	290 ± 20	430 ± 50	350 ± 20
	24	2.1 ±0.3	0.01 ±0.01	3.7 ±0.4	14 ± 2	26 ± 3	120 ± 10	420 ± 40	580 ± 10	510 ± 20
	36	13 ± 1	0.20 ±0.04	17 ± 2	34 ±15	35 ± 2	150 ± 20	570 ± 30	790 ± 20	720 ± 20
7.5										
	48	28 ± 2	0.2 ±0.1	39 ± 7	38 ± 2	38 ± 3	180 ± 20	660 ± 30	960 ± 40	840 ± 40
	60	28 ± 2	0.20 ±0.07	45 ± 5	54 ± 3	42 ± 4	210 ± 20	760 ± 30	980 ± 30	920 ± 30
	72	28 ± 3	0.18 ±0.07	49 ± 6	60 ± 4	52 ± 3	230 ± 20	830 ± 20	1020 ± 90	1030 ± 40

Table 7. (cont'd)

pH	Time (hour)	n =	K_{eq}, $\underline{M} \times 10^{13}$ 2	3	4	5	6	7	8	9	10
	12		0.3 ±0.1	0	0.91 ±0.09	130 ± 20	240 ± 40	310 ± 30	380 ± 50	370 ± 20	390 ± 20
	24		0.4 ±0.2	0	1.2 ±0.2	130 ± 10	230 ± 30	300 ± 30	400 ± 20	410 ± 10	430 ± 10
	36		0.8 ±0.3	0	2.4 ±0.2	130 ± 10	210 ± 10	290 ± 10	430 ± 10	450 ± 20	490 ± 10
8.0											
	48		1.5 ±0.2	0	5.0 ±0.7	130 ± 10	200 ± 20	270 ± 30	470 ± 20	470 ± 30	440 ± 30
	60		6.0 ±2.0	0.05 ±0.01	13 ± 2	150 ± 10	200 ± 10	280 ± 20	500 ± 50	500 ± 10	590 ± 10
	72		9.0 ±2.0	0.016 ±0.006	20 ± 1	150 ± 30	190 ± 20	250 ± 50	500 ± 30	480 ± 30	600 ± 20

Table 7. (cont'd)

pH	Time (hour)	n = 2	3	4	5	6	7	8	9	10
		K_{eq}, M x 10^{13}								
	12	0.33 ±0.04	0	0.67 ±0.03	7.3 ±0.3	13 ± 2	35 ± 3	72 ± 3	85 ± 4	78 ± 3
	24	0.39 ±0.04	0	0.87 ±0.03	5.8 ±0.3	11 ± 1	43 ± 2	94 ± 4	120 ± 10	110 ± 10
	36	0.48 ±0.05	0	1.3 ±0.1	5.1 ±0.2	10.6 ± 0.7	53 ± 1	120 ± 10	150 ± 10	140 ± 10
8.8										
	48	0.78 ±0.03	0	1.8 ±0.2	5.6 ±0.3	11 ± 1	61 ± 7	140 ± 20	170 ± 10	160 ± 10
	60	0.98 ±0.01	0	2.3 ±0.2	5.2 ±0.4	12 ± 1	70 ± 2	140 ± 30	190 ± 10	190 ± 10
	72	0.72 ±0.03	0	2.4 ±0.2	5.7 ±0.3	13 ± 1	76 ± 4	170 ± 30	190 ± 20	190 ± 10

Table 7. (cont'd)

pH	Time (hour)	K_{eq}, M x 10^{13} n = 2	3	4	5	6	7	8	9	10
	12	0.21 ±0.03	0.005 ±0.002	0.44 ±0.04	7.9 ±0.4	12 ± 1	17 ± 2	18 ± 1	20 ± 1	21 ± 1
	24	0.26 ±0.02	0.007 ±0.001	0.59 ±0.04	7.0 ±0.2	8.8 ±0.4	17 ± 2	20 ± 1	21 ± 1	21 ± 1
	36	0.30 ±0.05	0.007 ±0.001	0.80 ±0.06	5.3 ±0.1	7.0 ±0.3	17 ± 1	20 ± 1	21 ± 1	21 ± 1
10.0										
	48	0.29 ±0.04	0.008 ±0.001	0.86 ±0.09	4.4 ±0.2	6.3 ±0.4	16 ± 2	20 ± 1	21 ± 2	21 ± 2
	60	0.30 ±0.03	0.006 ±0.002	0.83 ±0.10	3.6 ±0.3	5.6 ±0.4	15 ± 2	19 ± 1	20 ± 1	21 ± 1
	72	0.29 ±0.02	0	0.87 ±0.10	3.1 ±0.2	4.9 ±0.2	14 ± 2	19 ± 2	20 ± 2	20 ± 2

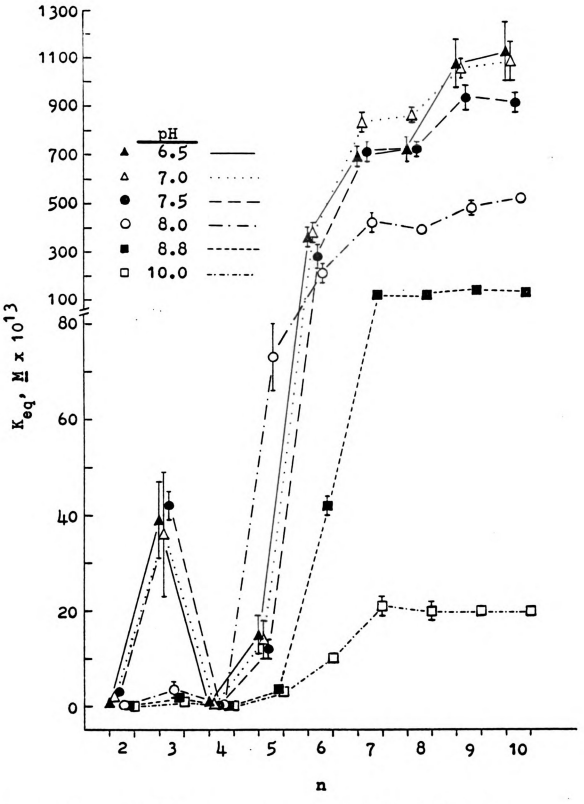


Figure 20. - Equilibrium constants for the oxidation of N,N,N-trimethylaminoalcohols as a function of pH and n at 36 hours at room temperature

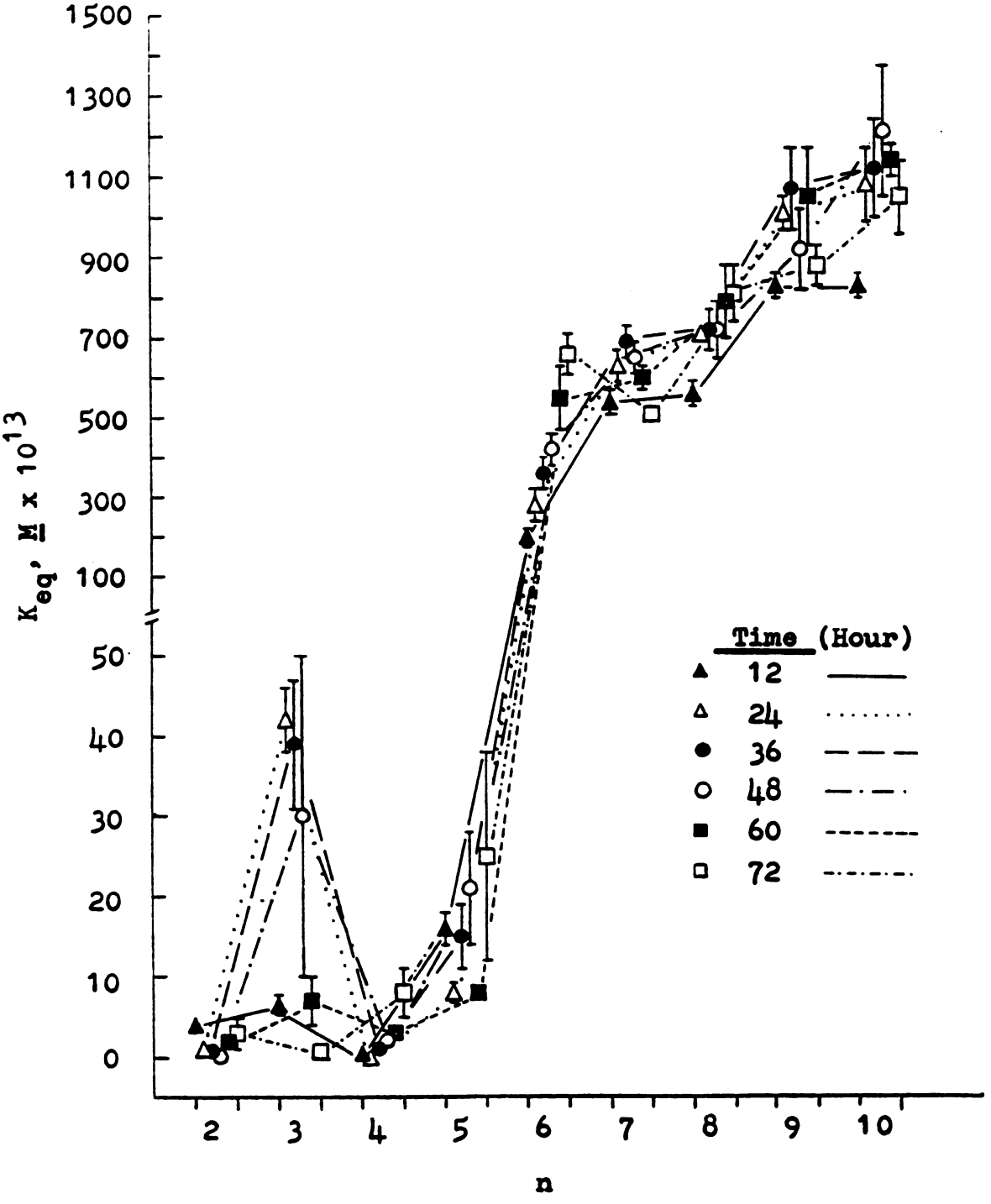


Figure 21. - Equilibrium constants for the oxidation of N,N,N-trimethylaminoalcohols as a function of time and n at pH 6.5 at room temperature

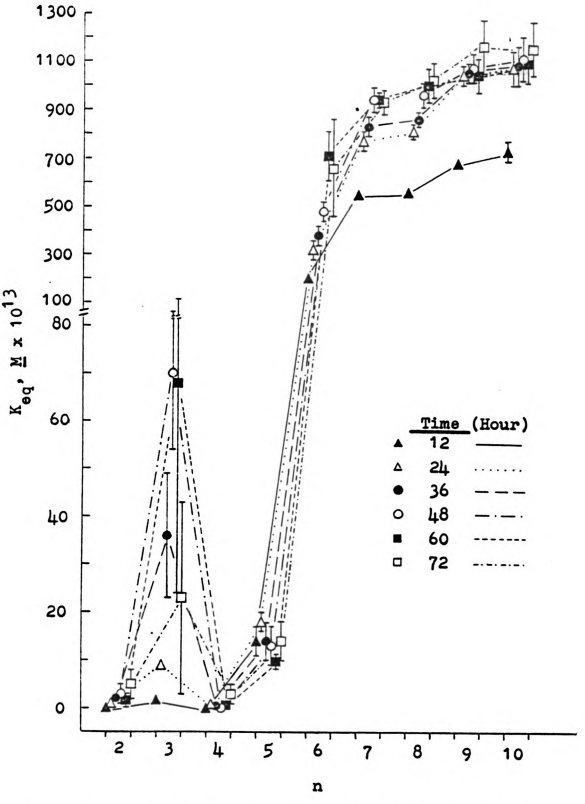


Figure 22. - Equilibrium constants for the oxidation of N,N,N-trimethylaminoalcohols as a function of time and n at pH 7.0 at room temperature

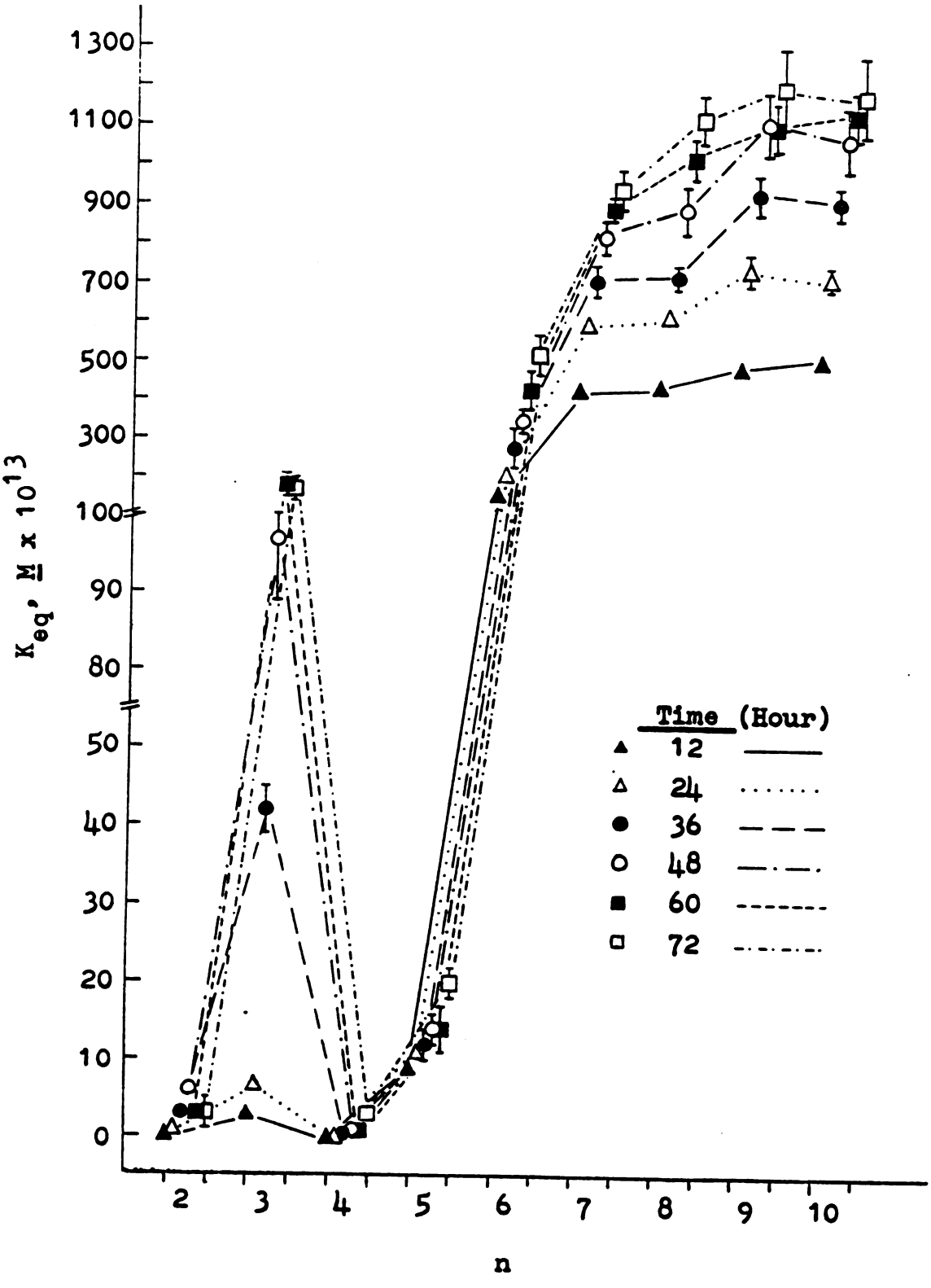


Figure 23. - Equilibrium constants for the oxidation of N,N,N-trimethylaminoalcohols as a function of time and n at pH 7.5 at room temperature

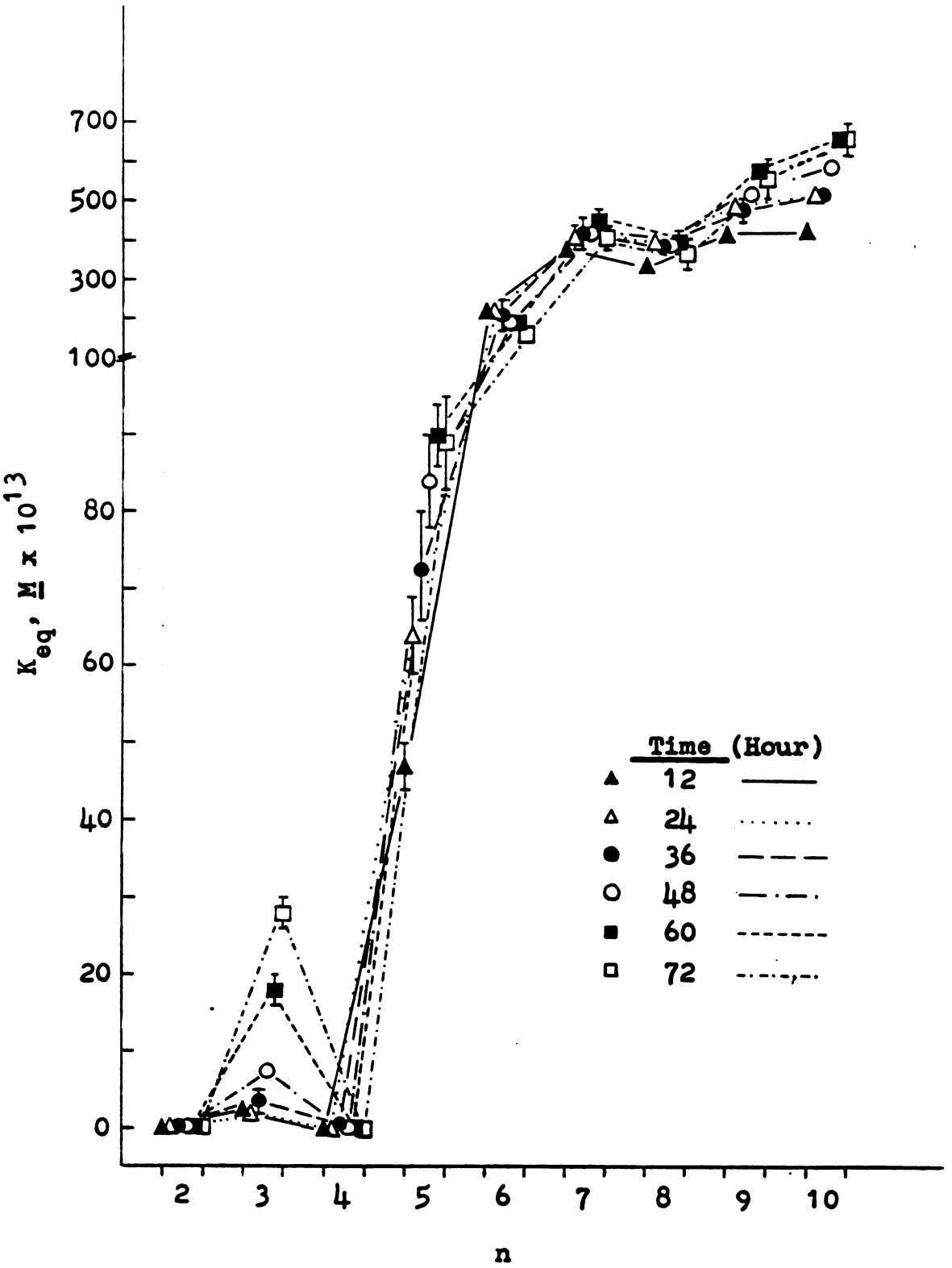


Figure 24. - Equilibrium constants for the oxidation of N,N,N-trimethylaminoalcohols as a function of time and n at pH 8.0 at room temperature

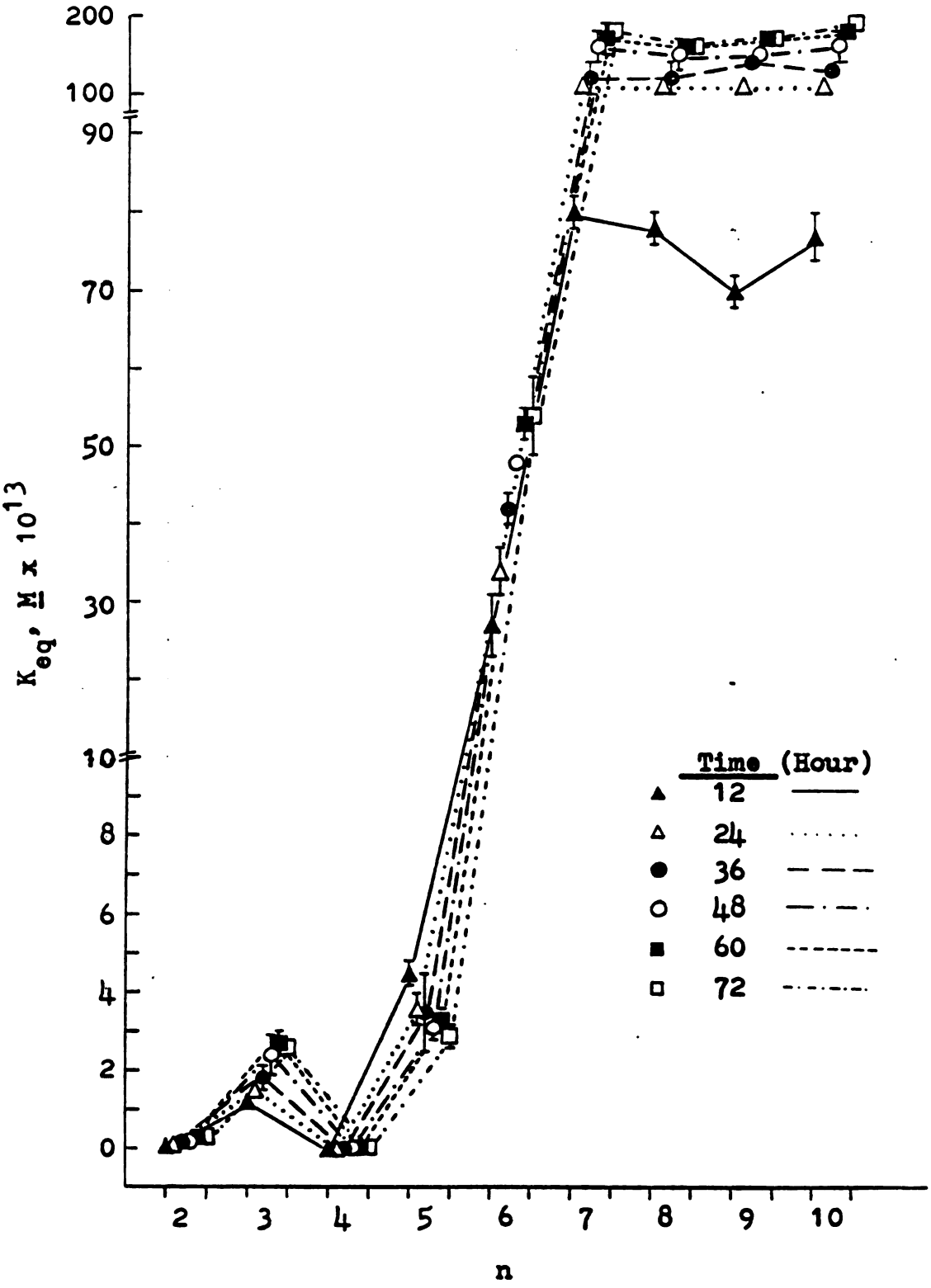


Figure 25. - Equilibrium constants for the oxidation of N,N,N-trimethylaminoalcohols as a function of time and n at pH 8.8 at room temperature

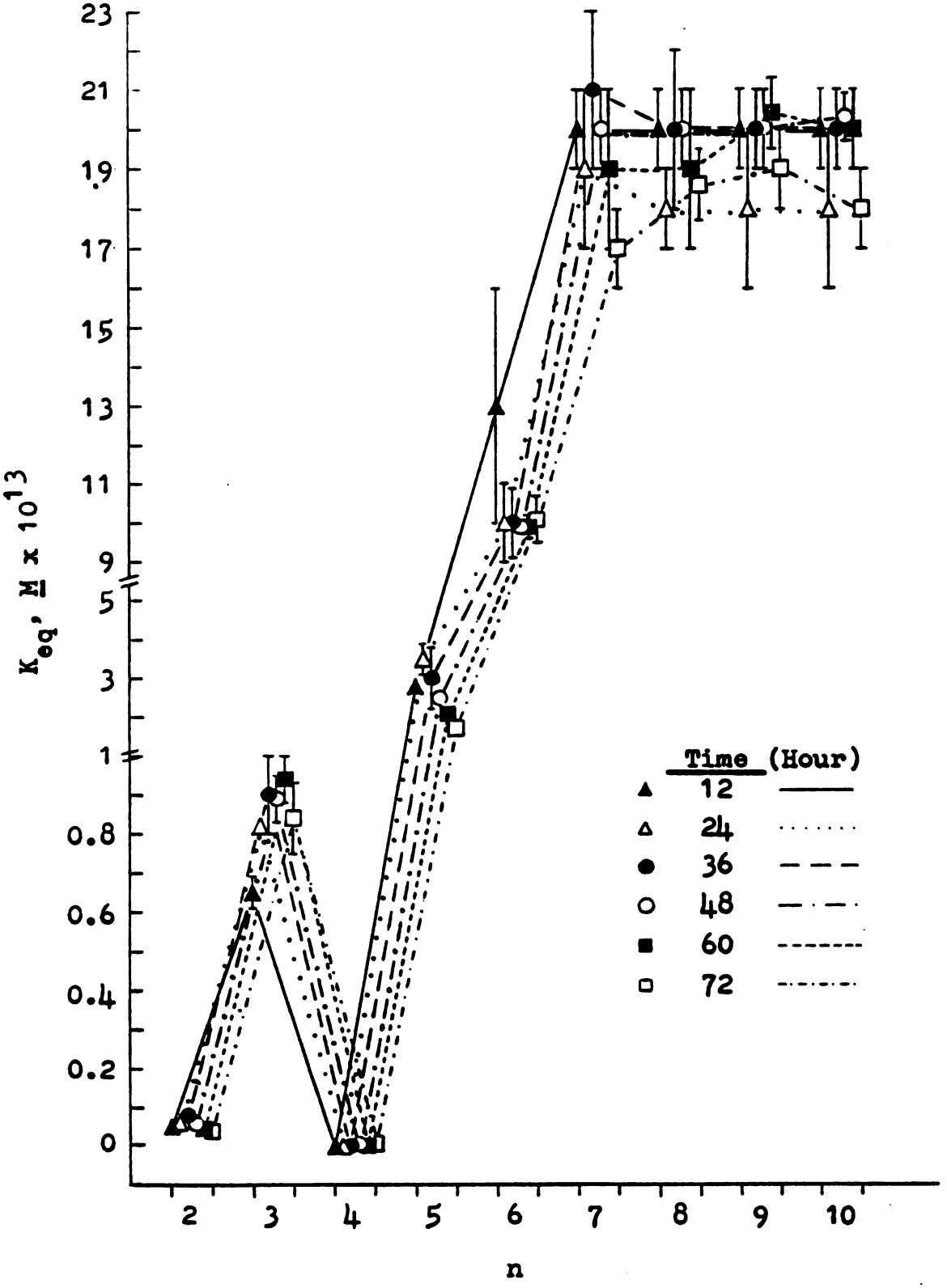


Figure 26. - Equilibrium constants for the oxidation of N,N,N-trimethylaminoalcohols as a function of time and n at pH 10.0 at room temperature

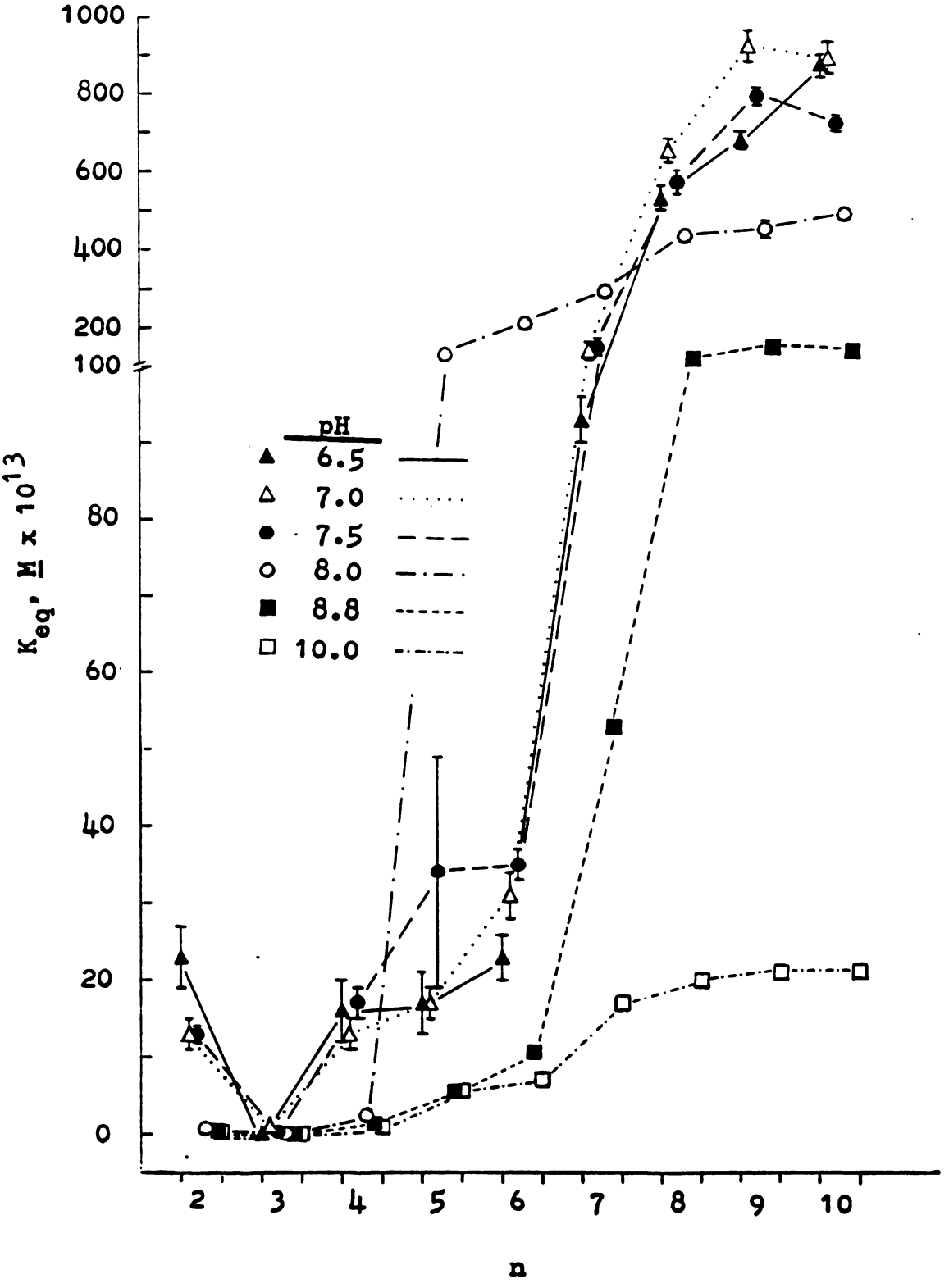


Figure 27. - Equilibrium constants for the oxidation of N,N,N-triethylaminoalcohols as a function of pH and n at 36 hours at room temperature

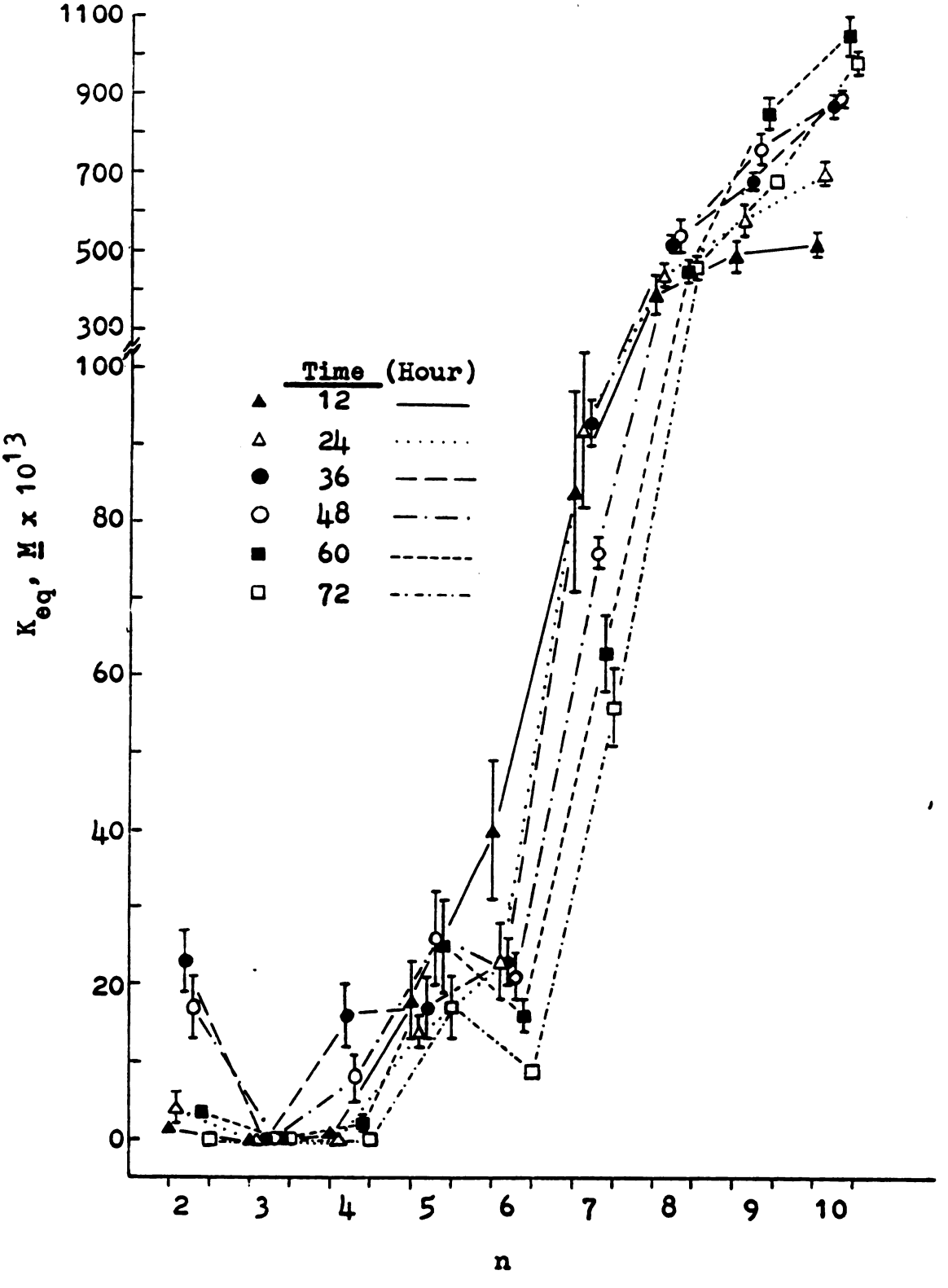


Figure 28. - Equilibrium constants for the oxidation of N,N,N-triethylaminoalcohols as a function of time and n at pH 6.5 at room temperature

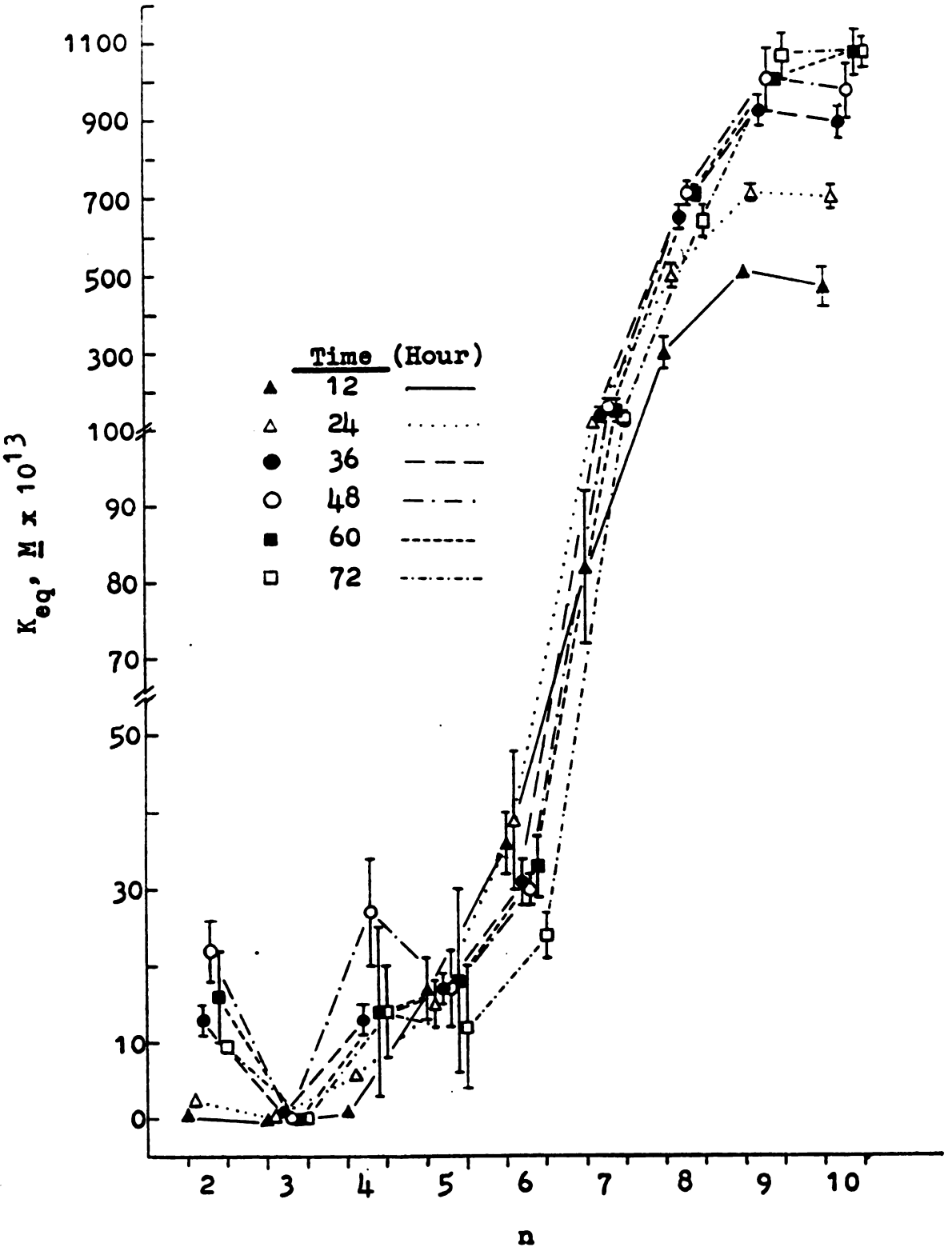


Figure 29. - Equilibrium constants for the oxidation of N,N,N-triethylaminoalcohols as a function of time and n at pH 7.0 at room temperature

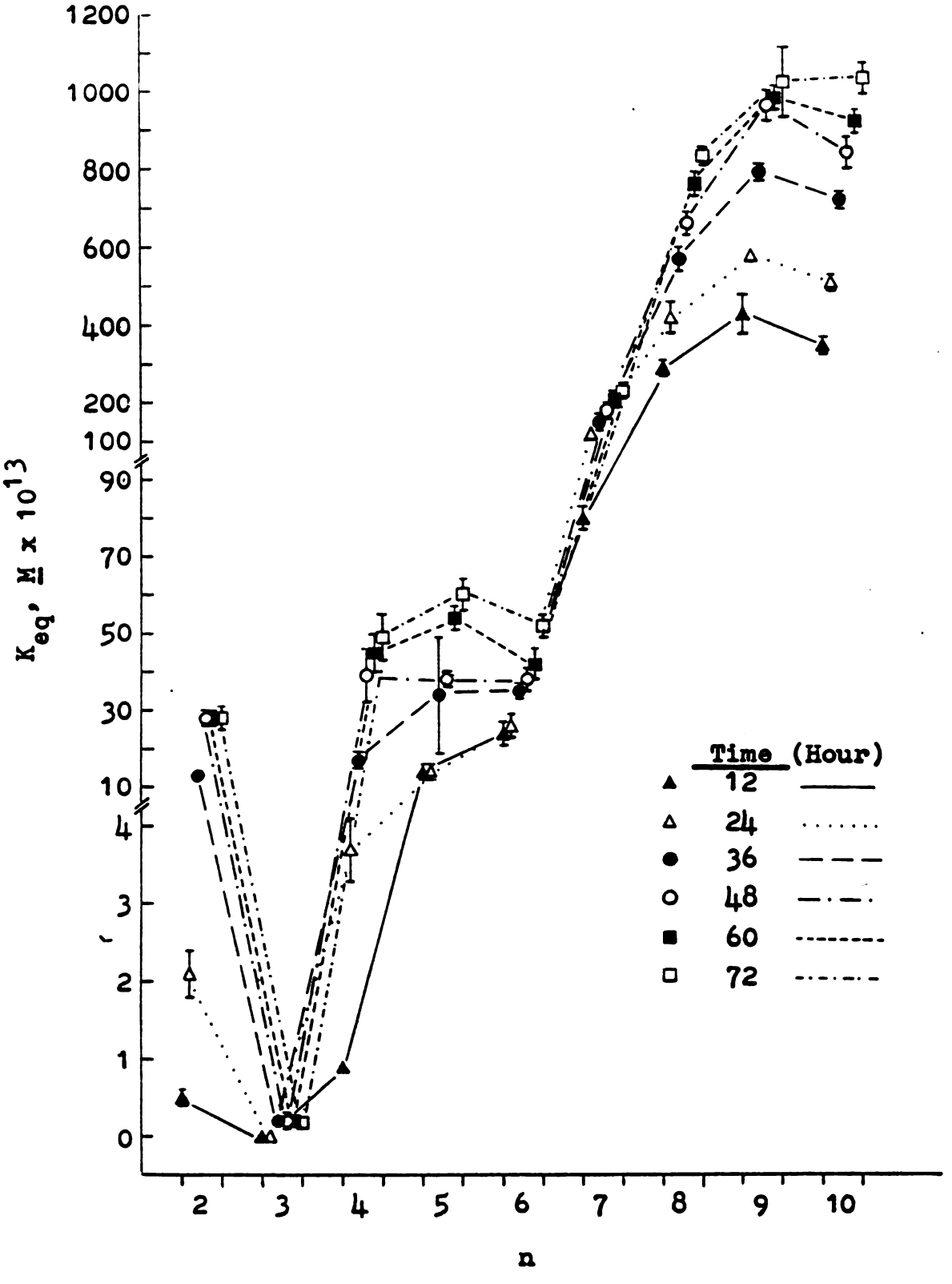


Figure 30. - Equilibrium constants for the oxidation of N,N,N-triethylaminoalcohols as a function of time and n at pH 7.5 at room temperature

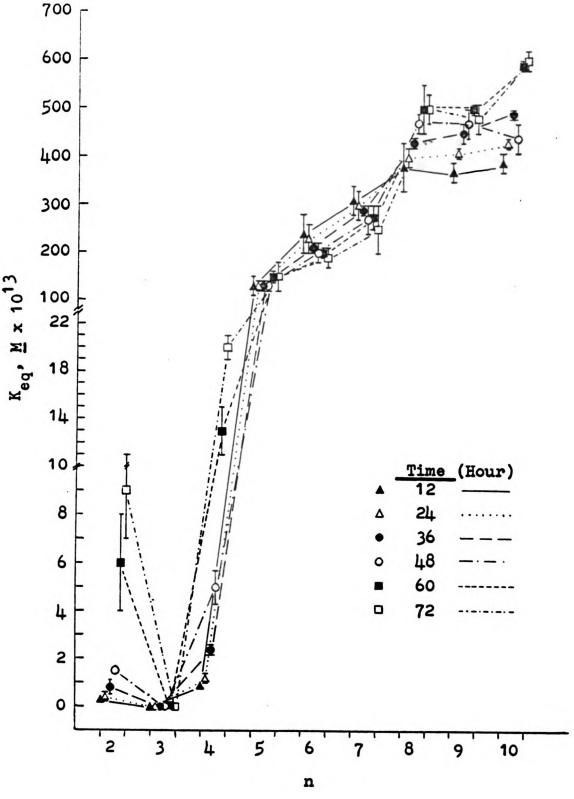


Figure 31. - Equilibrium constants for the oxidation of N,N,N-triethylaminoalcohols as a function of time and n at pH 8.0 at room temperature

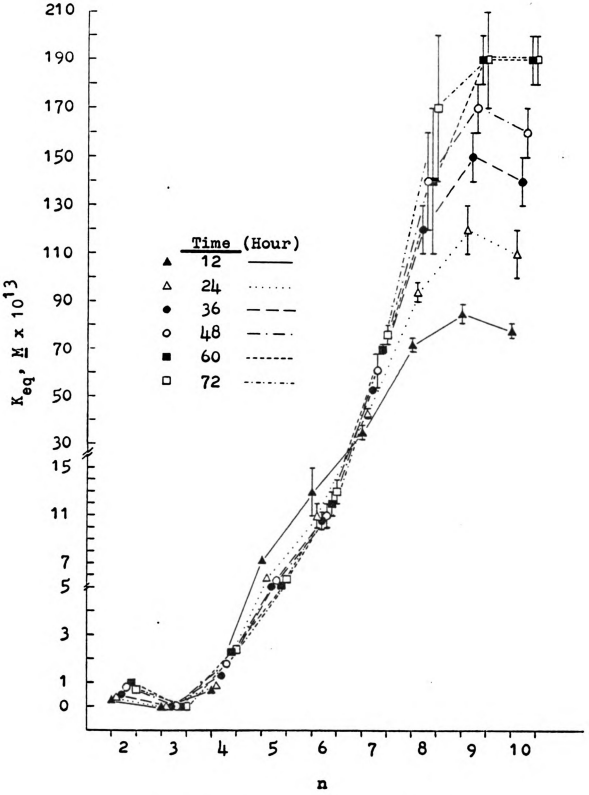


Figure 32. - Equilibrium constants for the oxidation of N,N,N-triethylaminoalcohols as a function of time and n at pH 8.8 at room temperature

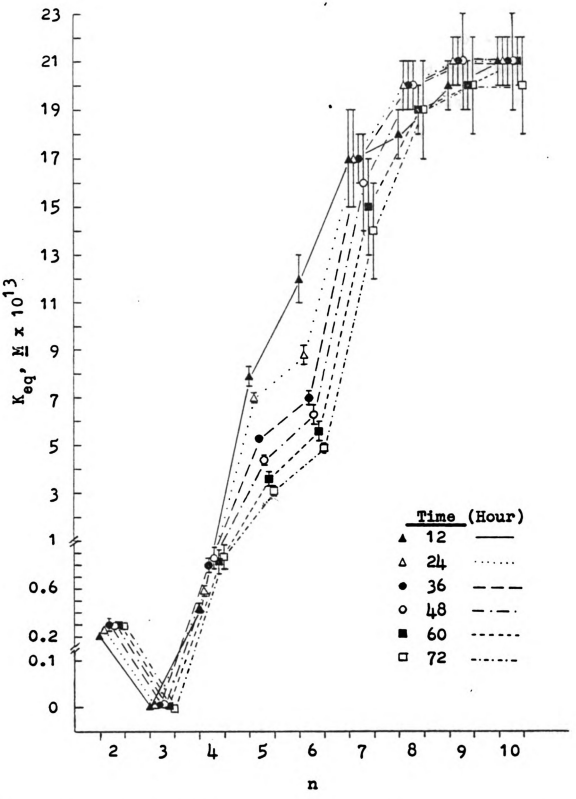


Figure 33. - Equilibrium constants for the oxidation of N,N,N-triethylaminoalcohols as a function of time and n at pH 10.0 at room temperature

The results showed that most of the substrates had reached equilibrium within the three day period; and by the end of the third day, the equilibrium constant had remained relatively constant or already had started decreasing, thus indicating that some degradation had begun to take place.

Both series of the salts of the aminoalcohols showed significant changes in the equilibrium constant both as a function of alkyl chain length and of pH. The equilibrium constant of the N,N,N-trimethyl series increased as the alkyl chain of the aminoalcohol increased from C-2 to C-7 and remained relatively constant up to C-10, while that of the N,N,N-triethyl series increased from C-2 to C-8 and remained constant up to C-10. The equilibrium constants reached a maximum between pH 7.0 and 7.5 and then decreased as the pH was increased further.

It has been reported[1, 36] that for most primary aliphatic alcohols the equilibrium constant is relatively constant and has a value of about 8.0×10^{-12} M. However, the results obtained in this study are clearly inconsistent with the values reported. On the other hand, they are consistent with the results obtained by Kroon[24] with secondary aminoalcohols that showed that the equilibrium constant increased with increasing chain length. Furthermore, Pietruszko et al[37] observed the same trend with 2-enoic alcohols.

Theorell and coworkers[38-40] found that the dissociation constant of the binary complex of NAD^+ and HLADH favored the free oxidized coenzyme and enzyme at all pHs, whereas the

dissociation constant of the binary complex of NADH and HLADH favored the bound NADH at pHs lower than 9. In addition, all of the studies had indicated that the binary complex was favored over the ternary complex with primary aliphatic alcohols and aldehydes.[41] Under these circumstances, the equilibrium constant measured for primary aliphatic alcohols would in fact be a measure of the equilibrium between the free NAD^+ and the bound NADH in the system and would be independent of the structure of the substrate. Thus, this would explain the relatively similar equilibrium constant for primary aliphatic alcohols. Clearly, the data obtained by this work cannot be a measure of the equilibrium between the free NAD^+ and the bound NADH. It is worth pointing out that Winer and Theorell,[42] and Theorell and McKinley-McKee,[39] working with saturated straight chain fatty acids and amides as inhibitors of HLADH, showed that the binding of the inhibitor to the binary complex of coenzyme and enzyme was greatly enhanced when the chain length was increased. In fact, in a few cases, the dissociation constant favored the ternary complex over the binary complex. Also the dissociation constant of the ternary complex with primary aliphatic alcohols decreased as the chain length was increased, although the binary complex was still favored in all cases studied.

We suggest that hydrophobic interaction plays an important role in the binding of the substrate to the binary complex and may affect the relative stability of the ternary

complex with respect to the binary complex. Conceivably, in some cases, it could even make the ternary complex more stable than the binary complex. The equilibrium constants would then depend on the structure of the substrate and might be a measure of the equilibrium between the ternary complex and another identity. We suggest that as the length of the carbon chain of the aminoalcohol derivative is increased, the stability of the ternary complex increases.

The effect of pH on the equilibrium constant cannot be explained satisfactorily. It would seem from equation 4 that at higher pH values, the equilibrium would favor the oxidation of the alcohols and increase the equilibrium constant. However, it is also known that at higher pH values, NAD^+ undergoes a side reaction to form a pseudobase,[43, 44] and at pH 10.0, the EE-dimer of HLADH, which is responsible for the oxidoreduction of non-steroid substrates, is gradually transformed to other isomers.[45] These two factors may explain the decrease in the equilibrium constant as the pH is increased from 7.5 to 10.0.

In conclusion, this work has shown that: 1. The quaternary ammonium salts of several aminoalcohols can be used as substrates for HLADH. 2. The initial velocities and equilibrium constants obtained at six pHs and at room temperature are consistent with the premise that hydrophobic interactions affect this enzymatic reaction significantly. 3. These substrates could be used as probes for the hydrophobic substrate binding pocket of the enzyme. The potential

use of these substrates, or structurally similar ones, to elucidate the role of the catalytic zinc in this reaction, or to study other alcohol dehydrogenases must await further experimentation.

EXPERIMENTAL

Instrumentation

Proton NMR spectra were recorded either on a Varian T-60 NMR spectrometer or a Bruker WP 250 NMR spectrometer.

Ultraviolet spectra for enzyme activity studies were obtained with a Beckman DB-G spectrometer equipped with an analog converter connected to a Sargent SR recorder.

The pH of the buffers was adjusted with an Instrumentation Laboratory Model 245 pH meter.

Mass spectra were obtained with a Finnigan GC-MS mass spectrometer.

Melting points of the quaternary ammonium halides of the aminoalcohols were determined with a Hoover capillary melting point machine.

Materials

Crystalline horse liver alcohol dehydrogenase and the oxidized form of nicotinamide adenine dinucleotide Grade III were purchased from Sigma.

The compounds N,N-dimethyl-2-aminoethanol, N,N-dimethyl-3-amino-1-propanol, N,N-diethyl-2-aminoethanol, N,N-diethyl-3-amino-1-propanol, 6-chloro-1-hexanol, 1,5-pentanediol,

1,7-heptanediol, 1,8-octanediol, 1,9-nonanediol, 1,10-decanediol, and iodoethane were obtained from Aldrich.

Synthesis

A. Preparation of Chlorohydrins

I. 4-Chloro-1-butanol.[46]

$$+ HCl \longrightarrow ClCH_2CH_2CH_2CH_2OH$$

A 500 ml. three-necked round-bottomed flask containing 130 ml. (114 gm. or 1.58 moles) of tetrahydrofuran was equipped with a reflux condenser, a thermometer dipping into the liquid, and a bent glass tube arranged to introduce gaseous hydrogen chloride near the bottom of the flask. The condenser was connected to a soda lime tube to trap the hydrogen chloride gas that might escape.

The tetrahydrofuran was heated to boiling in an oil bath, and a slow stream of hydrogen chloride gas, produced by dripping concentrated sulfuric acid onto a mixture of sodium chloride and concentrated hydrochloric acid, was bubbled into the liquid. The temperature increased slowly at first, and then more rapidly. The reaction was stopped when the temperature remained practically constant at about 110° after six hours of heating. The liquid turned yellowish brown at this point.

The liquid was cooled and the unreacted tetrahydrofuran was removed by fractional distillation. The chlorohydrin was collected by vacuum distillation at 81-82°/14mm Hg.

II. Other chlorohydrins from the diols.[47]

$$HO\text{-}(CH_2)_n\text{-}OH + HCl \longrightarrow HO\text{-}(CH_2)_n\text{-}Cl + H_2O$$

$$n = 5, 7, 8, 9, 10$$

A special apparatus shown in Figure 34 was set up for these preparations.

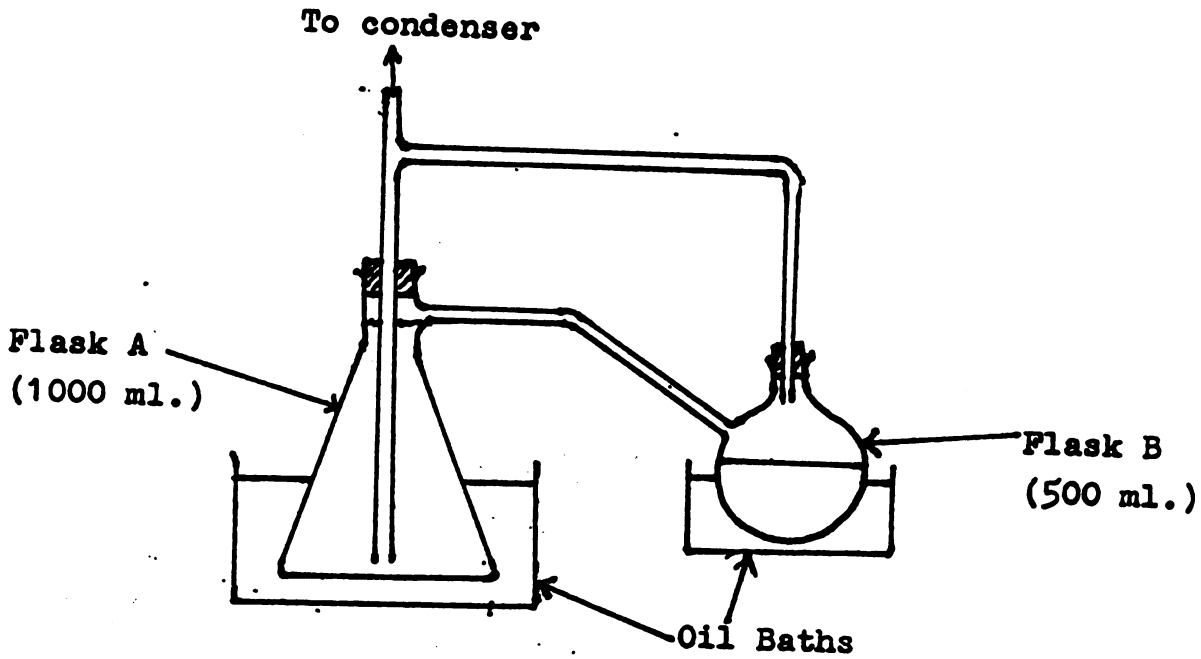


Figure 34. - Apparatus for the preparation of chlorohydrins from diols

About 0.89 mole of the diol was put in reaction flask A together with 9.5 moles of concentrated hydrochloric acid, 130 ml. of water, and 55 ml. of toluene.* Flask B contained 350 ml. of toluene with boiling chips. Both flasks were heated by oil baths, Flask A being kept at 95° and Flask B at 140-145°. As the reaction proceeded, the product chlorohydrin was continuously extracted and removed by toluene flowing through the reaction mixture. After 12-24 hours of heating, the organic phase had turned brown and the reaction was stopped. After the flasks were allowed to cool, the organic phase in Flask A was forced out into Flask B by adding water through a long stem glass funnel. The aqueous phase was then extracted twice with 100 ml. portions of toluene. Toluene was removed from the combined organic mixture by either distillation or low pressure rotary evaporation. The remaining liquid was fractionally distilled at low pressure to obtain the chlorohydrin. Table 8 gives the yield and distillation temperature and pressure of the chlorohydrins.

* Since nonamethylene glycol and decamethylene glycol are quite soluble in toluene, ligroine (b.p. 100-110°) was used as the extracting solvent instead of toluene.[48]

Table 8. - Yields and Boiling Points of the Chlorohydrins ($HO-(CH_2)_n-Cl$)

n	b.p. ^oC/mm Hg	% Yield
4	81-82/14	66.0
5	100-104/9	20.0
7	122-124/12	57.0
8	130-140/10	75.0
9	146-148/14	60.0
10	185-189/15	46.0

B. Preparation of N,N-dialkylaminoalkanols

I. 4-Dimethylamino-1-butanol.

$$HO-(CH_2)_4-Cl + (CH_3)_2NH \longrightarrow (CH_3)_2N-(CH_2)_4-OH + HCl$$

This aminoalcohol was prepared by a modified method of Nakajima.[49] An amount of 20 gm. of dimethylamine was placed in a 250 ml. three-necked round-bottomed flask equipped with a dry ice/acetone condenser, a separatory funnel, and a magnetic stirrer. The flask was cooled to below 5^o by an ice-water bath. To the amine was added slowly 22 gm. (0.2 mole) of butamethylene chlorohydrin through the separatory funnel while the temperature was maintained at below 5^o. After the addition was complete, the reaction vessel was allowed to warm up to room temperature. This warming allowed low boiling substance to evaporate. The remaining

liquid residue was vacuum distilled to obtain the product 4-dimethylamino-1-butanol, as a colorless viscous liquid.

II. 5-Dimethylamino-1-pentanol.[49]

$$HO\text{-}(CH_2)_5\text{-}Cl + (CH_3)_2NH \longrightarrow (CH_3)_2N\text{-}(CH_2)_5\text{-}OH + HCl$$

A mixture of 12.3 gm. (0.1 mole) of pentamethylene chlorohydrin and 25.0 gm. of a 40% aqueous solution of dimethylamine was allowed to react in a 100 ml. round-bottomed flask with a condenser and magnetic stirrer. The reaction mixture was heated to 60^{o} for about 4 hours until it became homogeneous. The solution was then distilled until the internal temperature reached 180^{o}. A concentrated solution of potassium hydroxide in methyl alcohol was added to the cooled reaction mixture. The precipitated potassium chloride was filtered off. After the addition of water, ether was added to the biphasic mixture. The organic layer was removed and saved. The aqueous layer was extracted three times with 50 ml. portions of ether. The organic layers were combined and dried with anhydrous magnesium sulfate. After removal of the low boiling ether and methyl alcohol by rotary evaporation, the remaining light brownish liquid was distilled to obtain the product, 5-dimethylamino-1-pentanol, as a colorless liquid.

III. N,N-Diethylaminoalkanols.

$$HO\text{-}(CH_2)_n\text{-}Cl + (CH_3CH_2)_2NH \longrightarrow (CH_3CH_2)_2N\text{-}(CH_2)_n\text{-}OH + HCl$$

$n = 4$ to 10

The procedure used for the synthesis of these amino-alcohols was the one used by W. W. Hartman.[50] In a typical experiment, 0.2 mole of diethylamine was refluxed in a 100 ml. round-bottomed flask equipped with dropping funnel, condenser and magnetic stirrer. To the refluxing diethylamine was added dropwise 0.1 mole of the chlorohydrin. After the addition was complete, the mixture was heated further for 8 to 12 hours. During this time, a white crystalline precipitate began to form and the liquid turned brown. The heating was stopped and the contents were allowed to cool. Addition of 5.0 ml. of 16.4 N sodium hydroxide to the cooled solution caused the precipitation of sodium chloride. After 10 ml. of water was added to dissolve the sodium chloride, 50 ml. of ether was added to the mixture. The organic layer was separated from the aqueous phase and the aqueous layer was extracted three more times with 50 ml. portions of ether. The combined ether extracts were dried over anhydrous potassium carbonate by mechanical stirring until the turbidity disappeared. The potassium carbonate was removed by filtration, and the ether was removed by rotary evaporation. The remaining brownish liquid was vacuum distilled to obtain the desired product. Table 9 shows the boiling points and percentage yields of the N,N-dialkylaminoalkanols prepared.

Table 9. - Boiling points and percentage yields of N,N-Dialkylaminoalkanols

$(CH_3)_2N-(CH_2)_n-OH$

n	b.p. ^oC/mm Hg	% Yield
4	55-57/2	45.5
5	62-63/3	48.0

$(CH_3CH_2)_2N-(CH_2)_n-OH$

n	b.p. ^oC/mm Hg	% Yield
4	92-95/4.8	33.6
5	114/14	55.0
6	72-74/0.04	70.0
7	146-148/7	72.0
8	140-141/6	68.0
9	135-137/1	65.5
10	133-134/4	63.0

C. Preparation of Quaternary Ammonium Halides of Aminoalcohols

I. Quaternary ammonium chlorides.

$$HO-(CH_2)_n-Cl + (CH_3)_3N \longrightarrow (CH_3)_3N^+-(CH_2)_n-OH\ Cl^-$$

The following is a typical procedure for the preparation of quaternary ammonium chlorides.

An amount of 0.02 mole of chlorohydrin was allowed to react with either a 25% aqueous solution of trimethylamine (n = 6, 8) or with neat trimethylamine (n = 7, 9, 10) that was produced by distilling the trimethylamine from a 25% aqueous solution and condensing it with the aid of a dry ice-acetone bath.

With the 25% aqueous solution of trimethylamine, the reaction mixture was refluxed for about 24 hours. Low boiling material was removed by rotary evaporation. The remaining liquid solidified on standing and was recrystallized from ether-acetone solutions. The salt was dissolved in acetone on a steam bath. To the acetone solution was added ether until it became slightly cloudy. White crystalline solid was obtained on cooling.

With the neat trimethylamine, equimolar amounts of the amine and chlorohydrin were allowed to react. The liquid trimethylamine was placed in a 100 ml. round-bottomed flask with a dry ice/acetone condenser and a dropping funnel. The chlorohydrin was slowly added to the amine. After the completion of the addition, the reaction mixture was allowed to react for 6 hours at room temperature. Low boiling material was removed by rotary evaporation and the resulting white solid was recrystallized from ether-acetone solution.

II. Quaternary ammonium iodides.

$$(CH_3)_2N\text{-}(CH_2)_n\text{-}OH + CH_3I \longrightarrow (CH_3)_3N^+\text{-}(CH_2)_n\text{-}OH\ I^-$$

n = 2, 3, 4, 5

$$(CH_3CH_2)_2N\text{-}(CH_2)_n\text{-}OH + CH_3CH_2I \longrightarrow (CH_3CH_2)_3N^+\text{-}(CH_2)_n\text{-}OH\ I^-$$

$$n = 2 \text{ to } 10$$

The following is a typical procedure for the preparation of the quaternary ammonium iodides of various aminoalcohols.

The N,N-dialkylaminoalcohol (0.02 mole) was put in a 250 ml. round-bottomed flask equipped with condenser, dropping funnel and magnetic stirrer. Anhydrous ether was then added and the solution was heated to about 75° in an oil bath. An equimolar amount (0.02 mole) of the alkyl iodide was added dropwise to the solution. After the addition was complete, the heating was maintained for another 2-3 hours. The reaction mixture was then allowed to cool to room temperature. Low boiling substances were removed by rotary evaporation. The white solid that was formed on standing was recrystallized from ether-acetone solution.

Table 10 gives the melting points and percentage yields of the quaternary ammonium halides of the aminoalcohols, and Tables 11 to 14 the proton NMR and mass spectral data.

Qualitative identification of quaternary ammonium salts[51]

The test for quaternary ammonium salts was performed as follows: a sample of the salt (0.1 gm) was dissolved in water and excess concentrated nitric acid was added followed by 1.0 ml of 0.1 N aqueous silver nitrate. A

white precipitate of silver halide was formed. Excess amount of 1.0 N sodium hydroxide was added until the solution was alkaline and the precipitate redissolved. The mixture was extracted twice with ether and separated. Ether was dried over anhydrous sodium sulfate and evaporated away. A positive test for quaternary ammonium salts is indicated when no residue remained after ether was removed.

Table 10. - Melting points and percentage yields of quaternary ammonium halides of aminoalcohols $(R_3N^+-(CH_2)_n-OH\ X^-)$

R = CH_3-	n	X	m.p. ^oC	% Yield
	2	I	270-272	82
	3	I	198-200	80
	4	I	130-132	75
	5	I	130-135	65
	6	Cl	171-175	47
	7	Cl	201-205	40
	8	Cl	195-200	38
	9	Cl	218-223	41
	10	Cl	195-202	31

R = CH_3CH_2-	n	X	m.p. ^oC	% Yield
	2	I	subl. 283	76
	3	I	239-241	62
	4	I	137-140	30
	5	I	90-94	49
	6	I	----*	--
	7	I	77-80	44
	8	I	58-62	40
	9	I	70-72	38
	10	I	85-90	39

*This was never isolated as a solid. It was a very viscous, clear liquid.

Table 11. - 250 MHz proton NMR spectral data for $(CH_3)_3N^+-(CH_2)_n-OH\ X^-$

$$(\underset{a}{CH_3})_3N^+-\underset{b}{CH_2}(\underset{c}{CH_2})_m\underset{d}{CH_2}OH\ X^-$$

		Chemical Shifts (δ) and Splitting Patterns			
n	X	a	b	c	d
2	I	3.04,s,(9H)	3.35,m,(2H)		3.90,m,(2H)
3	I	2.98,s,(9H)	3.27,m,(2H)	1.87,m,(2H)	3.53,t,(2H)
4	I	2.97,s,(9H)	3.20,m,(2H)	1.44-1.69,m,(4H)	3.48,t,(2H)
5	I	2.95,s,(9H)	3.17,m,(2H)	1.25-1.67,m,(6H)	3.44,t,(2H)
6	Cl	2.94,s,(9H)	3.15,m,(2H)	1.24-1.64,m,(8H)	3.44,t,(2H)
7	Cl	2.93,s,(9H)	3.14,m,(2H)	1.22-1.61,m,(10H)	3.43,t,(2H)
8	Cl	2.93,s,(9H)	3.14,m,(2H)	1.19-1.61,m,(12H)	3.42,t,(2H)
9	Cl	2.93,s,(9H)	3.13,m,(2H)	1.17-1.60,m,(14H)	3.42,t,(2H)
10	Cl	2.95,s,(9H)	3.13,m,(2H)	1.15-1.60,m,(16H)	3.42,t,(2H)

Splitting patterns: s, singlet; t, triplet; m, multiplet;
Number of hydrogens for each group is given in parenthesis
Internal standard: acetone (δ = 2.05), Solvent: D_2O

Table 12. - 250 MHz proton NMR spectral data for $(CH_3CH_2)_3N^+-(CH_2)_n-OH\ I^-$

$$(\underset{a}{CH_3}\underset{b}{CH_2})_3N^+-\underset{c}{CH_2}(\underset{d}{CH_2})_m\underset{e}{CH_2}OH\ I^-$$

	Chemical Shifts (δ) and Splitting Patterns				
n	a	b	c	d	e
2	1.10,t,(9H)	3.23,q,(6H)	3.18,m,(2H)		3.80,m,(2H)
3	1.10,t,(9H)	3.15,q,(6H)	3.10,m,(2H)	3.77,m,(2H)	3.54,t,(2H)
4	1.10,t,(9H)	3.12,q,(6H)	3.05,m,(2H)	1.45-1.59,m,(4H)	3.47,t,(2H)
5	1.09,t,(9H)	3.13,q,(6H)	3.03,m,(2H)	1.24-1.55,m,(6H)	3.44,t,(2H)
6	1.10,t,(9H)	3.12,q,(6H)	3.00,m,(2H)	1.23-1.53,m,(8H)	3.44,t,(2H)
7	1.12,t,(9H)	3.14,q,(6H)	3.00,m,(2H)	1.22-1.54,m,(10H)	3.45,t,(2H)
8	1.10,t,(9H)	3.10,q,(6H)	2.97,m,(2H)	1.18-1.50,m,(12H)	3.43,t,(2H)
9	1.10,t,(9H)	3.10,q,(6H)	2.98,m,(2H)	1.15-1.50,m,(14H)	3.43,t,(2H)
10	1.10,t,(9H)	3.10,q,(6H)	2.98,m,(2H)	1.15-1.50,m,(16H)	3.43,t,(2H)

Splitting patterns: s, singlet; t, triplet; q, quartet; m, multiplet
Number of hydrogens for each group is given in parenthesis
Internal standard: acetone (δ = 2.05), Solvent: D_2O

Table 13. - Chemical Ionization Mass spectral data for $(CH_3)_3N^+-(CH_2)_n-OH\ X^-$

		m/e									
n	X	m	m - 1	m + 1	m + 2	m + 15	m + 29	m + 41	m + 1 - 18	other	
2	I	89 (3)	88 (16)	90 (100)	91 (5)		118 (5)	130 (2)	72 (59)	142 (9) 143 (96)	(CH_3I) (CH_3I + 1)
3	I	103 (5)	102 (22)	104 (100)	105 (8)		132 (13)	144 (4)	86 (26)	142 (10) 143 (91)	(CH_3I) (CH_3I + 1)
4	I	117 (6)	116 (29)	118 (90)	119 (6)		146 (6)	158 (2)	100 (37)	142 (10) 143 (100) 73 (23)	(CH_3I) (CH_3I + 1) (m - 44)
5	I	131 (5)	130 (28)	132 (100)	133 (8)	146 (21)	160 (11)	172 (2)	114 (32)	142 (5) 143 (54) 87 (6)	(CH_3I) (CH_3I + 1) (m - 44)
6	Cl	145 (6)	144 (35)	146 (100)	147 (10)		174 (14)	186 (2)	128 (13)		
7	Cl	159 (7)	158 (37)	160 (100)	161 (10)	174 (4)	188 (15)	200 (2)	142 (14)		
8	Cl	173 (7)	172 (37)	174 (100)	175 (11)		202 (14)	214 (2)	156 (11)		

Table 13. (cont'd)

		m/e								
n	X	m	m - 1	m + 1	m + 2	m + 15	m + 29	m + 41	m + 1 - 18	other
9	Cl	187 (8)	186 (44)	188 (100)	189 (13)	202 (2)	216 (16)	228 (2)	170 (15)	
10	Cl	201 (8)	200 (47)	202 (100)	203 (13)	216 (4)	230 (17)		184 (16)	

m represents the mass ion of the dealkylhalogenated quaternary ammonium halide of the aminoalcohol (N,N-dialkylaminoalkanol)

Conditions: Reactant gas, methane; temperature, 200°; and pressure, 0.3 Torr

Relative intensities are given in parenthesis.

Table 14. - Chemical Ionization Mass spectral data for $(CH_3CH_2)_3N^+-(CH_2)_n-OH\ I^-$

	m/e									
n	m	m - 1	m + 1	m + 2	m + 15	m + 29	m + 41	m + 1 - 18	other	
2	117 (5)	116 (21)	118 (100)	119 (7)		146 (13)	158 (4)	100 (32)	156 (8) 157 (69) 86 (16)	(CH_3CH_2I) $(CH_3CH_2I + 1)$ (m - 31)
3	131 (6)	130 (21)	132 (100)	133 (8)		160 (19)	172 (2)	114 (13)	156 (6) 157 (57) 100 (9) 86 (25) 72 (2)	(CH_3CH_2I) $(CH_3CH_2I + 1)$ (m - 31) (m - 45) (m - 59)
4	145 (9)	144 (31)	146 (100)	147 (9)		174 (14)	186 (2)	128 (34)	156 (10) 157 (99) 100 (13) 86 (30)	(CH_3CH_2I) $(CH_3CH_2I + 1)$ (m - 45) (m - 59)
5	159 (8)	158 (29)	160 (84)	161 (9)		188 (18)	200 (2)	142 (17)	156 (9) 157 (100) 100 (10) 86 (26)	(CH_3CH_2I) $(CH_3CH_2I + 1)$ (m - 59) (m - 73)
6	173 (9)	172 (29)	174 (100)	175 (12)		202 (13)	214 (2)	156 (11)	157 (2) 86 (18)	$(CH_3CH_2I + 1)$ (m - 87)

Table 14. (cont'd)

n	m/e: m	m - 1	m + 1	m + 2	m + 15	m + 29	m + 41	m + 1 - 18	other
7	187 (6)	186 (25)	188 (72)	189 (9)		216 (11)	228 (1)	170 (8)	156 (10) (CH_3CH_2I) 157 (100)(CH_3CH_2I + 1) 100 (8) (m - 87) 86 (21) (m - 21)
8	201 (7)	200 (29)	202 (73)	203 (10)		230 (11)	242 (1)	184 (8)	156 (10) (CH_3CH_2I) 157 (100)(CH_3CH_2I + 1) 100 (8) (m - 101) 86 (27) (m - 115)
9	215 (7)	214 (29)	216 (69)	217 (10)		244 (10)	256 (1)	198 (7)	156 (10) (CH_3CH_2I) 157 (100)(CH_3CH_2I + 1) 100 (8) (m - 115) 86 (25) (m - 129)
10	229 (5)	228 (24)	230 (52)	231 (8)		258 (7)		212 (5)	156 (9) (CH_3CH_2I) 157 (100)(CH_3CH_2I + 1) 100 (6) (m - 129) 86 (19) (m - 143)

m represents the mass ion of the dealkylhalogenated quaternary halide of the aminoalcohol (N,N-dialkylaminoalkanol)

Conditions: Reactant gas, methane; temperature, 200°; and pressure, 0.3 Torr

Relative intensities are given in parenthesis

Activity of quaternary ammonium halides of aminoalcohols with HLADH

Horse liver alcohol dehydrogenase was freshly prepared daily for enzyme activity assays. Aqueous solutions of NAD^+ were kept frozen for no longer than three days. Water used for the preparation of solutions was deionized and twice distilled. Buffer solutions were prepared as described in Methods in Enzymology.[52] The pH was adjusted with an Instrumentation Laboratory pH meter. For pH 6.5, 7.0 and 7.5, 0.05 M sodium phosphate was used; for pH 8.0, 0.05 M Tris.HCl was used; and for pH 8.8 and 10.0, 0.5 M glycine/NaOH was used.

The crystalline enzyme was reconstituted just prior to use by dissolving 25 mg of the enzyme in 20 ml. of 0.05 M sodium phosphate buffer, pH 7.0. All 15 mM stock solutions of NAD^+ were prepared by dissolving 0.1 gm of the coenzyme in 10 ml. of water. Throughout the experiments 45 mM substrate stock solutions were used. Activity assay solutions were made up of 0.1 ml NAD^+ stock solution, 0.4 ml. of substrate stock solution, 0.2 ml. of enzyme stock solution, and the appropriate amount of buffer to bring the final volume to 3.0 ml. The final concentrations of NAD^+ and substrate were 0.5 mM and 6.0 mM respectively. The amount of enzyme used for each assay was 0.5 unit or 0.25 mg.

The initial activity of the substrates with the enzyme was measured by the increase in the UV absorbance at 340 nm due to the reduction of the pyridine ring of the nicotinamide

adenine dinucleotide. The increase of the absorbance for the first three minutes was monitored on a Sargent SR recorder. Initial velocities of substrates were determined from the best straight lines drawn as close to the initial increase in absorbance as possible. At least six runs were made for each substrate at each pH. Solutions minus the enzyme were used as references (blanks) in all the runs. All activity assays were performed at room temperature.

Equilibrium studies were run under the same conditions as the initial velocity experiments. Ultraviolet absorbance at 340 nm was measured at twelve hour intervals up to three days and then at the seventh day. Triplicate samples were run at the same time for each substrate at each pH at room temperature. The entire experiment was again repeated. In addition to the usual blank (solution minus enzyme), the absorbance of a solution containing enzyme and coenzyme but no substrate was also monitored. The absorbance was calculated to be that of the sample with enzyme, coenzyme and substrate less that of the sample with only enzyme and coenzyme.

The equilibrium constant for the oxidation of alcohols by NAD^+ and HLADH as represented in equation 1 was calculated by using equation 4,

$$Keq = \frac{x^2 \quad (H^+)}{(NAD^+)_0 - x)\ (R_1R_2CHOH)_0 - x)} \qquad \text{(eq. 4)}$$

where $(NAD^+)_0$ is the initial concentration of the coenzyme; $(R_1R_2CHOH)_0$ the initial concentration of the alcohol; and x the concentrations of NADH and $R_1R_2C=O$ at equilibrium. The concentration of NADH was calculated from the absorbance at 340 nm by using the reported extinction coefficient of 6.22×10^6 cm^2/mole.[53] The proton concentration was fixed by the pH of the buffer used. It was further assumed that the extinction coefficient at 340 nm is independent of pH.

APPENDIX

APPENDIX

Nuclear Magnetic Resonance Studies of Cadmium-111-substituted Horse Liver Alcohol Dehydrogenase

Cadmium-111 nmr studies of cadmium-substituted horse liver alcohol dehydrogenase were initiated in 1977 in order to elucidate the role of zinc in the enzymatic reaction and to study changes at the active site of the reaction. The cadmium-substituted enzyme was found to be 60% less active than the native enzyme containing zinc.

The Cadmium-111 NMR spectra were recorded under various conditions. The concentration of the totally cadmium-substituted enzyme ranged from a low 30 μM to a high of 580 μM with or without 96% enriched cadmium-111 (purchased from Oakridge Laboratory). The natural abundance of cadmium-111 is about 12%. High enzyme concentration, desirable for nmr studies, was maintained by adding 1.0 M urea at the metal exchange stage and during the subsequent removal of extraneous cadmium ion from the system. The NMR spectra were usually taken under the following conditions: 0.045 M sodium phosphate buffer, 1.0 M urea with 10% D_2O as an internal lock, pH 6.0; spectral width of 20,000 Hz; pulse anglė of 4 microseconds (9 to 12°); no delay to a pulse delay of 0.2 seconds; and the number of transients from 130,000 to 290,000.

Unfortunately, under none of the conditions tried were we able to observe any Cadmium NMR signals. After one and a half years of work the project was abandoned.

In March, 1980, several months after this project was discontinued, Bobsein and Myers[31] published a communication on Cadmium-113 NMR spectra of totally cadmium-substituted horse liver alcohol dehydrogenase. Their conditions were: 0.29 m$\underline{M}$ 90% enriched cadmium-113-substituted HLADH; 0.05 $\underline{M}$ sodium phosphate buffer, pH 7.5 with D_2O added; pulse angle, 70^{o}; spectral width, 20,000 Hz; pulse delay of 3 seconds; and 24,000 scans. The spectra showed two distinct signals, one for each kind of cadmium.

NMR-wise, there is not much difference between cadmium-113 and cadmium-111 (our probe for the Bruker 180 is just out of the range for cadmium-113 NMR), although cadmium-113 is about 10 to 15% more sensitive than cadmium-111. Therefore, our failure to observe any nmr signal cannot be attributed to differences between cadmium-111 and cadmium-113. The most probable cause is an error in estimating the T_1 of the cadmium in the enzyme system from the T_1 determined from a solution of cadmium chloride and glycine at a ratio of 1:4. The wrong estimate of T_1 probably led to inadequate delay time between pulses that in turn led to saturation.

This area of cadmium NMR spectroscopy of the enzyme certainly merits further study since the ability to observe the cadmium signals enables possible elucidation of the role of the metal in the catalytic action of the enzyme.

Experimental

Cadmium-111 NMR spectra were taken with a Bruker WH 180 NMR spectrometer at 38.16 MHz equipped with a 20 mm probe. The cadmium content, after metal exchange, in the enzyme was determined by atomic absorption. Protein concentration was determined by the Lowry method[54] with crystalline HLADH as the standard.

Metal exchange (cadmium for zinc) was accomplished by a modification of Vallee's method,[30] the exchange was carried out at 4^{0} and the buffer solutions for dialysis were kept under a continuous flow of argon. The following is a typical experiment: A solution of crystalline HLADH in 0.045 M sodium phosphate buffer, pH 7.0 was dialized against a liter of the same buffer for 12 hours. The enzyme solution was then dialyzed against a liter of 0.2 M sodium acetate buffer, pH 5.5, with 0.1 mM cadmium chloride added* for 72 hours with two changes of buffer. After the metal exchange, extraneous cadmium was removed by dialyzing the enzyme solution with a liter of 0.045 M sodium phosphate buffer, pH 6.0, for 24 hours with one change of buffer. The enzyme solution was concentrated, whenever needed by ultrafiltration with an Amicon ultrafiltration cell.

*at high concentrations of enzyme (0.5 mM or 40 mg/ml, or higher), 1.0 M urea was added to the buffer solution to stabilize the enzyme.

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