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Synthesis and Spectroscopic Probing of Novel Derivatives of Polyethylenimine, a Matrix for Catalytic Groups

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SYNTHESIS AND SPECTROSCOPIC PROBING OF NOVEL DERIVATIVES OF POLYETHYLENIMINE, A MATRIX FOR CATALYTIC GROUPS

BY

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DEDICATION

Dedicated to my mother,
Mildred Killian Ondeck, whose
respect for education inspired
pursuit of this degree

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VITA

The author, Elizabeth Ondeck Myatt, was born on September 6, 1958, in Chicago, Illinois.

Her elementary and secondary educations were obtained in the public schools of Riverside, Illinois. In 1975 she completed her high school education.

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INTRODUCTION

The rate enhancements attributed to enzymes typically are in the range of 10^{10} but may be between 10^{6} and 10^{14}. Several physical and chemical factors contribute but their relative and absolute contributions remain uncertain for many enzymes. This is probably due in part to the fact that different enzymes exploit different catalytic mechanisms but also there is often an inadequate understanding of the basic processes involved. There still remains doubt as to which single factor, if indeed there is one common to all enzymes, is most responsible for catalysis, although a number of attempts to dissect out contributions to the enzymic process have been made. Commonly quoted rate enhancement values of 10^{2} to 10^{3} have been attributed to general acid or general base catalysis,[1] but 10^{5} has been reported for chymotrypsin by the active site histidyl imidazole serving as a general base.[2] General acid or base catalysts acting intramolecularly in small molecule reactions cause up to 10^{4} rate enhancements when dissolved in ethanol, though in only a few cases has so large an effect been observed.[3] An acceleration of even 10^{3} would otherwise be achievable only when there is low interference from the surrounding medium. Electrostatics, be they of the fixed or instantaneous dipole-dipole type, are more strongly felt as the dielectric constant of the medium is reduced. However, highly polar media stabilize reactions involving transient charge development, as do ionic side chain groups when properly aligned. Hydrogen bonding media stabilize charge development as well, and may stabilize or destabilize

nucleophiles through solvation or polarization, respectively. Groundstate stabilization and transition state destabilization are both deleterious to catalysis (p. 28).

Entropy factors must be important in enzymic catalysis and practically all synthetic catalytic schemes consider them, if nothing else. There are two general types of entropy catalysis:

- Binding to increase the probability of encounter between reactive groups and substrate(s)
- Optimal static juxtapositioning for cooperativity, enantioselectivity, and presumably substrate specificity

Interaction of small molecules with enzymes or binding proteins such as serum albumin is characterized by the saturation phenomenon involving complex formation.[4] The highly selective adsorption of substrated by enzymes is in part responsible for their rate effects. Such specificity is as yet unachievable in synthetic macromolecular systems. However, even nonspecific substrates, modifying reagents, and dyes associate with binding proteins, enzymes, and some macromolecular models via Michaelis-Menten kinetics with a binding step preceeding the catalytic steps.

The saturation binding phenomenon for synthetic macromolecules can be affected by polymer and substrate hydrophobicity. Electrostatic effects may promote binding. Polymers without hydrophobic components do not form a molecular association prior to affecting reactions of small molecules. However, electrostatic repulsions of a polyion may exclude coions, and often preclude formation of stable hydrocarbon-enriched subdomains containing only a few, poorly solvated ionic groups. Since such domains are

believed to be of importance in enzyme catalysis, it often is the aim of those who design model systems to mimic this type of microenvironment.

Synthesis and analysis of synthetic catalysts whose structure is designed to capitalize on one or a few contributions of enzymic catalysis are not only desirable in their own right (since this direction in synthetic macromolecular catalysis has already proven to be a promising one[178]) but should contribute, albeit indirectly, to our understanding of the quantitative values assignable to a particular factor or group of factors by a macromolecular catalyst in an aqueous solution. Also, snythetic catalysts can illuminate the effects macromolecules have on the properties and chemistry of small molecule cosolutes covalently attached or noncovalently bound to them.

The Reaction: Cleavage of Carboxylic Esters

The carbonyl carbon is among the most frequently encountered of functional groups in organic chemistry and biochemistry. Among the biological reactions that involve carbonyl carbons are aldol condensations, esterolysis, amidolysis, reductions, and the reverse reactions. Since esterolysis is among the most studied and best understood of organic reactions, it has been used extensively to assess and analyze kinetically the efficiency and mechanism of both synthetic and natural nucleophilic catalysts.

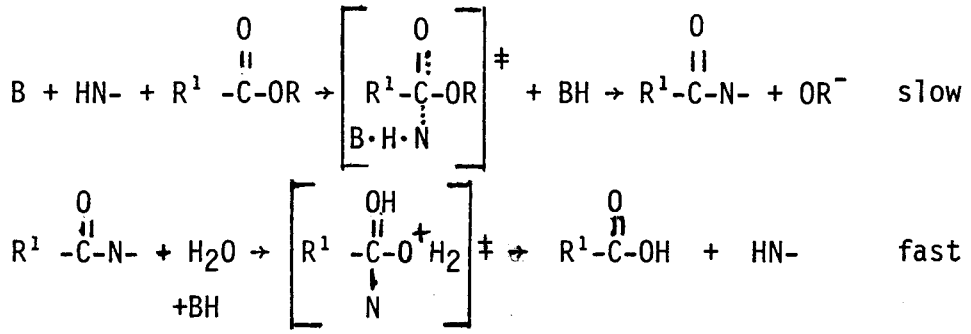
Edwards has enumerated seven factors as being of importance in determining the nucleophilic rate of a reaction:[5]

1. The charge potential of the nucleophile and the electrophile
2. Chemical nature of nucleophile (substituent effects, too)

3. Chemical nature of electrophile
4. Nature of solvent
5. Recession of the nucleophile's electrons to its back side (bond strength in product)
6. Nature of the leaving group
7. The alpha nucleophile effect: two electronegative atoms attached to one another show unusually high reactivity.

Because the carbonyl carbon is coordinatively unsaturated and possesses a substantial partial positive charge, it is classified as a "hard" electrophilic center. Addition to the sp^2 orbital of the electrophilic carbon is favored for basic, rather than polarizable nucleophiles.[5,6] Therefore, the relative importance of these factors is dependent on the peculilarities of the particular system under investigation.

General base-catalyzed nucleophilic hydrolysis of esters occurs by the following mechanism:

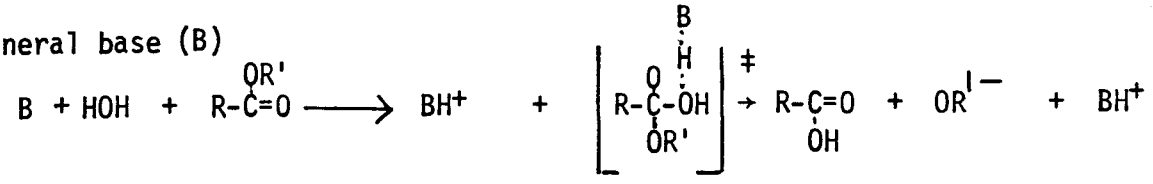


Acylation must be rapid relative to direct hydrolysis and the acylated nucleophile must be more susceptible to hydrolysis than was the original ester in order for the nucleophile to be catalytically effective. An efficient nucleophile equals or exceeds the leaving group in basicity.[6] If

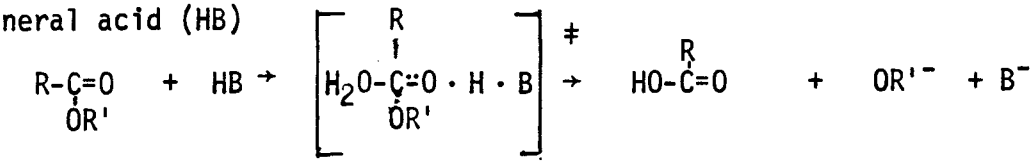
the nucleophile is substantially more basic than the leaving group, transacetylation will be rapid but deacylation of the nucleophile is slow.

General acid and general base catalysts acting independent of any enzyme nucleophiles are also encountered in biochemistry. They are also proposed mechanisms for some model system studies (p.64). Therefore, these mechanisms are also worthy of brief consideration. General rather than specific acid-base catalysis of esters will be considered since enzymes utilize the general route due to the low concentrations of protons and hydroxyl ions at physiological pH. The mechanisms for general base and general acid catalysis are shown below:[7]

General base (B)



General acid (HB)



Both mechanisms facilitate formation of the bond between the oxygen atom of water and the carbon of the carbonyl group. The general base activates the oxygen by deprotonating it (thereby making it a stronger nucleophile),[8] whereas a general acid increases the electron deficiency on the carbonyl carbon, rendering it more susceptable to attack. In the hydrolysis of oxygen and thiol esters, the general base mechanism is preferred over the general acid mechanism due to its lower energy of activation.[6,9]

The Tetrahedral Intermediate and Its Importance in Catalysis

The involvement of a tetrahedral intermediate in nucleophilic

hydrolysis of esters and amides by enzymes has not been directly demonstrated with natural substrates due to its short half life (about 10^{-9} seconds) but would seem likely in both the acylation and deacylation steps since it is involved in esterolysis by small molecules having analogous functional groups.[6,10] Evidence for its involvement in chymotrypsin acylation has been accumulating since the 1960's.[11] High affinity aldehyde analogues of substrates specific for chymotrypsin associate with the active site serine to form a covalent hemiacetal[12] in which the central carbon is sp^3 hybridized.[13] Such association complexes have also been observed with the sulfhydryl protease papain.[14] With elastase, Richards reported tetrahedral detection by stopped-flow spectrometry with a paranitroanilide substrate.[15] It has recently been reported in the hydroxide hydrolysis of this same substrate by a polyethyleneimine derivative, the polyamine which is the subject of this work.[16] Therefore, a brief consideration of the tetrahedral intermediate should be made since the transition state must closely resemble it, and tetrahedral stabilization and/or catalysis of its formation or breakdown by a number of chemical forces are important mechanisms of rate enhancement. Whether facilitation of formation or breakdown is effective depends on whether tetrahedral formation or breakdown is rate-limiting, which in turn depends on the strength of the nucleophile, the stability of the leaving group, the presence of other catalytic groups, and the relative stabilization of reactants, transition state, and products by the surrounding medium.[17]

Whether formation or breakdown is rate-limiting may be predicted by consideration of the relative pKs of the nucleophile and the leaving group.

Tetrahedral formation (i.e. nucleophilic attack) is rate-limiting when the leaving group has a pK which is lower than that of the nucleophile.[6] The tetrahedral intermediate shows little bond development with the nucleophile and resembles an adduct of the reactants. Such a reaction is particularly responsive to the presence of general base catalysts for nucleophile activation. In the esterolysis of paranitrophenylacetate, tetrahedral formation is frequently rate-limiting since the alkoxyl portion, paranitrophenol, is a good leaving group (pK about 7.0) and therefore easily expelled.[17] Tetrahedral breakdown (i.e. expulsion of the leaving group) is rate-limiting when the pK of the nucleophile is lower than that of the leaving group.[6] Since the leaving group is a relatively poor one in this case, it is subject to acid catalysis, which will facilitate its expulsion by protonating the leaving group. In this case, the tetrahedral intermediate resembles products with a high degree of bond development between nucleophile and electrophile. Amidolysis is typically characterized by such kinetics.[17]A marked sensitivity of rate to substituents on the alkoxyl portion of the ester (leaving group) is characteristic of reactions for which tetrahedral intermediate breakdown in esterolysis is rate-determining.[18,19]

If protonation/deprotonation reactions are occurring, they may or may not be rate-limiting. When nucleophilic addition preceeds proton transfer and is rate-limiting, the observed reaction rate is not substantially the result of general acid catalysis. However, proton transfer may present the largest of energetic barriers with subsequent heavier atom rearrangements occurring subsequently and more rapidly. Rates of proton transfer depend on the medium and occur much more rapidly when transfer is between amines

and paranitrophenol in aprotic solvents.[19] Aprotic solvent effects on acid-base reactions will be discussed in a subsequent section. (p.12). On the other hand, hydroxylic solvents may partake in the transfer or at least dominate its kinetics through solvation.[20]

Even if protons are not transferred in concert with (general acid base catalysis) or prior to (specific acid-base catalysis) the nucleophilic attack, they may participate in the reaction through "solvation catalysis."[21] Protic solvents, particularly water, may hydrogen bond to the carbonyl oxygen and further increase the partial positive charge on the electrophilic center.[22] Alternatively, protic-type solvation catalysis may be due to hydrogen bond donation by the solvent to the leaving group.[23] Proton-containing nucleophiles are subject to yet another type of "solvation catalysis", that by dipolar aprotic solvents. For example, the nucleophilicity of aliphatic amines is increased by DMSO, which serves as a proton acceptor and further polarizes the N-H bonds.[24] The catalytic activity of a highly polar solvent, S_1, may be realized more fully if that solvent is diluted with an excess of less polar solvent, S_2, since the latter will diminish S_1-S_1 interactions permitting S_1's effect to approach more closely its instantaneous dipole moment.[25] A continuum of possibilities exist for solvent or cosolute interaction with reactants and transition state, from no participation to partial or complete covalency.[20,26]

The oxyanion of trypsin is stabilized by some 200 water molecules upon conversion of the proenzyme to the enzyme and as a specific tripeptide substrate is bound. The total energy afforded by the water is 14.2 kcal over the Michaelis Menten complex plus 5.8 kcal over the zymogen.[27]

Solvation's importance would seem even greater for analogous small molecule reactions in aqueous solution. Ritchie has concluded from his theoretical work on anion-cation bimolecular reactions in solution that motion along the reaction coordinate is purely a solvent motion, and that even in this monopole- monopole reaction, monopole-dipole interactions dominate and dictate the nature of the transition state.[28] Solvation catalysis is an important reason for the difficulty in choosing "uncatalyzed" rate constants for comparison to enzymic ones.

Yet another mechanism of decreasing the free energy of activation is by electrostatic stabilization of the transition state. Transition state formation may be characterized by charge development, neutralization, or dispersion.[29] Anionic nucleophiles are neutralized by acylation whereas neutral ones acquire a partial positive charge in the transition state in the absence of general base catalysts. The carbonyl oxygen also undergoes a change in formal charge: specifically, it takes on a negative charge. Therefore, whereas anionic nucleophiles undergo a charge dispersion (with varying degrees of resonance stabilization depending on the pi bonding properties of the nucleophile), neutral nucleophiles form quasizwitterionic transition states with charge aquisition. Charge development is stabilized in solutions of high dielectric constant or by fixed proton-donating/ accepting or ionic groups. Such groups in enzymes often are located in a microenvironment of otherwise low dielectric constant where charges are particularly strong (see enzyme theory section). The reaction of two neutral molecules is the only one of the S_N2 reactions anticipated to be accelerated by increases in solvent polarity.[29] One example of such a reaction

is that in which tertiary amines are quaternized with an alkyl halide. However, charge dispersion, and even more so charge obstruction, is favored in polarizable or less polar solvents where ionic reactants are destabilized relative to the charge-dispersed, resonance rich transition state between an anionic nucleophile and a neutral or cationic electrophile.[30]

Electrostatic stabilization of substrate charge development may occur via ion pair formation with an oppositely charged group[31] or by interaction with a hydrogen-bonding moiety.[22] Both appear to be a relatively important mechanisms in enzymic catalysis. An appropriately positioned "oxyanion hole" exists within the active site of the serine proteases for stabilization of the developing negative charge on the carbonyl oxygen atom via hydrogen bond donation.[32] In the case of lysozyme, there is a true electrostatic stabilization of the developing carbocation by a neighboring carboxylate.[33]

The energetics of a reaction are determined by the free energy of the reactants and of the products as well as of the transition state. Electrostatic destabilization of the substrate may be catalytically important in some enzymic reactions. For example, enzymes catalyzing the transfer of a methyl group from the positively charged S-adenosyl methionine donor may destabilize the donor by placing it near a positive charge in the active site.[34] Anionic nucleophiles are also very reactive in poorly solvating media;[29] this will be discussed further in the following section. It may well be that substrate destabilization and transition state stabilization are but two sides of the same coin.[34]

Solvent Effects on Nucleophilicity

Nucleophiles must be partially desolvated prior to their reaction with an electron-deficient center.[35] Partial desolvation is necessary whether attack is on a hard electrophile such as carbonyl carbon[36] or on a soft one such as an alkyl halide.[29] The energetics of desolvation of the electrophilic center generally appear to affect the S_N2 reaction far less.[37] Rather, the magnitude of the energetic cost of desolvation is largely determined by the nature of the solvent and the nature and charge of the nucleophile. Properties of the solvent that affect its solvating strength include hydrogen accepting/donating properties, dielectric constant, and specific effects such as aromaticity. Inert solvents interact with ions by means of their dipole moments.

Some neutral nucleophiles such as triethylamine, when reacted with an alkyl halide, show a reactivity which is strongly affected by the dielectric constant of the medium but virtually independent of the hydrogen-bonding properties. Indeed, water is among the best solvents for the Menschutkin reaction.[38]

Anions are solvated strongly by both dipolar aprotic and protic solvents but are very insoluble in hydrocarbon ones. The strength of solvation by a hydrogen bond donating solvent increases dramatically with increasing charge density (i.e. decreasing nucleophile radius) whereas the strength of solvation by dipolar aprotic solvents is less for all anions and increases more gradually with charge density.[39] The stabilization of anions by protic solvents increases the free energy of activation barrier and slows their reactivity.[36] For the anionic nucleophiles F^- and N_3^- variations in reactivity with a change in solvent are over several powers

of ten, despite no change in dielectric constant. Through a series of small anions, there is a reversal in ordering of nucleophilic reactivity as the medium is changed from the gas phase to a protic solvent.[40]

Both Parker[29] and Frank[41] consider that the large cost of nucleophile partial desolvation is principally due to the unfavorable enthalpy of bound solvent-nucleophile dissociation. According to Sabatino, the interaction between an anion and hydrogen bonding solvent molecules is a uniquely strong one.[42] Arnett disagrees, and attributes the energies of desolvation to cooperative interaction changes of the bulk solvent which anion desolvation induces.[43] pK values of nucleophiles determined in aqueous media are highly perturbed relative to their intrinsic (i.e. gaseous) values, he says, due principally to solute effects on water structure.[43] A carboxyl-containing small molecule, o-carboxy benzaldehyde diethyl acetal, titrates at pH 11 in 82 + % dioxan, making it a strong nucleophile.[44]

Little is known about solvent effects on nucleophilicity in solvents of low dielectric constant, due in large part to solubility problems;[45] these are most severe for charged solutes. The magnitude of the difference in nucleophilic reactivity between neutral and anionic forms of imidazole and benzimidazole is 10^3 in water but 10^6 in a micellar environment.[46]

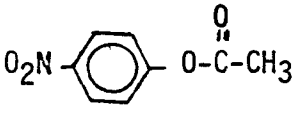
Solvent Effects on Acid-Base Catalysis in Nucleophilic Esterolysis

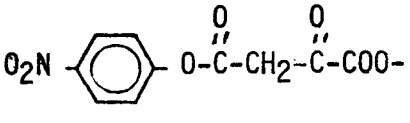
Despite an anticipated activation of nucleophiles in solvents of low polarity, aminolysis of paranitrophenylacetate by imidazole, piperidine,

and other amine nucleophiles in such media is very slow. The rate of this reaction was shown to be increased by a factor of 1200 upon addition of tetra-n-hexyl ammonium benzoate hemihydrate,[19] which serves as a general base by accepting a proton from the nucleophilic nitrogen atom, and thereby permitting tetrahedral breakdown, which is prohibitively slow with an N-protonated amide.[47] Consistent with this mechanism is the observed dependence of esterolytic rate on leaving group substituents; 2,4 dinitrophenylacetate was found to exceed paranitrophenylacetate in reactivity by a factor of 980.[19] Such sensitivity to a second electron-withdrawing group on leaving the group indicates that tetrahedral breakdown, (e.g. N-protonated amide deprotonation), rather than formation, is rate-limiting, unlike the aqueous reaction.

The fact that the carboxylate is so efficient compared to piperidine, which in aqueous solution is 10^3 better as a base than are carboxyls, indicates a 10^{10} reversal in relative basicity of the two with a solvent change from water to toluene. Indeed, at high tetra-n-hexyl ammonium benzoate concentrations, general base catalysis was observed to be so effective that tetrahedral formation becomes rate-limiting. However, catalysis by the benzoate was minimal when the water concentration reached 2 M. Therefore, this marked catalytic effect of the carboxylate requires that the solution be nearly anhydrous.

The rate of esterolysis of two esters, PNPA and paranitrophenyloxalate, by imidazole in toluene was compared.





PNPOx reacted faster then PNPA by a factor of 1400 due to intramolecular general base catalysis, despite negligible or deleterious inductive and steric effects with the oxalate-containing ester.[48] Even in aqueous media, the adduct between esters with poor leaving groups and weakly basic nucleophiles return with great frequency to starting materials.[10,47,49]

The potential importance of solvent effects on the strength of and need for carboxyls as general base catalysts in enzymology would seem plausible in light of the fact that the catalytically important Asp-102 of chymotrypsin is buried in a hydrophobic pocket and that water expulsion from the active site may be induced by substrate binding.[50]

Schiff base hydrolysis is also subject to general base catalysis. When the reaction is carried out in the absence of the base, the transition state is believed to involve charge dispersion, but when base is added, the transition state formation entails charge destruction. Therefore, a reduction in solvent dielectric constant would be expected to favor the base-catalyzed reaction even more than the uncatalyzed one. Indeed, the effects of lowering the dielectric constant and adding a base are found to be more than additive, with a 250-fold increase in Schiff base hydrolysis under both conditions.[51] Reduction of the dielectric constant alone by carrying the reaction out in 70% dioxane increases the reaction rate 11-fold. Addition of the base to the aqueous reaction accelerates it by a factor of 2.5. The synergistic effects of reducing the active site polarity and adding base (or acid) catalysts are suggested as a plausible contribution to enzymic catalysis.

Ion Pair Formation and Aggregation State of Nucleophiles and General Base Catalysts

While poor solvation of nucleophiles, electrostatic and general acid/base catalysts in solvents of low dielectric constant might be expected to enhance their reactivity, self-aggregation, ion pair formation, or association via hydrogen bonding so frequently observed in such media may or may not mitigate realization of the anticipated activation. For instance, a contact ion pair may be considered an unionized species having a more or less polorized bond.[52] Association by salt formation or by hydrogen bond formation is important in that only the ionic, unprotonated/unassociated forms of many nucleophiles or catalysts are reactive. In the S_N2 reaction of a series of alkali halides with R_3CX, reactivity of the halide was related much more directly with the salts' dissociation constant in the solvent, acetone, than on the intrinsic nucleophilicity of the particular halide since only the dissociated species reacts.[53] Dissolved $Na^+ OR^-$ was nucleophilically active as OR^- only when dissociated.[54]

Hydrogen bond formation without ionization has been reported not only between weak acids and bases but between strong ones as well in aromatic solvents such as benzene [55], this would certainly affect their ability to act as nucleophilic, acid, or base catalysts. For example, it is believed that triethylamine and benzoic acid form both 2:1 and 1:1 complexes in benzene but are not ionized.[55] In DMSO, these same solutes ionize but remain associated with a dissociation constant of 1.4×10^{-4} M. Triethylamine and benzoic acid ionize in the solvents acetonitrile and chloroform, but

unusual species are formed as reflected in the absence of a sharp R_3NH^+ peak in the IR and its replacement by a broad and general absorbance attributed to uncertainty of proton location.[56] The nature of the interactions is not only highly solvent-dependent but also highly dependent on the nature of the solutes. No such unusual species are formed in chloroform or acetonitrile when phenols rather than benzoic acid are used, and the effect is much less pronounced when primary or secondary amines are used rather than tertiary ones.[57]

Use of coordinatively saturated cations precludes hydrogen bond formation with anions and also reduces anion association through steric factors. Alternatively, an ion may be activated by effective removal of its counterion through addition of a chelating agent. Tetrahexylammonium benzoate was found to be a much more effective catalyst for ester aminolysis in toluene than was octadecylammonium benzoate.[19] In Menger's study on paranitrophenyloxalate esterolysis by piperidine, an estimated rate enhancement by the carboxylate of 10^6 was predicted had the counterion for the carboxylate been a tetraalkylammonium cation rather than piperidine-H^+.[48]

Self-aggregation of acid/base catalysts or nucleophiles is less deleterious to their reactivity than are H^+ or salt bonding. The general base reactivity of tetra-n-hexyl ammonium benzoate discussed previously was linearly related to its concentration, indicating that it is not affected by its own self-aggregation. Such is the case with imidazole as a nucleophile.[58] Imidazole forms linear complexes in toluene and benzene, but true rate constants of esterolysis are unaffected by this. The spontaneous rate

of paranitrophenylcaprylate esterolysis by the hydroxyl ion is approximately the same as paranitrophenylacetate, although the former forms micelles.[59] Also, the nucleophilicity of the primary amine of decylamine is independent of its aggregation state.[60] However, this simple generalization is not steadfast, particularly for charged reactive groups (See Micelle Catalysis).

Functional Groups of Enzymes

It is useful to consider only those moieties of the active as that may be classified as primary.[61] Primary groups form covalent bonds with the substrate or interact with it through hydrogen bonding, polarization, or electrostatic stabilization of charged intermediates or the transition state. Of the organic functional groups used by enzymes, only those that could be used as covalent catalysts (i.e. nucleophiles) will be considered since this is the nature of the synthetic catalysts synthesized in this laboratory. Also excluded are the cofactors, some of which have been studied extensively in this and other model systems.[62]

Functional groups of amino acids which could potentially serve as nucleophiles are the primary aliphatic amino group of lysine, the methoxyl group of serine, the pyridyl nitrogen of histidine, the phenolic oxygen of tyrosine, the thiol group of cysteine, and the oxyanion of the acidic amino acids aspartate and glutamate. Carboxylates, hydroxyls, and thiols are substantially more nucleophilic when in their anionic form. There are two ways whereby they could be maintained as such if that form is unstable at the operating pH: firstly, they may exist as an ion pair within the active site with pKs which may differ substantially from the aqueous one.

Secondly, a second moiety in the active site may serve as a general base and upon binding of the substrate, deprotonate and thereby activate the nucleophile. Both mechanisms appear to be employed in vivo. A very dramatic example of pK perturbation in protein native structure is in the protein lactalbumin, some of whose carboxyl groups titrate as much as 4.3 units away above the aqueous pK or denatured pKs of the aspartyl and glutamyl groups.[63] One enzyme utilizing pK perturbation via ion pair formation is lysozyme, whose active site glutamate-35 titrates at pH 6.5.[64] It interacts with a positively charged ammonium group. The nucleophilic lysine within the active site of acetoacetate decarboxylase titrates 4 pH units below that of N-propyl lys.[65] Enzymes utilizing general base catalysis include the serine proteases trypsin, chymotrypsin, and elastase. The thiol proteases may also employ general base catalysis, although it has also been suggested that substantial pK perturbation maintains the nucleophilic cysteine in its anionic form at neutral pH.[66]

The higher the pK of small molecule nucleophiles of a single class, the more nucleophilic they are (generally as anions only) but the more alkaline must be the pH of the solution to generate a substantial fraction of the reactive form. The relation of k_{nucl} and pK_{nucl} does not hold up in enzymes or some model systems, as will be seen. Despite ambient pKs, groups such as the active site thiol of Streptococcal proteinase are 10^2 more reactive to nonspecific modifying reagents than are small molecule thiols of comparable pK.[67]

In biological systems, nitrogen most often serves as a general acid/base catalyst.[68] Imidazole, with a pK of about 6, is well suited for this

under physiological conditions. A characteristic of its heteroaromatic ring system is its ability to protonate and deprotonate with relative ease at either nitrogen of the ring, permitting it to serve first as a base, then as an acid, in a charge-relay system.[69]

Histidine's monoprotonated, neutral imidazole is a covalent, nucleophilic catalyst in facile reactions such as phosphate ester hydrolysis or phosphate transfer reactions. Enzymes operating by this mechanism include nucleoside diphosphate kinase, phosphoglycerate mutase, succinyl-CoA synthetase, histone phosphokinase, and the acid phosphatases.[70] Aryl sulfatases also employ neutral histidyl imidazole as a nucleophile towards the electron-deficient sulfur atom of sulfate esters.[71]

Free neutral imidazole is capable of acting as a nucleophile towards carbonyl carbon of activated phenyl esters despite histidine's apparent failure to do so in the active sites of enzymes.[72] Imidazole serves only as a general base in hydrolysis of esters whose leaving group pK exceeds imidazole by three or more units.[73] In vivo, active site imidazole generally activates nucleophiles or facilitates tetrahedral decomposition.[74] The pyridyl nitrogen shows a 400-fold increased reactivity relative to primary amines of equal pK.[75] Explanations offered for this enhanced nucleophilicity include the following:

1. decreased geometric demands in the reaction due to steric constraints on the nucleophilic nitrogen
2. poorer amine solvation
3. increased s character on the nonbonding orbital.[6]

Imidazole covalently attached to synthetic macromolecular systems,

either micellar or polymeric, may also function as a nucleophile towards labile electrophilic centers. The unprotonated, anionic form of it and of the related synthetic compound, benzimidazole, are highly nucleophilic in aqueous solution possessing high pK_2 values (14.5 and 12.2, respectively).[76,77] These anions are therefore difficult to generate at appreciable concentrations in all but highly alkaline solutions unless chelated to a metal.[78] They acylate rapidly but may require general bases or weak acid to deacylate rapidly;[79] in vitro, tertiary amines have been shown to be quite effective in this capacity.[80]

The primary amino group of lysine serves as a nucleophile towards carbonyl carbon in enzymic reactions involving Schiff base formation such as decarboxylation of acetoacetate[81] and aldol condensations.[82] In the former, enamine tautomerization stabilizes deprotonation of the alpha carbon, generating the highly basic enolate ion, which can more readily expel carbon dioxide. The intermediate has been trapped by reduction with sodium borohydride to form isopropyl lysine.[81,83]

The phenolic oxygen of tyrosine is also a potential nucleophile. It has been demonstrated that phenolates catalyze acetylimidazole deacylation by nucleophilic displacement in a reaction which is the reverse of phenyl acetate esterolysis by imidazole.[80] In vivo, the only known case in which tyrosine acts physiologically as a nucleophile is in a regulatory capacity rather than a catalytic one. Glutamine synthase is subject to reversible, covalent modification by adenylate intermediates;[84] addition of ATP to glutamine synthase rapidly inactivates it by formation of a tyrosyl-AMP enzyme.[84,85]

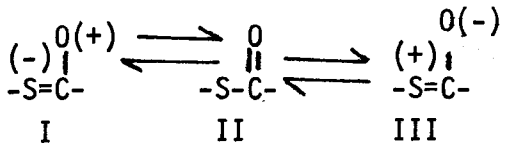
Usually carboxyls act as general acid or base catalysts within enzyme active sites. Alternatively, the oxygen atom of glutamate or aspartate's carboxyl group may attack carbonyl or phosphoryl electrophiles to form an anhydride or phosphoanhydride intermediate. The carboxylate group is only weakly basic, however, and thus is a poor nucleophile. Its nucleophilic attack on esters requires that the leaving group be a very good one. For example, if the pK exceeds 8.4, only the protolytic type of ester hydrolysis will occur in aqueous acetone.[86] The enzymes pepsin and renin have been shown to possess acid pH optima, and pepsin operates under such conditions. Pepsin is known to contain an essential carboxylate in the active site.[87] An anhydride intermediate has been postulated, but directly demonstrated only with synthetic sulfite substrates.[87] Proposed mechanisms for carboxypeptidase[88] and lysozyme include anhydride mechanisms as well but have yet to be unequivocally demonstrated. Covalent, reversible modification of a carboxylate through phosphorylation by ATP in both the Na^+/K^+ ATPase and Ca^{++} ATPase is believed to be the event inducing the conformational changes responsible for cation translocation against their concentration gradients.[89]

In small molecule reactions, the nucleophilic rate of reaction between alcohols and hard electrophilic centers depends on the fraction present as alkoxide ion.[54] Since serine -OH has a very basic pK in water,[90] it may be activated by general bases and this is true in the serine proteases as well. "Partial" generation of the alkoxide by a concertive interaction with imidazole or other bases decreases the enthalpy of activation compared to the reaction of RO^-.[91] This is because the desolvation

contribution to $\Delta H^{\ddagger}$ is smaller when the charge density on oxygen is less, as in the general base reaction. For both the small molecule and the chymotrypsin reactions, ROH is favored over H_2O and RO^- over OH^- by a factor of 100 in carboxylic acid esterolysis. (This assumes that the effective molar concentration of serine is 55M.)[92]

Thiolytic rates towards PNPA,[93] ethylene oxide,[94] chloroacetamide,[95] and maleic anhydride[96] are directly proportional to the fraction of mercaptan present as anion in the absence of general base catalysts. In a series of aliphatic thiolates, nucleophilicity increases as the pK increases.[96] Thiophenol shows a positive deviation, but within a series of aryl thiols the pK-nucleophilicity relationship holds.[96] Carboxylates increase sulphydryl pKs whereas ammonium cations lower and mix with the thiolate pKs.[97] As a general class, the thiols have a low beta value, indicating a high nucleophilicity for their basicity relative to amine or oxygen nucleophiles.[98] The relative nucleophilicity of RO^- and RS^- towards both PNPA and phenylisocyanates[99] in aqueous solution favors the mercaptide. The mercaptides are particularly reactive towards electrophilic sulfur, i.e. disulfides and their mono- and dioxide oxidation products.[100]

However, to be an effective catalyst, a nucleophile must show facile deacylation. Thiol ester hydrolysis is more favored thermodynamically since the thiol ester is less subject to orientation demands imposed by the greater resonance stabilization available to oxygen esters.[101] The third resonance form is unstable for the thioester since S is a poorer pi donor than is O.



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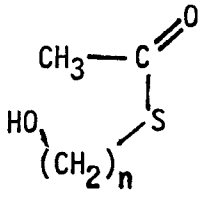
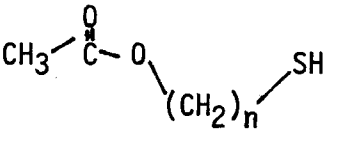
The differences in susceptibility to hydrolysis may not be large, however. The thioester is characterized by a lower electronegatvity and higher polarizability.[102] Rylander and Tarbell have suggested that in deacylation inductive and polarization effects balance resonance effects, making oxygen and thiol esters approximately equal in their susceptibility to nucleophilic attack by hydroxide.[103] Intramolecular transesterification in the following small molecule between RO^- and RS^- favors formation of the oxygen





ester.[104]

In the hydrolysis of oxygen and thiol esters, the entropy of activation is highly important and essentially controls the reaction rate. It is more positive for the oxygen ester. Two explanations offered for this are the greater resonance stabilization for the oxygen ester leading to a more rigid transition state and greater hydration of the oxygen ester's transition state since oxygen is more electronegative than sulfur.[104]

Since sulfur is a relatively large atom, it is less strongly solvated relative to oxygen and therefore might be expected to be less susceptable to solvent effects. Indeed, in the ordering of thiolphenols it was found that the pK-k_{nucl} plot is the same whether the solvent is xylene, aqueous

acetone, or water.[96] The more rapid acylation by maleic anhydride of small molecule thiols as the solvent dielectric constant is increased has been attributed to an increase in acidity constant of the nucleophile.[96]

Jencks and Carruiolo have plotted the nucleophilic reactivity of a large number of small molecules towards PNPA versus their pK. While the plot is a fairly scattered one, substantial positive deviations are observed for three physiologically significant groups: thiols, imidazole, and the hydroxyl of serine. These moieties are employed at a high frequency in enzymic catalysis of energetically difficult reactions.[79,105]

Enzyme Catalysis

Several theories have been put forth as to which single factor is the most important in the rate enhancement of reactions by enzymes. These include both entropic and enthalpic contributions. Entropic factors include both approximation of catalyst and reactant through strong substrate binding and proper juxtapositioning of a number of primary catalytic groups of the enzyme with the substrate.[73] Enthalpic factors include general acid/base catalysis,[9] utilization of enzyme-substrate binding energy to decrease the free energy of activation,[124] and electrostatic stabilization of the transition state,[33] or indeed any process which alters the making or breaking of bonds. Compensatory changes in the energy of activation have also been proposed as an explanation of enzyme catalysis.[138] According to this theory, there is an obligatory coupling of the catalytic act to hydration changes of charged groups of the enzyme upon binding of substrate, as reflected in volume of activation changes. If energetically favorable, these may provide the required energy of activation.

Theoretical work has not resolved the question as to the relative and absolute importance of these factors. All theories seem plausible from either a thermodynamic, kinetic, or quantum mechanical view. But only the relatively weak dispersion forces are understood from a fundamental molecular approach.[106] Also, for so many enzymes the active site composition, let alone structure, remains unknown. Active sites, like globular proteins themselves, have essentially no symmetry, complicating structural analyses for crystalline or solution conformation.

The theories of enzyme catalysis also differ as to the plausibility of their being mimicked in rigid small molecule or synthetic macromolecular catalytic systems. Whereas compensatory energy of activation changes, utilization of binding energy to promote transition state formation, or even multifunctional catalysis would intuitively (or practically in the case of multifunctionality) appear nearly impossible to recreate, general base or acid catalysts can enhance reaction rates by as much as 10^4 intramolecularly, one tenth the factor quoted for this contribution to amidolysis by the serine proteases.[2]

Enzyme Mechanism I: Cooperativity in the Geometrically Fixed Active Site

A factor which has played a central role in enzymology is cooperativity between two or more functional groups.[107] For termolecular reactions in solution, the favorable enthalpy contribution of general base catalysts is largely unrealized due to the unfavorable entropy cost of bringing the three molecules together.[108] Such was observed in the esterolysis of phenyl esters by imidazole or hydrazine. In the case of enzymes,

however, the entropic cost has been paid in large part during the original synthesis of the protein.[73,109]

Among the most studied mechanisms is the "charge-relay" or "catalytic triad" system of the serine proteases.[110] The nucleophilic serine hydroxyl is acted upon by the imidazole ring of His-57 as a general base catalyst, generating the alkoxide simultaneous with its attack on the carbonyl carbon of the susceptable peptide bond. The imidazole acts as a general acid in deacylation. It is maintained in its active forms by interacting with the carboxyl group Aspartate-102.[111] Experimental evidence indicates that tetrahedral decomposition in chymotrypsin's reaction with paranitroanilide esters is accompanied by two concerted, not sequential, proton transfers.[15] In chymotrypsin Asp-102 is buried in a hydrophobic pocket and interacts with the polarizable ring of His-57 even in the absence of substrate.[50] The rate enhancement to amidolysis attributed to general base catalysis by histidine has been estimated to be as high as 10^5. A reduction in activity of this magnitude has been observed when the nitrogen of His-57 is methylated.[2] Yet the triad mechanism has been disputed.[112]

The thiol proteases papain, ficin, and bromelain appear to share in common with the serine proteases general base catalysis by imidazole of an active site histidine for nucleophile activation.[113] In enzymes such as ficin the active thiol is twenty times more reactive towards nonspecific alkylating agents such as chloroacetate or bromoacetate than are small molecule thiols of equal pK.[114] This is due at least in part to general base catalysis by imidazole, as occurs with the serine proteases. A conformationally equivalent histidine is absent from streptococcal

proteinase, however.[115] Also the active site sulfhydryl titrates freely at pH 8.6.[114] An aspartate conformationally equivalent to that in the active site of papain is absent from ficin and bromelain.[116] Even in papain, its catalytic function, if any, remains unclear. It very likely is involved in substrate binding. Thus, the catalytic triad of serine proteases has no analogy in the sulfhydryl proteases.[117]

The activity of papain is dependent on ionizations at pH 4 and about 8.[118] The pK of the active site cysteine in papain remains unclear. It is probably about 8.4 (reporter group titration[119]) although some evidence such as pH- reactivity experiments with 2,2-dipyridyl disulfide indicate an unusually low pK of about 4.[66,120] If so, this is a dramatic example of pK perturbation not accompanied by loss in the activity normally associated with pK depression characteristic of small thiol chemistry.

Urease is a remarkably efficient enzyme, capable of rate enhancements on the order of 10^{14}. Although its mechanism is far from well understood, six of its 24 thiols have been determined to be "essential" for activity. Exposure of the enzyme to 2,2'-dipyridyl disulfide seemed to indicate that the essential ones react as uncomplicated thiolate ions, inactivated by a conformational change occurring at pH 9.5.[121]

It has been estimated that the effective concentration of imidazole within the active site of chymotrypsin would have to be 20,000 M in order to explain the rate enhancements seen with this enzyme strictly in terms of cooperativity.[1] Koshland has calculated that five functional groups would have to participate in order for a rate enhancement of 10^{18} to be achieved. While it is possible, both seem unlikely and would be quite

impossible to demonstrate or mimic in model systems.[122]

Enzyme Mechanism 2: Harnessing of Binding Energy

The specific and strong binding energies of enzymes for their substrates have led many enzymologists, from Pauling[123] to Jencks, Lienhard, Page, and Wolfenden[124] to suggest that this energy is utilized to lower the free energy of activation of reactions, as reflected in the increase in k_{cat}/K_m. This theory has been considered a new version of the older theory of substrate distortion and destabilization.

By binding substrate more tightly when it is in the transition state configuration, the free energy of activation is lowered. It has been argued that very tight binding of the ground state substrate would stabilize its complex with the enzyme and thereby increase the free energy of activation. By forming new and energetically favorable interactions with the enzyme as the transition state is formed, the binding energy is used to increase k_{cat} rather than decrease K_m. Tight binding decreases the energy of the E-S complex and thus increases the activation energy barrier.[125] In a series of synthetic substrate esters of the general structure R-CH(NHAc)-CO_2Me, increasing k_{cat}/K_m values towards chymotrypsin are seen as the size of the radical is increased.[126] Increasing radical size of m-hydroxybenzoic acid esters increases k_{cat} rather than decreasing K_m.[127] Inhibition constants of formanilides also increase with increasing hydrophobic character.[128]

An accurate determination must be made of the free energy of binding in order to suggest that it drives the reaction. Page has noted that the

free energy of transfer of a substrate methylene group to the active site of amino-acyl synthase is -3kcal/mol as compared to transfer of the same group from water to a nonpolar liquid, which is associated with a free energy change of about -1.0 kcal/mol.[129] He has suggested that more accurate estimates can be made for binding energies if they are approximated by the free energy of transfer of the group from water to a nonpolar solid rather than liquid. Presumably, solvent-solvent interactions are not disturbed to as great an extent. The number of water molecules that must be displaced from the active site of chymotrypsin has been estimated at 16, a fairly small number.[130] A second explanation offered for the disparity between transfer energy and binding energy is that more and possibly stronger dispersion interactions may be available for the E-S complex than for the highly dynamic solvent.[129]

Enzyme Mechanism 3: The Electrostatic Theory

The pronounced accelerative effect of salts on reactions in poorly solvating media (benzene, diethyl ether, etc.) has led a number of enzymologists to the electrostatic theory of enzyme catalysis. The classic study of Swain and Brown involved tetramethyl glucose mutarotation in benzene.[131] The first order process of this reaction proceeds at an imperceptibly slow rate. Addition of either pyridine or phenol only mildly promotes the reaction but when added together the reaction proceeds rapidly as a third order process. 2-pyridone, a relatively weak bifunctional acid-base catalyst, accelerates the reaction by a factor of 7000. Salts which are unable to serve as proton acceptor-donors also accelerate the mutarotation, a

reaction involving a charged transition state.[132] Therefore, it has been proposed that ion pair formation with either salts or general acid-base catalysts promotes and stabilizes the charged transition state in poorly solvating media/microenvironments.[133]

The rate of ionization of p-methoxy-neophyl p-toluenesulfonate is promoted by addition of 0.1 M lithium perchlorate to a varying extent depending on the ionizing power of the solvent. Addition of it to the very nonpolar solvent diethyl ether results in a rate enhancement of 10^5.[134] S_N2 displacements by lithium chloride also proceed rapidly in media of low dielectric constant despite the latter's poor ionizing ability.[135]

It has been observed by solution chemists that for reactions having polar transition states, rates are faster in select solvents (nonaromatic, nonhydrogen-bonding) if performed in media of low polarity with a small amount of a highly polar solvent than if performed in pure polar solvent. The catalytic role of the highly polar solvent is more nearly reflective of its instantaneous dipole moment since solvent-solvent interactions are reduced and the transition state can more effectively compete for the polar solvent molecules and thereby be stabilized by them.[25]

Levitt and Warshel have pointed out the importance in distinguishing between bulk and microscopic dielectric constants.[33] They have performed ab initio calculations on the active site of lysozyme including bound water molecules and have suggested that a rate enhancement of 4×10^6 be attributed to stabilization of the carbonium ion intermediate by Asp-52's carboxylate relative to unionized aspartate. Stabilization of isolated charges by fixed charges and induced dipoles of the enzyme, they find, are equivalent

to the energy of solvation. Therefore, enzymes can stabilize ions as well as water can. Moreover, they stabilize ion pairs much better due to their exclusion of bulk water and possibly their orienting of bound water molecules. In aqueous solutions, water dipoles are not oriented or fixed to optimize ion pair stabilization, and solvent-solute interactions, particularly of the first hydration shell, destabilize ion pairs. According to Levitt and Warshel, the enzymes' fixed dipoles stabilize ion pair formation between substrate or transition state and some ionized group of the enzyme. This decreases the free energy of activation, enhancing the reaction rate.

Molecular orbital studies on small molecules placed in the active site of chymotrypsin which are similar to the catalytic moieties (formic acid, imidazole, and methanol) of the catalytic triad have led Nakagawa et al. to conclude that the protonation states of these three groups proposed for the catalytic triad mechanism are not the most stable.[136] They also have interpreted their results in support of the electrostatic theory of enzyme catalysis.

The objection raised to the electrostatic hypothesis is that enzymes operate in water, not benzene. In aqueous solution rate enhancements comparable or even approaching those seen in benzene have never been observed. If the active site were sufficiently water-depleted, however, such rate enhancements might be achievable. Perutz has commented on the exclusion of charged and polar amino acids from the active site with the exception of those involved in the special functions of substrate binding and catalysis.[137] Recall also that globular enzymes have highly compact conformations in solution, possibly comparable to solids in their density.[129]

Enzyme Mechanism 4: Coupling the Energetics of Transfer Processes to the Catalytic Act

With the advent of more and more techniques capable of monitoring the dynamics of enzyme domains, support is growing for a theory in enzymology which explains catalysis through coupling of energetically favorable motions distant to the active site to the energetically difficult ones occurring in the active site.[138] Evidence for this has come from determining the dependence of rate on pressure. The relation between reaction rate changes and pressure changes is given as follows:

$$k_p = k_o \, e^{-PV^{\ddagger}/RT}$$

where k_p is the rate at increased pressure, k_o is the reaction rate at one atmosphere pressure, and other terms are given their common definition.

From relative rate data, the volume of activation can be calculated. This gives some indication of the direction of transfer processes occurring during the rate-limiting step. The conformation is often found to be loosened as the E-S complex is formed.[139] For example, exposure of a previously buried carboxylate group permits release of its electrostricted bound water. A decrease in volume results. As is evident from the equation, decreases in the volume of activation are associated with a decrease in the free energy of activation. Such energetically favorable transfer processes need not be at the active site but they must be coupled in an obligatory manner to the activation event in order to be catalytically relevant. Hammes suggests that full exposure of a polar peptide linkage may

increase the rate seven-fold, and full exposure of a carboxylate may increase it 2000-fold.[140] This theory provides an explanation for enzymes to be macromolecular, despite the fact that only a few amino acids directly participate in the chemical conversion.

Micelle Structure and Properties

Micelles are one type of synthetic macromolecular system that have been used, sometimes quite successfully, to effect reactions in solution. Unlike enzymes, the macromolecular aggregate is an inter- rather than an intramolecular one. Consideration of the static and dynamic conformation and properties of micelles is therefore of particular importance in attempting to understand micelle effects on cosolubilizate reactions and properties.

Micelle formation occurs at a certain concentration of surfactant known as the critical micelle concentration (CMC). The critical micelle concentration is relatively constant for a given surfactant, and depends little on the nature of the ionic head group or the structure of the counterion.[141] The aggregation number (number of surfactant molecules per micelle) is also approximately constant in all but very dilute solutions. Both electrostatic and hydrophobic interactions are important in stabilization of the micellar structure. Without counterion binding the electrostatic repulsions between ionic head groups would be too great to permit aggregation. Increasing the salt concentration decreases the CMC, presumably by screening the mutual charges in the micelles.[142] However, the importance of hydrophobic interactions is reflected in the fact that surfactants

whose aliphatic tails are seven carbons or less do not form aggregates at a well defined concentration, but rather remain dispersed in solution.[143]

Implicit in the concept of micelle formation is the view that the majority of hydrocarbon-water interfaces are eliminated. Consistent with this are ^{1}H-NMR results which indicate that alkyl chain-water contact is very small.[144] A hydrocarbon solubilizate in a micellar solution shows a partial molar volume very close to that in a hydrocarbon solution, rather than an aqueous one, confirming the hydrocarbon nature of the micellar interior.[145,146] Nonpolar solutes such as cyclohexane have little effect on the structure of the micelle, as reflected in unaltered rheological and spectroscopic properties of the micelle,[147] whereas partly polar solubilizates do alter micelles structure since incorporation is into the vicinity of the outer Stern layer.[148] The hydrophobic amphoteric molecules para-nitrophenyl hexanoate, methyl orthobenzoate,[149] and naphthol[150] decrease the CMC of CTABr, promoting micellization. Certain charged dyes decrease the CMC of NaLS.[151] The degree of penetration of small aromatic solubilizates is increased on substitution with alphatic chains.[152] These results suggest that there is in fact a partitioning of the hydrocarbon core from the bulk solution and that nonpolar solutes may enter this core. Counterions appear to retain their hydration spheres,[153] as do the ionic head groups.[154]

Stability as well as formation of altered microenvironments within macromolecules is also critical in design of catalysts. As early as 1936, Hartley appreciated the liquid-like, rather than the solid-like nature of the micellar core.[155] This implies a diffusion-controlled Brownian motion

in solution. Particularly with the emergence of physical techniques having time resolutions on the nanosecond time scale, such as NMR and quasi-elastic light scattering, the dynamic nature of micelles is being realized. Our current view of the micelle is that of a dynamic protrusion and exchange of monomers from the micelle, with an entry rate that is diffusion controlled and an exit rate which is determined by the partition coefficient of the incorporated species.[156] This has been further supported by a study on the phosphorescence quenching of the solute 1-bromonapthalene by a quencher whose charge was the same as that of the ionic head group, guaranteeing its exclusion from the micelle. Despite incorporation of the bromonapthalene into the micelles, its phosphorescence was effectively quenched, indicating that the bromonapthalene is not strictly localized within the micelle, but also spends at least 10^{-6} second periods in the bulk medium.[157] By NMR techniques, it has been demonstrated that counterions also undergo a rapid exchange with their unbound counterparts.[158] Even when a hydrogen on carbon seven from the ammonium group is substituted by a functional group such as imidazole, carbon-13 spectroscopy indicates its random orientation.[159]

Micelle Catalysis

Effects of micelles on reactions of solubilizates may result from electrostatic field effects of their ionic headgroups or from hydrophobic effects contributed by the hydrocarbon chain. Micelle catalysts, like other catalytic systems, may promote reactions by approximation (i.e. increasing the effective concentrations of reactants at the micelle) or by

facilitation of the actual bond making and bond breaking processes. Examples of the latter have classically included covalent nucleophilic/electrophilic catalysis, general acid/base catalysis, reactant destabilization, and/or transition state stabilization.

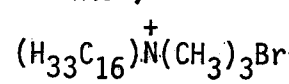
The rate enhancements attributable to micelle catalysis generally do not exceed 10^2 or 10^3, although they may reach 10^5. That unimolecular reactions are rarely enhanced by more than a factor of 10 suggests that approximation accounts for a major portion of rate enhancements in the more studied bi- or termolecular reactions.[142] Ion binding is predominantly entropy-driven, and as such is relatively insensitive to temperature.[142]

Micelles may have no effect on solution reactions if:

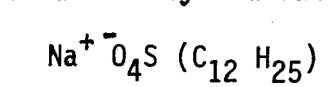
1. neither reactant's distribution in solution is significantly altered by the micelles.
2. only the unbound form of reactant is reactive.
3. unfavorable medium effects on the intrinsic rate constant just counterbalance the positive approximation ones.[160]

As in any catalytic system, the efficiency observed depends on the reaction chosen to assess it.

Micelles used as catalysts are generally ionotrophic; they are amphipaths whose polar head groups carry formal, fixed charges. Frequently used surfactants include the cationic n-cetylammonium bromide or chloride (CTABr or CTACl) and the anionic sodium dodecyl sulfate (SDS).



n-cetyl ammonium bromide

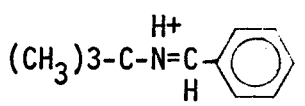


sodium dodecyl sulfate

Theories based on classical electrostatics accurately predict the observed

effects of ionic micelles on the thermodynamic equilibria of ionic reactions.[161] Due to the local electric fields set up by micelles, reactions between two solutes of like charge will be enhanced by a surfactant whose charge is opposite that of the reactants, since the micelle enhances the probability of encounter by countering their coulombic repulsion. Reactions between oppositely charged reactants may be anticipated to be inhibited by addition of micelles of either charge type, since one reactant will be incorporated and the other excluded from the micelle, thereby decreasing their probability of encounter. The maximum effect seen in such reactions occurs at surfactant concentrations which are just slightly above the critical micelle concentration, since this minimizes competition that counterions of the surfactant itself present to ionic reactants.[162]

The electrostatic effects of ionic micelles also predictably alter the real or apparent pKs of small molecule solubilizates. Specifically, forms which are oppositely charged to the micelle occur at more ambient pH. The dye shown below possesses a pK of 6.55 in aqueous solution, 4.96 in cationic micellar solution, 7.02 in anionic micellar solution, and 5.0 in nonionic micelles.[163]



The electrostatic origin of micelle effects also may be observed in micellar effects on uncatalyzed, bulk solution rates. The buffer-catalyzed reaction rate of ionic reactions in micelle solutions reflects their bulk ion activities.[164] For example, the buffer-catalyzed rate of PNPA

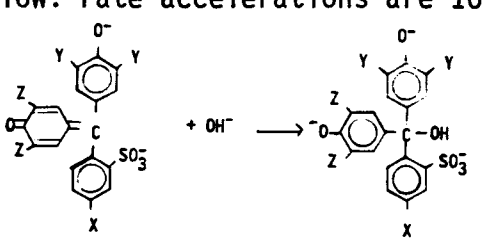
hydrolysis by hydroxide may be decelerated by addition of CTABr which sequesters OH^-.[165] This effect is greatest at low salt concentrations, since hydroxyls of water represent a larger proportion of the total anion content.

Hydrophobicity may also serve as a driving force for reactant-micelle association. Generally, in promotion of bimolecular reactions by micelles, both reactants must be either hydrophobic or counterionic to the micelle or both.

Since reactions of ions promoted by ionic micelles are in effect reactions of counterions and counterion binding is entropy-driven (i.e. temperature-independent),[142] it is often assumed the micelle catalysis reflects itself in $\Delta S^{\ddagger}$, at least in ionic reactions. However, the electrostatic theory of Hartley has limited predictive ability with regards to micelle effects on reaction rates and equilibria.

An early study by Duynstee and Grunwald showed up to an 80-fold rate enhancement of alkaline (OH^-) fading of organic dyes by the presence of CTABr.[146] Both rate and equilibrium positions of the reaction of deprotonation are in a direction and to an extent predictable by Hartley's electrostatic model.[161] However, the absorption spectrum in the presence of the CTABr micelles is shifted, suggesting its incorporation into the micelle.

Sodium dodecyl sulfate is effective in promoting the ligand formation reaction shown below: rate accelerations are 10^6 with the ligand shown below.



The equilibrium position of the reaction again was in accordance with Hartley's model. However, the second order rate constant is highly dependent on the hydrophobic character of the ligand. The catalytic effect of the micelle is reported to be by a reduction in the enthalpy of activation.[166]

Work by Kurz is interesting in that the seemingly electrostatically driven reaction of a proton with anionic micellar ester is due to a decreased enthalpy of activation rather than being entropically driven.[167] The monalkyl sulfate esters are subject to proton, water, and hydroxide hydrolysis. Long chain esters form anionic self-contained micelles whereas small ones do not. Comparing the cleavage rate by these 3 routes of methyl, ethyl, amyl, decyl, lauryl, and octadecyl monosulfates revealed that micellization inhibits the hydroxyl reaction, has no effect on the water reaction, and enhances the proton reaction by as much as 80 fold for the octadecyl ester relative to the methyl ester. In the proton reaction, temperature effects indicate enthalpic contributions of micelles to transition processes. This despite the fact that electrostatics promote reactant association. Electrostatics in micelle catalysis can appear in the enthalpic term,[168] even though it has been generally assumed in the past that electrostatics reflect themselves in $\Delta S^{\ddagger}$, not $\Delta H^{\ddagger}$.[169]

Micelle Catalysis: Ester Hydrolysis

The hydrolysis of carboxylic esters in the presence of micelles may be through:

1. direct hydroxide attack
2. nucleophilic displacement by solubilized small molecules

3. cosolubilization of hydrophobe-sustituted nucleophiles with quaternary ammonium surfactants

4. direct covalent fixation of the functional group(s) onto an ammonium surfactant either at the head group or on the aliphatic chain

Enhancements thus far achieved in PNPester hydrolysis range from 1.6×10^{0}[149] to 5×10^{5}[46]. Nucleophiles include hydroxyl ion, imidazole, benzimidazole, thiol, hydroxamate, and peroxide. CTABr micelles are mildly catalytic through their sequestering of hydroxyl ions. This catalytic effect is greater the larger the alkyl portion of the ester.[170] Behme et al. reported that CTABr micelles enhanced PNPA hydrolysis by 65% and PNPhexanoate by 5 fold at pH 10.07.[149]

Martinek and coworkers have reported that when benzimidazole is cosolubilized with the substrate esters in CTABr at pH9, rates of PNP exceed by 10^5 those observed in the presence of CTABr alone.[46] Imidazole is not so effectively activated. However, benzimidazole is more hydrophobic and has lower pK_1 and pK_2 values than imidazole. The marked rate accelerations have been attributed to generation of the benzimidazole anion, since N-methylation eliminates the catalytic effect.

By kinetic analysis, they have dissected out the three catalytically important contributions to their remarkable 10^5 rate accelerations:

10^1 due to favorable effects of the micelle on k_2

10^2 due to approximation effects

10^2 due to pK perturbation of benzimidazole

Martinek has pointed out that only the reaction of the anion, not that of the neutral nucleophile, entails charge destruction as the transition state is approached.

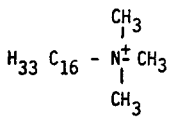
Whereas the differences in nucleophilic reactivity of benzimidazole anion and neutral benzimidazole in water are 10^3, an estimated 10^6 difference is anticipated when placed in a micellar microenvironment.

Neutral imidazole or benzimidazole in CTABr or SDS-nucleophile systems were both reported ineffective in catalyzing PNPester hydrolysis since for these systems the favorable contribution of localization is accompanied by a pronounced decrease (by 10^2) in k_2, due apparently to deleterious effects of the micelles on the polar transition state. Martinek has suggested that a major reason for the frequent failure of hydrophobic model systems is their inability to stabilize charged or partially charged intermediates.[46]

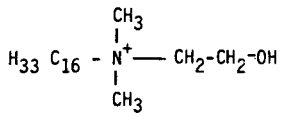
The imidazole group may be more stably localized in CTABr micelles if derivatized with a hydrophobic component. Gitler and Ochoa-Solano studied PNPesterolysis by N-myristoyl-L-histidine in the presence of excess CTABr.[165] The log of both a rapid, nonproductive preequilibrium binding step and the second order rate constant were linearly related to substrate chain length. The nonproductive binding was hypothesized to result from substrate binding to CTABr enriched domains of the micelle devoid of histidyl residues. The second order rate constants towards PNPA and PNPhex are 377 and 7310 M^{-1} min^{-1}, respectively. Even after correction for the fraction of histidyl residues in the active basic form, a k_2 increase of 442 cal/mol per methylene group in the substrate's alkoxyl portion is seen. As with the proton-catalyzed monoalkyl sulfate reaction, the free energy of binding provided by hydrophobic contributions is purported to decrease the free energy of the transition state directly, since the process is second

order, not a complexation one, and substrate binding occurs much more rapidly than turnover. Electrostatic stabilization of the transition state by CTABr is postulated by the authors. The absorption spectrum of acetyl n-myr-L-his is slightly shifted relative to acetyl imidazole in water (from 248 to 245 nm), suggesting partial water depletion of the micellar intermediate.

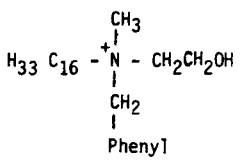
Moss and coworkers substituted one or two of the quaternary ammoniummethyls of CTABr with methylimidazole and/or hydroxyethyl groups:[171]



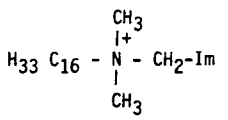
I



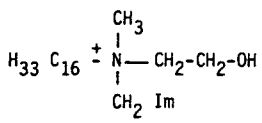
II



III



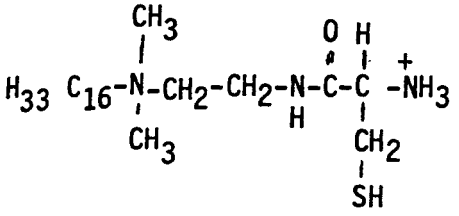
IV



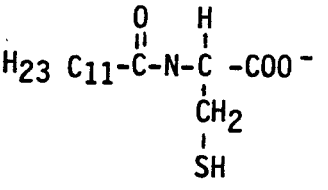
V

The functional groups are an intrinsic part of the cationic micelles. The activity of IV exceeded that of I,CTABr, by a factor of 1200. IV exceeds a 1:1 comicelle of IV and III as well as the bifunctional surfactant V. Again, increased ImH acidity to generate Im^-, substrate association, and transition state stabilization were cited as reasons for the 10^3 rate enhancement of IV, although no attempt was made to sort out the relative importance of each.

Moss also synthesized the first self-contained thiol surfactant in which the primary amine of the ammonium salt below is acylated with cysteine.[172] The alpha amine reaction is 10^2 times slower than the thiol:



VI As-Cys-HCL



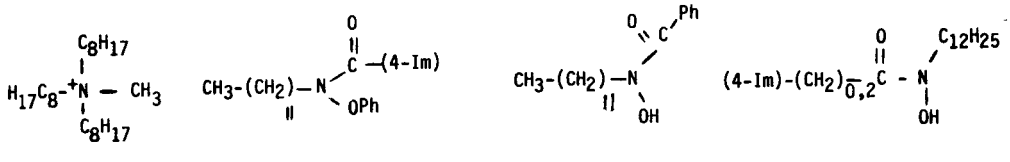
dodecanoyl cysteine

and thus does not interfere in acylation to a significant extent. The surfactant exceeds CTACl hydrolysis of PNPA by a factor of 1860 and the buffer reaction by 29,700 (pH=8.0). They observed a 20 fold increase in PNP release by the presence of CTACl alone, a greater value than that reported by Behme.[149] The thiolcatalyzed reaction is accelerated by a decrease in its acidity constant induced by the CTACl. Dodecanoyl cysteine is not active towards PNPA.[173]

Compared to ficin and dodecanoyl cysteine/CTABr, As-Cys is intermediate in effectiveness towards PNPA. The enzyme exceeds by 29x the CTABr Cys-C12 reaction, which itself falls short by a factor of 3 the activity of VI.[172,174]

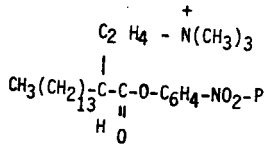
Trioctylmethylammonium chloride (TMAC,I) contains 3 hydrophobes per head group rather than the normal one.[175] This surfactant forms unusually small aggregates as a consequence of the increased substitution. Hydrophobic imidazole (II) and hydroxamate (III) derivatives were more activated by TMAC than by CTABr at all pHs studied:[176]

I II III IV

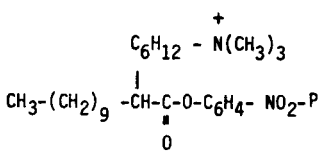


The hydroxamate surfactant, III, is a thousand fold more active in the presence of CTABr than is a nonhydrophobic hydroxamate. In the bifunctional surfactant IV, the acyl hydroxamate is rapidly hydrolyzed by imidazole, possibly in the anionic form although the solution pH is but 9.0.[176] So efficient was the transacetylation that the acetyl-hydroxamate intermediate was not detected spectroscopically. The bifunctional surfactant/CTABr reaction exceeded that of chymotrypsin; $k_{turnover}$ is more than 5000 fold greater than that of imidazole. Despite rapid, intramolecular transacetylation, the model system is not strictly bifunctional, that is, cooperative. The process is sequential, not concertive.[159]

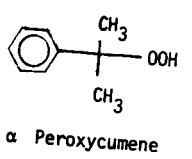
Rates of hydrolysis by cosolubilized nucleophiles of ammonium surfactants having the PNPester covalently attached to them are of interest since in this case localization of the electrophile near the cationic head group is guaranteed. The esterolysis of I and II by alpha peroxycume (III) is 80 and 24 fold faster than with P-NO_2-phenylacetate in water, respectively:[177]



I

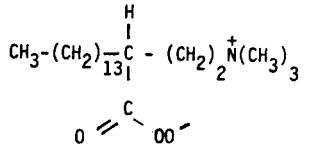


II



α Peroxycumene

III



IV

I has a shorter distance of cationic N to the susceptible carbonyl carbon presumably effecting greater electrostatic stabilization of the transition state.[178]

More impressive rate accelerations are achieved when the nucleophile

has amphipathic character as well. Substrate I was hydrolyzed by IV $1.2x10^3$ more than anticipated but not through a perturbation in peroxide pK. Such rates are observed when both are well below their critical micelle concentrations. Hydrophobic ion pairs are presumed to form, however. This suggests the possible adequacy of simple model systems in amphipathic (micelle) catalysis. The observed rate acceleration at submicellar concentrations is without precedent for this reaction, although it has been observed previously in photochemical redox system.[178]

Polymer Catalysis

Polymers as synthetic macromolecular catalysts offer advantages over micelles in that there is no minimal catalyst concentration necessary for an observed effect on solute kinetics. The reaction may be monitored under conditions of excess substrate or excess polymer, a necessary prerequisite for establishing whether kinetics are of the second-order or Michaelis-Menten type. Polymers are the most easily immobilized of the three although this may or may not cause a loss of activity. Immobilized H^+ sulfonate is more effective in peptidolysis than is a mineral acid solution of equal pH.[179] Maximal rates for a particular reaction may be greater in the presence of polymers than micelles. Both micelles and polymers often compare favorably to enzymes in terms of cost and chemical stability.

The disadvantages of polymer catalysts include a complex kinetic profile since multiple catalytic sites usually exist on a single polymer chain. Even if Michaelis-Menten kinetics are observed, the possible presence of a second-order process cannot be ignored. The catalytic ability and free energy of binding are often less than that observed in the

presence of micelles.[180] Some polymers, such as polyvinyl imidazole, are insoluble in purely aqueous solutions or are of limited solubility in water. Analytical chemistry for monitoring the step-wise modification of polymers has lagged behind other areas of analytical chemistry and great concern has been expressed regarding the degree of polymer modifications.[181]

Synthetic poly alpha amino acids or copolymers of two amino acid types most closely resemble the proteinaceous enzymes in their backbone structure. The polyvinyls represent the largest class of polymers tested as catalysts. They possess the ethylene group as a repetitive unit, in which one hydrogen of every other carbon is replaced by a different group which could be hydrophobic, ionized, polar, and/or catalytically active (e.g. imidazole). Two or three monomeric types differing in R group combine to form copolymers. The polyiminomethylenes are a unique class of polymers recently synthesized by van der Eijk.[182] Polyethyleneimine is a highly branched polyamine whose derivatives have been the topic of this research.

Polyelectrolyte Structure and Its Consequences to Small Molecule Reactions

Most synthetic polymer catalysts, like most biopolymers, are polyelectrolytes. A useful approach to understanding polymer effects on ionic reactions in aqueous solution has been to consider the polyelectrolyte effect itself[183] and then treat solubilizate chemistry as a manifestation thereof. The polyelectrolyte effect sets in when the polymer's charge density (ξ), as determined by the fraction of ionizable groups that are charged and the spacing between them, equals or exceeds the critical density (ξ_{crit})

of that polymer.[184] The Brownian motion of a fraction of the counterions is so retarded as to display a self diffusion coefficient of zero: these are the condensed counterions. Uncondensed counterions and coions also display reduced self diffusion coefficeints as a consequence of the electrostatic fields which the polyions set up. If the charge parameter on the polymer is below its critical value, there is no polyelectrolyte effect and both co- and counterion display equal self diffusion coefficients. When the charge density exceeds its critical value, the polyelectrolyte behaves as if (ξ) equals (ξ_{crit}), maintaining the net charge of the polyion at its critical value.[185] The critical charge density is independent of the structure of the counterion. Substitution of sodium by acridine dye counterions in DNA has no effect on the polyion's electrophoretic mobility.[186]

When the polyion possesses a linear structure and an extended conformation, an appropriate model would be that of a cylinder or rod. Lifson and Katchalsky have applied the Boltzmann-Poisson theory to such a model and Manning has developed the theory of counterion condensation which also has been successfully applied to polyelectrolytes approximating rods in solution.[183] Mathematically, this model is easier to treat since interactions between two segments as well as interactions between two separate chains (approximated experimentally at low polyelectrolyte concentration) may be ignored without undue introduction of error. The rod model is not applicable at low charge density of soft polyelectrolytes or at high salt concentration where the ionic atmosphere around a single fixed charge is much smaller than the distance between neighboring charges.[187] This

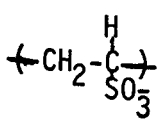
will be discussed in the following section.

Consequences of the polyelectrolyte effect on reactions of cations and anions are readily predictable and often substantial,[188] unlike simple saline effects.[189] In general, polyanions would be expected to enhance reactions between two positively charged molecules by increasing their probability of encounter, but decelerate reactions between oppositely charged species by sequestering one reactant and excluding the other. Polycations would be expected to promote reactions between two anions but inhibit those between oppositely charged solutes. As predicted, the proton-catalyzed hydrolysis of cationic esters is indeed accelerated by polyanions.[179,190] The magnitude of these electrostatic effects depends on the valency of reactant(s) and product(s).[191] Conversion of reactant to a higher valency product results in "product inhibition" insofar as the product is preferentially bound.[192]

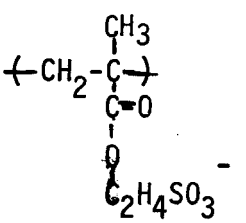
The coulombic effects of polyelectrolytes on ionic[193] reactions are strongest for polymers having a high charge density. The ligand exchange reaction shown below

$$Co(NH_3)_5Cl^{2+} + Hg^{2+} \longrightarrow Co(NH_3)_5H_2O^{3+} + HgCl^+$$

is increased by 176,000 in the presence of 5×10^{-5} M polyvinyl sulfonate (PVS) and 24,700 by polymethacryloxyethylsulfonate (PMES).[194]



Polyvinylsulfonate (PVS)



Polyvinyl methacryloxy ethyl sulfonate (PMES)

The polymer concentration giving the maximum rate enhancement of ionic reactions is predictable from Manning's polyelectrolyte theory. At high polymer concentrations, bi- or higher order reactions are less accelerated by the polymer due to their separation from one another on separate polymers or polymer domains.[193] The separation between the backbone and the charged group in the latter is greater, decreasing its charge density. Increasing the rod radius may also decrease the polyion effect. The catalytic activity of polyions in ionic reactions is lost by addition of simple salts, which displace reactants from the polyion domain.[194]

The pK values of ionic groups in polyelectrolytes are on the average shifted relative to the pK of the monomer unit in a direction to stabilize the neutral form. This reduces intramolecular coulombic repulsions. The ionizations of both polymethacrylate and polyamino acids were found to be minimal at a pH equal to the pK of the monomer unit.[195]

A consequence of the polyelectrolyte effect is a real or apparent perturbation of solute pK in a direction to stabilize the form carrying an opposite charge.[196] The pK perturbation of solubilizates is important for more than promoting protonation/deprotonation reactions. For instance, in esterolysis, only the unprotonated forms of nucleophiles may be active. Also, rates of esterolysis are sensitive to the pK of the leaving group. This has been offered as an explanation for enhanced phosphorolysis of aryl phosphates in the presence of polyethyleneimine.[197]

Since reactants frequently are counterions as well, it is relevant

to consider the nature of polyion-counterion association. The fact that Nagasawa and Eguchi were successful in monitoring counterion condensation by following changes in the refractive index of a sodium chloride solution of polyacrylate at a charge density exceeding its critical one is indicative that the counterions are able to penetrate at least partially the polymer hydration sphere.[198] An attempt to determine exchange rates for free and bound counterions by fluorescence quenching was also quantitatively unsuccessful due to changes in solvent structure in the immediate vicinity of the polymer.[199] Such observations have led many investigators to treat counterion condensation like ion pair formation, although a different model, one in which the polymer is viewed as a plane with fixed charges and the counterions as a second plane of opposite charge, also correctly accounts for the observed activity coefficients in these solutions.[200] Whether higher order (lamellar or lattice) structures or less defined counterion association occurs in some ionomers remains unclear.[201]

Polymers Not Approximating the Rod Model: Hydrophobic Domains and Macromolecular Catalysis

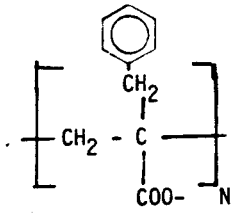
Ion condensation is not peculiar to cylindrical rod-shaped polyions. Planar[202], and more importantly here spherical systems also have characteristic critical charge densities and condense a constant amount of counterions at finite but low salt concentrations. While some have argued that the extent and nature of the condensation is quantitatively identical to that of extended, rod-shaped polymers,[203] others disagree because

1. there is a nonuniform distribution of metals in polycations[204]

2. it is theoretically plausible that there exist within organic polymers such as polymethacrylate higher order, lattice-like structures when the polymer has a low charge density.[201]

3. while the rod model accurately predicts trends in counterion binding with changes in degree of ionization of the branched ionogenic polymer PEI, it underestimates the absolute amount bound.[205]

Condensed conformations would be more likely to exclude a part of the polymer domain from the bulk solution. Since hydrophobic effects are believed important in binding and catalysis of reactions by enzymes, design of synthetic macromolecular catalysts often are directed in an attempt to incorporate this property as well as electrostatic ones into the polymer matrix. Electrostatics increase water solubility. Hydrophobic domains do indeed exist within a number of polyelectrolytes, however: When determining the pK perturbation of organic dyes by polyions, not only did titration behavior change, but also spectral properties (i.e. maximum wavelength of absorption and extinction coefficient) were altered, suggesting incorporation of the dye into a micromedium of reduced dielectric constant.[190] The polymer shown below, PVBMA, alters the fluorescence spectrum of noncovalently bound probe molecules in a direction indicative of reduced dielectric permittivity as well.[206]



Polyvinylbenzyl methacrylate (PVBMA)

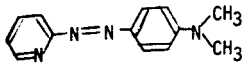
As has been reported in micelle catalysis,[146] cationic polymers can promote the alkaline (OH^-) fading of cationic dyes despite coulombic

repulsion between macromolecule and dye since their association is driven by hydrophobic interactions. Such was found using polyvinyl pyridine which had been partially quaternized with n-cetyl bromide.[206] Hydrophobic components of polymers enhance their catalytic ability for more reaction types than simple protonation/deprotonation reactions, however. Substantial rate enhancements for ligand bond formation between transition metal cations and organic molecules have been reported,[207] as will be discussed shortly in this section. Esterolyses are also frequently promoted by hydrophobic components in the polymer. For example, polystyrenesulfonate is more effective than a mineral acid solution of the same pH in hydrolyzing cationic esters.[179] The increased efficiency of the protic hydrolysis increases with increasing alkoxyl radical on the substrate.

In general, both electrostatics and hydrophobicity are necessary components of catalysts. The polyanion polyphosphate inhibits ligand bond formation of transition metals with hydrophobic organic molecules whereas polystyrenesulfonate promotes it.[193] PEI, a ionogenic polycation, is not very efficient in aryl phosphate hydrolysis at pH 9 due to its low charge density.[197] PEI actually inhibits pyrophosphate hydrolysis by OH^- despite coulombic considerations probably because of tight complexation of the multivalent anion to the polycation.[208]

Quantitative dissection of hydrophobic and electrostatic contributions to polymer[207] and micelle[209] catalysis is currently an area of intense interest to researchers in these fields. Ligand bond formation between the ligands I & II shown below and Co++ or Ni++ were accelerated by factors of

81.6 and 60.8 respectively in the presence of styrene sulfonate-methacrylate copolymers.[207]



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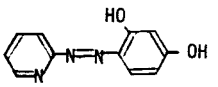
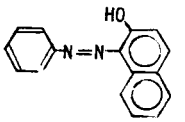


II



III

Manning's electrostatic theory for extended, linear polyions was found to correctly predict the polymer concentration giving the maximum effect (at which all reactant ions are bound to the catalyst), but the amplitude of the ligand exchange reaction rate was dependent on the strength of the hydrophobic interaction between polymer and ligand. (III > I >II)[207]

With increasing hydrophobic character of the polymer Michaelis-Menten kinetics may be observed.[210] A clear demonstration of saturation kinetics requires that one show pseudofirst order kinetics both at high polymer concentration and high substrate concentration. This is unattainable in micelle catalysis since at high substrate concentration, micelle structure is altered and at high micelle concentration, the simultaneous addition of countersalts precludes the catalytic effect. Other requirements for demonstration of Michaelis-Menten kinetics in synthetic systems are:

1. there must be sufficiently large differences between catalyzed and uncatalyzed rates.
2. the K_m must be sufficiently low so that the polymer concentration and the substrate concentration need not be prohibitively large since their solubility may be limited.

The likelihood of observing Michaelis-Menten kinetics depends on the hydrophobic content not only of the polymer but also of the substrate as well. The differences in K_ms in a series of substrates differing only in aliphatic content has been used to calculate differences in the free energy of binding of a substrate to catalyst. In the section on PEI catalysis,

it will be noted that from PNPA to PNPcaprylate, 10^2 differences in K_m are observed with micelles but 10^3 differences are observed in binding to partially benzoylated PEI. This derivatized polymer falls between micelles and chymotrypsin in binding caproate as opposed to acetate esters (see page 82).

There have been some reports of binding through Michaelis-Menten complex formation on the basis that product formation and release levels off at certain substrate concentrations. The actual reason for this was that polymer groups were participating covalently in a second order process with the substrate, and were only slowly regenerated due to slow deacylation rates. A specific example of this commonly made error will be given in the section on polyvinyl catalysis.

Enzymes are linear in primary structure but globular in conformation. They manifest Michaelis-Menten kinetics.[4] Therefore, their gross electrostatic properties are frequently ignored. Yet some nonenzymic proteins, both natural (e.g. heparin[211]) and synthetic (e.g. poly-L-lys and poly-L-glu[212]) behave as typical polyelectrolytes. Poly-L-glu and poly-L-lys take on an alpha helical conformation upon uncharging.[212] Poly-L-glu whose gamma carboxylates have been modified with the benzyl group more nearly resembles globular proteins than polyions in its physicochemical properties.[213] Yet polyelectrolyte characteristics of natural proteins are not so few and isolated. In 1905, Sorensen reported on the "protein error" observed in colorimetric assays.[214] It was found to depend on the formal charge of dye and protein. A shift in dye pk was found to occur in the

direction of countercharge stabilization. The dye pK shift could be eliminated by protein hydrolysis (i.e. it requires macromolecularity). Subsequently, the polyelectrolyte properties of spherical proteins were reviewed.[215] Even enzymes not uncommonly possess an excess of charge type.[216]

Poly alpha Amino Acids

The synthetic homo poly-alpha-amino acids, copolymers thereof, or modified poly alpha amino acids are most closely related structurally to the natural catalysts, enzymes. They have been studied both for their ability to bind small molecules and their capacity to catalyze simple reactions, particularly PNPA hydrolysis. The four types of poly alpha amino acids which have been studied can be grouped into four classes:

1. simple poly alpha amino acids
2. copolymers of two amino acid types
3. polyamino acids "capped" at termini with another amino acid or dipeptide
4. polyamino acids with modified R groups

Gamma benzyl poly glutamate is one member of the last class. It was alluded to briefly in the previous section on polyelectrolyte binding and hydrophobics.[213] A second member of this class is slightly cross-linked (through R amino groups) polylysine. This derivitization enhances the polymer's capacity and affinity to bind the anionic dye methyl orange.[217] Only the catalytic (nucleophilic) ability of poly α amino acids are considered here, although binding is an important and often poorly mimicked aspect of catalysis.[210] Cooperativity[218,219] and enantioselectivity[220] have also been goals that remain inadequately met.

Merrifield and Woolley were among the earliest to explore use of synthetic poly alpha amino acids as catalysts for ester hydrolysis.[221] They synthesized polyhistidine and polyhistidine capped with L-glu or the dipeptide L-seryl L-glu on the carboxy terminus. The highest activity was seen with poly-L-his-L-glu, some twelve times more active than poly-L-his, which in turn showed about a nine-fold increased activity over L-his. The results were quoted per mg. catalyst, and it has been pointed out that a more accurate analysis of the data would be to correct for differences in imidazole pks.[222] Katchalski's study on poly-L-his led him to conclude that the activity observed was about that of histidine, which is lower than that of free imidazole.[223] A linear correlation between rate and mole percent imidazole further suggested that the observed activity was simply that of imidazoles diluted by an inert polymer without imidazole-imidazole cooperation or activation by the backbone.

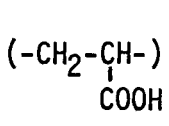
In an effort to mimic the sulphydryl enzymes, which at the time were believed to operate by general base catalysis from a carboxylate, not histidine, Komai and Noguchi synthesized Copoly (L-cys, L-glu).[224] Two pH optima were observed in contrast to other polymer catalysts studied previously. The activity of pH 7.1 was about equal to that of poly-L-cys, suggesting that the carboxyls make no contribution at the higher pH optimum. At pH 6.1, the lower pH optimum, the activity of the copolymer exceeded that of poly-L-glu at pH 5.2, its maximum, by a factor of 20. The K_ms are about equal at 10^{-2}M at both pHs. This activity could not be seen by simply adding poly-L-glu to a solution of poly-L-cys. The activity maxima occur at pH values near those at which the polymer possesses its

most compact conformation as determined by ORD and molecular rigidity studies (pH 5.9 and 7.4).[224] The pKs of carboxylate and sulphydryl in the copolymer when corrected to 0 concentration of NaCl were 5.38 and 10.08, respectively. Copoly L-cys, L-asp showed no activity. The system is sensitive to small structural differences in the monomer units. The authors site a related study in which copoly (tyr-glu) and copoly (tyr-asp) are compared for hydrolytic ability.[225] Again, the aspartyl-containing polymer was ineffective. The glutamyl-containing polymer had activity at pH 6.1 but not 7.1.

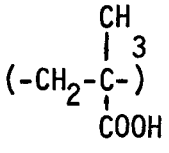
The Polyvinyls

Polyacrylates

The acrylic and methacrylic acid vinylic units shown below differ



polyacrylic acid (PAA)



polymethacrylic acid (PMA)

only with regard to addition of a methyl group. However, this difference profoundly affects the polymer's properties. The binding constant for small cationic molecules is enhanced at least by a factor of 100.[210] At low degrees of dissociation (alpha less than or equal to 0.2) PMA takes on a condensed conformation and is able to bind neutral polycyclic aromatic hydrocarbons. The condensed conformation further promotes binding of auramine-O, a positively charged dye. Binding of this dye promotes polymer condensation at alpha values slightly below the transition point.[226]

It is also at fairly low degrees of dissociation that the carboxylates

of PMA function as nucleophiles in bromide displacement from alpha-bromoacetamide.[227]

$$P\text{-}COO^- + Br\text{-}CH_2\text{-}CONH_2 \xrightarrow[Br^-]{} P\text{-}COOCH_2\text{-}CONH_2 \xrightarrow{H_2O}$$

$$P\text{-}COOH + HO\text{-}CH_2CONH_2$$

In general, however, carboxylates are weak nucleophiles and have not been used with frequency as covalent catalysts.

Very recently, micromedium effects of polyacrylates on ester hydrolysis have been considered. Hydrolysis of two neutral activated esters, p-methoxyphenyl dichloroacetate and P-methoxyphenyl 2,2 dichloropropionate, by water is strongly inhibited upon addition of PMA but not PAA or polyvinylpyrrolidinone.[226]

$$RCCl_2CO_2C_6H_4Me\text{-para} + H_2O \longrightarrow RCCl_2CO_2H + HOC_6H_4Me\text{-para}$$

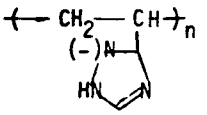
R=H or CH_3

PMAA's inhibitory effect disappears as the degree of ionization increases and the polymer takes on an extended conformation. Inhibition is also eliminated by addition of urea. PAA and PVPyrrolidinone do not form hydrophobic microdomains although the former may influence the hydrogen-bonding network of surrounding water.[228]

The inhibition differs from that of binary solvent systems for which changes in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are opposite in sign, large, and partially compensating. PMA increases $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. This has been taken to indicate that whereas the binary solvent system inhibits by initial state solvation, the polymer inhibits by transition state destabilization, that is, through poor hydration of the polar activated complex.[218]

Polyvinyl Triazole

Polyvinyl triazoles I, whose reactive forms are anionic, are less reactive as covalent catalysts than are small molecule analogues II towards phenyl esters of either the positive, neutral, or anionic type.[229]



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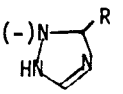
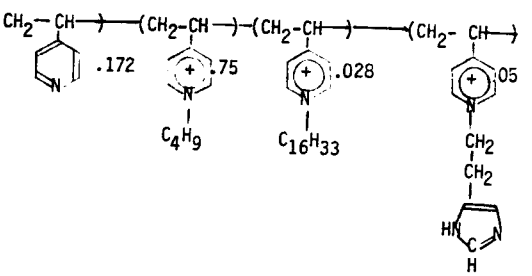


II

Polyvinyl Pyridine

Polyvinyl pyridine has been more extensively studied as a nucleophile. The pyridyl nitrogen shows a nucleophilic reactivity which is comparable to that of imidazole. The reactivity of the pyridyl nitrogen is reduced in PVP relative to that of ethylpyridine.[230] If some of the polymeric pyridines are quaternized with long-chain hydrophobes, reactivity towards the anionic ester NABA but not the neutral PNPA is enhanced relative to the low molecular weight analogue.[231] NABA esterolysis is maximal at 75% neutralization, where sufficient positive charge promotes binding while most pyridyl nitrogens are unprotonated and thus nucleophilic.

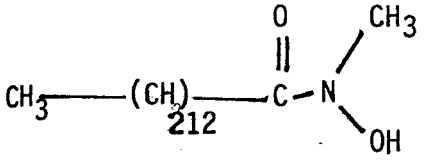
Partially and variously quaternized polyvinylpyridine was prepared by Ise and coworkers.[232] All but 17% of the pyridyl nitrogens were quaternized with butyl (62-75%), cetyl (2.8%), ethyl imidazole (histamine) (5-7%), and propionate (0-8%) groups.



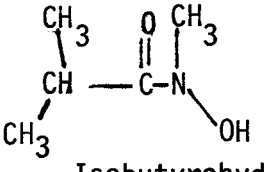
polyvinylpyridine partly quaternized with n-butyl, n-hexadecyl, and ethylimidazole groups

k_2 towards NABA and PNPA of the polymer shown were 0.25 and 0.26 min^{-1} respectively. Michaelis-Menten kinetics were reported with all three substrates used: PNPA, PNPPr and NABA.[232] Comparison of two polymers differing in quaternizing substituents revealed an inverse relation between tight binding and esterolytic ability. Such a relationship between k_{cat} and K_m had been reported previously for NABA hydrolysis by a copolymer of 1-vinyl-2-methylimidazole and 1- vinyl pyrrolidinone [233,234] (see below).

PVP quaternized extensively with methyl (96%) and lauryl (3 + %) halides is capable of activating an exogenously added hydrophobe-substituted hydroxamate, I, much more than do cationic micelles.



N-methylmyristohydroxamate (I)



Isobutyrohydroxamic acid (II)

For the PVPR$^+$ /I reaction with PNP hexanoate as substrate and at low salt (μ = 0.008) the k_a equals 145000 M^{-1} sec^{-1}, corresponding to $k_{a_{cat}}$ = 94.7 sec^{-1}. Towards PNPAc the k_a = 9800 M^{-1} sec^{-1} compared to 32 M^{-1} sec^{-1} for butyrohydroxamate anion.[233] Towards PNPA, CTABr/II micelles have a k_2= 6.67 sec^{-1} and k_{cat}= k/K_d= 4,830 sec^{-1} M^{-1}. The large rate acceleration is attributed to partial desolvation of the hydroxamate anion through tight ion pair formation with the polymer ammonium cations. This permits ionization despite a local decrease in polarity.

The oxime group (R_2-C =N-OH) is strongly nucleophilic but possesses a high pK value of 12. Kabanov quaternized PVPyridine with an oxime-

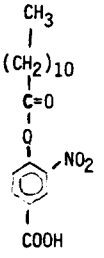
containing radical group and found a substantial reduction in pK to a value of 8.5 with retention of nucleophilicity.[234] A small molecule analogue also manifested a decreased pK (9.6). The polymer's activity toward anionic esters was only one half less than that of chymotrypsin, with a true second order rate constant of 1000 M^{-1} sec^{-1} at pH 8. The less the extent of quaternization, the greater the polymer's activity. This could not be accounted for by pK alterations with alterations in percent quaternization since the observed oxime pK was independent of its fractional content in PVP .

PVImidazole

Imidazole in its monoprotonated, neutral form is a weak base (pK=6.5) and therefore is only capable of nucleophilic displacement towards activated esters such as thiol esters and phenyl esters. Imidazole exceeds polyvinylimidazole in reactivity until the pH is made sufficiently alkaline for 80+% of the imidazoles to be monounprotonated. At this point, the polymer surpasses imidazole in nucleophilic reactivity towards PNPA.[235] While it has been suggested that this is due to generation of the highly reactive unprotonated anionic form of the diazole,[236] the generally very high pK_2 (an estimated 14.5) of this moiety indicates that intramolecular general base catalysis may be operating.[237] A more favorable enthalpy of activation for the reaction (3.7 kcal/mol vs. 7.0 kcal/mol) is consistent with this since it indicates differences in bond making/breaking processes. Polyvinyl benzimidazole exceeds benzimidazole activity towards NABA by a factor of 4 over a wide pH range (pH 4-10).[238] This may be at least partly understood by

consideration of the relative pK's of the imidazole and benzimidazole groups. Protonated benzimidazole has a pK of 3.5 and the benzimidazole anion is generated at pH 12.2[77]

Initially it was suggested that esterolysis of NABS by PVIm proceeded through complexation,[239] although it has since been shown that the observed rate plateau is due to slow deacylation.[240] Michaelis-Menten kinetics are observed between PVIm and long-chain analogues of NABA such



as the C_{12}-C_{18} analogues.[241] The maximum k_{PVIm}/k_{im} achieved with this substrate was 390 and was observed in a low alcohol solution. Using a series of acyl substrates, it was concluded that the transformation from a second order process to a complexation one paralleled the hydrophobic content of the substrate. The K_m for the C_{12} analogue in a 20% ethanol solution is 0.38×10^{-4} M. As the percent ethanol is increased to 50%, K_m increases to 4.53×10^{-4} M, hydrolytic activity decreases, and the polymer takes on an expanded conformation as reflected in an increased solution viscosity.[242]

In a series of neutral paranitrophenyl esters, acylation was found to be rate-limiting for short chain substrates whereas deacylation constitutes the slow step for C_{12} and C_{18} analogues. Yet for all PNP esters, deacylation is more rapid than the nonpolymeric reaction, suggesting intramolecular catalysis in the polymer.[243]

A plot of the free energy of binding of NABA to partly methylated

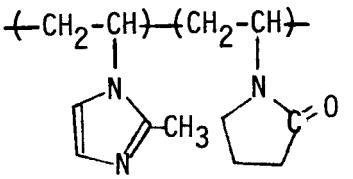
PVIm versus the free energy of activation of the esterolysis generated a linear plot. This suggests that rate enhancement of the slow step for this substrate, namely acylation, is largely due to approximation (less negative $\Delta S^{\ddagger}$) with no essential differences in the polymer's chemical process and that of the small molecule model.[244]

Vinylic Copolymers

Copolymers consisting of two or more vinyl units permit simultaneous incorporation of two different catalytically active species, or may enhance hydrophobic binding to functional polymers through hydrophobic or ionic moieties. Alternatively, pendant radical groups may themselves be chemically modified subsequent to polymerization, again with groups which are either catalytic or of importance for the binding and microenvironmental properties which they confer. Several such examples were presented in the discussion of polyvinyl pyridine. Copolymers will be considered at this time.

Im-containing Copolymers

Vinyl-2- methyl imidazole-vinylpyrrolidinone copolymers were prepared by Kunitake et al.[245]



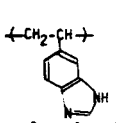
Polyvinyl pyrrolidinone is catalytically inert but has a capacity to bind small neutral molecules. The copolymer was reported to manifest saturation kinetics with anionic NABA, having a K_m for this substrate of 9-11 nM. (Recall that with PVIm, second order kinetics were observed with this same small ester.) The smaller the Km, the smaller is K_3

possibly due to inacessibility of substrate to the imidazoles buried within the hydrophobic microdomains. The copolymer was reported to follow second order kinetics with PNPA.[245,246] Variation in the percent vinyl imidazole revealed that at greater than 16%, heterogeneous catalytic sites were present on the polymer as reflected in a nonlinear Lineweaver-Burke plot.

Seryl and tyrosyl alcohols are among the catalytic moieties in the active sites of a number of enzymes. For this reason, several investigators have explored the properties of vinylimidazole copolymerized with vinyl alcohol or vinyl phenol.[247] The alcohol-containing copolymer is only slightly more active (by 1.2 to 1.7) than PVIm towards PNPA but the phenolic copolymer shows a k/k_{im} of 63 towards ANTI under mildly alkaline conditions. The proposed mechanisms include both general base catalysis and tetrahedral intermediate stabilization.

Copolymers of imidazole and acrylate exceed the small molecule analogue γ-4(5)-imidazolyl butyrate in the hydrolysis of the cationic ester ANTI, but not in PNPA or NABA hydrolysis.[248] As the content of vinyl imidazole is increased from 12-93 molar percent, pK_{im} decreases from 9.4 to 5.1.[249] Substitution of acrylate by sulfonate eliminates catalysis. At pH 9.0 and in 28.5% EtOH, the k_{cat} of the acrylate-imidazole copolymer equals 15 1/mol min, whereas the sulfonate containing copolymer has a k_{cat} of 0 1/mol min.[248]

Poly (5(6)-vinylbenzimidazole)(I) is less active in cationic ester hydrolysis than benzimidazole,(II) the greatest inhibition being observed with small aliphatic esters and at higher pHs.



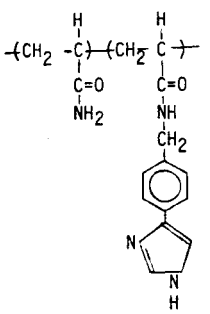
I

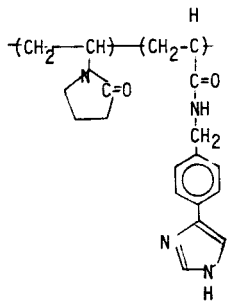
II

When copolymerized with acrylic acid, the k_{cat} in buffered 30% methanol

relative to that of benzimidazole varied from -149 liters/mole-min to + 192 $^{l}/_{m}$ min, the greatest solvolysis being observed with the C_8 ester. Whereas in 30% propanol the activity increases with increasing % acrylate, the reverse is observed in 30% methanol.[250]

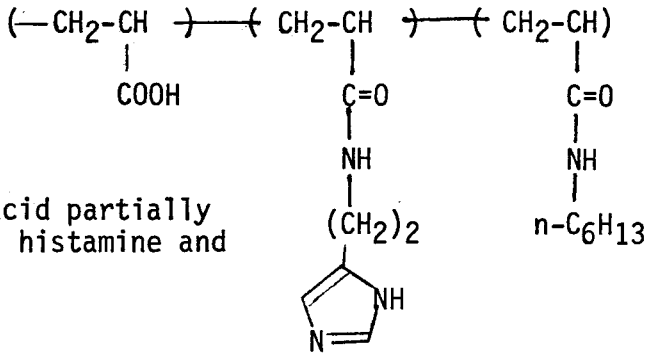
Modification of carboxylates in PAA-Polyacrylamide and PAA-polyvinylpyrrolidinone copolymers with phenyl imidazole generates two polymers differing only slightly in structure with the latter showing greater hydrophobic character through its pyrrolidinone ring.[251] After correcting for ΔpK, it was concluded that the favorable substrate binding contribution is mostly offset by an increased $\Delta G^{\ddagger}$. Again linearity in the plot of $\Delta\Delta G_{binding}$ versus $\Delta G^{\ddagger}_{NABA}$ reveals little or no enhancement in catalytic efficiency with the increased hydrophobicity. Rather, hydrophobic interactions between polymer and substrate are lost in the transition state.





Modification of some of polyacrylate's carboxyl groups with histamine and n-hexyl amine resulted in a polymer which bound PNPA prior to hydrolysis with a K_m= 1.8 mM. Although k_2 is less than that of k_{cat} of imidazole (5.0×10^{-3} sec $^{-1}$ for the terpolymer), k_{2/K_m} is more than 6 times greater than the k_{cat} of imidazole. The authors suggest that the polarity of the transition state is not favored in the hydrophobic microdomains of

the polymer.[252]



polyacrylic acid partially amidated with histamine and n-hexylamine

Thiol-containing Copolymers

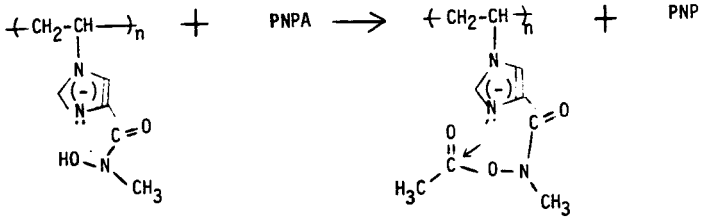
The imidazole group is believed to serve as a general base in thiol as well as serine proteases. In an attempt to recreate such bifunctionality, Overberger and coworkers synthesized copolymers of vinyl thiol and vinyl imidazole.[253] Their activity was compared to that of polyvinyl mercaptan, polyvinylimidazole, and low molecular weight analogues of the copolymer. Substrates include PNPA, NABA, ANTI, and 3-nitro-4-dodecanoyloxybenzoic acid. The activity depends on the amount of imidazole in its neutral form and the amount of mercaptan present as the thiolate anion. Their pKs are 7 and 9, respectively. Thus, there is little or no pK perturbation and the increase in activity seen with increasing pH is due principally to an increase in the concentration of thiolate. Activity of polyvinyl mercaptide was less than that of the model compound 2,5 dimercaptohexane. Activity of the copolymer only slightly exceeded the sum of imidazole and thiolate contributions indicating little if any of the anticipated cooperativity. Indeed, a 1:1 copolymer showed a minimum of activity relative to others. The long chain anionic ester exceeded slightly the acetoxy substrate in hydrolytic rate by the copolymer contrary to that seen in the

presence of the small molecule analogues, indicating a slight contribution of polymer structure to substrate association.

Starodubtsev and Kabanov synthesized vinyl mercaptan and 3-vinyl pyridine copolymers and quaternized to varying extents the pyridyl nitrogens with methyl, ethyl, and carboxymethyl halides.[254] Relatively independently of the polymer structure, sulfhydryl pKs were depressed two to three units. Reactivity towards both PNPA and NABA was greatest in those polymers quaternized to a lesser extent and with the neutral methyl or ethyl radicals.

Two Functional Groups in one Vinylic Unit

Manecke and coworkers synthesized n-vinylimidazole-4-carbohydroxamic acid, whose structure and proposed mechanism are shown below.



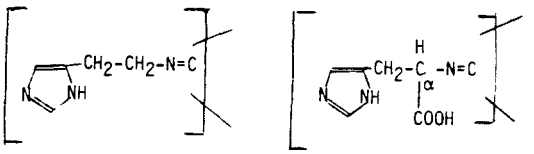
As with Kunitake's trioctylammonium micelles, imidazole should catalyze hydroxamate deacylation. Rather than having two functional groups in separate vinylic monomers, both imidazole and hydroxamate are covalently attached to one monomer to form the pendent radical of this unique homopolymer. It was designed to minimize the steric limitations precluding bifunctionality in polymers. However, its activity only slightly exceeded that of imidazole or benzylhydroxamate alone.[255]

Polyiminomethylenes

An interesting new class of polymers has been synthesized and tested

for esterolytic activity by Van der Eijk and coworkers.[182,220,256] These are the poly(iminomethylenes). Monomers may be obtained which are optically active and some remain so upon polymerization. The intrinsic regidity of these macromolecules which might enhance cooperativity and their potential for enantioselectivity were decisive factors in the authors' choice of this polymer. Two papers were published on esterolytic activity. The first did not address the question of stereoselectivity, but rather was concerned with preliminary assessment of catalytic capacity.

Polycarbyhistamine and polycarbyhistidine are shown here



1 2

The carboxyl containing polymer showed a second order rate constant of 24.9×10^{-2} 1 mol^{-1} sec^{-1} and under the conditions of excess substrate the k_{acyl} was determined to be $8.5 \pm 1.0 \times 10^{-2}$ 1 mol^{-1} sec^{-1}. This is about 400 times more active than histidine. Yet its pK of 6.0 is about the same as that of histidine.

The reactivity of polycarbyhistamine, whose pK is also about 6.0, was quite low, lower in fact than the analogous polyvinyl polymer, poly-vinyl imidazole-poly-vinyl acetate. Using the highly reactive substrate 2,4 dinitrophenylacetate, an acetylated imidazole intermediate was directly detected with only this polymer, although such a mechanism is believed to occur with both polymers and both substrates.

The linearity of substrate concentration versus velocity suggests that binding as a separate step does not occur, unlike binding

proteins and most enzymes.

A plot of $alpha_{im}$, the fraction of imidazoles in the nucleophilic, monoprotonated form, versus velocity is linear until alpha exceeds 0.8, at which point the reaction rate deviates in a positive direction. The authors do not believe that this represents generation of the highly nucleophilic anionic form, as the pH need not be very high in order for $alpha_{im}$ to be equal to 0.8. Rather, they suggest general-base cooperativity of imidazoles. Other explanations are tenable; the authors cite that the decrease in local positive charge may relieve unfavorable interactions with the positive charge on the central carbon of the tetrahedral intermediate.

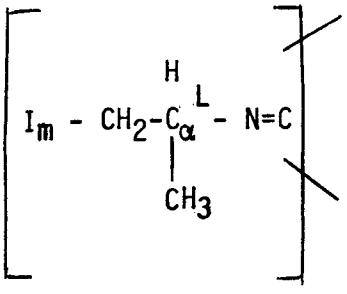
Deacylation rates were studied using the highly reactive substrate 2,4-dinitrophenyl acetate. Hydroxyl ion, water, or imidazole might potentially catalyze the deacylation, but the linearity of rates versus (OH^-) is indicative that the reaction proceeds by hydroxyl assisted deacylation. Moreover, the magnitude of the initial burst in imidazole acylation suggests that an insignificant amount of imidazoles are nonacylated and available to catalyze the deacylation. Not surprisingly, then, deacylation rates of the polymers are about equal to that of acetylimidazole.

The conclusions which may be reached from this first paper on the polyiminomethylenes are as follows:

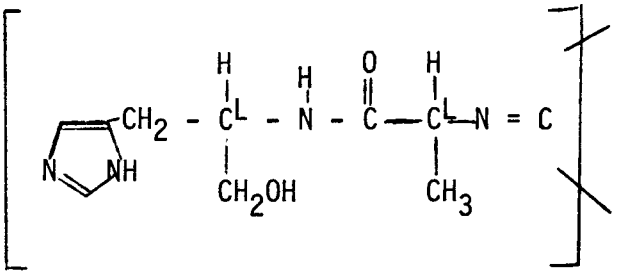
1. Activation of imidazoles in polymer 2 is not due to pK perturbation. Cooperativity with carboxylates remains a tenable possibility.
2. No cooperativity or rate enhancement of deacylation was observed here.
3. Previous structural work suggests that pendant catalytic groups

protrude outward from the backbone, and thus would not be expected to be desolvated.[256] The kinetics appear to be consistent with this view.

The second paper published on the catalytic activity of poly (iminomethylenes) was aimed at making an enantioselective catalyst.[220] Previous polymers racemized upon polymerization, and therefore two novel ones which were able to retain a predominance of left- or right-handedness were studied. Their structures are shown below.



1 poly (L-carbyl-α-methylhistamine)



2 poly (D-carbylalanyl-L-histidinol)

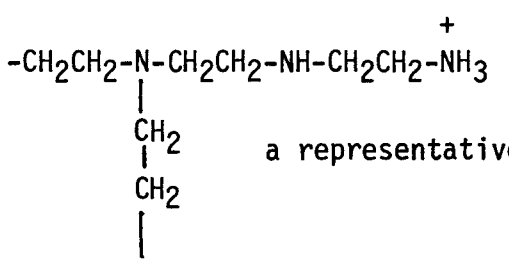
The 2nd order rate constant for 1 was found to be 190×10^{-2} $dm^3\ mol^{-1}\ sec^{-1}$ towards 2,4 dinitrophenyl acetate. That of 2 was 410×10^{-2} $dm^3 mol^{-1}\ sec^{-1}$ at pH 7.9. The pK of the imidazoles in 1 equalled 5.5 while those of 2 were 5.8. Again, a positive deviation of rate versus $alpha_{im}$ was seen at alpha values greater than 0.8. The activity of 1 is approximately the same as that of the small molecule model compound histamine. The activity of 2 is approximately 5 times greater. If this increased activity is due to interaction with the hydroxymethyl group, then the polymer provides an interesting model for imidazole-hydroxymethyl interaction, an interaction important in enzymes such as chymotrypsin and trypsin, but little studied in synthetic systems with the exception of work of Overberger with

polyvinylimidazole-polyvinylalcohol.[247]

Regarding stereoselectivity, 2 is slightly more active towards L-enantiomers of amino acid esters (by 35%) than D enantiomers. This specificity is not observed in dipeptides, and therefore may be due to the helicity or handedness of the polymers.

Polyethyleneimine

Polyethyleneimine is a highly branched homopolymer synthesized by acid-catalyzed polymerization of aziridine (ethyleneamine).[257] Primary, secondary, and tertiary amines are present in a relative ratio of 0.25: 0.5:.25.[258] It is the only branched polymer used as a matrix for catalysis. The molecular weight of the polymer used in this research is 60,000 (1,400 monomers, $-CH_2-CH_2-NH$),although other sizes are also available. A portion of the polymer is schematically represented below.



a representative portion of polyethylenimine (PEI)

The polymer shows a peculiar potentiometric titration curve extending over a wide pH range.[259] Even at pH 2.0, only 70% of the amines protonate. Unlike polylysine or PAA, the titration does not reduce to the monomeric type at high salt concentrations. As with other polycations, an increase in solution pH is observed with increasing salt concentration. The average amine pK is 8.45 at high salt concentrations, 8.27 at 0.02 M KCl, and 7.47 at 0 M KCl. This depression relative to small molecule

monoamines of either the primary, secondary, or tertiary type has been attributed to nearest neighbor effects. Oligomeric amines also often have reduced pK_{NH}[260] $\Delta pK/\Delta\alpha$ is insensitive to salt concentration, however, as a consequence of PEI's compact conformation.[259]

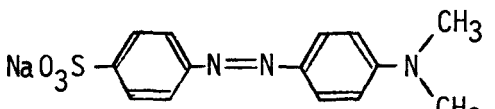
Colloidal titration of branched PEI with polyvinyl alcohol sulfate in the pH range of 3 to 11 generates a plot of alpha versus pH which is unlike the potentiometric titration curve.[261] The latter is smooth whereas the former shows three plateaus, one in the pH range of 3 to 5, the second from 7.5 to 8.5, and third from 10.5 up. This has been ascribed to primary, secondary, and tertiary amine dissociation, respectively, although only a small fraction (3 out of 25%) of the tertiary amines could be titrated presumably because of steric hindrance.

Counterion condensation by PEI differs from that by PAA and polyvinyl amine.[205] The extent to which the linear polymers effectively remove counterions from the bulk solution is dependent on the local charge density but not on nearest neighbor interactions or the macromolecular configuration. Branched PEI, on the other hand, effectively removes a larger fraction of counterions at the same polyion concentration and degree of dissociation. In PEI, the macromolecular structure enhances the degree of counterion binding. PAA and PMA show no such dependence, despite the fact that the latter takes on a compact conformation at alpha values less than 0.2.[210] Polystyrene sulfonate counterion binding is also unaffected by slight polyion cross-linking.[262]

Compared to a number of other polymers including PVA $\{CH_2\text{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}\}$,

Polymethyl ether $(\!(CH_2\text{-}CH\ (OCH_3))\!)_n$, PAA, PMAA, and PV Pyrrolidinone, PEI is highly hydrated as reflected in an unexpectedly large solute contribution to the complex permittivity.[263] This has been suggested to be due to the diffuse axial motion of "bound" and no doubt hydrated OH^- ions.

Binding capacity is an important prerequisite to design of a synthetic macromolecular catalyst. It is at this point that so many polymers and other synthetic models fail.[210] PEI even when unmodified compares favorably to the general binding protein of serum bovine serum albumin in uptake of methyl orange (sodium p-dimethyl-aminophenyl azobenzene-p-sulfonate), an aromatic anionic dye.[264]



methyl orange

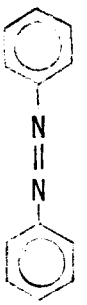
When a fraction of the primary amines of PEI are acylated with hydrophobes, the polymer's ability to bind the small anionic dye is enhanced substantially. Substitutions need not be extensive, and substantial increases are seen when a mere 2 to 10% carbobenzoxytyrosine are placed on primary amines. About 10% butyryl to lauroyl derivatized PEI show increasing hydrophobe content. Lauroyl PEI compares favorably to BSA at 1/200 the concentration despite the fact that 40% of BSA's amino acids are classified as hydrophobic.[264,265]

PEI or hydrophobe-PEI binding is a highly concertive process and in fact the underivatized polymer exceeds substituted PEI in extent of cooperativity despite its lower affinity.[267]

Binding studies on PEI were elaborated by studying binding of smaller

molecule anions and neutral molecules.[269] p-nitrophenolate, azobenzene, and nitroaniline were compared to methyl orange. Their structures are shown below.

Nitrophenolate



Azobenzene

Nitroaniline

The polymer charge properties were also modified by acylation (for partial neutralization) or introduction of carboxylic acid residues simultaneous with hydrophobe introduction onto PEI primary and secondary amines. The neutral azobenzene and nitroaniline are bound with 4-fold higher affinity by C_{12}- PEI than by BSA. PNP and paranitroaniline are both bound by lauryl PEI to a significantly lesser extent than are methyl orange or azobenzene, indicating the importance of hydrophobicity to affinity. The azobenzene molecule is 1/30 as well bound as methyl orange. Since these two molecules differ only in the presence or absence of a sulfonate group, an estimated 2 to 3 kcal/mol per charge unit has been ascribed to electrostatics. This is a larger $\Delta\Delta G_b/\text{-}CH_2\text{-}$ than reported for micelles.[266]

50% acetylation of lauryl PEI reduces its affinity for methyl orange. The simultaneous introduction of hydrophobe and carboxylate is accomplished by reacting PEI with dodecyl succinic anhydride, this also reducing the polymer's positive charge density. Yet modification by either of these routes is not sufficiently deleterious to reduce the polymer's binding

capacity to the level of BSA.[266]

Immobilized PEI used for ion exchange chromatography compared favorably to other polyethylene polyamines in selectively adsorbing anions such as chromate or metallic cations such as Fe^{+3} and Cu^{+2} .[267]

An absorption spectrum of laurylated PEI-dye solutions reveals incorporation of the dye into hydrophobic environments when bound up to the extent of 100 molecules of methyl orange per 10^5 gram of polymer. This is characterized by a maximal absorption at 420 nm as opposed to 470 nm in water or in the presence of PVPyrrolidinone or polylysine. When the amount bound reaches or exceeds 150 molecules of methyl orange per 10^5 gram of laurylated PEI, a new absorption peak is seen, with a maximum at 375 nm, which was ascribed to stacked dye molecules. It is also at this point that further binding is found to be cooperative.[264]

Shifts in fluorine NMR are sensitive to the local environment. In an effort to understand the structure of alkylated PEI, 5.5% of the amines were derivatized with 10,10,10-trifluorodecanoyl PNP.[268] The binding properties towards methyl orange are intermediate between hexyl and lauryl PEI, indicating that the slightly fluorinated polymer is not substantially different from aliphatic derivatives. Two peaks were observed in the ^{19}F NMR spectrum. Neither is sufficiently downfield to be considered in an environment which is PEI core-like or water-depleted. The more downfield of the two is in a micelle-like environment and the other is well exposed to solvent.

Klotz has recently probed by ESR spin labelling and fluorescence the dynamics of microenvironments in PEI, 10-24% lauryl PEI, and quaternized

laurylated PEI. PEI's effects are compared to those of a micelle, CTACl.[269] Spin labels (pyrene butyric acid) were studied when noncovalently bound and when covalently fixed to primary and secondary amines. In the secondary substituted polymer a bifunctional nitroxide radical was also used which cross-linked the polymer slightly. Covalency of attachment proved necessary for induction of changes in the ESR properties of the nitroxide radical. Its fixation onto 25% lauryl PEI but not native PEI caused an increase in the rotational correlation time of a fraction of the nitroxides from 10^{-10} seconds to 10^{-9} seconds. Proteins increase it to 10^{-8} seconds. Since some radicals are unaffected by the polymer, the results again indicate the presence of two microenvironments on the derivatized polymer, one solvent-like and one more highly restricted and presumably hydrocarbon-enriched. Quaternization reduces the fractional content of the latter as a result of electrostatic repulsion. Changes in the percent lauryl groups from 5 to 50 smoothly affect spin label properties, indicating that hydrocarbon core formation is not a cooperative process. The fraction of nitroxide radical in the polymer microenvironment is greater for the primary substituted PEI than for the cross-linked one.

The binding of the fluorophore 4-(1-pyrene) butyric acid to quaternized laurylated PEI occurs at a hydrophobe concentration two orders of magnitude lower than that required in micellar solutions and is complete at an alkyl to probe ratio of 5, compared to 20 for CTACl.[269]

Reactions of Polyethyleneimine

Decarboxylations and esterolyses are subject to the presence of nucleophiles. These may be primary, secondary amines, hydroxyl, or other nucleophiles. The primary amines of PEI participate covalently in sulfate,[270] phosphate,[271] and carboxylic ester hydrolysis[272] and in disulfide cleavage.[273] Secondary amines are inert to phosphorolysis[274], but covalently catalyze sulfate and carboxylic esterolyses, though at a slower rate.[275] Both primary and secondary amines covalently participate in alpha decarboxylations and aldol condensations. Tertiary as well as secondary and primary amines may serve as general base catalysts in reactions of the hydroxyl ion, water molecule, or even polymer functional groups. The efficiency of base catalysis is not enough to rapidly regenerate the covalently modified amido-sulfate, phosphate, or acyl - derivatized amines of PEI. Therefore primary and secondary amines are frequently inactivated when the polymer is to be used for catalysis of esterolyses as opposed to decarboxylation/aldol condensation reactions.

Selective modification of primary amines may be accomplished by reducing the Schiff base which they alone form with acetone to make N-isopropyl amines, which are somewhat sterically hindered amines.[276] The secondary amines are sufficiently unreactive to participate appreciably in covalent capacity over the time course often studied. Both primary and secondary amines may be converted to the tertiary state by reduction of the Schiff base which they form with formaldehyde.[277] Quaternization also inactivates the amines and simultaneously fixes the polymer's positive charge. Quaternizations are usually done with methyl iodide or methyl bromide and less frequently with the ethyl halides.[278] Dimethyl sulfate has also been

used.[279]

Amines may also be reacted with long chain alkyl halides. Most PEI catalysts contain such hydrophobes. If substitution is less than 25%, there remain some primary amines. Modification of PEI with hydrophobes alone proceeds to about 50% amine. While such extensive substitution may increase its catalytic ability, the difficulties encountered in solubility of the polymer may outweigh the advantages.[280] Optimization of hydrophobe content appears to vary with the reaction catalyzed.[281]

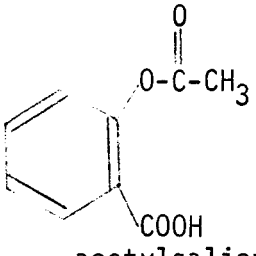
As with micelle catalysis, if the reaction is to be catalyzed by a nucleophile other than the hydroxyl ion or polymer amines, it may be covalently attached to the polymer or simply may be added to the solution as a free, cosolubilized reactant. In the latter case, electrostatics or hydrophobicity may drive their association.

Towards carboxylic esters and alkyl halides, primary aliphatic amines are more reactive than secondary amines,[275] and tertiary amines are least reactive of all. This ordering of reactivity is retained in PEI despite the amine-amine activation characteristic of both oligo- and polyamines.[282] Despite reduction in pK, nucleophilicity is retained. Amine-amine activation has been reported to range from 20 to 200 fold in acylations,[283] and this has been attributed to intermolecular general base catalysis.[284] Klotz, however, reported a mere four fold rate enhancement of PEI amines in branched, unmodified PEI relative to propyl amine in n-acylation by PNPA.[272]

Using linear, benzoylated PEI, Pshezhetskii et al studied PNP-carboxylic esterolysis by polymer amines.[285] Partially alkylated (C_4-C_{16}) PEI showed rapid acylation rates due to both pK perturbations stabilizing

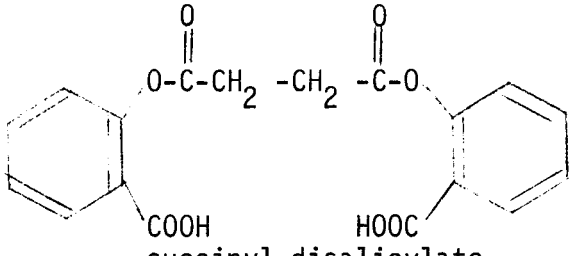
the unprotonated, nucleophilic form and to increased binding through hydrophobic binding, which increases with increasing radical size in polymer and substrate/solubilizate. Relative to small molecule amines, PEI shows 10^4-10^7 enhanced reactivity towards long chain esters.[286] For low molecular weight amines, the rate decreases with increasing substrate size. Both the small molecule and the PEI reactions have the same temperature dependence and therefore energy of activation, indicating that the positive contribution of binding contributed pre-exponentially with no essential differences in the elementary steps of the two reactions. The acylated amines are too stable to deacylate in the absence of catalysts.[272] Unreacted primary amines on substituted branched PEI show a 50 fold enhancement in acylation by $PNPC_5$ compared to propyl amine.

While PEI amines are more reactive than small mono- or even oligoamines towards the activated esters such as PNPA or PNPC12, it is of interest to compare the polymer's reactivity towards less labile esters. Two such esters are acetylsalicylate (aspirin) and succinyl-disalicylate (diaspirin).



acetylsalicylate

I



succinyl-disalicylate

II

For both, acylation of C12 PEI or PEI amines is more rapid, by 10^3 and 10^4-10^5 for I and II, respectively, than it is with methylamine.[287] With these

esters, alkylation of PEI with either isopropyl or lauryl groups has no effect. Deacylation is also unaffected by the presence or absence of pendant groups on the polymer.

The primary and secondary amines of linear, 10% benzylated PEI catalyze cleavage of the disulfide bond in Ellman's reagent, 5,5' dithiobis (2-nitrobenzoic acid) via nucleophilic attack, forming a protonated sulfenamide intermediate.[273] Its deprotonation is necessary for regeneration of the polymer. In the presence of 10% benzyl PEI 10^6 fold rate enhancements are achieved over that of small molecule amines of comparable pK; this corresponds to a first order rate constant with 26M effective molar concentration. The pH of the solution was 8.8, where more than 90% of the amines are present in the basic, reactive form. At this pH, Ellman's reagent has two negative charges. One to two amino groups per 800 were sulfenated with an estimated 1 to 2% of the benzyl groups near these reactive amines contributing to binding and/or catalysis. The Km for Ellman's reagent was found to be 11 M, compared to 1 M for papain and chymotrypsin.[288] The authors note that amine attack on disulfides entails formation of a zwitterionic transition state from neutral molecules; its formation is not favored in hydrophobic environments. The Bronsted plot indicates that in the transition state the bond between S and N is half complete.

In the decarboxylation of oxaloacetate (OAA), octyl-PEI compared favorably to poly-L-lysine or PEI.[289] Poly-L-lysine has the highest charge density due to the high pK values of its amines, but the k_2 of the reaction is slow. Although the transition state is believed to have one negative charge as does the OAA, there is charge dispersion in the transition

state, favored by a decrease in dielectric constant.(P.10) The solvent effect is not expected to be as dramatic as for reactions in which transition state formation entails charge destruction, however. However, dielectric effects have been offered to explain the positive contribution of the octyl groups to the observed rate. A second decarboxylation reaction study by Klotz and Scarpa is that of (N-phenylcyano acetate). The maximal aqueous rate enhancement was 1300 fold over the spontaneous rate. The maximum reported for this reaction in the presence of micelles is 100 fold.[290]

The same reaction was then carried out in increasing DMSO/H_2O solutions. Eventually, as the DMSO content reached and exceeded 70%, PEI surpassed C_8PEI in decarboxylation catalysis. This is due in part to amine activation by the dipolar aprotic DMSO further polarizing N-H bonds and in part to charge fixation in C_8PEI which may be deleterious to the charge-dispersed transition state in DMSO.[289]

Anilide amidolysis only occurs with hydroxide or the water molecule. Therefore, the polymer amines do not interfere. Hydrolysis of the anionic carboxytrifluoroacetanilide shows a change in rate-limiting step from tetrahedral breakdown to tetrahedral formation upon addition of C_{12}-PEI to the solution.[16] This was not observed with exhaustively quaternized PEI derivatives suggesting that the polymer amines serve as general bases unless quaternized. k_2 is pH independent. The observed rates were up to 180-fold greater than the spontaneous one.

Partially benzoylated PEI has been reported to manifest Michaelis-Menten kinetics with a large number of substrates: PNPA, $PNPC_4$, $PNPC_5$, PNacetanilide, and PNbutyranilide.[291] The proposed mechanism is the direct

attack of hydroxide ion onto the ester/anilide assisted by general base catalysis from polymer tertiary amines. The polymer displays 10^5 better hydrolysis of $PNPC_5$ than does the small molecule model dimethylbenzylamine. The relative reactivities of acetate versus caproate esters is $2X10^5$, and it is concluded that the binding energy supplies nearly if not all the energy used to reduce the free energy of activation for longer esters. The difference in reactivity of the same series in the presence of CTABr-tetradecanoyl L-his is only 10. Benzoylated PEI is therefore intermediate between cationic micelles and chymotrypsin in the $\Delta\Delta G^{\ddagger}/\text{-}CH_2\text{-}$. The intrinsic viscosity of the polymer, as determined by sedimentation coefficients is decreased by the addition of substrates with greater than 3 carbons in their alkoxyl portions. The condensed conformation decreases K_m. However, the desorption rate becomes slow. n -cetyl acid is inhibitory in a competitive manner.

By alkylating with lauryl or hexadecyl and then exhaustively methylating the polymer, the kinetics of exogenously added nucleophiles may be studied without the interference of covalent participation by the polymer. Salicylaldoxime, a synthetic alpha nucleophile, was added to hydrolyze PNPA, PNPC, PNPL, and the anionic NABA. In the presence of lauryl -PEI, the maximum rate of PNPL hydrolysis exceeds the micellar one by a factor of 17 and is obtained at one-twentieth of the total lauryl concentration.[292] The polymer reaction does not require a certain "critical" catalyst concentration, but sets in as soon as it is added to the solution. The reaction is highly sensitive to salt; addition of 0.007M KCl decreased k_2 in half. This marked sensitivity to ionic strength contrasts with the

decarboxylation reaction, where the 10^{-3}M salt introduced as polyion with counterion did not significantly change the ionic strength of the buffer, which was 0.05M.

Lauryl hydroxamate esterolysis of PNPCarboxylates was studied in the presence of per-tertiary PEI that had been partially quaternized with C_{18} groups.[293] Addition of the hydrophobe-PEI^+ increases the hydroxamate-PNPA reaction 200 fold over that of PEI^+, which is essentially ineffective. Partial, rather than complete quaternization retains some tertiary amines. Their participation as general base catalysts was reported to catalyze deacylation by OH^- by a factor of 10.

Sulfate esters, even activated aryl ones, are generally not subject to polymer catalysis. PEI and its derivatives are an exception to this generalization. Sulfate esters of NO_2-catechol, NO_2-phenol, and dinitrophenol have been shown to be cleaved by water in the presence of 10-15% C12-PEI.[270,294] The polymer amines may simply be alkylated (preferentially occurring on primary ones) or first modified by reductive formylation, bringing all primary and secondary amines to the tertiary state, followed by alkylation with long chain hydrophobes (either C12 or C18). The second polymer was also 63% quaternized with methyl halide.

Kiefer reported 3.6×10^{12} rate accelerations in the esterolysis of PNPS by 20% C12 15% methylimidazole PEI compared to imidazole.[270] Kunitake failed to reproduce this rate enhancement with PNPS or NCS, reporting that the presence or absence of 14% imidazole on primary amines of 20% quaternized PEI had no effect on its catalytic ability. However, the DNPS substrate was cleaved 10^7 better than in the absence of polymer.[294] Kunitake observed that

8% C 18 PEI undergoes amidosulfation to the extent of 30% total amine content and that this covalent intermediate undergoes a slow turnover without prior complexation. The quaternized polymer, however, shows a saturation type of kinetics. The K_m increases with pH but so also does the k_2. The two effects balance to give a bell shaped pH-rate profile with a maximum at pH 8.0. This maximum is also observed in the hydrolysis of NABA, although in this reaction the nonquaternized, 25% C12 PEI exceeded the quaternized one in catalytic ability.

In the presence of a derivative of PEI in which 15% of its amines are substituted with methylene imidazole and the remaining primary amines (10%) with dodecyl halide, PNPA cleavage proceeds at 45 $M^{-1}sec^{-1}$ (pH 7.4).[295] This is more than 100 times faster than the reaction with imidazole. The acetyl imidazole polymer was detectable spectroscopically[296]; its decomposition occurred at a rate of 1 to 6x 10^{-3} sec^{-1}, which is ten times more rapid than the hydrolysis of acetyl imidazole.

PEI laurylated and reacted with ethylene sulfide to give a sulfhydryl PEI showed 100 fold increased activity towards PNPcaproate relative to small molecule thiols.[297] The total percent lauryl varied from 1-10% and the sulfhydryl content was 2.5% total amine, presumably primary amines. Even PEI with the nucleophile but without hydrophobes was reported to have 50% the activity of hydrophobe, sulfhydryl PEI. Activities were measured at pH 7.0 and 9.0 However, the acylated nucleophile was stable and did not undergo deacylation in the absence of polymer primary amines. The need for a general base catalyst was cited to facilitate acyl-S bond breaking.

Nuclear Magnetic Resonance of PEI

The proton NMR spectrum of branched PEI is unresolved and methylene hydrogens appear as a single rather sharp peak at2.3 ppm from TMS. The spectrum is rather independent of solvent, be it D_2O, $CDCl_3$, or d_6-DMSO. Amine protons are readily exchangeable by polymer dissolution in and lyophilization from D_2O (see figure 1).

The carbon-13 NMR spectrum of PEI may be most readily understood in terms of diads (-N-CH_2-CH_2-N-). Comparing the spectrum to that of linear PEI and oligoamines, tentative peak assignments of the methylene carbons were made by Lukovkin.[298] The deshielding by nitrogen atoms of methylene carbons to which they are located alpha decreases from tertiary to secondary to primary amine. Nitrogen deshielding of carbons to which they are beta increases with decreasing substitution on the nitrogen atom (1°>2°>3°). Thus, the three peaks most downfield at 71.5, 74.3, and 75.8 ppm correspond to carbons alpha to tertiary amines and beta to primary, secondary, and tertiary amines, respectively. The next three peaks upfield, located at 76.9, 79.9, and 81.4 ppm correspond to carbons alpha to tertiary amines and beta to primary, secondary, and tertiary amines, respectively. Finally, the two most upfield peaks (87.0 and 89.2) are assigned to carbons on primary amines and beta to secondary and tertiary amines, respectively. Peak assignments and chemical shifts are in good agreement with those later made by St. Pierre and Geckle.[299] The third and fourth peaks overlap substantially. Protonation of an amine nitrogen causes an upfield shift of

2 to 6 ppm in carbons to which it is beta and a one ppm upward or downward shift in alpha carbons.[300]

The nitrogen-15 NMR spectra of branched and linear PEI were published by Kricheldorf.[301] The linear polymer in alkaline solution has a single peak for primary amines and a signal for secondary amines which is partially resolved into two peaks. The branched polymer shows three broad signals (1°, 2°, 3° nitrogens) with no fine structure in acid conditions. In alkaline solution, the secondary amines are partially resolved into two peaks and the tertiary into three broad peaks. Thus, there are a total of six signals, which includes a single sharp primary nitrogen signal. Theoretically, there are nineteen possible nitrogen types, depending on the order (1°, 2°, 3°,) of gamma nitrogens. That there appear only six is due in part to insufficient resolution and in part to low percentages of some possible types, such as tertiary amine surrounded by tertiary nitrogens or secondary amine by strictly secondary amines.

Purpose

The objectives of this research are enumerated below:

1. To determine if multiple and complex synthetic steps can be performed on the polymer.
2. To develop exact analytical techniques for identification of synthetic intermediates and characterization of the final products in as definitive a manner as possible.
3. To introduce reporter groups in various microenvironments of the polymer, testing pK perturbation and attempting to monitor hydrophobicity with fluorescence and absorption spectroscopy.

4. To introduce the sulfhydryl and other nucleophiles onto nonsurface amines. These derivatives will thus be available for use as catalysts.

Previous modification of branched PEI with hydrophobes or catalytic groups had been onto primary amines or had involved their indiscriminant placement onto modified (i.e. pertertiary or partly quaternized) polymer amines. The spectral shifts reported for dye molecules in the presence of PEI derivatives did not involve covalent attachment of them onto the polymer, making it impossible to draw conclusions on their exact location.[264]

Synthetic Strategy

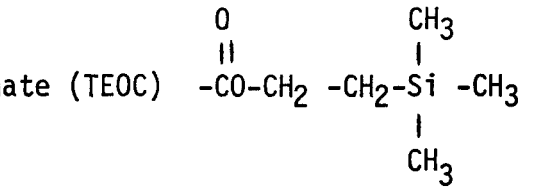
The following outlines the synthetic sequence employed most frequently in this work:

1. Blocking of primary and secondary amines with appropriate principle and differential blocking groups.
2. Alkylation of tertiary amines
3. Removal of the principle blocking group
4. Inactivation of these regenerated primary and secondary amines
5. Deblockage of the differentially blocked amines
6. Introduction of the nucleophile

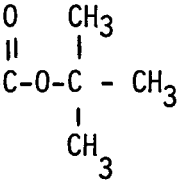
The role of the principle blocking group is to avoid substituting onto primary amines and a large percentage of the secondary amines. Any of the three blocking groups shown below could be the principle one used.

Trifluoroacetyl (TFA) $-\overset{\overset{O}{\|}}{C}-CF_3$

2-(Trimethylsilyl) ethyl carbonate (TEOC) (Fluka Chemicals)



tert-Butyloxy carbonate (t-boc) (Fluka Chemicals)



The trifluoroacetyl group is susceptable to gentle base or strong acid, neither of which attack the polymer.[302] The trimethyl silyl[303] and t-boc groups[304] are more susceptible to acid, such as trifluoroacetic acid, but are stable to base. The amido-TEOC bond is specifically cleaved by "naked" fluoride (tetramethyl ammonium fluoride).[303] The utility of a blocking group depends on how reactive it leaves nonacylated amines to substitution, especially in the quaternization of tertiary amines with hydrophobes.

One blocking group other than the principle one is reacted with the small fraction of remaining secondary amines; this is the site of nucleophile addition. This differential blocking group must be stable under conditions which remove the principle one, since these amines are regenerated subsequent to polymer amine inactivation. (See p.105) After both blocking groups have been reacted with the polymer, tertiary amines are reacted with the binding hydrophobe dodecyl iodide by quaternization in the presence of a sterically hindered base, diisopropylethylamine. Acetonitrile and nitromethane are especially suitable due to their high dielectric constants, which favor the Menschutkin reaction. Should the halide not be sufficiently reactive, a more active alkylating reagent, alkyl trifluoro methane

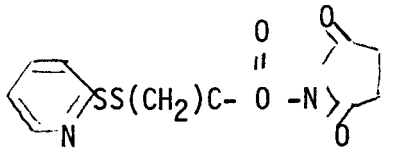
sulfonate, could have been utilized.[306] Its structure is shown below.

$$CF_3SO_2\text{-}OR$$

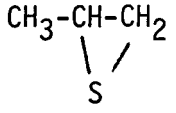
Reporter groups are also covalently attached by alkylation. Therefore, they may be added to primary, secondary, or tertiary amines. Following alkylation, the TFA group is removed with base.

The polymer's primary and secondary amines interfere with kinetics as mentioned previously and therefore they must be inactivated. Frequently this is done by exhaustively methylating the polymer. This modification also fixes and increases the local positive charge density to stabilize anionic forms of nucleophiles. However, quaternization also inactivates most nucleophiles including the sulphydryl group and imidazole. Since blocking groups are available for both primary and secondary amines, but not tertiary ones, nucleophile placement onto secondary amines is the best achievable route towards substitution of imidazole and mercaptans onto the nonsurface regions of the polymer. By differentially blocking a fraction of the secondary amines and later regenerating them subsequent to polyamine inactivation, functional groups may be kept active.

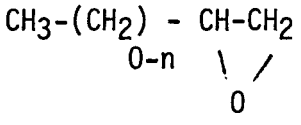
The marcapto group is our nucleophile of interest. It is a strong nucleophile for its basicity but remains little studied in model systems due its tendency to oxidize. It may be added as a final step after differentially blocked secondary amines are regenerated. SPDP, whose structure is shown below, is one agent for introducing the nucleophile onto the polymer.[310] Reducing agents and disulfide exchange reagents convert the disulfide into a free sulfhydryl.



Propylene sulfide also reacts with secondary amines as well as primary ones and is an alternative reagent for nucleophile placement.[297]



A third route for introducing the mercapto group is by conversion of it from an alcohol using a tosylating agent (phenylmethyl sulfonyl fluoride) and thioacetic acid (CH_3COSH).[310] The alcohol may be introduced onto the polymer with a 1,2-expoxide group in a reaction which is analogous to that of propylene sulfide. Long chain aliphatic epoxides are available. This permits simultaneous introduction of a hydrophobe, and such a hydrophobe is located in situ as a part of the "active site".



Chemical and Physical Analysis

Unfortunately, trifluoroacetylation is not readily followed by proton NMR, the principle analytical tool used in this work, since the group contains no hydrogens. Carbon-13 NMR is subject to nuclear Overhauser effects, precluding integration for quantitation of percent trifluoroacetylation. It is qualitatively useful, particularly in that through it one can

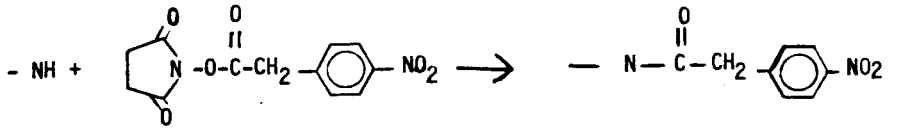
determine whether primary or secondary amines have been reacted with TEOC-O-Np or dit-butyl dicarbonate. Both the TEOC and t-boc groups are visible in proton NMR, each possessing nine or more protons per group upfield from the polymer backbone.

Chemical modification is an alternate analytical tool although great concern has been expressed as to the extent to which modification reactions proceed in polymers. Nonetheless, if different highly reactive modifying reagents are compared on a single polymer prep, there is greater confidence regarding degree of modification. The choice of modifying reagent entails the following considerations:

1. high reactivity towards primary and secondary amines
2. the test may be done in nonaqueous solvents since the blocked polymers are not water-soluble
3. the reagent can be quantitated easily and with high accuracy
4. the reagent does not undergo transacetylation with the blocking group

The second blocking group used on the polymer to protect the site of nucleophile addition may itself serve as a reagent for quantitating the amount of secondaries not blocked by the principle group. Both the TEOC and t-boc group are readily integratable by PMR. Also available is a reagent used in HPLC for labelling primary and secondary amines with a UV probe. This reagent is reacted in THF, an organic solvent in which all three blocked PEI derivatives have been found to be soluble.[305] Its reaction is shown below.

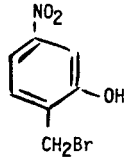
n-succinimidyl p-nitrophenyl acetate (SNPA)

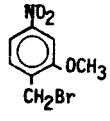


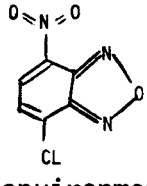
By reacting a single "blocked" polymer with a number of these reagents stepwise, their relative reactivity towards the derivatized polymer may be directly compared. In fact, this has been the means whereby I have been able to follow reactions on trifluoroacetylated polymers.

Another possibility to assessing degree of blockage is to quantitate the amount of amide groups, such as ^1H content or N-H stretching. The primary amines retain one proton each but amide protons are absent from modified secondary amines. Therefore, the N-H group should still be present in spectra of ^1H-NMR or FT-IR, though reduced by two-thirds relative to backbone signals. However, the amide peak in PMR is very broad and inconstant in size and shift. The FT-IR spectrum's N-H band is also broad and is highly subject to interference by bound water, a contaminant which is difficult to eliminate completely from spectral samples.

The polymer products described herein are compared to previously synthesized, primary derivatized ones by substituting amines of preference with Koshland I and II as well as with the fluorescence probe NBD-Cl.







These covalent probes together give information on pK and environmental polarity. The ionizable Koshland I has three possible absorption maxima. If ionized to form the phenolate, a maximum of either 405 or 395 nm is seen. When on a protonated amine (ammonium nitrogen), the reporter group

shows a maximum at 395 nm with an extinction coefficient of 18,000. As this amine of attachment deprotonates, the maximum shifts to 405 nm and its extinction coefficient at this wavelength is 20,000. The unionized phenol absorbs in the ultraviolet range, 310 nm, with an extinction coefficient of 10,000.[307]

Koshland II is not ionizable since the probe has been O-methylated. When transferred from hexane to DMSO, the wavelength of maximum absorption shifts from 288 to 317 nm. The extinction coefficient varies slightly but is about 10,000. Solvents of intermediate polarity show increasing wavelength of maximal absorption as their dielectric increases.[308]

The emission maximum of NBD-derivatized amines is also sensitive to the polarity of the solvent. Lancet and Pecht studied the emission maxima in numerous solvents and plotted these against the dielectric constant.[309] Their study also included fluorescence results obtained after substitution of NBD onto immunoglobulin proteins.

MATERIALS

Polyethylenimine 600 was provided by Dow Chemicals (Midland, Mn.). Trifluoroacetic anhydride and methyl trifluoroacetate were obtained from Alpha chemicals (Danover, MA.). Acetic anhydride was purchased from Mallinckrodt chemical works (St. Louis, MO.). Di-tert-butyloxy dicarbonate and 2-trimethylsilyl) ethyl carbonate, p-nitrophenyl ester (TEOC-O-Np) were obtained from Fluka A.G. (Buchs SG, Switzerland). The alkyl iodides were purchased from Eastman (Rochester, N.Y.). Dodecyl benzyl chloride was from Stauffer chemicals (Westport, CT.). Styrene, propylene, and decyl epoxides, propylene sulfide, diisopropylethylamine (DIEA), Koshland reagents I and II, thioacetic acid, tetrabutylammonium fluoride, and deuterated solvents were purchased from Aldrich (Milwaukee, WI.). N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) was obtained from Pharmacia Fine Chemicals A.B. (Uppsala, Sweden). Nitrobenzoxadiazole (NBD-Cl), N-succinimidyl p-nitrophenylacetate (SNPA), and n-methyl bis trifluoroacetate (MB-TFA) were from Pierce (Rockford, IL.). Piperidine, phenylmethyl sulfonyl fluoride (PMSF), 5,5' dithiobis-(2nitrobenzoic acid) DTNB), sodium arsenate, Dowex-1 X8-200, and Sephadex LH-20 were from Sigma Chemical Company (St. Louis, MO.). 2-(N-morpholino) ethane sulfonic acid (MES) was purchased from Calbiochem-Behring Corp. (La Jolla, CA.). Organic solvents were dried over 3,5, and 13 A molecular sieves purchased from Davison Chemicals (Baltimore, MD.). Thin layer chromatographic plates were from Whatman, Ltd. (Clifton, N.Y.) and Analtech (Newark, Delaware).

Methods

Preparation of Trifluoroacetyl PEI

For synthesis of 100mM of trifluoroacetyl PEI (TFA-PEI) (MW=116), 100ml of ethanolic stock solution of Cordova or Dow PEI 600 is rotoevaporated 1.5 hours at 45C redissolved in chloroform (Aldrich), and twice rotoevaporated to remove any small amounts of water. The dried solution is then dissolved in sufficient chloroform to bring the final volume to 100 ml. To this is added 75% total amine (75mM)* methyl trifluoroacete. The solution is shaken at room temperature (23°) for 24 hours. The polymer appears flocculent since it is insoluble in chloroform when derivatized. An additional 75% methyl trifluoroacetate is added after vacuum drying and then dissolving in 100 ml acetonitrile. Again the solution is shaken overnight at room temperature. At this point, approximately 10-25% of the total amines remain unblocked (they could be 1° or 2°). To further react the polymer the acetonitrile solution is cooled to -5°C and 10% total amine (7.5mM) of the sterically hindred base, diisopropylethylamine (DIEZ) is added followed by 1.5mM trifluoroacetic anhydride, also cooled to -15C. The solution is kept at this temperature for 24 hours. It is then vacuum dried to "flake" dryness (4+ hours at 45°C). The trifluoroacetylated polymer contains approximately 6% free secondary amines. (See p. 120) It is stored as a powder in the presence of dessicant at -4C. Correction of molecular weight for the presence of diisopropylethylamine was made in subsequent modification. PEI was irreversibly discolored, probably as a consequence of amine

* Stoichiometries of reagents or polymer substituents are most easily expressed as percentages or fractional equivalents of nitrogen. Recall that one nitrogen atom is present in each monomeric unit. 25% of these are 1° amines, 50% are 2°, and 25% are 3°.

oxidation whenever reacted at >10°C with strong trifluoroacetylating agents such as trifluoroacetic anhydride or n-methyl bis TFA.

Preparation of TEOC PEI

An ethanolic solution of PEI is vacuum dried from ethanol. It is dissolved in sufficient anhydrous chloroform to bring the solution to a one monomer molar concentration. After dissolving, it is cooled to 4°C. Molecular sieve dried diisopropyl ethyl amine (100% total nitrogen) is then added followed by 70% TEOC-O-Np. The solution is stirred for thirty minutes, then brought to room temperature where stirring is continued for 24 hours. For complete blockage of the amines, an additional TEOC-O-Np is then added and again the solution is stirred at 25-30°C for 24 hours. Unreacted TEOC-O-Np may be detected by thin layer chromatography against hexane, ethyl acetate 2:1 (R_f = 0.95).

Preparation of t-boc PEI

Ethanolic PEI is vacuum dried to dryness and dissolved in chloroform to make a one molar solution. To this is added 75% total amine (stoichiometric amount) of di-t-butyl dicarbonate slowly and dropwise with continuous stirring. It is stirred uncovered for 24 hours at room temperature to allow carbon dioxide release. Preliminary work indicated that ethanol itself may be a suitable solvent. To assure completion of reaction, an additional 25% di-tert-butyl dicarbonate is added. After 24 hours of additional stirring, the polymer is rotoevaporated and stored dry at -4°C.

Preparation of Acetyl PEI

Stock ethanolic PEI is vacuum dried at 50°C then dissolved to 1 M in

anhydrous ethanol. To this is added .75 equivalent of diisopropyl ethyl amine, which has been previously dried over 3A molecular sieve. The dissolved polymer solution is cooled in an ice bath to 8°C or less. To this is added 0.75 N equivalent of acetic anhydride. The solution is stirred continuously and 5 minutes later removed from the ice. Stirring is continued overnight. The polymer is cleaned by vacuum drying from ethanol, dissolving in water, and dialyzing against water and aqueous sodium chloride.

3° alkylation of 75% acetyl PEI

The dialyzed polymer is lyophilized, weighed and dissovled in sufficient absolute ethanol to make a one molar solution. To this is added .20 N equivalent of DIEA and then .25 alkyl halide. Methyl or ethyl iodide are left to react at 25°C; dodecyl iodide and dodecyl benzyl chloride are stirred at 55°C for 48 hours.

To purify the polymer, it is concentrated and run on a (10½ X 1") LH20- ethanol column in 10 monomer millimolar quantities. The polymer is again dialyzed and lyophilized.

Differential Blocking of Partly Blocked PEI

A < 75% TEOC PEI solution is vacuum dried from chloroform and run first on an anion exchange (Dowex-C1 AGX- 8 200) column in ethanol and then, after concentrating under low pressure, on an LH 20 ethanol column. The polymer is vacuum dried from ethanol and its weight is accurately determined. Sufficient THF is added to bring the polymer to a one monomer molar concentration. To this is added 0.4 N equivalents each of DIEA and di-t-butyl dicarbonate . After 48 hours, an additional 0.4 of the latter is added. Again, the solution must be stirred while open to the atmosphere

to permit carbon dioxide release. In the spectrum to be shown, the solution had been heated at 50°C overnight, but this is not necessary to maintain a homogeneous solution.

To clean for chemical analysis, the reaction mixture is vacuum dried, then dissolved in ethanol, and run on an LH-20 ethanol column. Ethanol may then be removed by vacuum drying from ethanol, then chloroform, another solvent in which the product is soluble.

For TEOC, t-boc, or SNPA acylation of free amines of trifluoroacetyl PEI, powdered TFA PEI is weighed (10-50mM) and then dissolved in acetonitrile (for TEOC or t-boc) or THF (SNPA). To this is added .2 equivalent DIEA. The DIEA has been dried previously over 3A molecular sieve to avoid base-catalyzed hydrolysis of the blocking agent. TEOC-O-Np, di-t-butyl dicarbonate, or SNPA are then added at an approximate two-fold excess. The TEOC and t-boc mixtures are left to react 24 hours at room temperature. The SNPA solution is shaken vigorously at 23°C for 30 minutes, then heated to 40°C for 20 minutes with constant stirring.

To purify the TEOC or t-boc derivatized TFA PEI, the reaction mixture is vacuum dried and then dissolved in ethanol. DIEA and side products or unreacted blocking agents may all be removed by LH-20 ethanol chromatography. Early fractions of the polymer are free of these. Unreacted TEOC-O-Np may be detected by TLC against hexane, ethyl acetate, 2:1.

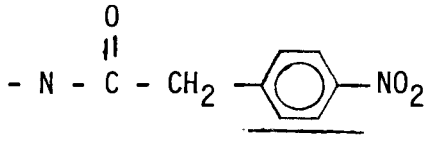
To remove the SNPA reagent, the solution mixture is vacuum dried, then dissolved in ethanol. Unreacted SNPA is relatively insoluble in ethanol. The cloudy solution is run first on Dowex-Cl (AGX8)/EtOH anion exchange column. Much of the unreacted SNPA remains at the top, and the

column is discarded after use. Polymer fractions are pooled, concentrated by vacuum drying then run on an LH20-ethanol column.

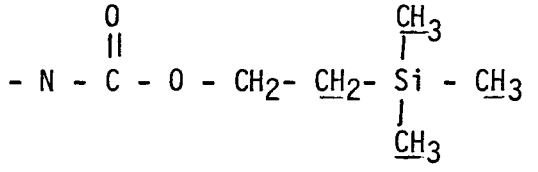
Spectral Analysis of Blocked PEI

With the exception of the trifluoroacetyl group, all of the modifying groups for primary and secondary amines mentioned above have protons whose chemical shift differ sufficiently from that of PEI to permit their quantitation by proton NMR. Underlined moieties of the groups shown below should give easily distinguishable peaks for trifluoroacetylated derivatives.

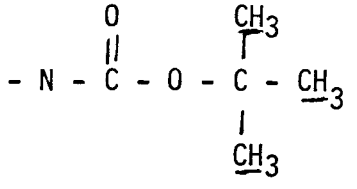
TFA-SNPA



TFA-TEOC



TFA-t-boc



Reaction of 1,2 epoxides with TFA PEI

A one monomer molar solution of TFA PEI is made in THF (nitromethane was also tested). 25% total N of DIEA is added, followed by 25% of the epoxide, either propylene oxide, styrene oxide, or decyl epoxide. For long chain alkanes, the solution is stirred with continuous heating to 55°C for 24 hours. The reaction should be run at room temperature for propylene

oxide, styrene oxide, and other smaller epoxides. To remove base and unreacted epoxode, the mixture is vacuum dried from THF, dissolved in a small amount of ethanol, and run on an LH20 column.

The reaction may be monitored by PMR, since with even the smallest of epoxides used, propylene oxide, a methyl group is present whose signal would be expected in the region of the alkanes, since no more electronegative element is attached to the carbon.

Tertiary Amine Quaternization of TFA PEI Derivatives

The polymer is dissolved as a 0.75 to 1.0 molar solution in acetonitrile. Base (DIEA) and alkyl iodide corresponding to 0.2 and 0.3 total amine, respectively, are added. The solution is heated at 55°C with stirring for 48 hours when long chain alkyl halides are used. Alkyl halides of only a few carbons need not be heated or stirred. Unreacted alkane is removed by LH20-ethanol chromatography, and, if necessary, the quaternization is repeated until a desired degree of substitution is achieved.

Unreacted dodecyl iodide or dodecyl benzyl chloride are assayed for by TLC against hexane (R_f = .75). The amount of n-alkane substitution is determined by PMR. With derivatized PEI the broadened backbone overlaps slightly with the methylene hydrogens of the aliphatic group, and the first methylene would be expected to contribute to the polymer backbone. Carbon-13 NMR is better resolved in general but not readily integrable. For a more thorough discussion of PMR of laurylated TFA PEI, see "Chemical Analysis of deblocked TFA PEI derivatives".

Base-Catalyzed Deblockage of TFA PEI Derivatives

The trifluoroacetyl group may be removed under strong acid or mild base conditions, but the TEOC- and t-boc amide bonds are subject to acid

only. Therefore, base permits differential deblockage by removing TFA groups only. PEI itself is not stable to strongly alkaline conditions but appears to be unaffected by ehtanolic piperidine at room temperature.

To deblock the trifluoroacetylated polymer, it is dissolved in 95% ethanol to a monomer concentration of 1/3 molar. To this is added 100% total amine each of base (piperidine) and deionized water. 24 hours later an additional 20+% water is added and this is repeated daily until a drop of the polymer is found to be freely soluble in water (5 or 6 days). Continuous shaking is necessary for polymers with substantial hydrophobe substitution. At this point, the solution is further diluted with water and dialyzed against 0.1 M NaCl, then pure deionized water. Extensively laurylated PEI may require acetate as a counterion, although this counterion is avoided if possible until after PMR spectra have been taken. The polymer may be concentrated by rotoevaporation at 50°C. It is then lyophilized in a preweighed bottle and stored with dissicant at -4°C.

Acid Deblockage of TFA PEI Derivatives

The weighed polymer is dissolved in 95% ethanol. To this is added enough HCl (37% solution) for 100% protonation of polymer amines. After 24 hours, the ethanol is removed by rotoevaporation and PEI is dissolved in glacial acetic acid. The solution is allowed to stand at room temperature for several days, up to one week, until the polymer is found to be freely soluble when added dropwise to water. The polymer solution is then carefully neutralized with NaOH and dialyzed against water. This removes trifluoroacetate, DIEA, and acetic acid.

Spectral Analysis of Deblocked TFA PEI

Carbon-13 NMR clearly demonstrates the presence or absence of trifluoroacetyl groups on PEI by the characteristic C=O or CF_3 peaks seen downfield. To prepare the polymer for such a spectrum, it is first passed through a Dowex AGX8 chloride anion exchange column to remove any unbound trifluoroacetate.

Removal of the trifluoroacetate blocking group and indirect evidence that laurylation was onto tertiary amines may be obtained in one step by acetylating quaternized PEI after the trifluoroacetyl groups have been removed. If hydrophobe placement is indeed onto tertiary amines, both primary and secondary amines should be available for acetylation. Indeed, two acetyl peaks must be seen in PMR when such a derivatization is performed.[275] This polymer can be compared to one in which acetylation precedes quaternization; both should give equivalent spectra.

Exhaustive Methylation of PEI Derivatives

The lyophilized polymer is dissolved in a two to one anhydrous solution of n-methyl pyrrolidinone, methanol or in anhydrous methanol and the solution is cooled to -4°C. To this is added 100% total amine of dried DIEA and then 1 equivalent methyl iodide. 8 hours later, the solution is brought to room temperature, flushed with argon, and an additional 1 equivalent CH_3I is added. The solution is shaken an additional 24 hours. The quaternized polymer is sparingly soluble in both of these solvent systems. Both base and unreacted methyl iodide are easily removed by dialysis of the product against water and an aqueous sodium chloride or acetate solution.

Spectral Analysis of Methylated PEI

The methyl groups of quaternized PEI have chemical shifts like those of the polymer ethylene units. Therefore, a second constituent whose PMR signal does not overlap and that is present both before and after methylation may serve as an internal reference. Integration of both peaks permits one to calculate the change in polymer backbone size and therefore the extent of quaternization.

Since all primary and secondary amines must be brought at least to the tertiary state in order not to react themselves, such amines may be assayed for by again lyophilizing the dialyzed polymer, dissolving in an anhydrous organic solvent, and attempting to acylate these amines with the most reactive of acylating reagents distinguishable in PMR. An alternative method would be to take an infrared spectrum. The N-H stretching band should disappear completely if the polymer is indeed completely quaternized. However, water also absorbs in this region of the spectrum and the quaternized polymer is known to be quite hygroscopic in nature.

Removal of t-boc from Secondary Amines of Quaternized PEI

The lyophilized, quaternized polymer is dissolved in a 2:1 solution of n-methylpyrrolidinone, methanol. To this is added a small amount of water, then a 3:1 excess of TFA acid over t-boc-ed amines. The solution is shaken and again dialyzed after 24 hours.

Removal of the TEOC group from Secondary Amines of Quaternized PEI Derivatives

The lyophilized polymer is quickly weighed and dissolved in HMPA, DMF (10:1) to a final monomer concentration of 0.5-0.75 M. The solution is

cooled to -4°C. Meanwhile, tetramethyl ammonium fluoride (0.3 millimole/millimole nitrogen) is diluted with a fraction of a milliliter of absolute methanol and this solution is also cooled to -4°C. Aliquots of F^- solution are slowly added to the polymer over a one to two hour period. Five to ten hours later the solution is brought to room temperature to allow carbon dioxide release. While deblockage was generally allowed to proceed overnight, complete evaporation of methanol was avoided by allowing only a small surface area to serve as the opening to the atmosphere, since the polymers were sometimes difficult to redissolve completely if permitted to dry.

Reaction of Newly Regenerated Secondary Amines with Episulfides

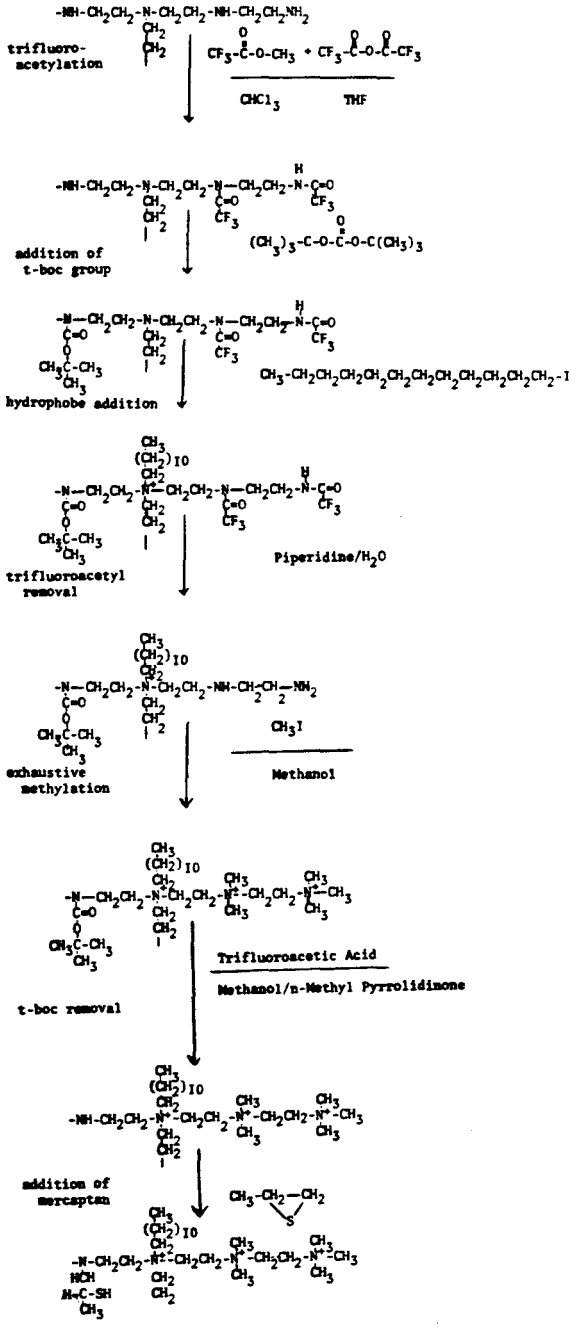
Excess methanol could be evaporated by briefly flushing the polymer solution with argon. Deionized water (3 ml minimum) is then added and the pH is brought to 7.2 with 1M NaOH. The polymer is lyophilized, then dissolved in absolute methanol and to this is added a volume of propylene sulfide corresponding to .1 equivalent N. This is stirred at 20°C for 3 to 4 hours under a slight positive pressure of argon. (Summarized in Scheme 1)

Assay of Mercaptan Content on the Polymer

Method I: DTNB reaction in presence of reducing agent and arsenate

The method of Zahler and Cleland[312] as sited by Birk and Klotz[297] was attempted. An excess of DTNB-H_2O solution (100 lambda of 40 mg/10 ml) was added to buffer blanks, reductant alone (dithioerythritol complexed for inactivation with arsenate), and polymer plus DTE-arsenate. All had a

Synthetic Scheme I



final volume of 3.0 ml. Several buffers were tested since most at a concentration of 0.02 M gave a large background absorbtion, even at neutral pH. Barbital, which is an excellent buffer for PEI, interfered least in the pH range of interest, i.e. 7-9.

Method 2: DTNB reaction on polymers followed by Paranitrothiophenol Release from PEI

The indirect method of Butterworth, Baum, and Porter[313] was modified to suit this system. 50 to 100 lambda aliquots of the thiolated polymer solution (0.6-0.8 M) are diluted in .02 M barbital, pH 8.7, and 100 lambda of stock DTNB (40 mg/10 ml H_2O) is then added. The reaction is allowed to proceed for at least ten minutes. Unreacted DTNB and released p-nitrothiophenolate are removed by dialysis against sodium acetate or sodium chloride, depending on the counterion desired. Excess salt is then removed by dialyzing against deionized water and the polymer is lyophilized in a preweighed bottle. The polymer is weighed quickly and diluted with one milliliter of water. Its absorption at 412 nm is read and subtracted from the A412 when the solution has been made alkaline (pH 11) with a drop of 1 M NaOH. Exposure to base releases the p-nitrothiophenolate previously bound to the polymer in a disulfide linkage.

Probe - Containing Polymers

Six polymer derivatives were synthesized containing the ionizable reporter group Koshland reagent I. In each case 3% total N of probe was added.

1. PEI with Koshland reagent I on tertiary amines.

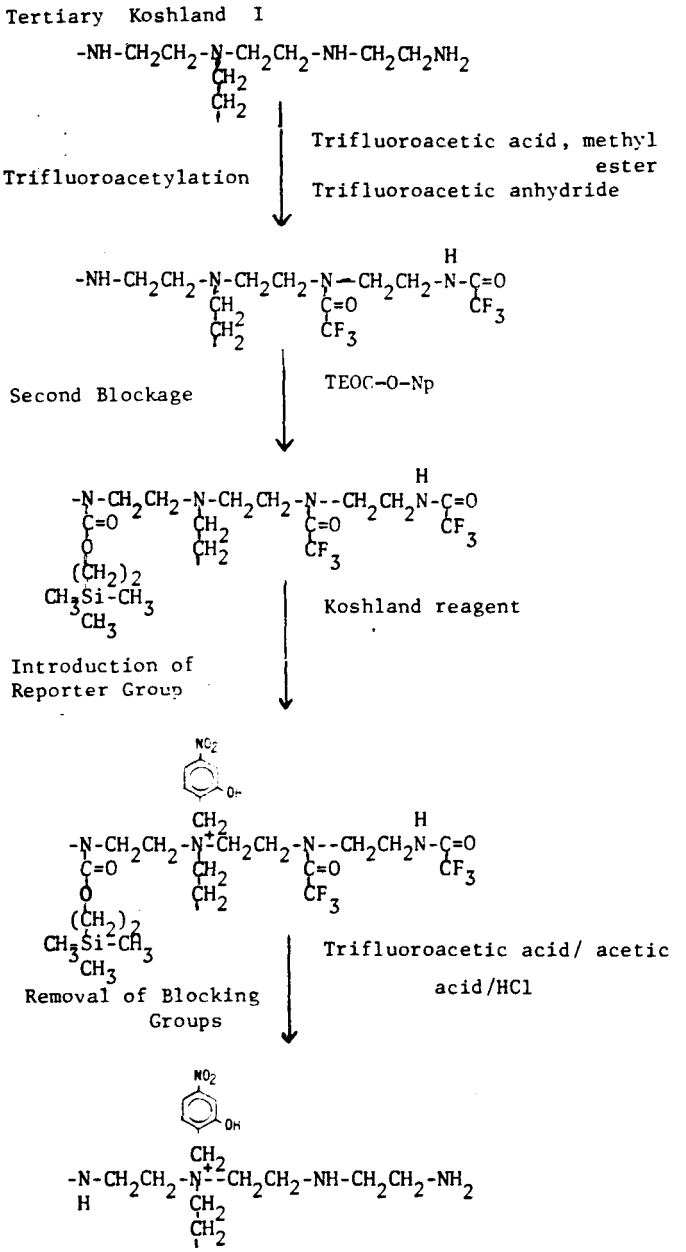
Prepared by blocking PEI with TFA (p. 95), then TEOC groups (p. 98). The polymer is then cleaned on LH-20/EtOH and Dowex-Cl/EtOH columns, dried by vacuum evaporation, dissolved to 1M in acetonitrile, and reacted with the reporter group (.05N equivalents) for 24 hours at 60°C. The polymer is then run on a Dowex-Cl/EtOH column to remove hydrolyzed and unreacted Koshland reagent I. It is then deblocked with acid (p.101) This is summarized in synthetic scheme 2.

2. PEI with Koshland reagent I on primary amines.
Prepared by drying the polymer by vacuum drying from chloroform. It is then dissolved in anhydrous ethanol to make a one monomer molar concentration. To this is added .03 N equivalents of Koshland reagent I. The reaction is allowed to continue at 20°C for 24 hours, and unbound reporter group is subsequently removed by ion exchange chromatography on a Dowex-Cl/EtOH column.

3. PEI with dodecyl benzyl groups and Koshland reagent I on tertiary amines.
Prepared by blocking PEI with TFA (p. 95), then TEOC groups (p.98), reacting with hydrophobe (p.100), then with Koshland reagent I (acetonitrile, 60°C, 24 hours as in 1 above). The polymer is purified by chromatography on a Dowex-Cl/EtOH column and an LH-20/EtOH column. It is then deblocked in acid (p.101). This is summarized in synthetic scheme 3.

4. PEI with dodecyl benzyl and Koshland reagent I on primary amines.
Prepared by dissolving PEI which has been vacuum dried from chloroform to 1M concentration in anhydrous ethanol. 0.1 N equivalent of dodecyl benzyl chloride is then reacted with the polymer at 60°C for 24 hours.

Synthetic Scheme 2



Koshland reagent I (.03 N equivalents) is then added, the reaction is allowed to proceed at 22°C for 24 hours, and the polymer is purified by ion exchange chromatography (Dowex Cl/EtOH) followed by LH-20/EtOH chromatography.

5. Quaternized PEI with dodecyl benzyl and Koshland reagent I on tertiary amines.

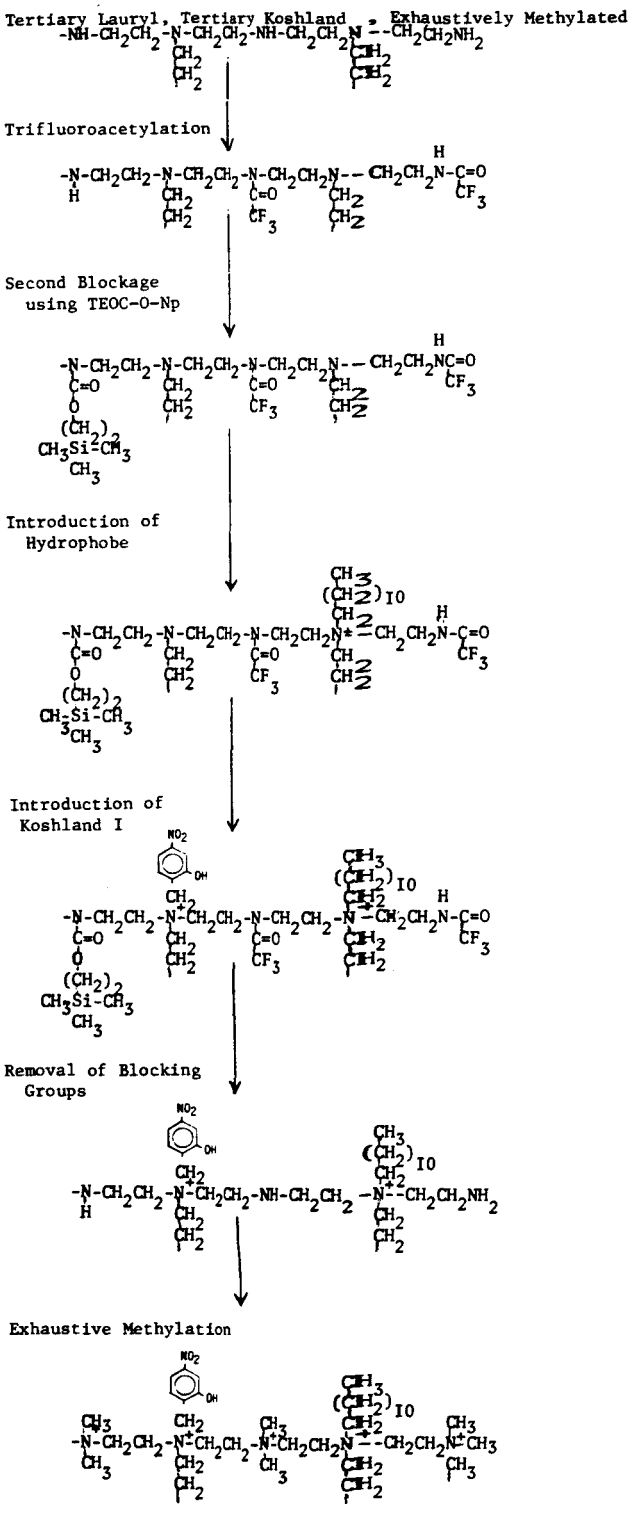
   Prepared by blocking the polymer with TFA, then TEOC groups (p.98), reacting with hydrophobe (p. 100), then with reporter group (.05 N equivalents) as in 1. Ion exchange chromatography (Dowex-Cl in EtOH) is followed by LH-20 /EtOH chromatography. The polymer is then acid deblocked (p.101) and subsequently exhaustively methylated (p.102) see synthetic scheme 3).

6. Quaternized PEI with Koshland reagent and dodecyl groups on secondary and/or tertiary amines.

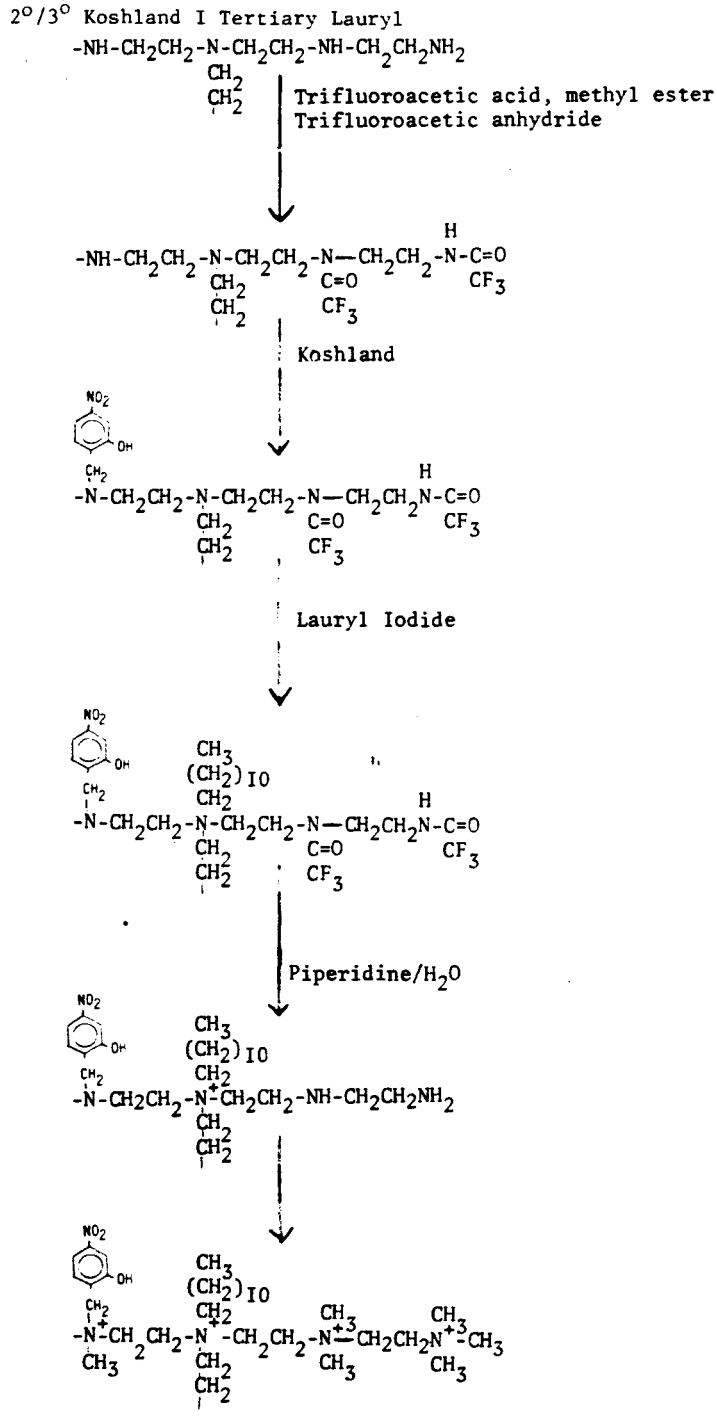
   Prepared by blocking PEI with methyl trifluoroacetate (p. 95), leaving 10-15% of the blockable amines free. The polymer is reacted as a 1 M acetonitrile solution with Koshland reagent I (0.05 N equivalents) for 24 hours at 60°C. Subsequently, 0.1 equivalent of dodecyl iodide is added and the reaction is continued at 60°C for 24 hours. Ion exchange chromatography (Dowex Cl in EtOH) is followed by LH-20/EtOH chromatography. The polymer is acid deblocked (p.101) and then exhaustively methylated (p.102). This is summarized in synthetic scheme 4.

The final degree of substitution of reporter group onto the polymers was between 0.5 and 1% total polymer amine. This was not surprising since the starting material is highly susceptible to water and

Synthetic Scheme 3



Synthetic Scheme 4



hydroxyl attack.

Two polymers were synthesized containing Koshland reagent II.

1. PEI with Koshland reagent II on primary amines followed by dodecyl benzylation of one per 2.5 of the remaining primary amines.
   The polymer was reacted as a 1 M solution in anhydrous ethanol first with Koshland II (.03 N equivalents) at 40°C for 24 hours followed by .1 N equivalent of dodecyl benzyl chloride, again reacted at 40°C for 24 hours. The product was purified by LH-20/EtOH chromatography.
2. Partially trifluoroacetylated PEI reacted on available amines with Koshland reagent II followed by laurylation.
   PEI was partially trifluoroacetylated with methyl trifluoroacetate (p.95). It was then dissolved to a 1 M concentration in acetonitrile. Koshland II is then added (.03 N equivalent) and the solution is allowed to react at 60°C for 24 hours. Dodecyl iodide (.1N equivalent) is then added and the reaction is continued at this temperature for an additional 24 hours. The TFA groups are removed by acid (p.101), and the polymer is exhaustively methylated (p.102).

For the fluorescence studies, a number of primary derivatized polymers were synthesized in a manner identical to those containing the Koshland reagents. Although the fluorophore is not ionic, the polymers were cleaned on both Dowex C1 200X columns and LH-20 columns since even the Dowex column material removed a good portion of the unreacted NBD-C1, and in so doing spared the LH-20 columns of undue contamination. These primary derivatized polymers included the following:

1. Primary NBD, (.01 equivalent) primary lauryl (.1 equivalent)
2. Primary dodecyl (.1 equivalent), then primary NBD (.02 equivalent)
3. Primary NBD (.01 N equivalent) primary dodecyl benzyl (.1 equivalent)
4. Primary dodecyl benzyl (.1 equivalent) followed by NBD (.01 equivalent) onto primary amines.

Two tertiary substituted polymers were also prepared.

5. Trifluoroacetylated, TEOC PEI (p.98) was prepared as a 1 M acetonitrile solution at 60°C with 0.1 N equivalent of dodecyl benzyl chloride and then for 24 hours with 0.01 N equivalent of NBD-Cl. Both blocking groups were removed simultaneously with acid (p.101).
6. The above polymer was quaternized with methyl iodide (p.102).

The cleaning procedures were LH-20/EtOH chromatography immediately following the laurylation and for the quaternized polymer dialysis against 0.1M NaCl and pure deionized water.

Spectrometric Determinations

The infrared spectrum was courtesy of Kate Martin of Loyola Lake Shore campus, chemistry department, and was performed on KBr pellets of the dried polymer using an IBM 98 F-T IR spectrometer interfaced with a Texas Instruments S-700 data terminal.

The carbon-13 spectra of the polymer derivatives were courtesy of Dr. Crumrine, also of the chemistry department on Lake Shore campus, Loyola University. They were performed on a multinuclear 24K Varian FT-80 NMR spectrometer operating with 10 mm broadband probe at 20.0 MHz and was operated with proton decoupling. All but one spectra are on PEI-600.

The proton NMR spectra were collected using a Varian 360-EL spectrometer. Approximate NMR shifts for polymer moieties are in Table I, Results.

A Perkin Elmer 320 spectrophotometer was used for studies on the Koshland reagents and for sulfhydryl assays based on Ellman's reagent.

The fluorescence data were collected on a Perkin-Elmer MPF-44B fluorescence spectrophotometer operating off of a Perkin-Elmer 150 xenon power supply. The slit width was 5 nm and the scanning speed 60 nm/min, with an excitation wavelength of 464 nm and an emission scan from 495 to 605 nm.

Blockage, Alkylation, and Deblockage

The proton NMR spectrum*of TFA-PEI is broadened relative to the original polymer and is poorly resolved into two peaks (Fig.'s 1 & 2). This broadened backbone is quite similar to that observed in a previously published spectrum of acetyl PEI.[296] While for that polymer it was suggested that the two partially resolved backbone peaks correspond to methylene hydrogens alpha to secondary and tertiary amides, evidence from this lab indicates that at least for the trifluoroacetylated polymer the spectrum is not so straightforward. PEI preps having 10% and 30% lauryl (leaving 15% and 0% free primary amines, respectively), when trifluoroacetylated, give spectra that differ little and without consistency in backbone fine structure when compared to TFA-PEI or acetylated PEI (Fig.'s 3 & 4). Even when there are no primary amines, as in 30% lauryl PEI, subsequent trifluoroacetylation gives a polymer whose PMR backbone is partially resolved into two peaks. Thus, to understand the origin of the backbone's broadness and make peak assignments for the two partially resolved peaks would require further research (Figs. 1 - 4).

By analytical techniques to be discussed shortly, it has been shown that methyl trifluoroacetate fails to react completely with the polymer. Even when present at a two-fold excess, as many as 25% of the amines remain unprotected. It will be shown later that these are secondary amines. Two additions of trifluoroacetic anhydride, 10% total amine each, further block the polymer, leaving an acceptably low (5-8%) number of the total

* Observed proton and carbon - 13. NMR shifts are summarized in Tables 1A and 1B and are presented on the following 2 pages for the reader's convenience.

TABLE 1A

Approximate ^{1}H NMR Chemical Shifts, denoted with Arabic numerals

PEI	PEI, trifluoroacetylated or acylated	1	2.2-3.8 ppm
	Amides of TFA PEI	2	8.3-8.8 ppm
	PEI, unmodified	3	2.6 ppm
	Quaternized PEI	4	3 ppm
n-alkanes	Terminal methyl group	5	.85 ppm
	methylenes not alpha to N of PEI	6	1.25 ppm
	benzyl of dodecyl benzyl	7	7.1 ppm
styrene oxide	methyl	8	1.3 ppm
	phenyl	9	8.2 ppm
propylene sulfide	methyl	10	1.2 ppm
TEOC	silylmethyls	11	0
	methylene alpha to carbonate O	12	3.85-4.3 ppm
	methylene beta to carbonate, alpha to Si	13	1.1 ppm
t-boc	methyls	14	1.3 ppm
Acetyl	methyls		
	on primary amine	15	2.3
	on secondary amine	16	2.1
Solvents			
acetonitrile		17	2 ppm
water		18	4.45 ppm
chloroform		19	7.1 ppm
ethanol		20	1,3 ppm
Koshland reagents	phenyl	21	7.9 ppm
SNPA	phenyl	22	8.2

Table 1B

Approximate ^{13}C NMR Chemical Shifts, denoted with Roman numerals

PEI	PEI, unmodified I	38-58 ppm
	PEI backbone, trifluoroacetylated or acylated I	38-58 ppm
trifluoro-acetyl	carbonyls II	154-162
	CF_3 quartet III	97, 110, 125, 138
t-boc	carbonyls IV	153-155
	central carbon of t-butyl function V	85-88
	methyls VI	30
TEOC	silyl methyls VII	0
	methylene alpha to Si VIII	20
	methylene alpha to carbonate IX	62
	carbonate X	155
solvents	DMSO XI	40
	Acetonitrile	
	CN XII	120
	methyl XIII	0
	chloroform XIV	73-77
	deuteroacetone	
	carbonyl XV	185
	methyl XVI	22-24
acetyl	carbonyl XVII	173
	methyl XVIII	22-24
hydrophobe	aliphatics XIX	27-30
	benzyl group XX	125-129

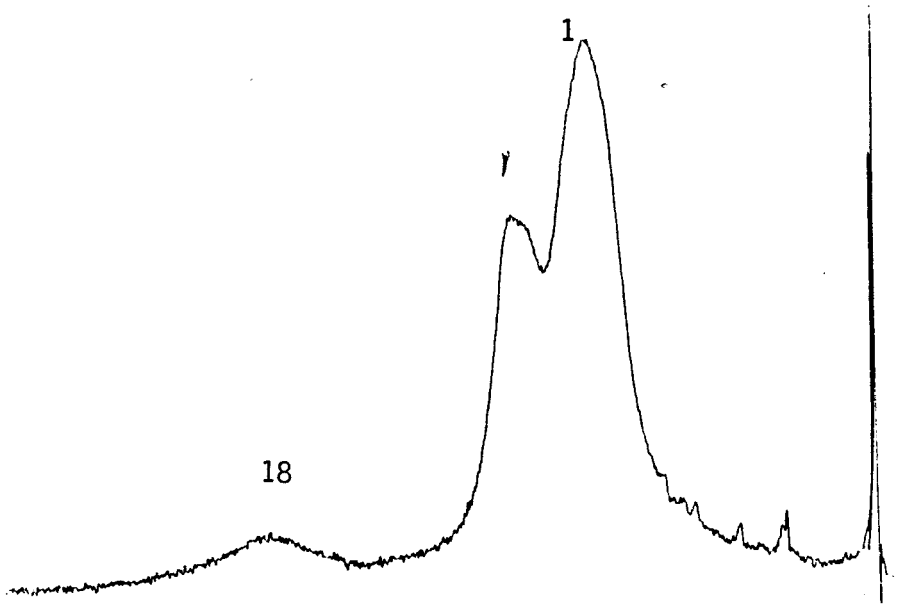


Figure 1. Trifluoroacetyl PEI d_6-DMSO/tms

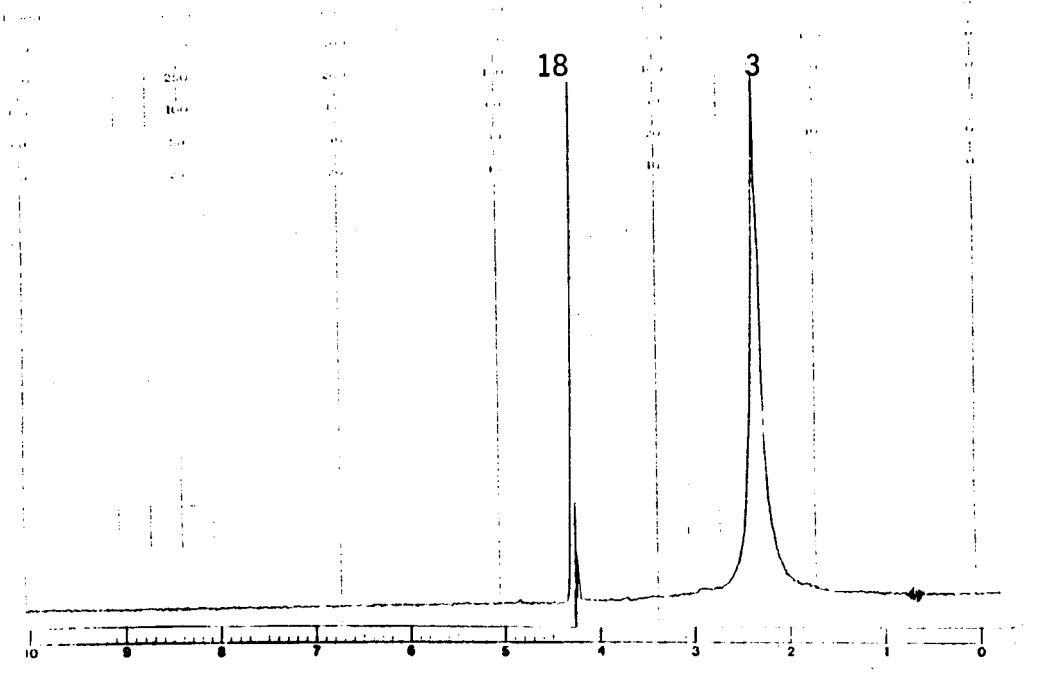


Figure 2. PEI-18 D_2O

Solvent - independent broadening of PEI's methylene hydrogens results from trifluoroacetylation.

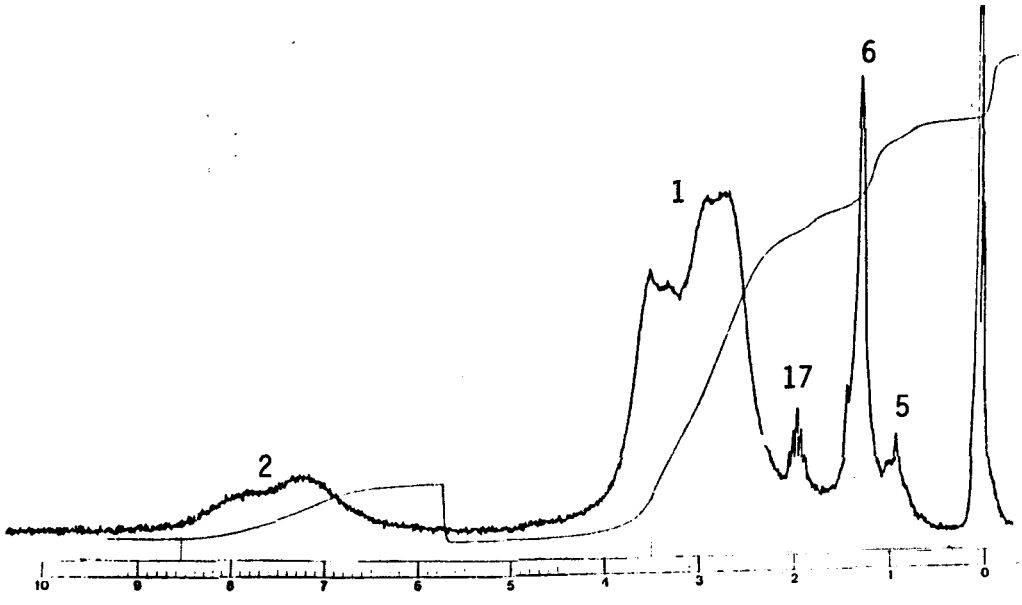


Figure 3. 10% $C_{12}H_{25}$(on 1° N) 75% TFA PEI $CD_3CN/_{tms}$

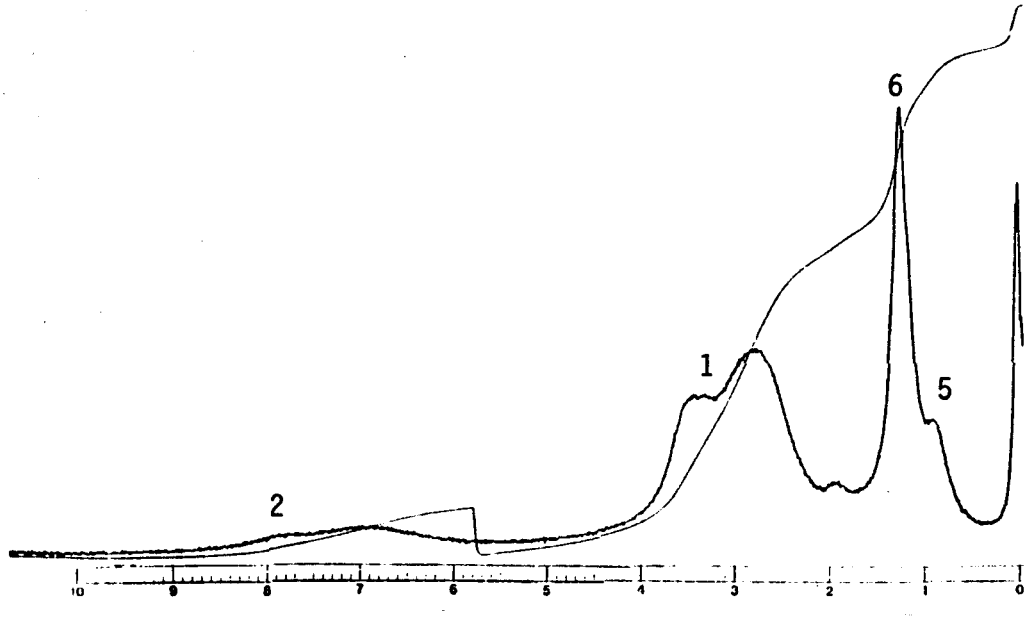


Figure 4. 30% $C_{12}H_{25}$ (on 1° N) 75% TFA PEI $CD_3CN/_{tms}$

The degree of primary amine substitution does not affect the shape of the broadened trifluoroacetyl PEI peak.

amines free. Trifluoroacetic anhydride and N-methyl bis (trifluoroacetate) (Pierce) are strong agents and the heat generated upon reacting with PEI is sufficient to discolor the polymer through amine oxidation. However, exothermic reactions are favored at low temperatures, and if carried out at -4°C, no discoloration results even if these strong trifluoroacetylating agents are the only ones used.

The 6 to 10% of the total blockable amines that remain free after trifluoroacetylation may be reacted with di-t-butyl dicarbonate or TEOC-Np (Figures 5 & 6). These blocking groups have proven to be more reactive than methyl trifluoroacetate or the anhydride toward both the original polymer and the partially trifluoroacetylated one. The t-boc and TEOC groups offer the advantage of being quantitatible by PMR (Figures 7 - 8). Even if the polymer's signal is broadened by prior trifluoroacetylation, an accurate integration is possible, particularly for the TEOC group (Figure 5). The t-boc's methyl hydrogens overlap slightly with the trifluoroacetylated backbone (Figure 6). Also, the methylene hydrogens alpha to the carbonate in the TEOC group are just downfield to the polymer and the two slightly overlap (Figures 5 & 8). However, this group may be substracted out of the backbone due to an accurate integration of the silylmethyls at 0 ppm (Figs. 5 - 8).

The carbon-13 NMR spectrum of trifluoroacetylated PEI clearly demonstrates that both primary and secondary amines have been blocked since there are two carbonyl carbons and two sets of CF_3 quartets (Figure 9). The backbone looses much of its fine structure relative to the carbon-13 spectrum of the original polymer (Fig. 10), reducing to three broad signals.

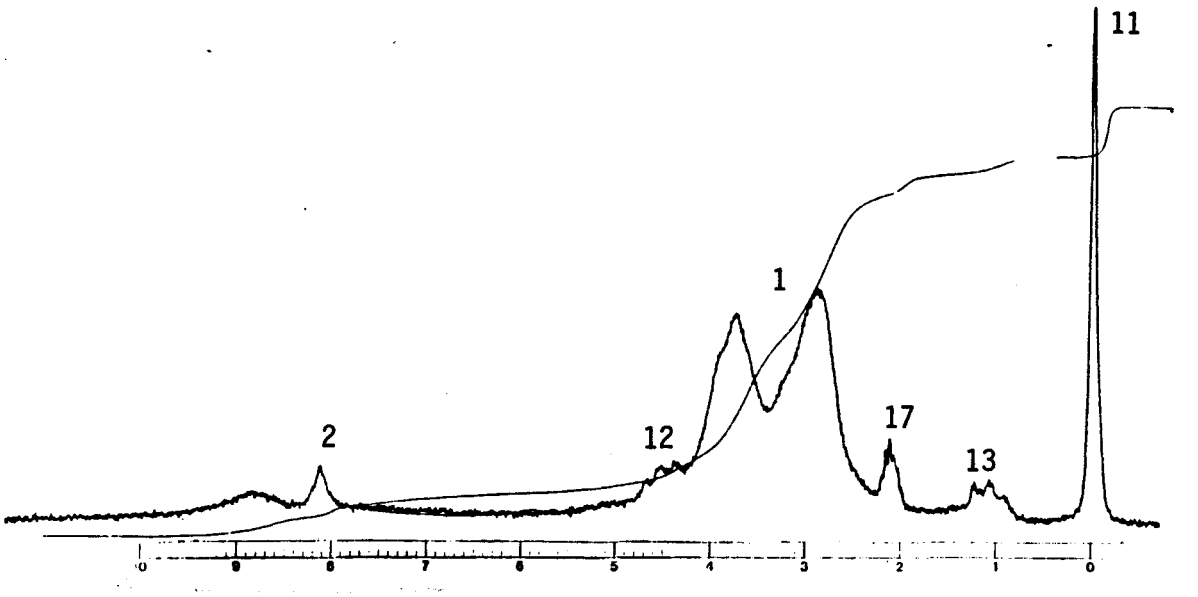


Figure 5. TFA - TEOC PEI CD_3CN

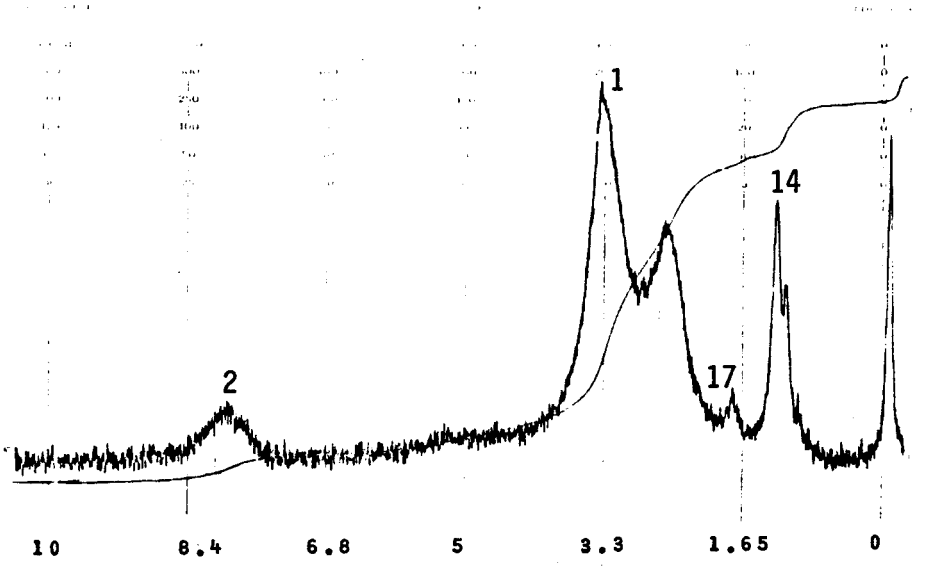


Figure 6. TFA - t-boc $CD_3CN/_{tms}$

Both the t-boc and TEOC groups remain integrable on trifluoroacetylated PEI as well as on PEI itself.

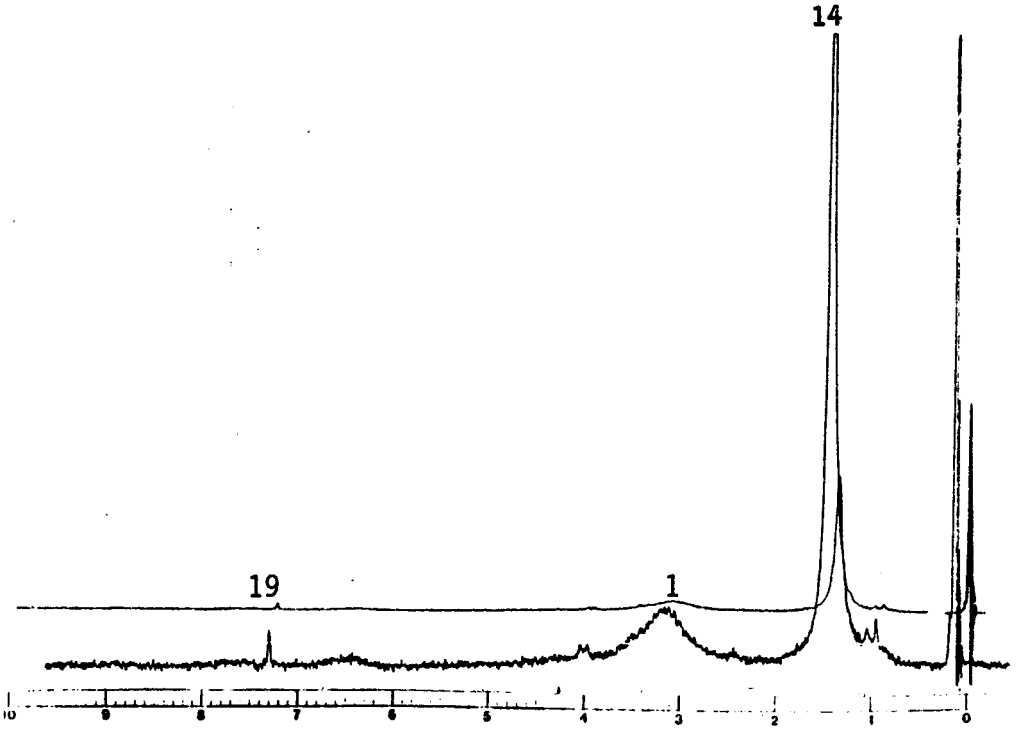


Figure 7. Per t-boc PEI + Koshland reagent 1 $CDCl_3$/tms

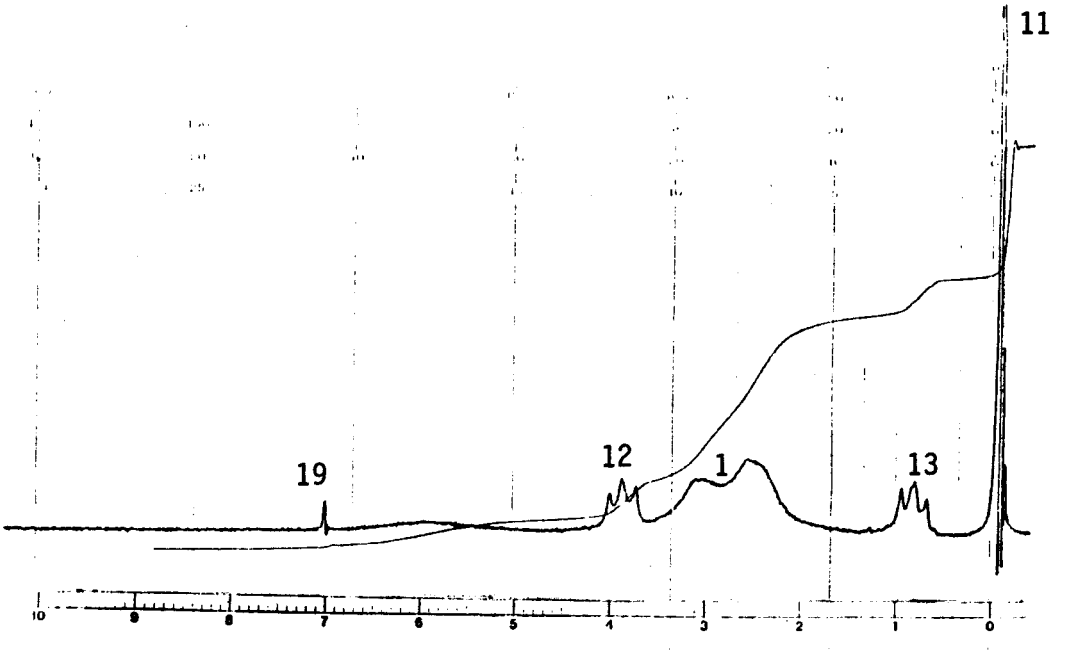


Figure 8. Per TEOC PEI $CDCl_3$

The t-boc and TEOC blocking groups give well resolved ^1H signals that can be readily integrated relative to the polymer backbone.

Due to low abundance and nuclear Overhauser effects, C-13 NMR is not a useful technique to determine percent trifluoroacetylation.(Figs. 9 & 10).

The ^{13}C NMR spectrum of t-boc PEI also shows two carbonyl signals (Figure 11). The backbone retains some of its fine structure, and six peaks are discernable. An even greater degree of backbone resolution is retained when the polymer has been reacted with TEOC-Np (Figure 12). Specific backbone peak assignments and indeed the potential of the t-boc and TEOC derivatives as analytical tools have not been exploited in this work. However, extensively trifluoroacetylated PEI that has been further blocked with t-boc gives a t-boc carbonyl signal corresponding to that on a secondary amine, indicating that this amine type resists trifluoroacetylation (Fig. 13).

Of the three blocked polymers, only trifluoroacetylated PEI may be further substituted on tertiary amines with alkyl halides. Whether steric or other factors are responsible for the inertness of t-boc and TEOC PEI is uncertain. However, it is evident from the three representative spectra shown that despite trying several solvents and heating all to 60°C for 48 hours, only TFA PEI will readily alkylate to a significant extent (Figures 14, 15, and 16).

Since only the TFA polymer will react further, a technique had to be found to quantitate the amount of trifluoroacetyl groups on PEI. Fourier transform I.R., ultraviolet spectroscopy, and finally PMR were employed. F.T.-I.R. spectra of the polymer are consistent with the assumed structure, but the amide protons overlap extensively with water, a contaminant not readily excluded from the polymer-KBr pellet (Fig. 1A). Since t-toc and

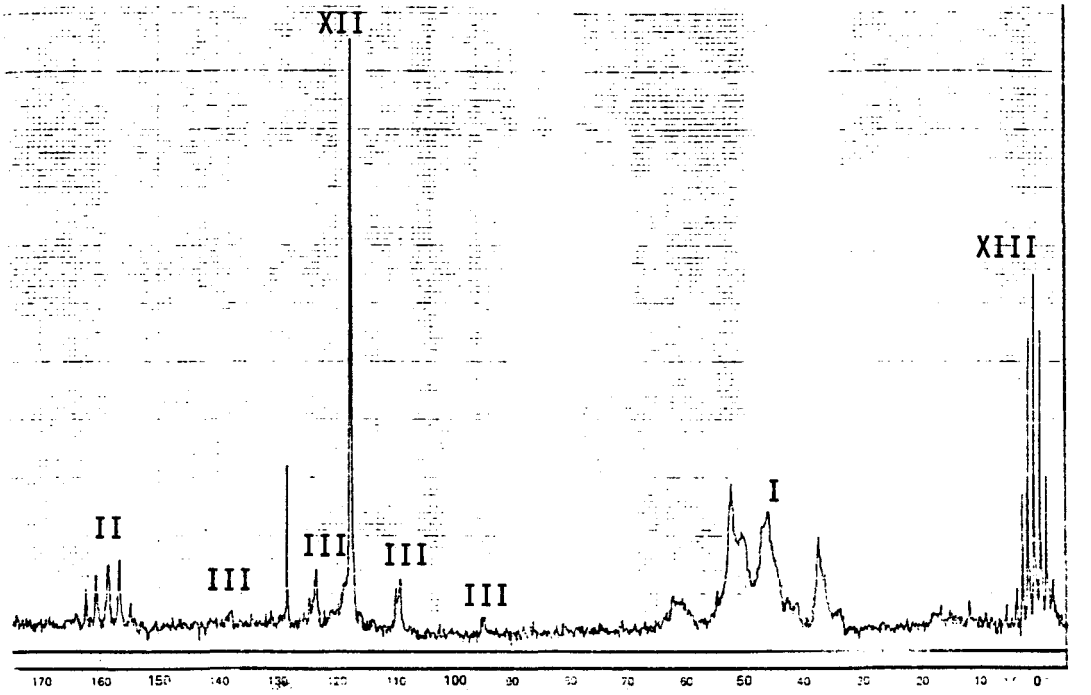


Figure 9. ^{13}C of 75% TFA 3°-CH_3 PEI CD_3CN

Two carbonyl carbon quartets and two CF_3 quartets are discernible. The backbone carbons are poorly resolved.

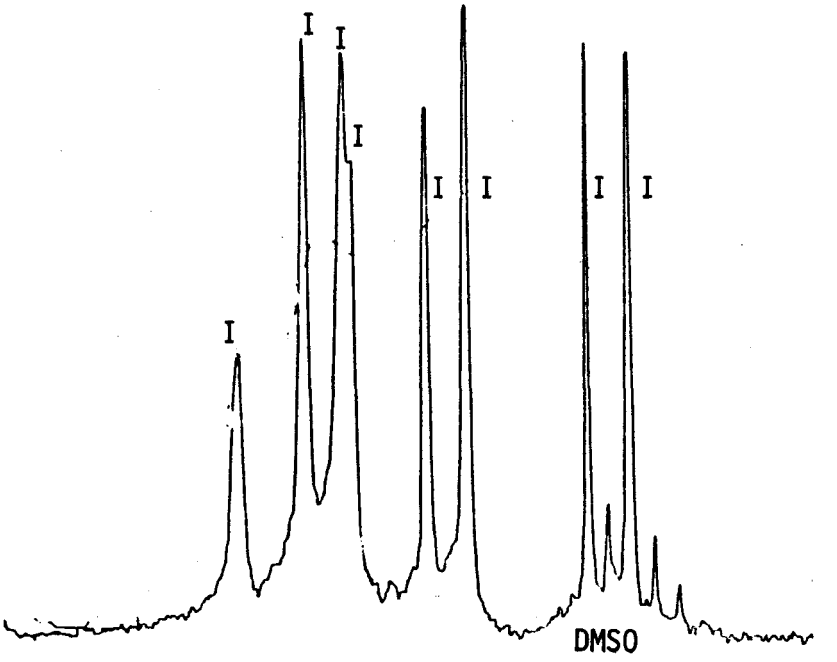


Figure 10. ^{13}C of PEI d_6 DMSO

The original polymer is resolved into 8 peaks in ^{13} C NMR depending on the amine type to which they are located alpha or beta.

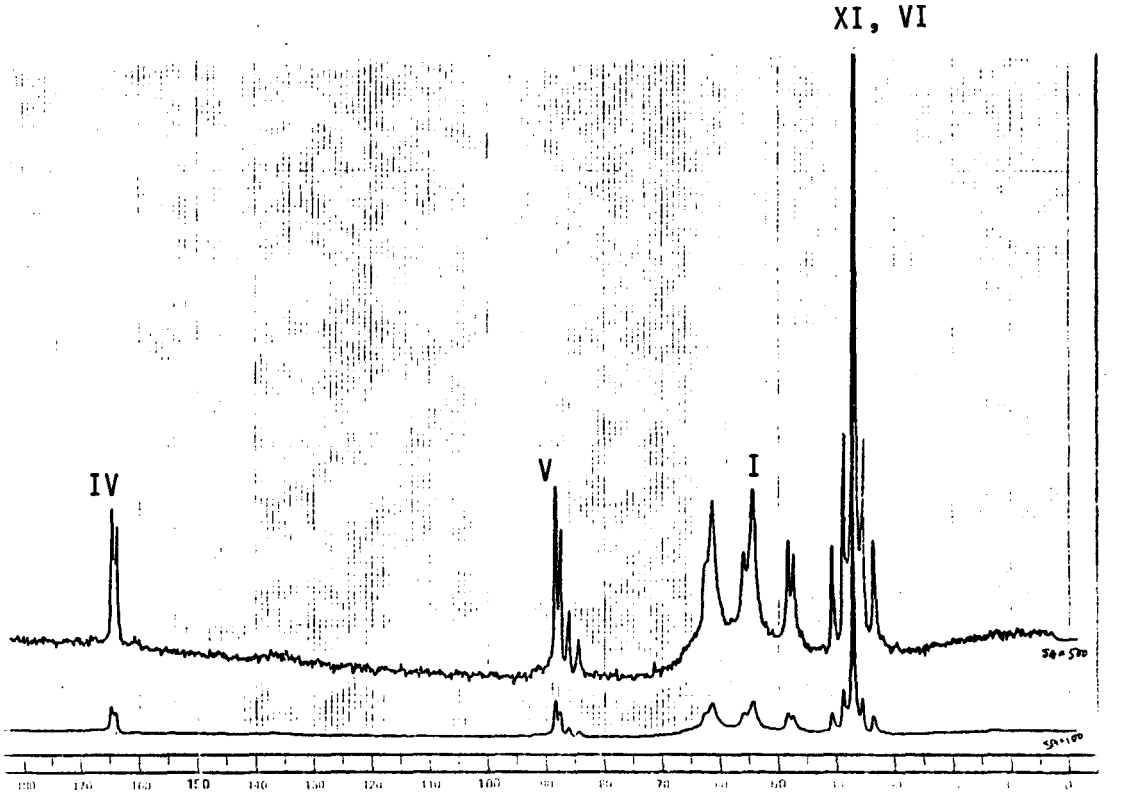


Figure 11. ^{13}C of Per t-boc PEI d_6DMSO

The polymer backbone remains rather well resolved, appearing in six peaks, in t-boc PEI. Two carbonyl carbon singlets and two doublets corresponding to the central carbon of the t-butyl function are discernible, depending on the amine type with which they have reacted.

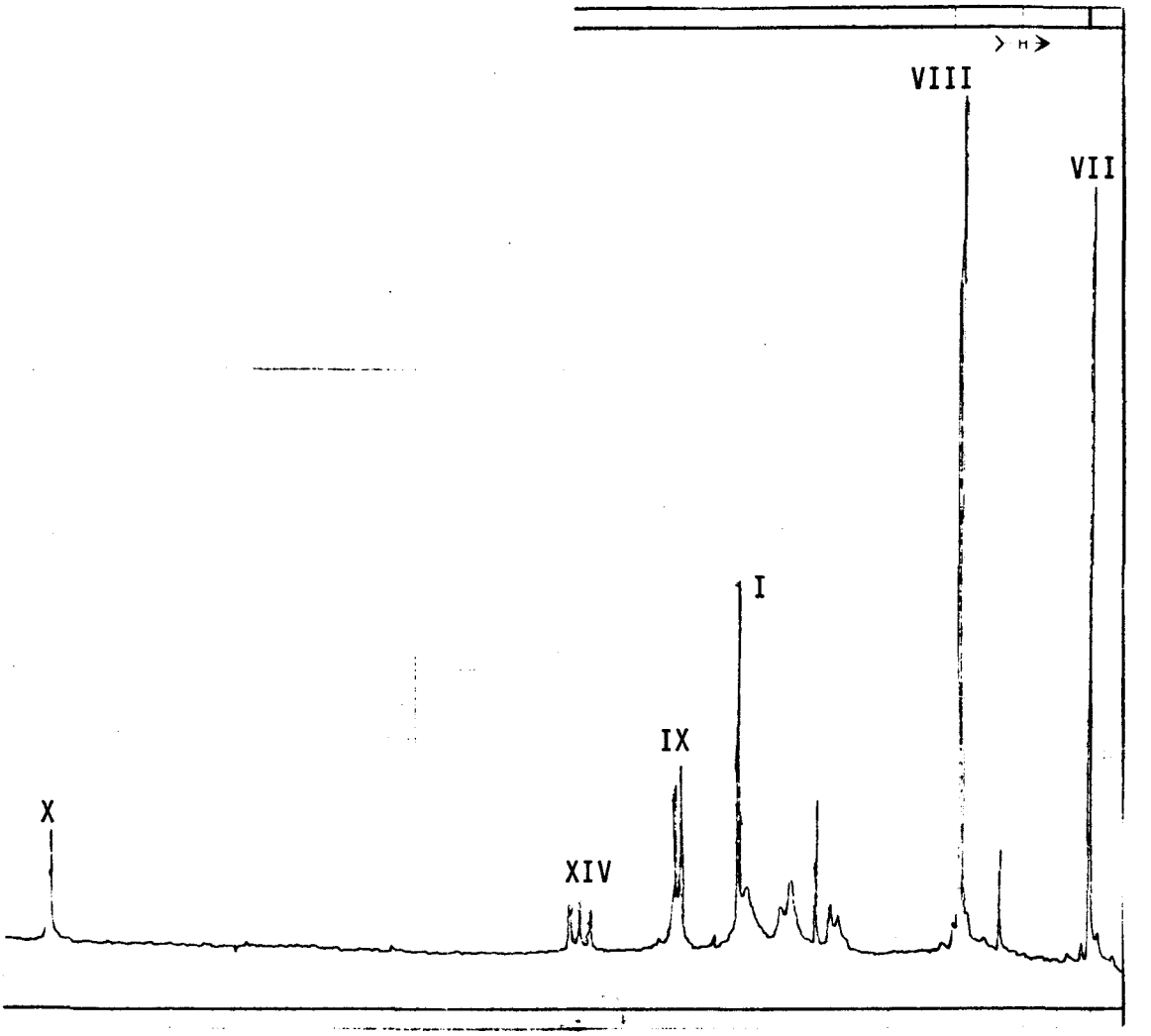


Figure 12. ^{13}C of Per TEOC PEI $CDCl_3$

The backbone remains well resolved upon TEOC blockage and various carbons of the blocking group are also easily discernible, though less informative with regards to amine type of attachment.

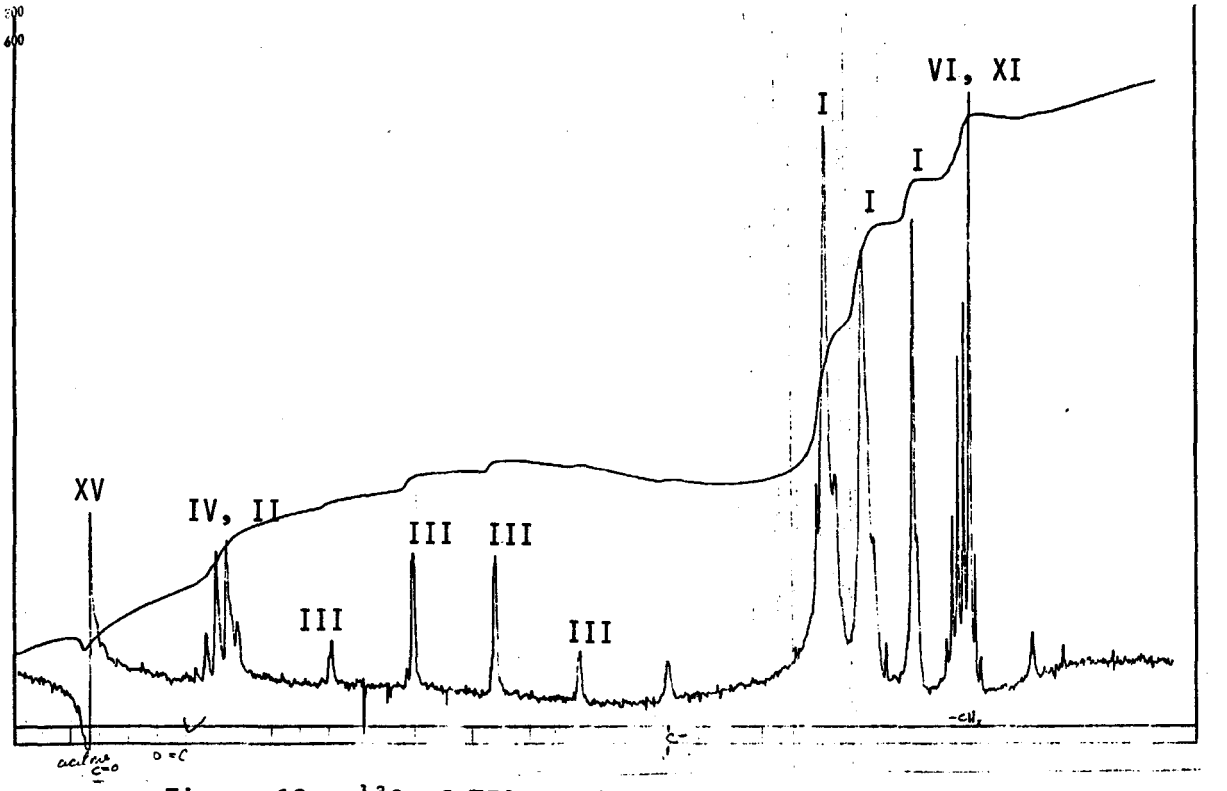


Figure 13. ^{13}C of $TFA_{.7}$ + $boc_{.05}$ PEI d_6 acetone

The central carbon of t-boc's t-butyl function and the carbonyl carbon of this differential blocking group indicate that the amine to which it is attached was a secondary amine; this type resists trifluoroacetylation.

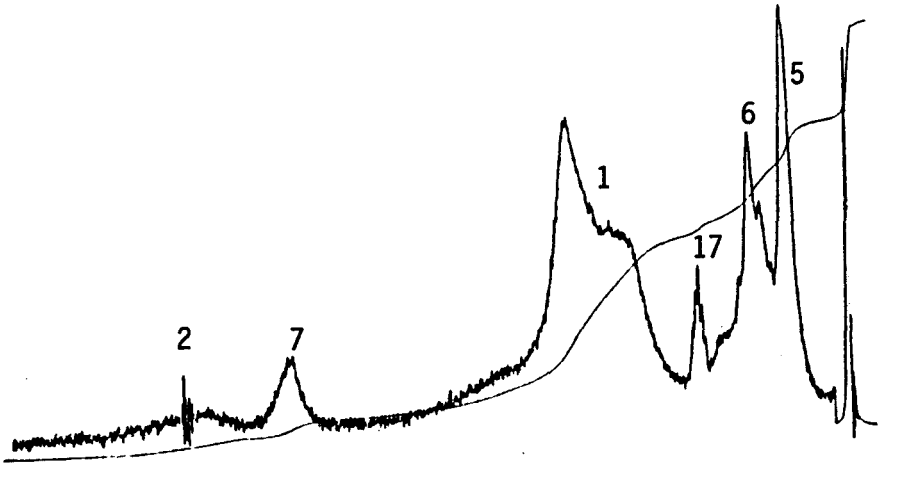


Figure 14. Trifluoroacetylated PEI reacted with dodecyl benzyl chloride CD_3CN/tms

TFA PEI can be substituted extensively with alkyl halides.

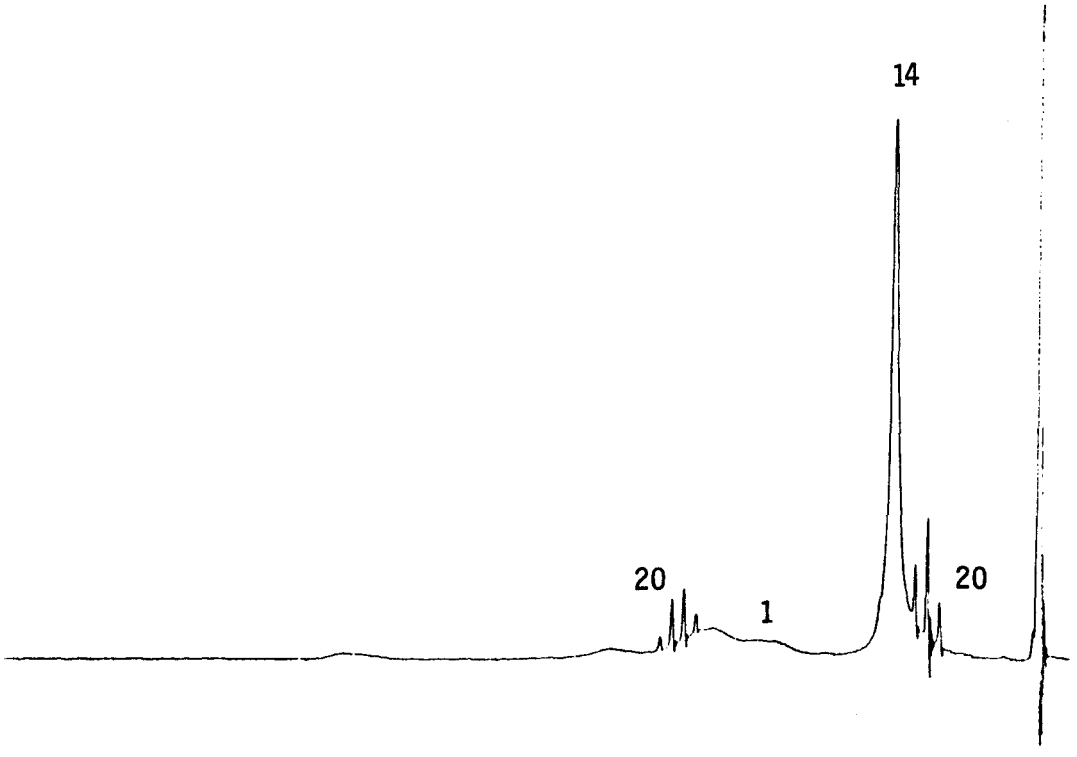


Figure 15. Per t-boc PEI 5% Koshland I 30% C_4H_9I
(EtOH present) d_6-DMSO/tms

t-boc PEI resists further substitution with alkyl halides; the methyl hydrogens of the blocking group appear as a single distinct peak which is not complicated by downfield overlap with possible aliphatic methylene hydrogens that would have been introduced upon quaternization.

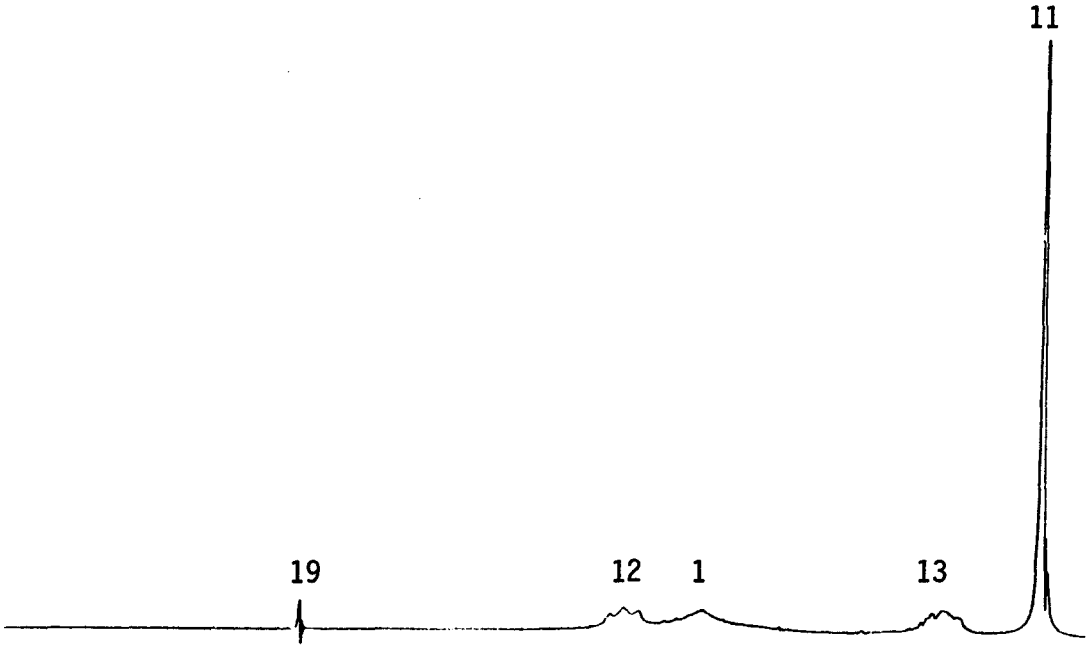


Figure 16. Per TEOC PEI Reacted with 30% dodecyl benzyl chloride in ETOH $CDCl_3$

TEOC - derivatized PEI also resists quaternization with alkyl halides.

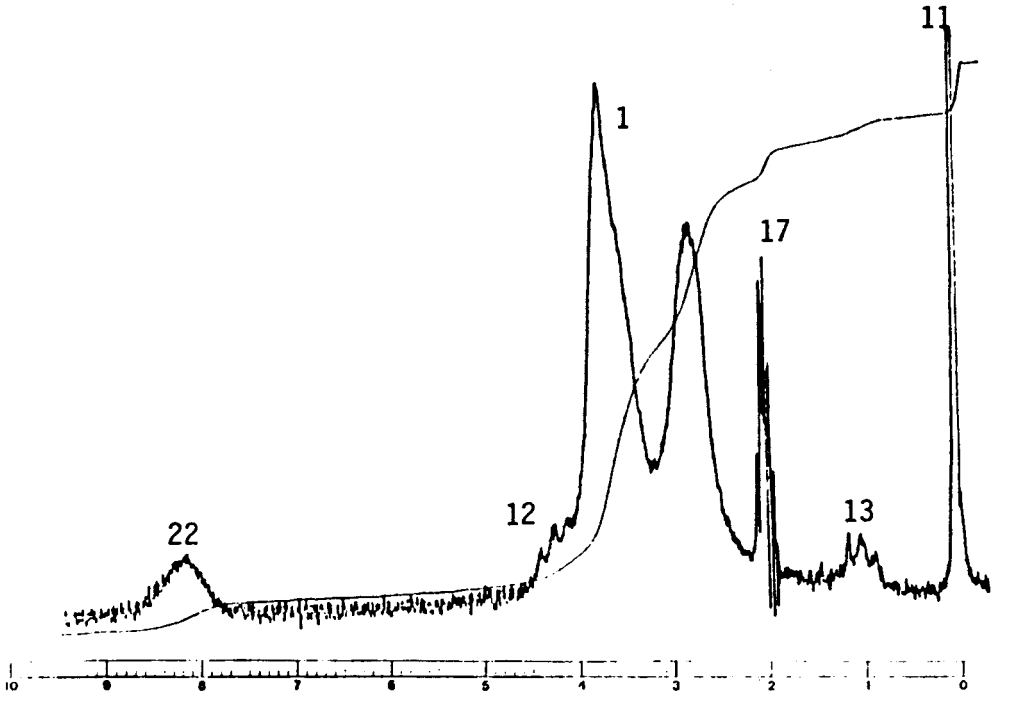


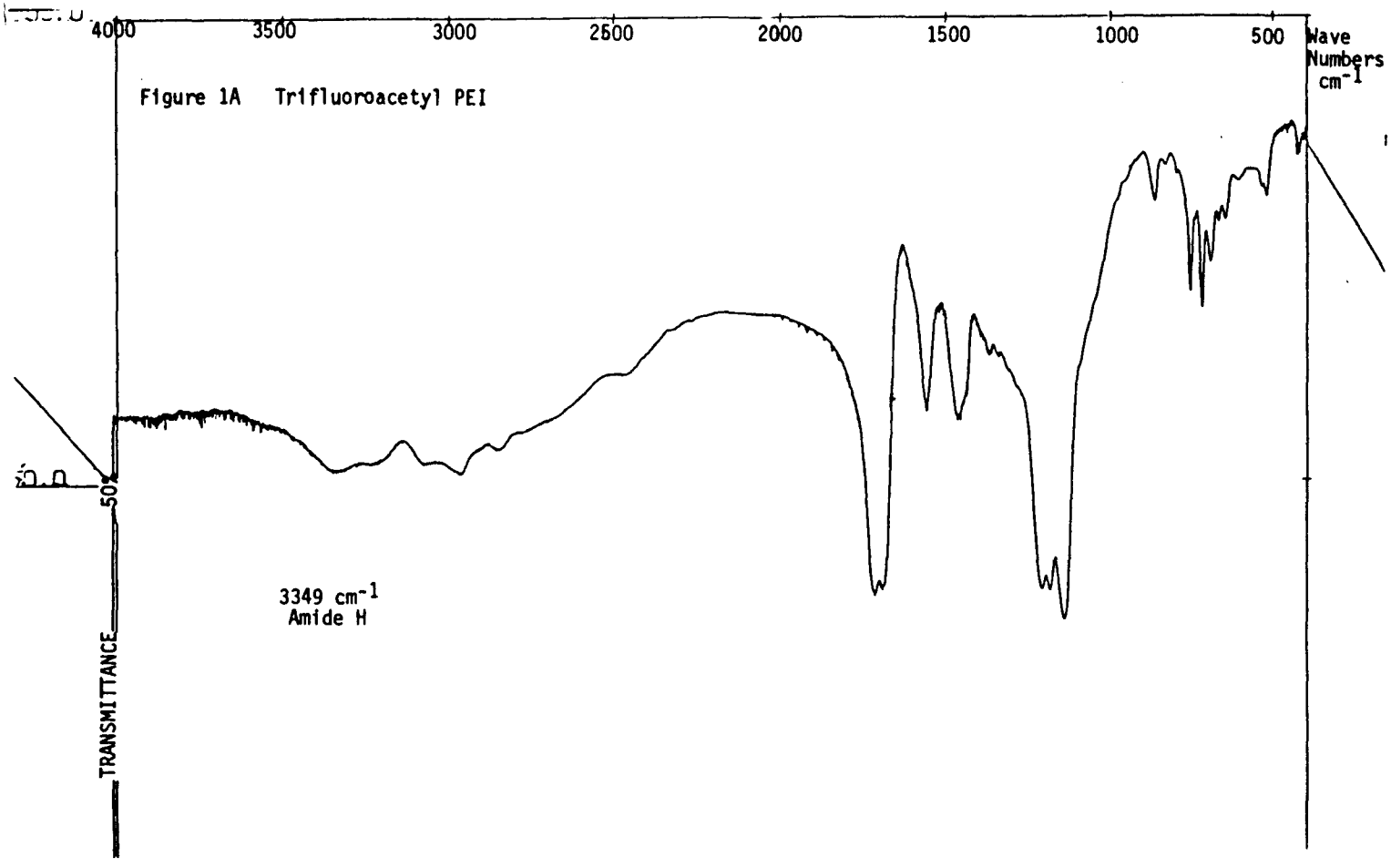
Figure 17. TFA PEI reacted with SNPA, then TEOC-O-Np CD_3CN

The reaction of SNPA with TFA is incomplete since there remain amines which can be reacted with TEOC-O-Np.

TEOC groups readily react with the polymer and are easily integrable in PMR, they have served not only as differential blocking groups but also as reagents for determining free secondary amines (Figures 5 and 6). Indeed, n-succinimidyl p-nitrophenyl acetate (SNPA), the one reagent now available for spectroscopic determination of both primary and secondary amines in organic solvents, fails to react with amines that later did react with TEOC-Np (Figure 17). t-boc-treated TFA PEI does not react with TEOC-Np, indicating that this blocking group is also a highly reactive one.

Trifluoroacetyl PEI can be substituted with dodecyl iodide (Figure 18) or dodecyl benzyl chloride (Figures 14 & 19) to the extent of 15% total amine or greater. If an acceptable amount of quaternization is not attained from performing the reaction once, the polymer may be cleaned on an LH20/EtOH column, rotoevaporated to dryness, and reacted again with the alkyl halide in the presence of base. One polymer (styrene-TFA PEI, Figure 20) was reacted two times with dodecyl iodide; slightly further substitution occurred during the repeat quaternization (Figures 21 and 22).

PEI in which all primary and secondary amines have been blocked as in TFA-TEOC or TFA-t-boc PEI can also be substituted extensively with dodecyl iodide or dodecyl benzyl chloride. The reaction is especially successful when carried out in acetonitrile at 50-60°C for 48 hours (Figure 23). The presence of 5 to 12% TEOC on TFA PEI does not adversely affect the subsequent quaternization of tertiary amines even though 75% TEOC PEI is itself inert. This also is true for the t-boc group. If t-boc is the differential blocking group to be used, spectral integration prior to laurylation is imperative since the t-boc and n-aliphatic groups give signals in the same


4000
3500
3000
2500
2000
1500
1000
500
Wave Numbers cm-1
Figure 1A Trifluoroacetyl PEI
50
TRANSMITTANCE
3349 cm-1
Amide H

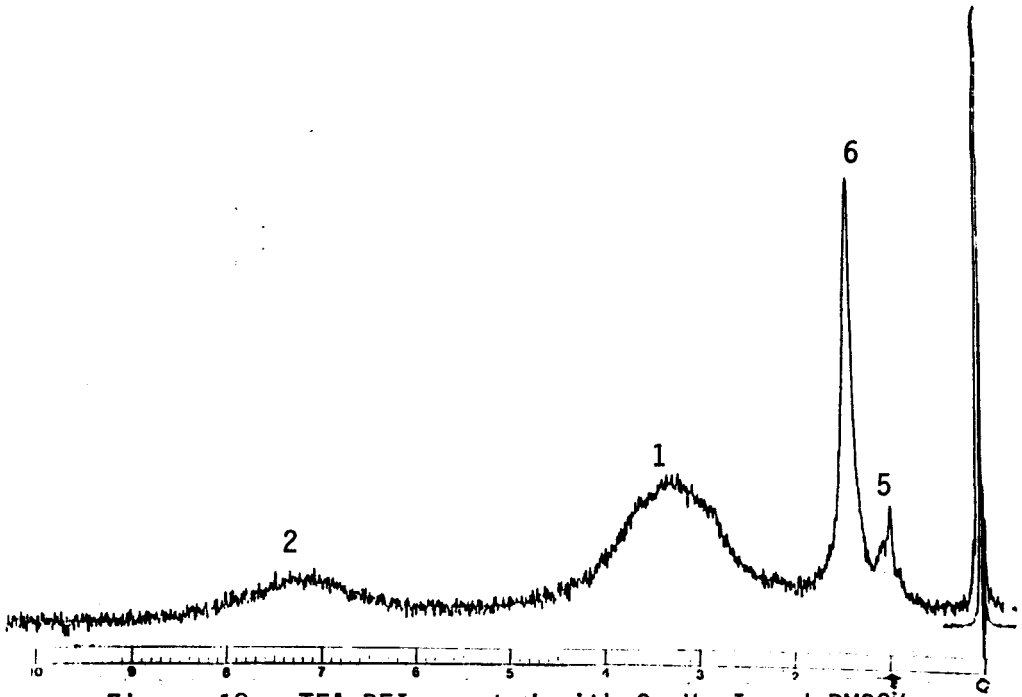


Figure 18. TFA PEI reacted with $C_{10}H_{21}I$ $d_6DMSO/_{tms}$

Extensive substitution of alkyl halides with TFA PEI may be obtained routinely.

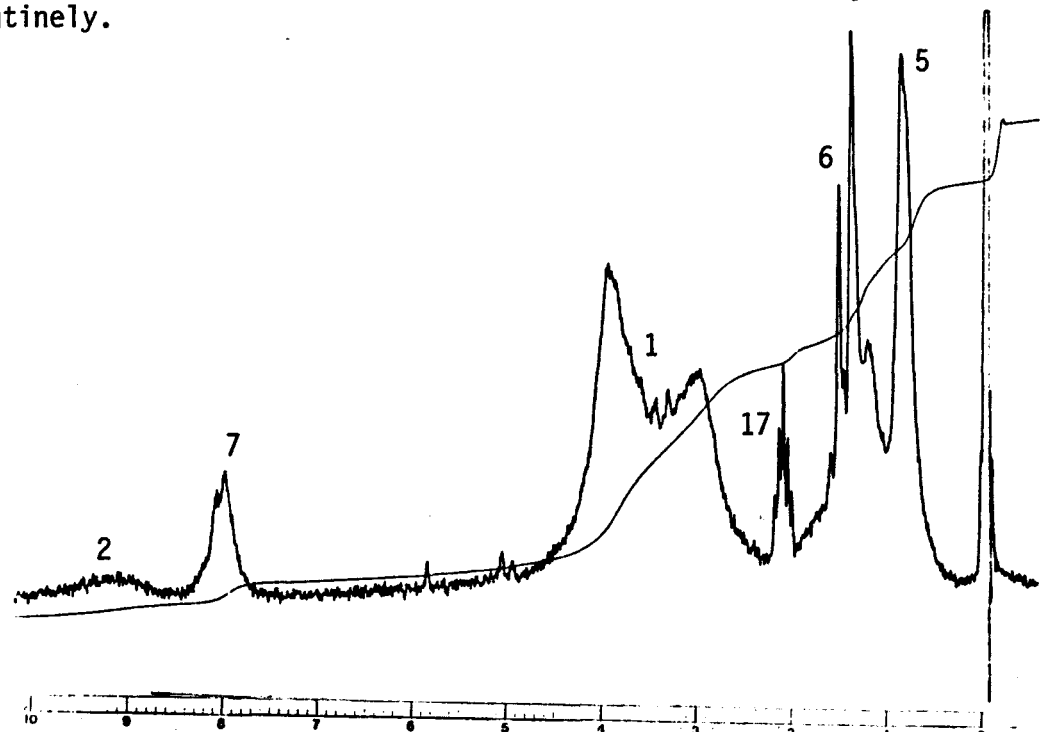


Figure 19. TFA PEI reacted with dodecyl benzyl chloride $CD_3CN/_{tms}$

TFA PEI also reacts substantially with benzyl chlorides.

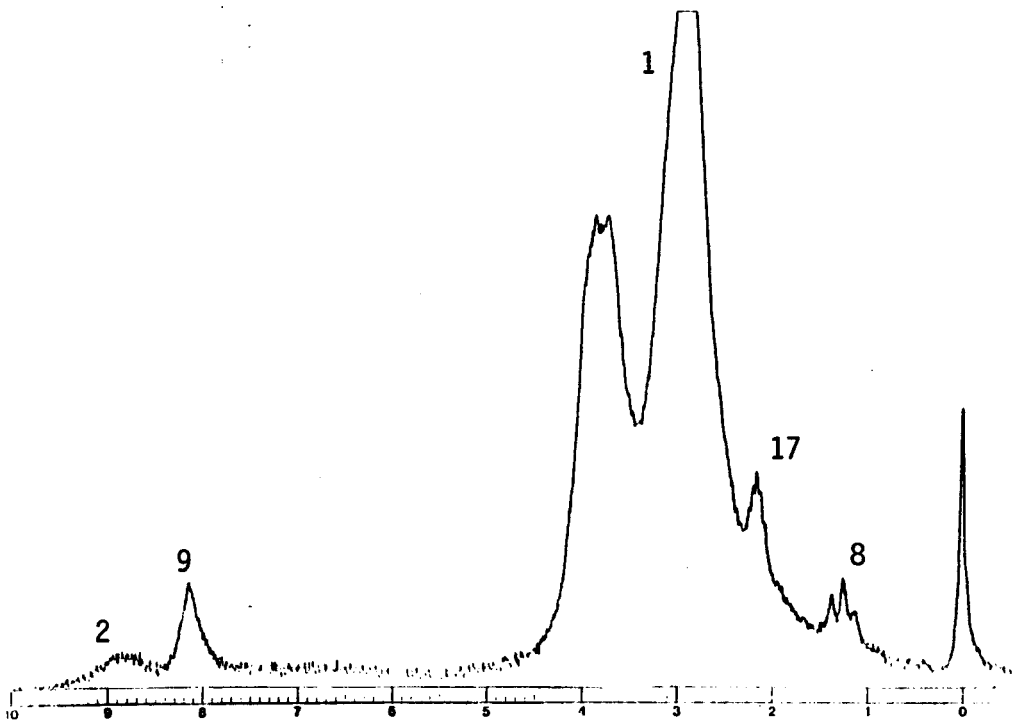


Figure 20. TFA PEI reacted on remaining 2°N's with styrene oxide $CD_3CN/_{tms}$

1,2-epoxides may be reacted with those secondary amines of TFA PEI that have resisted trifluoroacetylation.

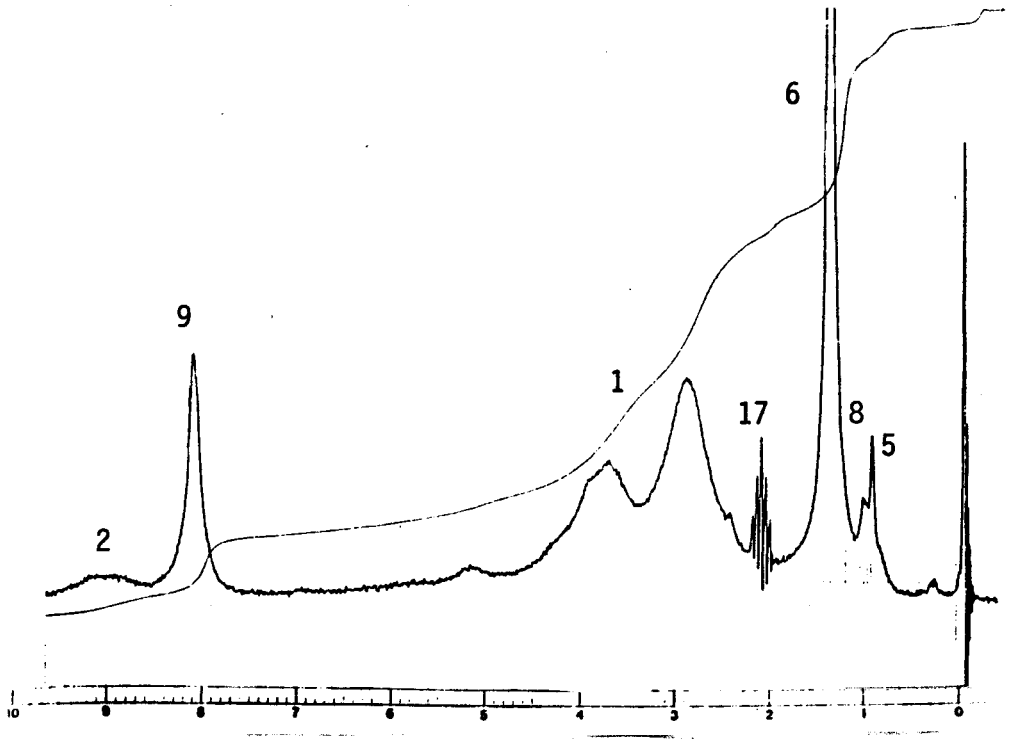


Figure 21. TFA-styrene oxide PEI reacted with $C_{12}H_{25}I$ $CD_3CN/_{tms}$

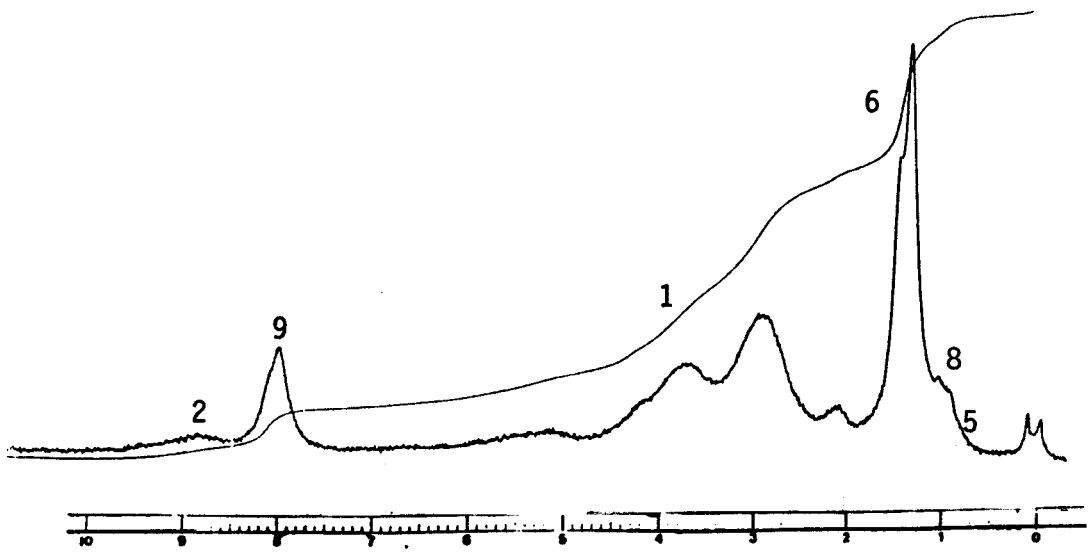


Figure 22. TFA-styrene oxide PEI reacted 2 times with $C_{12}H_{25}I$. $CD_3CN/_{tms}$

Repeated laurylations increase the degree of substitution if the degree of substitution is not sufficiently extensive after a single reaction.

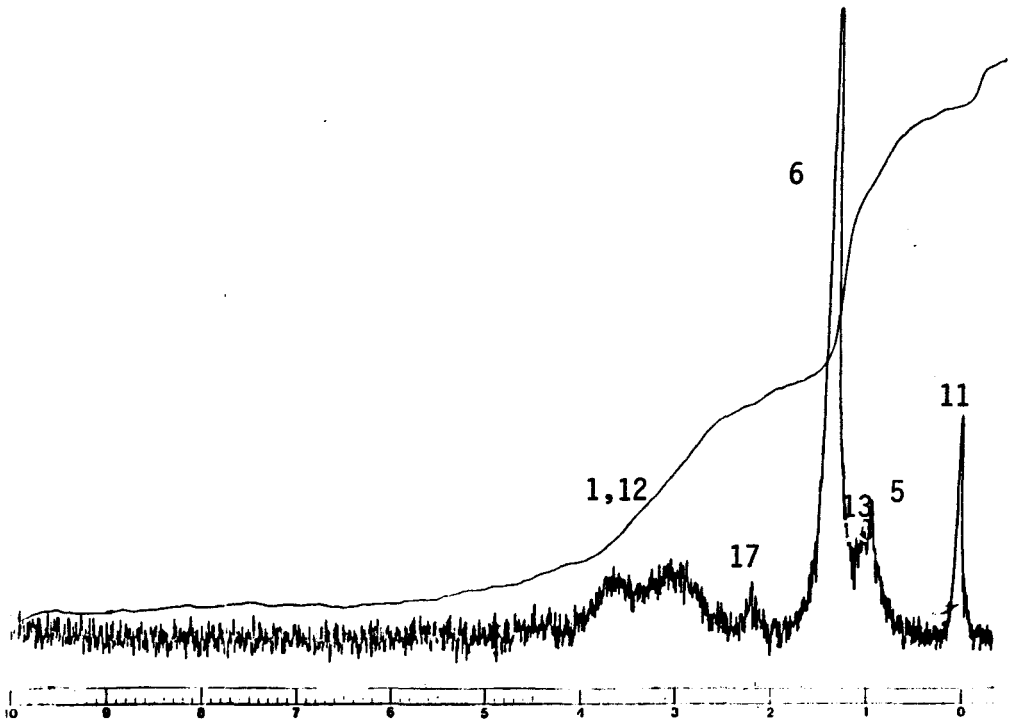


Figure 23. TFA- TEOC PEI reacted with $C_{12}H_{25}I$ CD_3CN

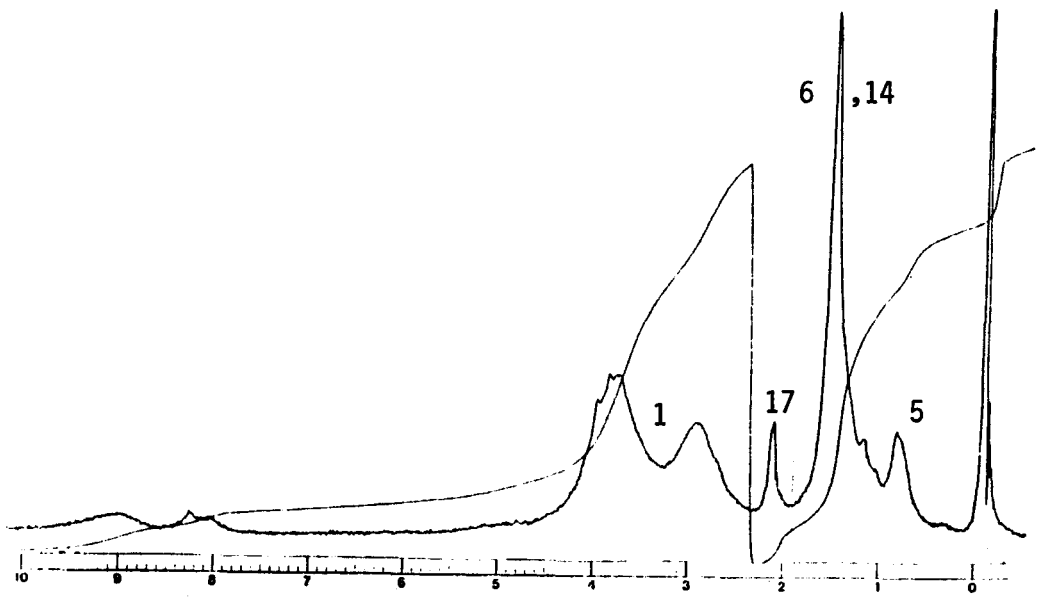


Figure 24. TFA- t-boc PEI reacted with $C_{12}H_{25}I$
$CD_3CN/_{tms}$

Small percentages of the t-boc and TEOC differential blocking groups on TFA-PEI do not impede quaternization with hydrophobes.

region of the spectrum (Figure 24). A technique for directly demonstrating that hydrophobe placement was onto tertiary amines was not found in this research; F.T.-I.R. failed since quaternary ammonium bands differ little in location from other polymer groups and the spectrum in general is broad. Either nitrogen-15 spectrometry or conductivity determinations in alkaline solution would appear to be the most likely techniques to directly demonstrate the presence of quaternary ammonium salts on the PEI.

The deblockage of TFA-PEI by piperidine is slow, as has been reported in the literature, and in the case of this polymer takes 5 or more days, with daily stepwise increments in water content. This avoids polymer precipitation. Extensively laurylated PEI must be shaken continuously with vigor during the deblockage to keep it in solution. If the reaction has not proceeded to completion and a carbon-13 NMR spectrum is taken, it is seen that primary amines, not secondary ones, have escaped deblockage (Figure 25). The backbone spectrum of completely deblocked TFA PEI differs little if at all from the original polymer (Figures 26 & 10). However, chloroform solubility is lost; this may reflect a more subtle, conformational change on the part of the polymer over the course of the reaction scheme.

Acid deblockage of TFA-PEI in HCl/acetic acid is rapid (less than 48 hours even when laurylated) but removes t-boc and TEOC as well as TFA groups from the polymer (Figure 27).

The ^{1}H and ^{13}C NMR spectra of acetyl PEI show two peaks each for acetyl protons corresponding to those that have reacted with 1° and 2° amines (Figures 28 & 29). Only one acetyl peak appears if primary amines are first eliminated, as in 25% lauryl PEI (Figure 30).

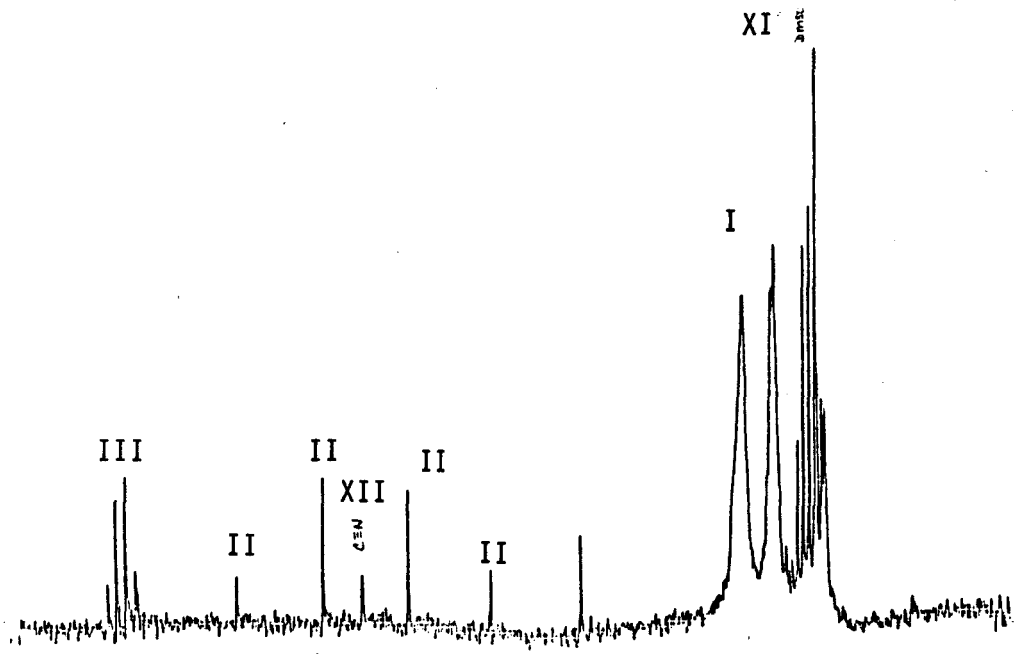


Figure 25. ^{13}C of partially deblocked TFA PEI CD_3CN/d_6 DMSO

Primary amines resist deblockage more than do secondaries.

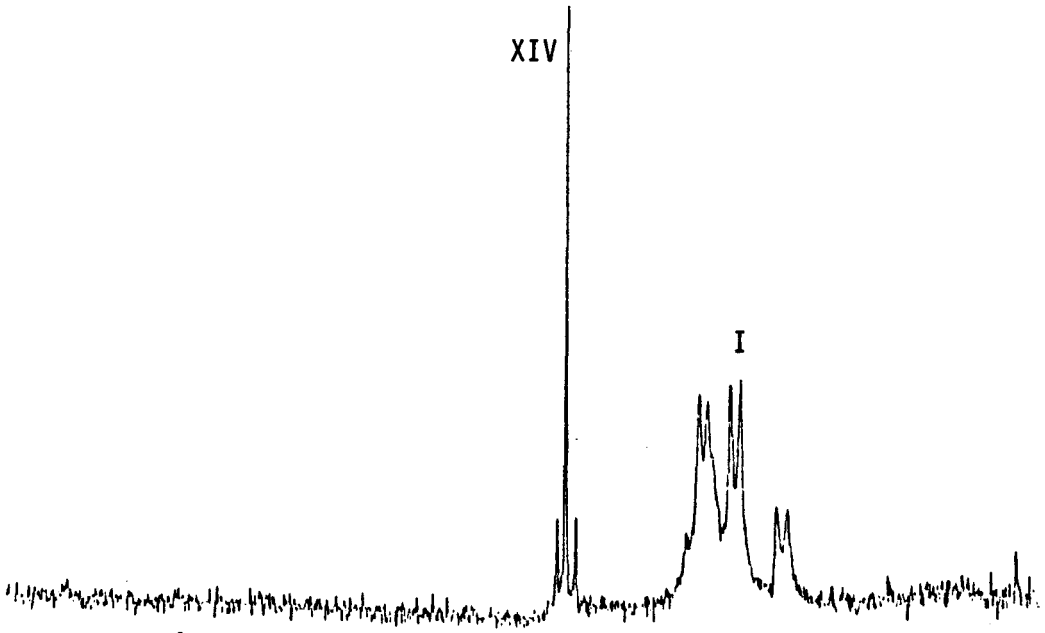


Figure 26. ^{13}C of Piperidine deblocked TFA PEI $CDCl_3$

Some resolution is lost upon removal of the TFA group but eight signals are still discernible.

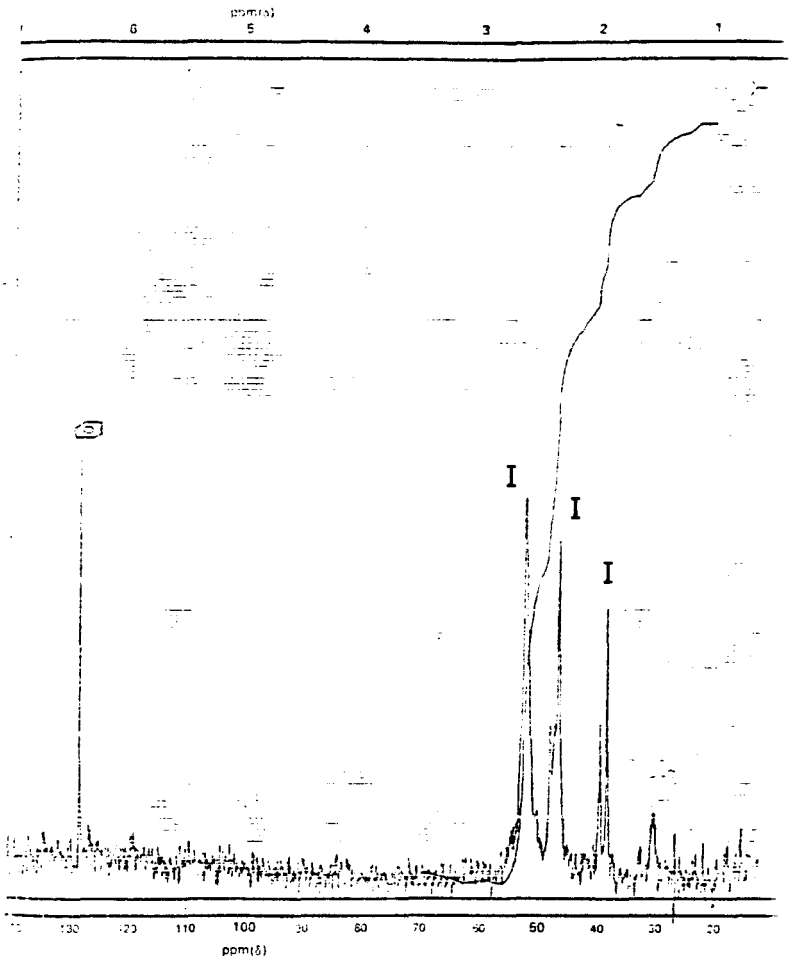


Figure 27. ^{13}C of acid deblocked TFA PEI D_2O

Acid deblockage has a less deleterious effect on backbone resolution.

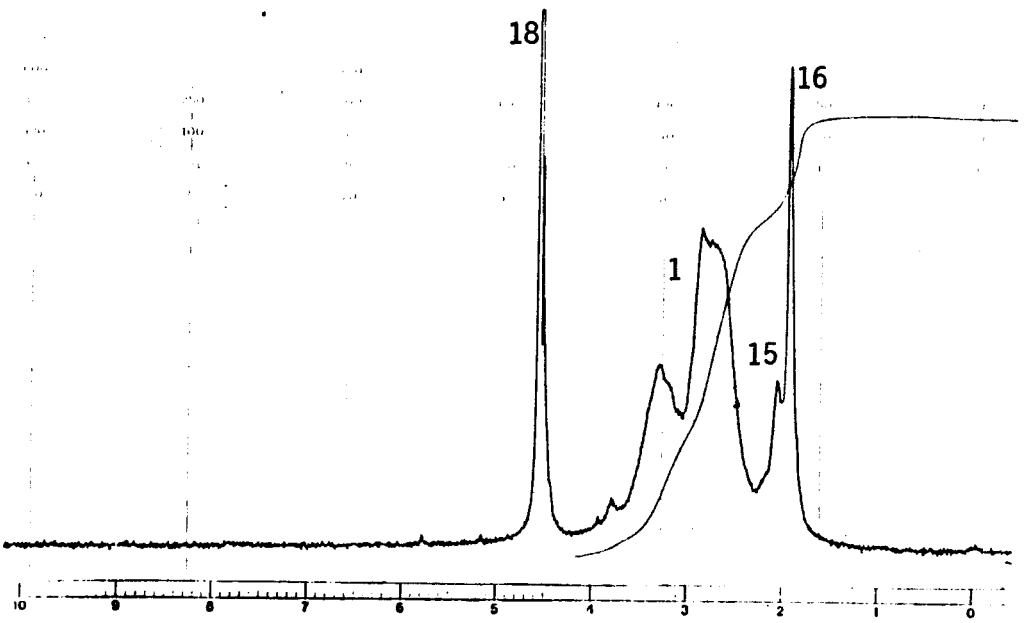
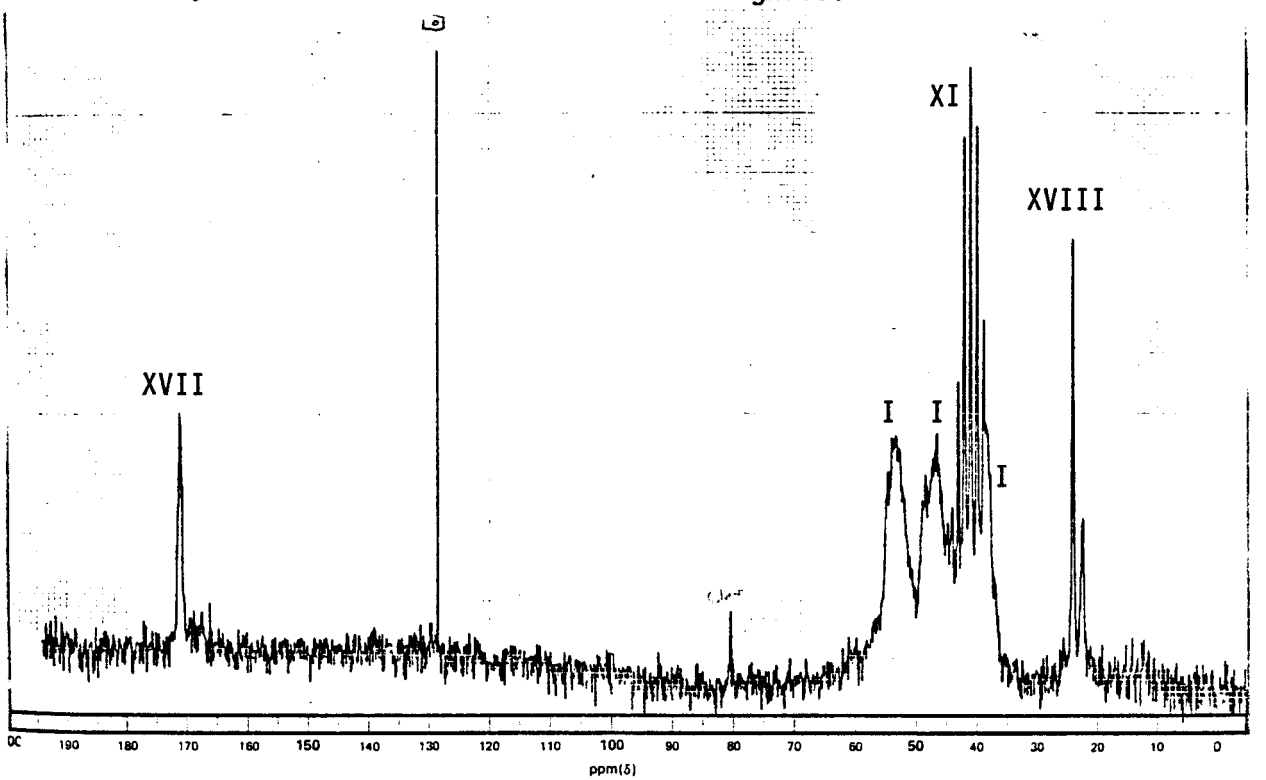


Figure 28. Peracetylated PEI $D_2O/_{tms}$

Two acetyl peaks are seen in PMR depending on the amine to which they have been reacted, unlike TEOC and t-boc ^1H NMR signals.



F Figure 29. ^{13}C of peracetylated PEI d_6 DMSO

Two acetyl methyl signals appear but the carbonyl peak is not resolved into two signals. The backbone is reduced to three broad signals.

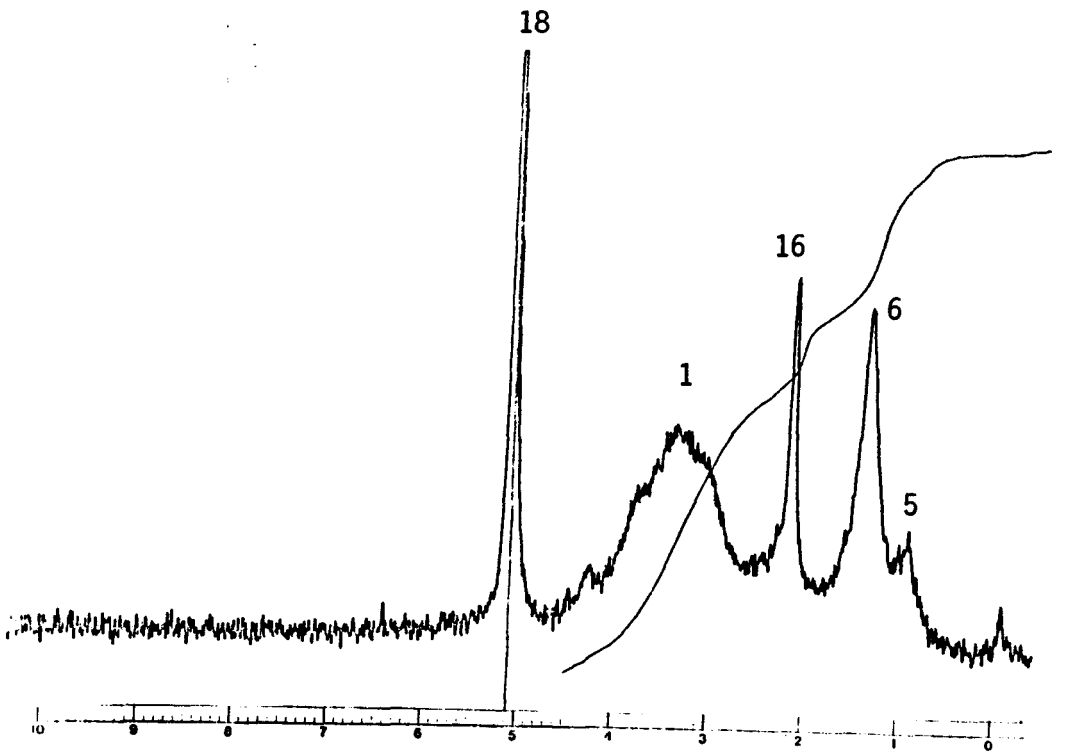


Figure 30. 25%$C_{12}H_{25}$ (on $1^{o}N$) PEI, acetylated $D_2O/_{tms}$

The primary amines preferentially react with dodecyl iodide and in so doing are converted into secondary amines. Subsequent acetylation gives a polymer having one acetyl signal.

Acetylation of piperidine deblocked, C_{12} quaternized TFA-PEI gives indirect evidence for TFA removal and for placement of lauryl groups onto tertiary amines since two acetyl peaks should appear. A derivative of this type is also of interest for its similarity of structure to tertiary laurylated TFA-PEI. Comparison of figure 31 to 32 shows the acetylated product of deblocked TFA-C_{12} to be very similar to directly acetylated, then alkylated PEI. It is interesting that direct quaternization of acetylated PEI, which was performed in ethanol, proceeded to about the same extent as quaternization of TFA-PEI in acetonitrile (Figures 32 and 14). Both TFA-PEI and acetyl PEI react to about the same extent with SNPA (Figures 33 & 34). The ^{13}C spectrum of acetyl-3* C_{12} PEI is poorly resolved in either of the two solvents used for collecting the spectra (D_2O and d_6DMSO) since concentrated solutions had to be made and the polymer eventually precipitated out (Figures 35 & 36).

Exhaustive methylation of PEI from which the trifluoroacetyl groups have been removed increases the size of the polymer backbone peak in PMR and, as expected, results in a downward shift. By integrating the peak and comparing it to the size of trimethylsilyl or lauryl peaks present before and after the total quaternization, the extent of this reaction may be determined. Typically 93% full quaternization is achieved (Figures 37 & 38). That no secondary amines remain has been demonstrated by attempting to react the product with the most reactive and easily quantitated of our acylating reagents, TEOC-Np. No substitution occurred (Figure 38). All amines have been taken to at least the tertiary state.

F^- or acid deblockage subsequent to quaternization regenerates the

Substitution with hydrophobes appears to have been on tertiary amines since acetylation of deblocked TFA-C_{12}PEI proceeds extensively and gives rise to two acetyl signals in the PMR spectrum.

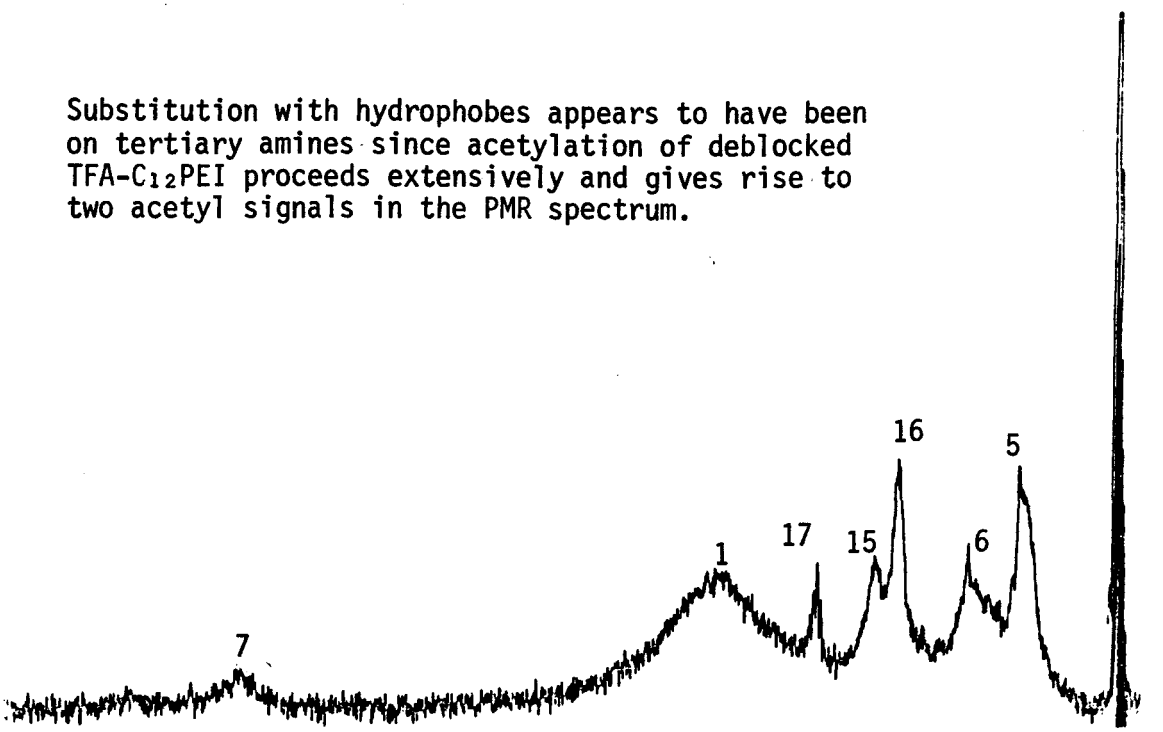


Figure 31. TFA PEI reacted with dodecylbenzyl chloride; deblocked then acetylated (residual acetonitrile present) d_6DMSO/tms

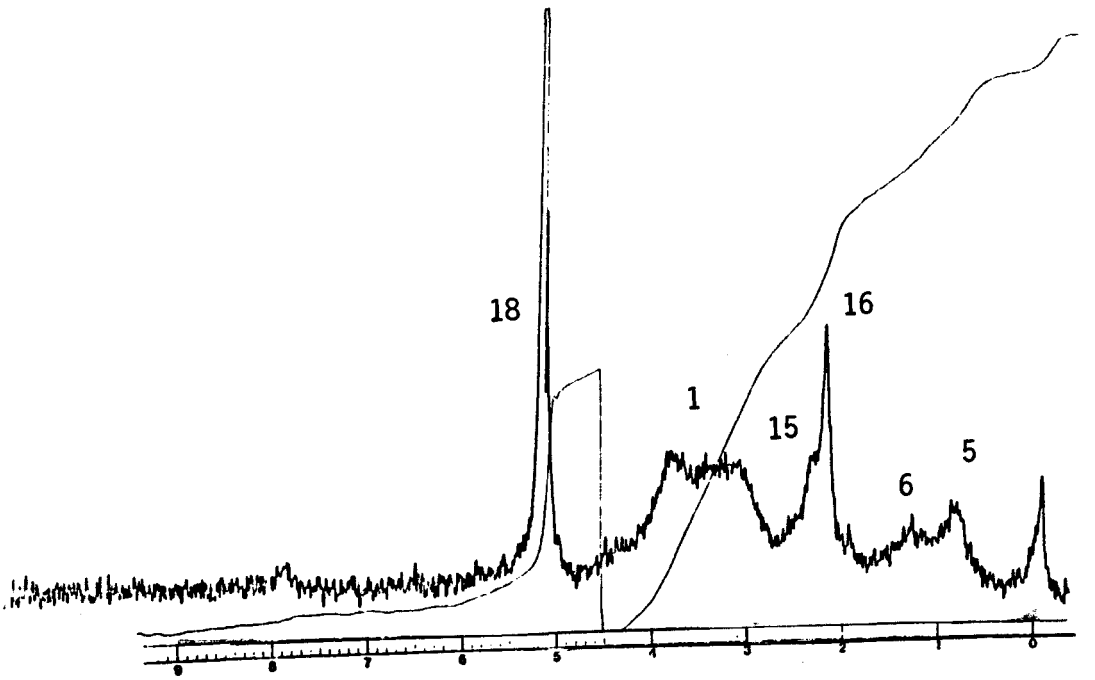


Figure 32. Peracetylated PEI reacted with $C_{12}H_{25}I$ D_2O/tms

Direct hydrophobe addition to acetyl PEI gives a polymer whose PMR spectrum is similar to acetyl PEI which had been quaternized when trifluoroacetylated.

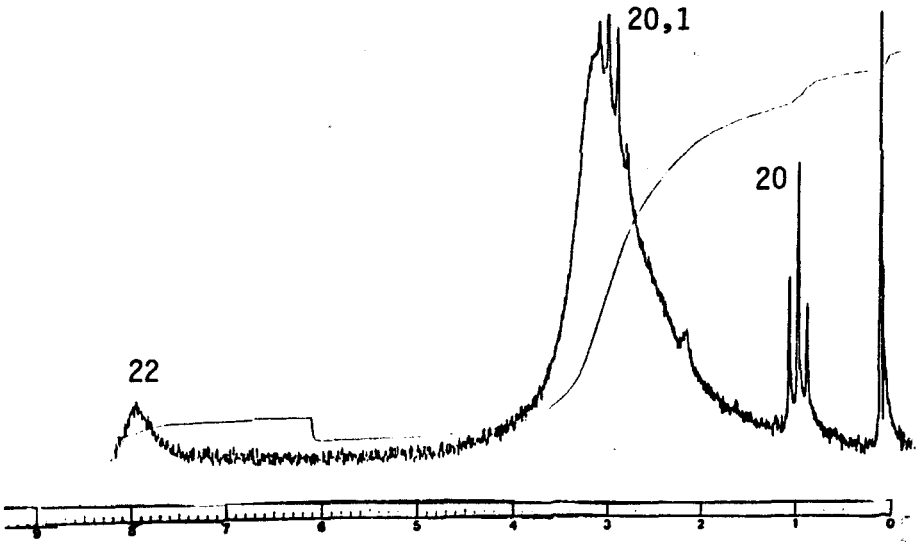


Fig. 33. TFA PEI reacted with SNPA (residual E+OH present) DMSO/$_{tms}$

The aromatic protons of the SNPA reagent are distant from the backbone of TFA PEI.

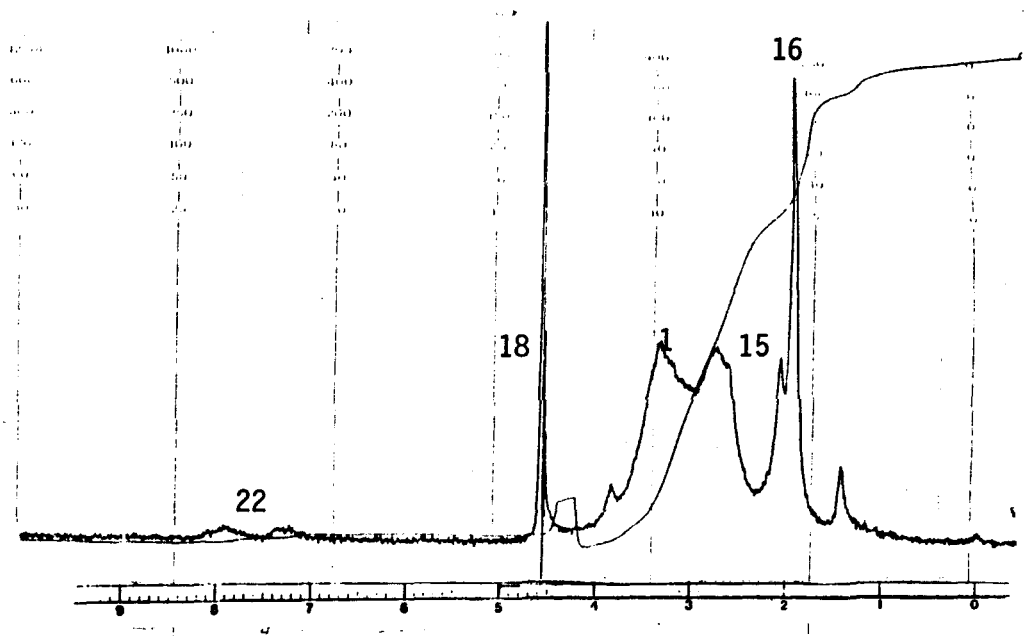


Fig. 34. Acetyl PEI reacted with SNPA $D_2O/_{tms}$

Acetylation of PEI also leaves a small number of amines free for amidation with SNPA.

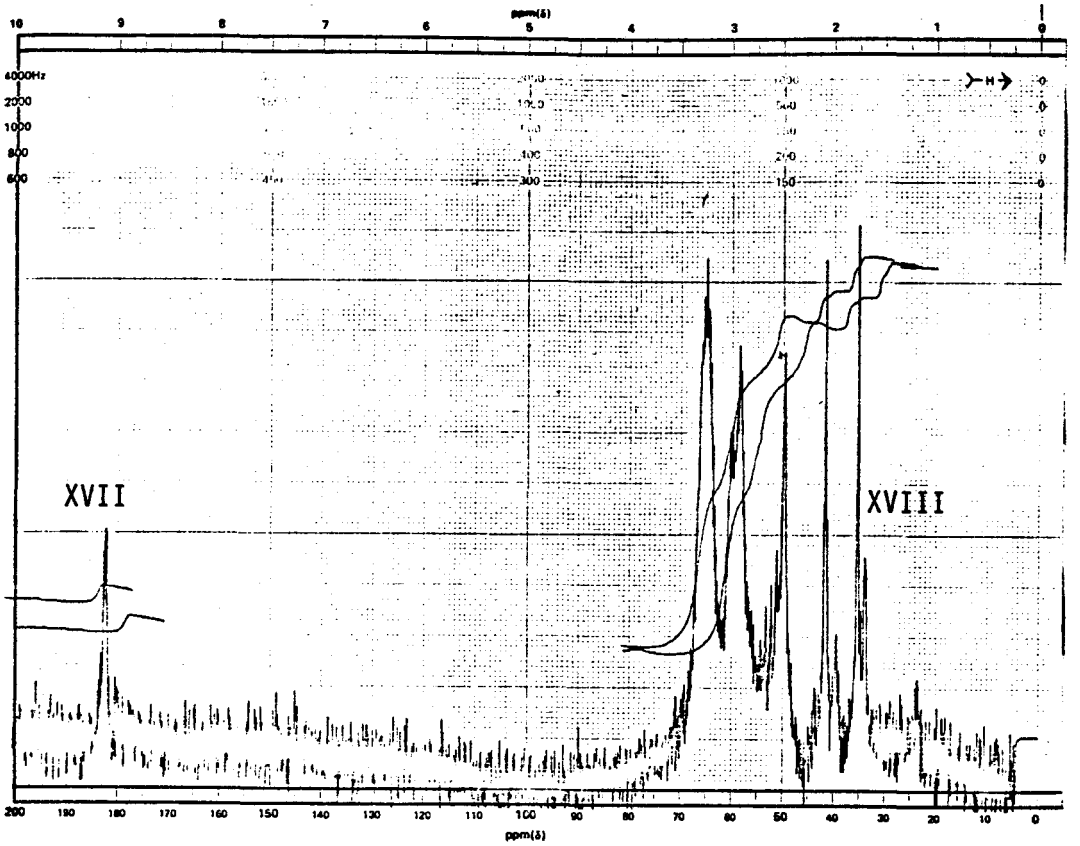


Figure 35. ^{13}C of Acetyl PEI reacted with dodecyl iodide D_2O

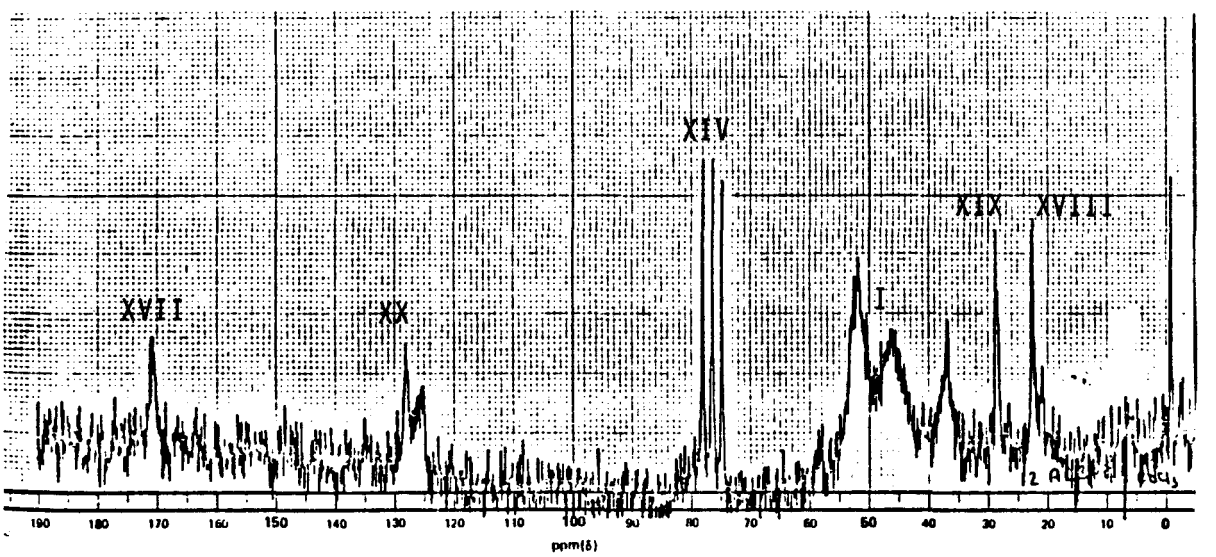


Figure 36. ^{13}C of Acetyl PEI reacted with dodecyl benzyl chloride $CDCl_3$

The aliphatic signals of hydrophobes appear between the acetyl methyl signal and the polymer backbone.

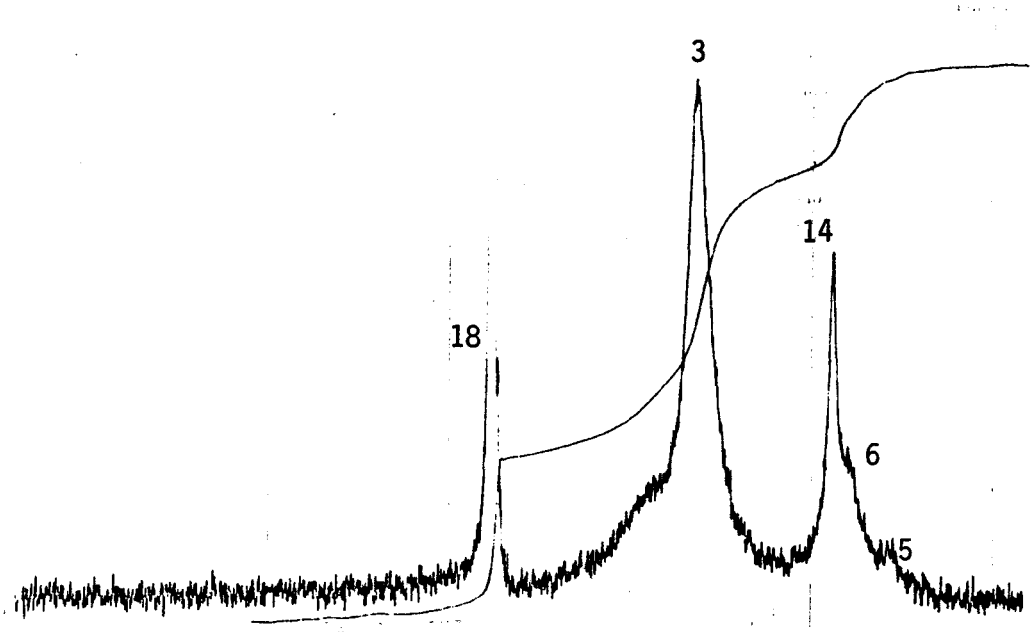


Figure 37. TFA 5% t-boc PEI 1-2% lauryl; deblock in piperidine D_2O

Piperidine selectively removes the TFA groups while leaving the t-boc group intact.

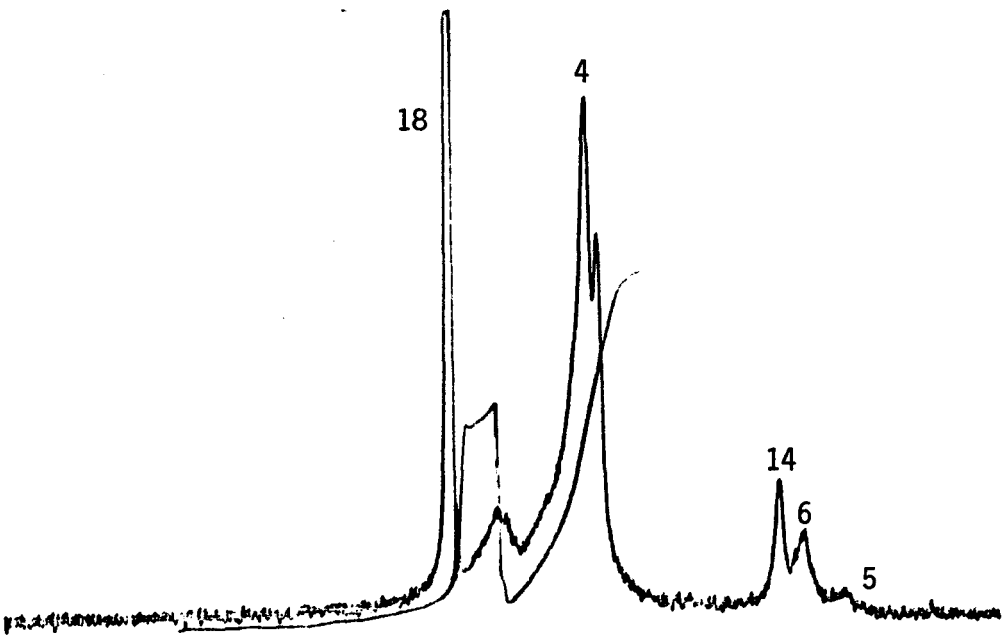


Figure 38. TFA 5% t-boc PEI 1-2% lauryl; deblock in base and methylated. Then subject to TEOC-O-NP D_2O

"Exhaustive" methylation essentially eliminates primary and secondary amines, rendering the polymer inert to further substitution with TEOC-O-Np.

differentially blocked secondary amines. TEOC or t-boc removal is quantitative as demonstrated by disappearance of their peaks in PMR spectra (Figures 39-42).

In placing a nucleophile on these newly regenerated secondary amines, several routes may be followed. Possibly the simplest is to react them with propylene sulfide. The methyl group of the propylene moiety is distinguishable in PMR (Figure 43). However, to determine the percent sulfhydryls, the polymer must be tested with a rapidly quantitated reagent such as Ellman's reagent, since the active sulfhydryls might easily oxidize to form disulfide crosslinks.

In performing the DTNB assay directly (see methods) it was found that some buffers promoted the dissociation of the DTE-arsenate complex, resulting in an unacceptably large background absorption. Even in the best of buffers, sodium barbital, the background absorbance was still so high (for example 0.3 O.D units at 10^{-5} M DTE), that the polymer contribution could not be accurately determined. Moreover, the polymer itself catalyzes DTNB breakdown and surprisingly the chromophoric product showed an absorption maximum at 430 nm, not 405-412. This would often return to the characteristic maximum upon sitting in the reaction medium for several days.

The problems of polymer catalyzed and reducing agent induced release of p-nitrothiophenolate could be circumvented by isolating the derivatized polymer and then releasing the chromophore with base as described in method two. Fortunately, absorption properties were not altered by the polymer under these conditions.

The most recently synthesized polymer which had been differentially

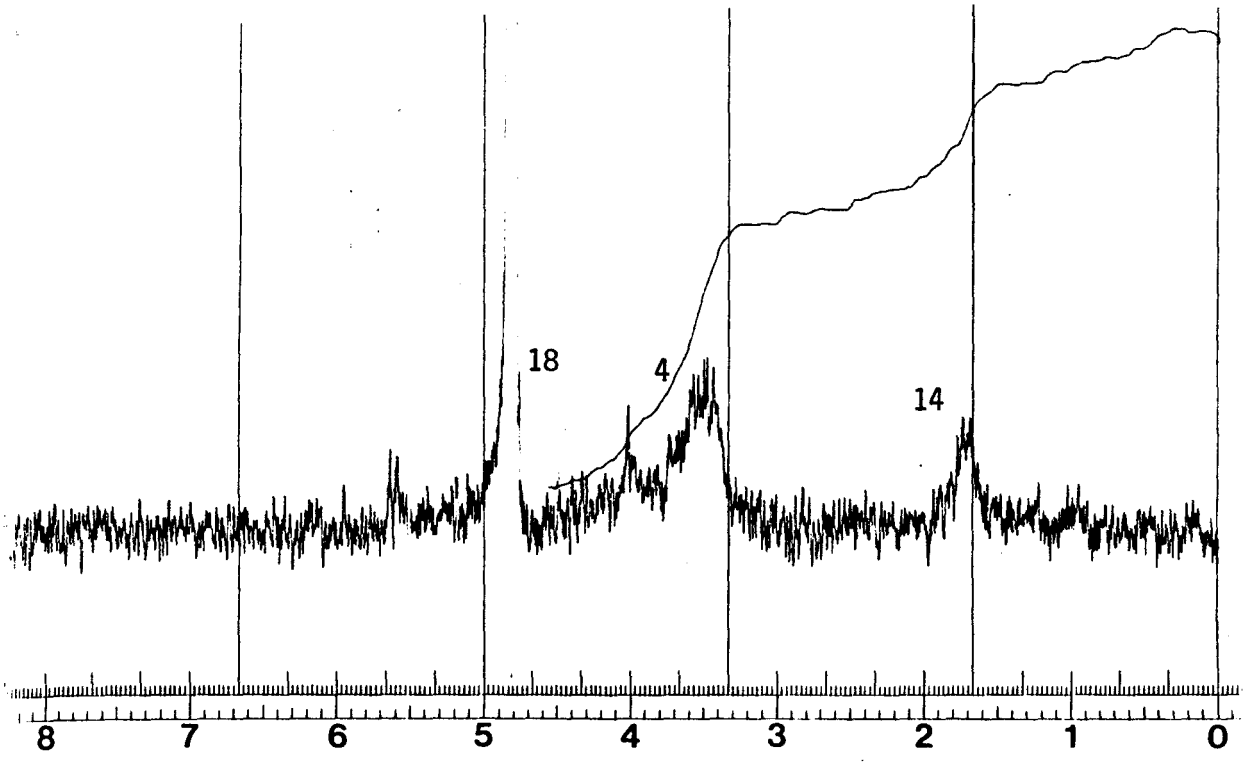


Figure 39. TFA t-boc PEI; base deblocked and methylated D_2O

The t-boc group is still visible after exhaustive methylation.

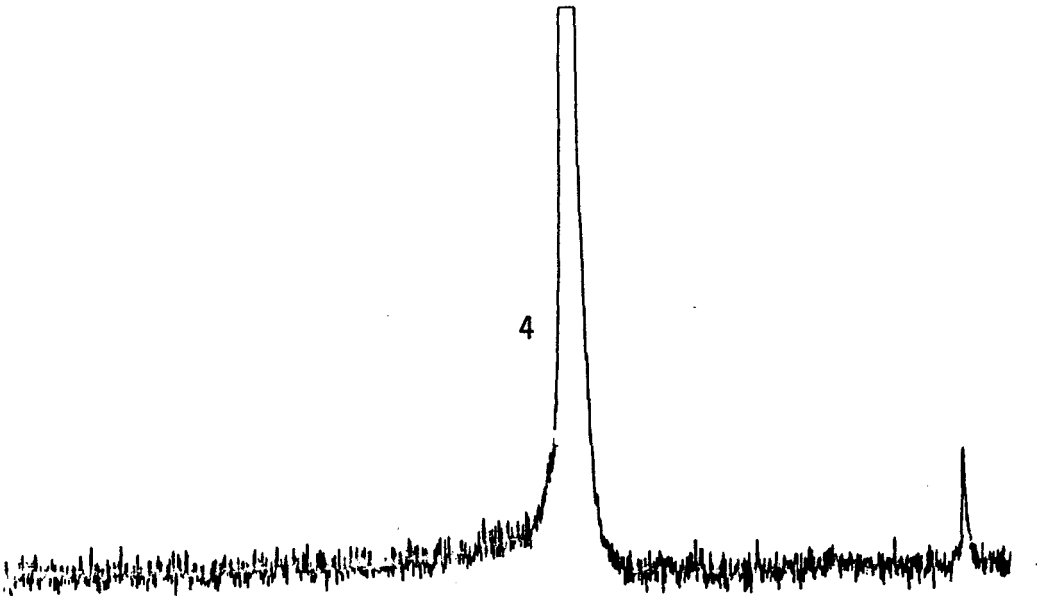


Figure 40. TFA tboc PEI, deblocked in base, methylated and subjected to acid. $D_2O/_{tms}$

Trifluoroacetic acid removes t-boc groups from quaternized PEI.

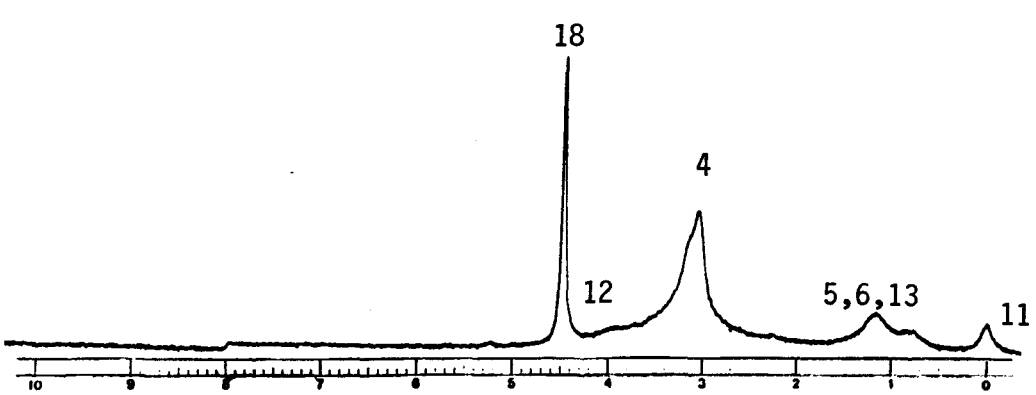


Figure 41. TFA-TEOC PEI, 3° Laurylated; Piperidine Deblocked and Exhaustively Methylated. D_2O

Base deblockage leaves TEOC as well as the t-boc (Fig. 37) groups intact.

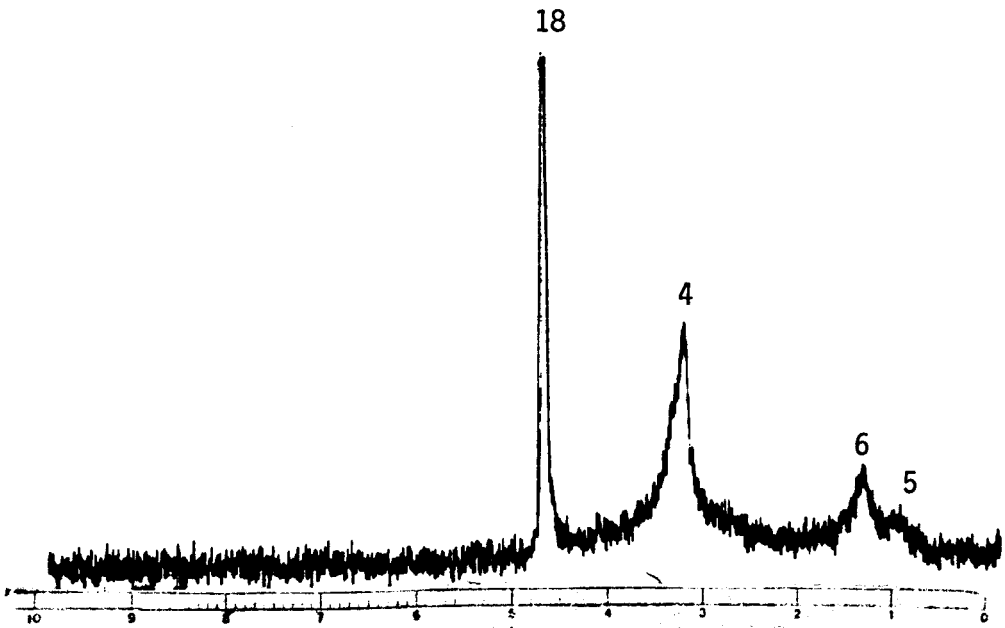


Figure 42. 2° TEOC 3° Lauryl PEI, exhaustively methylated; F^- deblocked in HMPA/DMF D_2O

The TEOC group can only be removed using "naked" fluoride.

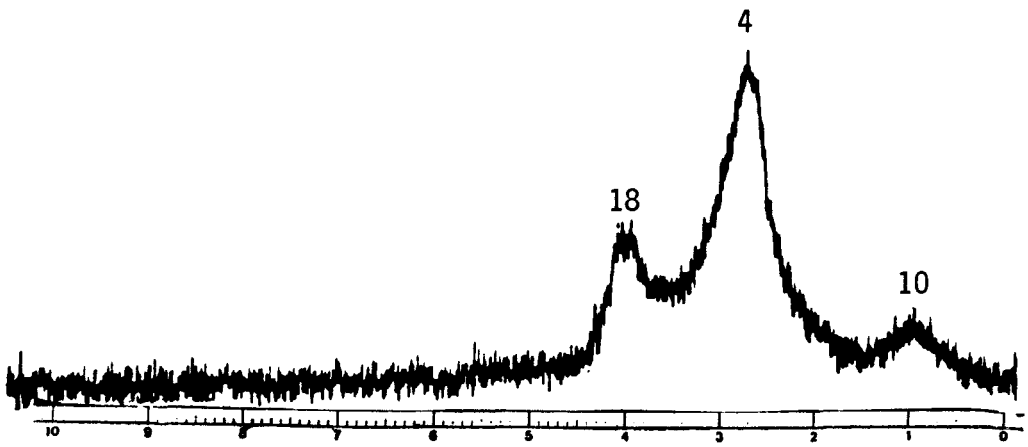


Figure 43. Exhaustively methylated 2° t-boc, acid deblocked; reacted with propylene sulfide. D_2O, DMSO/tms

The episulfides will react with newly regenerated secondary amines of quaternized PEI.

blocked with the TEOC group and deblocked subsequent to quaternization with methyl iodide for sulfhydryl placement has been carefully monitored at each step of the synthesis by PMR spectroscopy. Figures 44 through 48 illustrate the products of the stepwise synthesis as performed on a single polymer preparation. This particular derivative was especially interesting in that only a limited number of the reactive amines were available for the TEOC reaction, and both the laurylation and the quaternization with methyl iodide proceeded extensively. Figure 44 shows the TFA-TEOC polymer subsequent to laurylation of tertiary amines. In Figure 45 the TFA groups have been removed and the polymer dissolved in deuterium oxide. When the laurylation is as extensive as this, the polymer cannot be accurately integrated by PMR in D_2O due to signal overlap. Figures 46 and 47 are of the same polymer and were taken after exhaustive methylation. Figure 46 was in deuterated DMSO (some water appears around 5.2 ppm due to the hygroscopic nature of polymer and DMSO) whereas Figure 47 was recorded.in D_2O. In Figure 47, the peak at 6.65 ppm is an unidentified contaminant. Polymers such as this which have been extensively laurylated on tertiary amines and exhaustively methylated frequently show a peculiar tendency to broaden the water peak. In the final frame, Figure 48, the same polymer was subjected to benzoyl chloride in anhydrous methanol to verify that there are no free secondary amines. Indeed, the baseline is flat in the region of the spectrum corresponding to phenyl hydrogens. At the lower concentration of polymer seen in this spectrum (80mg/0.5 ml versus 140 + mg/0.5 ml) the peaks appear better resolved from one another due to better solubility at this concentration.

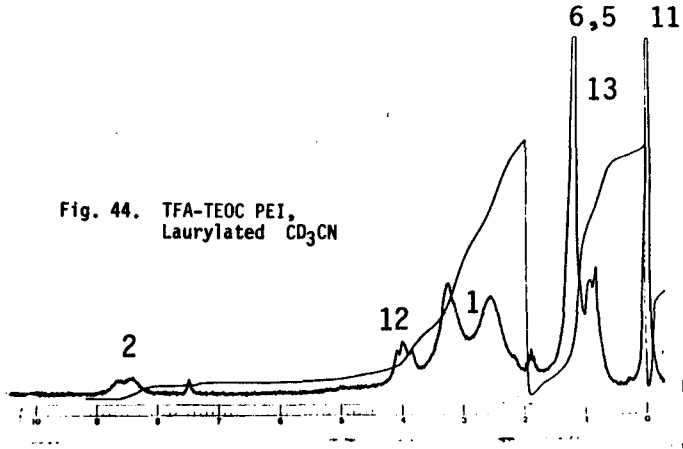


Fig. 44. TFA-TEOC PEI, Laurylated CD_3CN

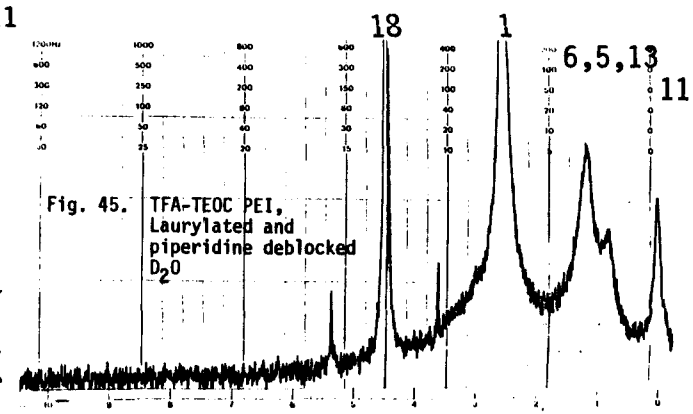


Fig. 45. TFA-TEOC PEI, Laurylated and piperidine deblocked D_2O

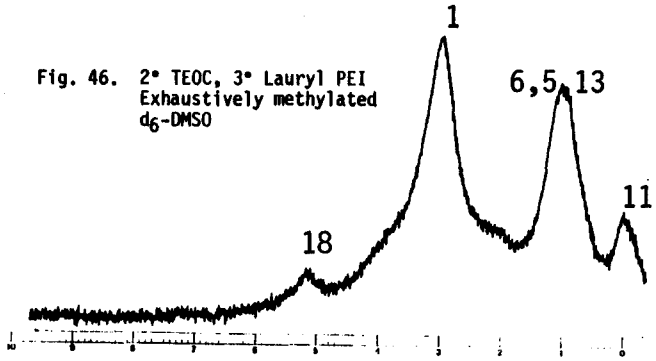


Fig. 46. 2° TEOC, 3° Lauryl PEI Exhaustively methylated d_6-DMSO

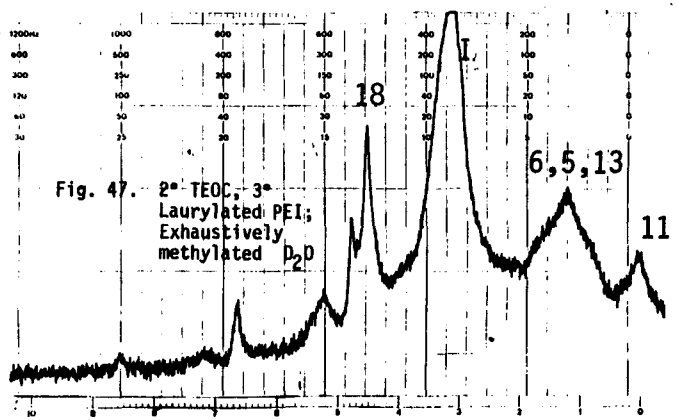


Fig. 47. 2° TEOC, 3° Laurylated PEI; Exhaustively methylated D_2O

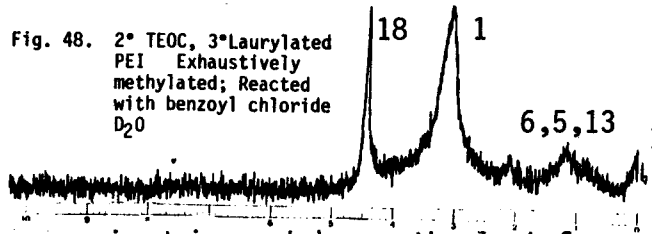


Fig. 48. 2° TEOC, 3°Laurylated PEI Exhaustively methylated; Reacted with benzoyl chloride D_2O

The stepwise synthesis is summarized in part here; the last frame indicates that prior to removal of the differential blocking group, there are essentially no primary or secondary amines available for acylation with benzoyl chloride.

Introduction and Assay of the Mercaptan

Spectrum 42 is the same polymer after removal of the TEOC group by "naked" fluoride. The newly regenerated amines were substituted to a slight extent with propylene sulfide as described in the methods section. The amount of mercaptan was consistently found to be 0.2% total monomer content. The content of sulfhydryl may be increased to 0.268% if precautions are taken to fully reduce the polymer immediately before performing the DTNB assay, although such small amounts of PEI were used that even this 33% difference may be due to inaccuracies of weighing particularly from differences in hydration.

Assay I: Direct determination of sulfhydryl content

Polymer weight: 0.8 mg into 1 ml

Background absorbance at 412 nm: .08

Absorbance in base .142

Molar percent sulfhydryl: 0.2%

Assay 2: Determination after reduction with dithioerythritol

Polymer weight: 0.8 mg into 1 ml

Background absorbance at 412 nm: 0.069

Absorbance in base: 0.152

Molar percent sulfhydryl: 0.268%

Polymers Containing Reporter Groups

The polymers used for the Koshland studies were analyzed by proton NMR spectrometry and absorption spectroscopy in the visible range. PMR allowed the syntheses to be monitored with the exception of determining the amount of reporter group present, since only one percent or less

substitution was desired. Proton NMR spectra of the partially trifluoroacetylated polymers having been substituted with hydrophobe and reporter group and then deblocked illustrate the absence or near absence of a phenyl signal since so few are present. (Figures 49 and 50). To add a five-fold excess of Koshland reagent I is not at all unreasonable since the chemical is highly susceptable to hydrolysis by water and hydroxide ions,[315] and these are present in trace amounts even when the solvent used in anhydrous methanol or acetonitrile.

The spectra of the six PEI derivatives containing the Koshland I reagent at various pHs are shown in Figures 51-56. The absorption spectra were scanned from 500 nm to about 250 nm. The polymer scatters light at these shorter wavelengths. The wavelength of maximum absorption for the phenolate and its optical density at each pH are summarized for each polymer in Tables 2-7. The total amount of nitrophenolate was determined at 400 nm using Westheimer's published extinction coefficient of 8,000, since the nitrophenol species has no absorption at this wavelength (eqn.3). Using the phenolate concentration determined above, the anticipated absorption at 320 nm due to the ionized species was calculated. Upon subtraction of this from the total absorption observed at 320 nm, the contribution of the unionized nitrophenol was found. Since its extinction coefficient at 320 nm is also published[314], the nitrophenol concentration could be obtained using Beer's law (eqn. 6).

Beer's law $A_\lambda = \varepsilon_\lambda c l$ $\quad c = \frac{A_\lambda}{\varepsilon_2 l}$ $\quad l = 1$ cm

400 nm = isosbestic point of the two phenolate species

$$A_{400nm} = \varepsilon_{400} [RNHR' + RNH_2^+R']$$

eqn 1 $$A_{400} = \varepsilon_{400}^{\phi\text{-}O^-} [\phi\text{-}O^-] + \underbrace{\varepsilon_{400}^{\phi\text{-}OH} [\phi\text{-}OH]}_{0}$$

eqn 2 $$A_{400} = \varepsilon_{400}^{\phi\text{-}O^-} [\phi\text{-}O^-]$$

eqn 3 $$\frac{A_{400}}{\varepsilon_{400}^{\phi\text{-}O^-}} = [\phi\text{-}O^-]$$

eqn 4 $$A_{320} = \varepsilon_{320}^{\phi\text{-}O^-} [\phi\text{-}O^-] + \varepsilon_{320}^{\phi\text{-}OH} [\phi\text{-}OH]$$

eqn 5 $$A_{320} - \varepsilon_{320}^{\phi\text{-}O^-} [\phi\text{-}O^-] = \varepsilon_{320}^{\phi\text{-}OH} [\phi\text{-}OH]$$

eqn 6 $$\frac{A_{320} - \varepsilon_{320}^{\phi\text{-}O^-} [\phi\text{-}O^-]}{\varepsilon_{320}} = [\phi\text{-}OH]$$

eqn 7 $$[Probe] = [\phi\text{-}O^-] + [\phi\text{-}OH]$$

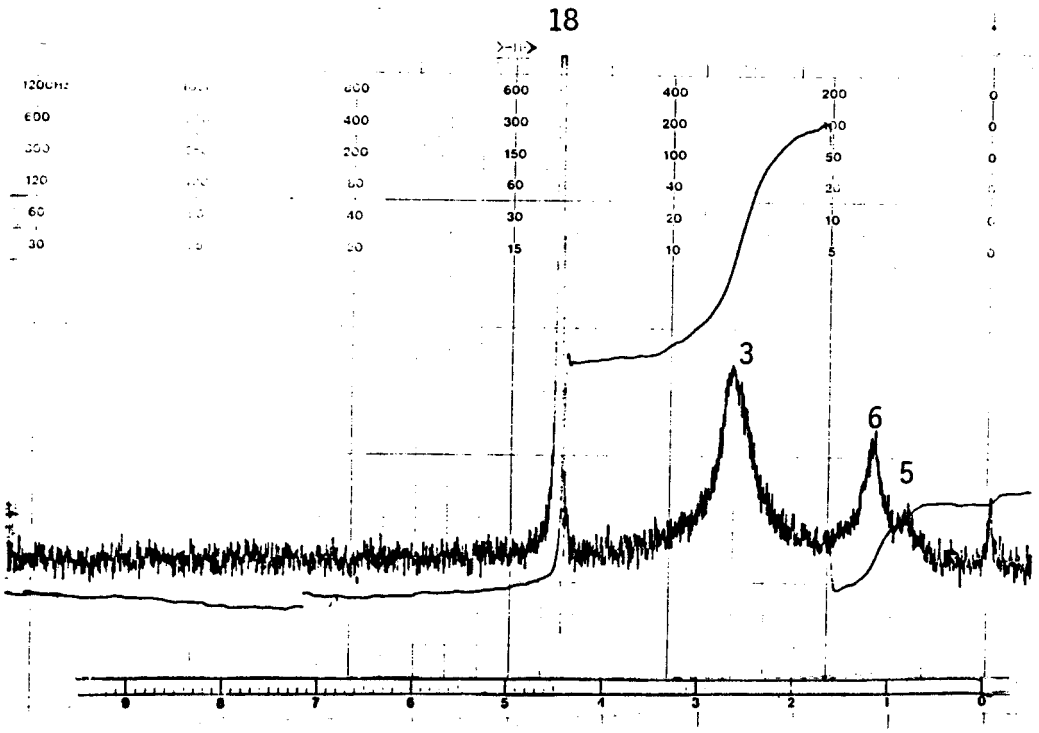


Fig. 49. $TFA_{.65}$ Koshland I $2°\text{-}3°C_{12}H_{25}$, Acid deblocked D_2O

The polymer has about 10% total amine of hydrophobes.

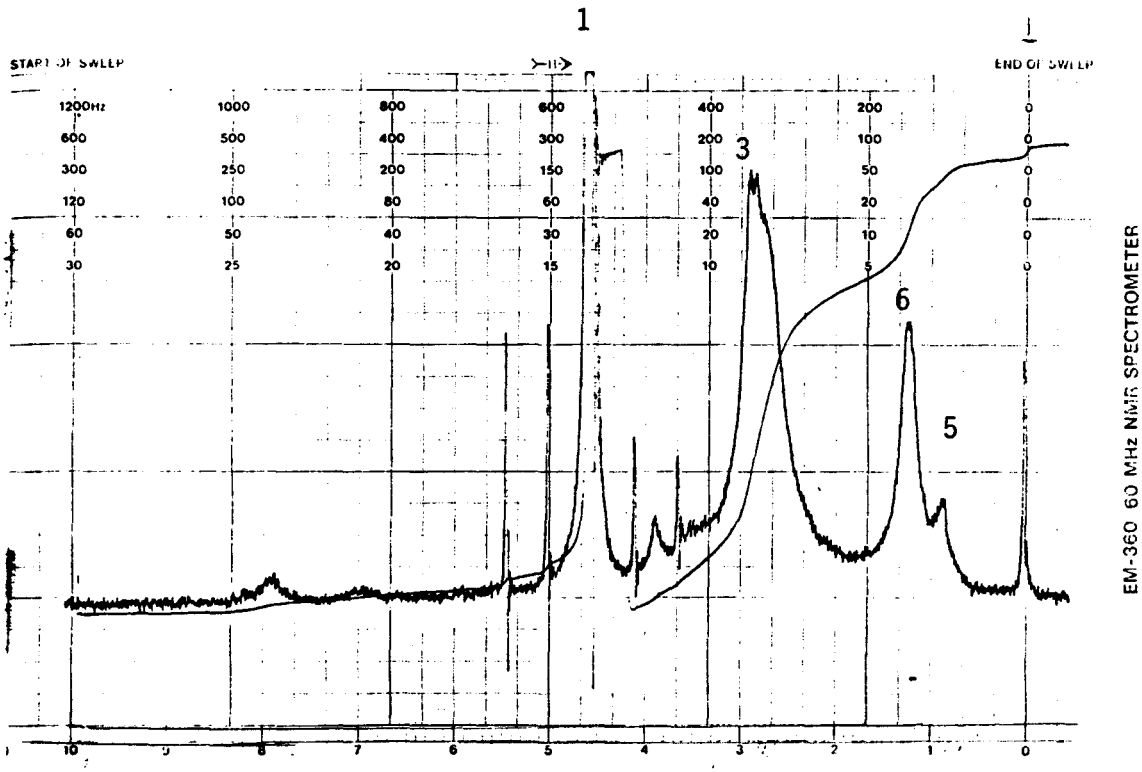


Fig. 50. $TFA_{.65}$ Koshland II $2°\text{-}3°C_{12}$, Acid deblocked D_2O

This polymer was also reacted to the extent of 10% total amine with hydrophobes.

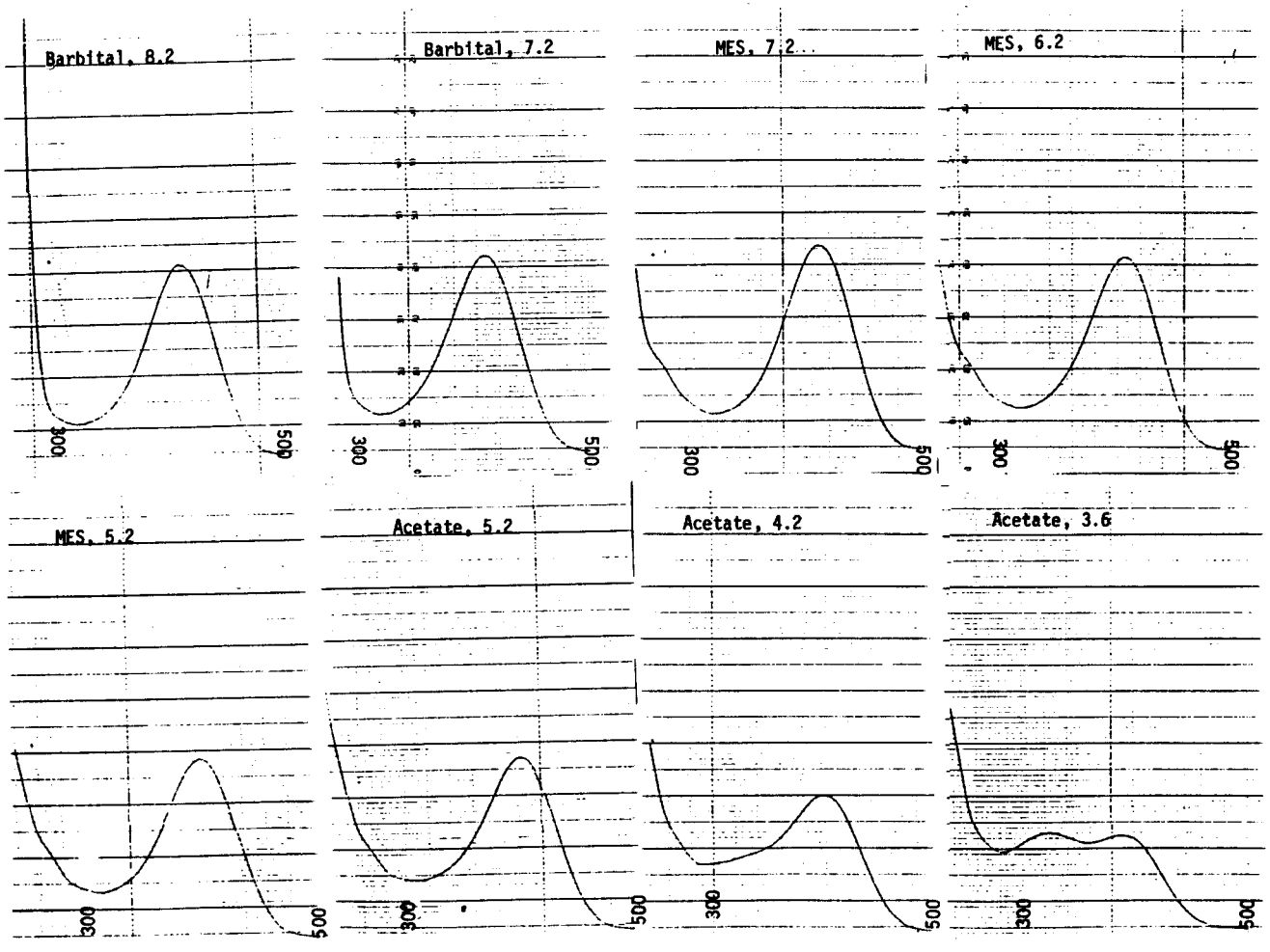


Fig. 51. TFA-TEOC PEI 3° Koshland I; Acid Deblocked Polymer conc. = 0.5 mg/ml

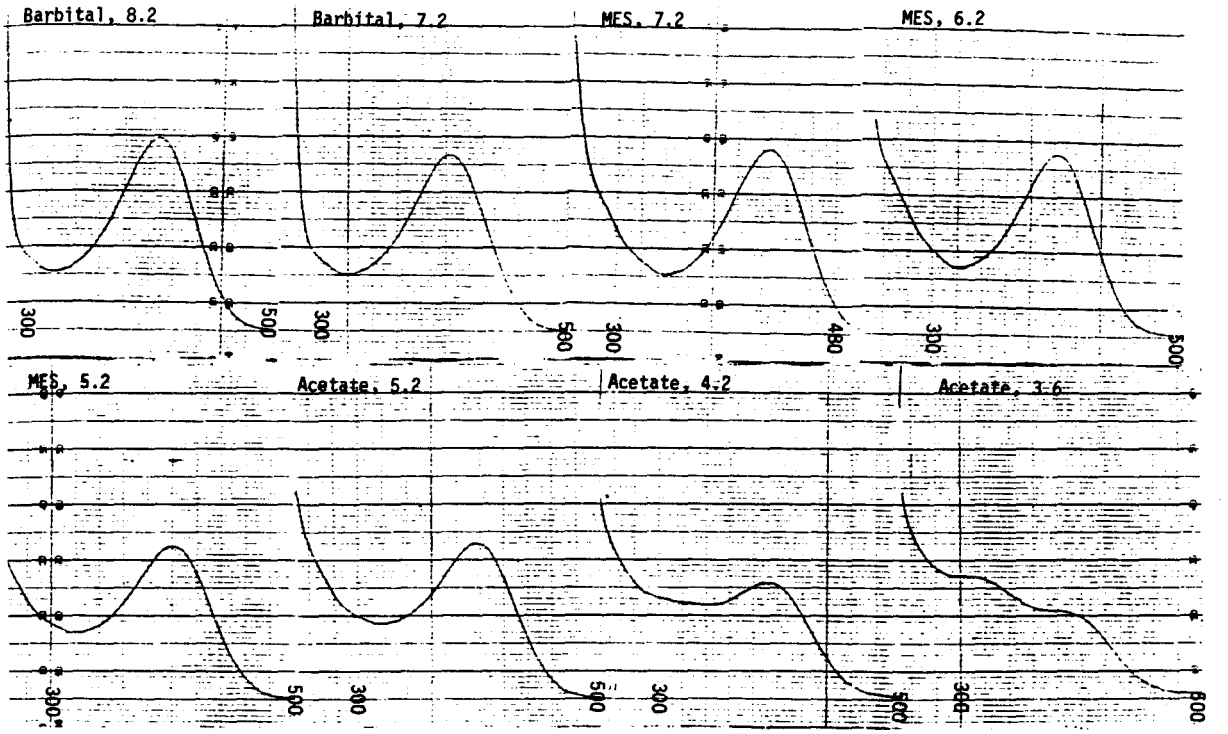


Fig. 52. Koshland I on Primary amines Polymer conc. = 0.2 mg/ml

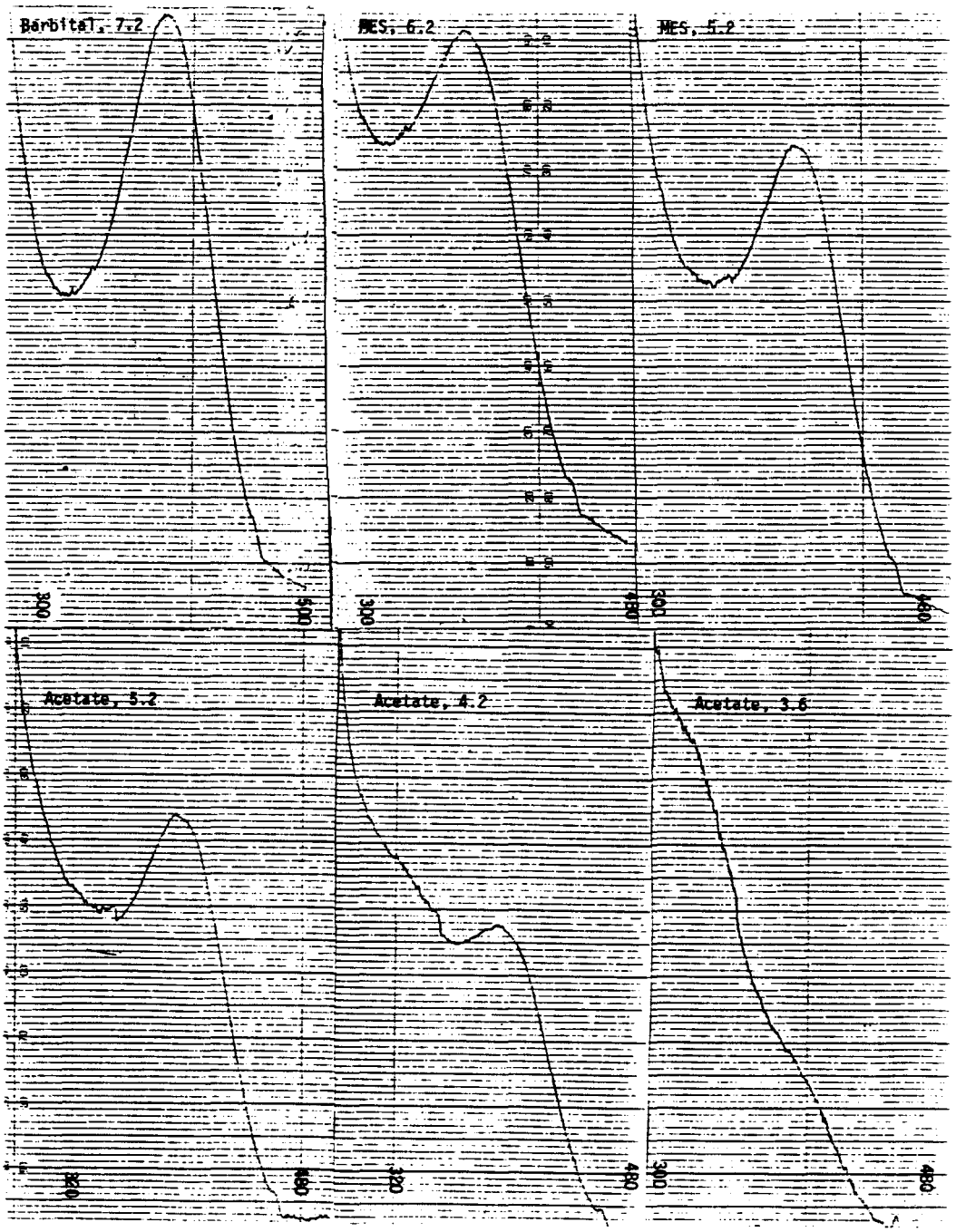


Fig. 53. TFA-TEOC 3° Dodecylbenzyl, Koshland I, Deblocked Polymer
conc. < 0.04 mg/ml due to difficulties in solubility

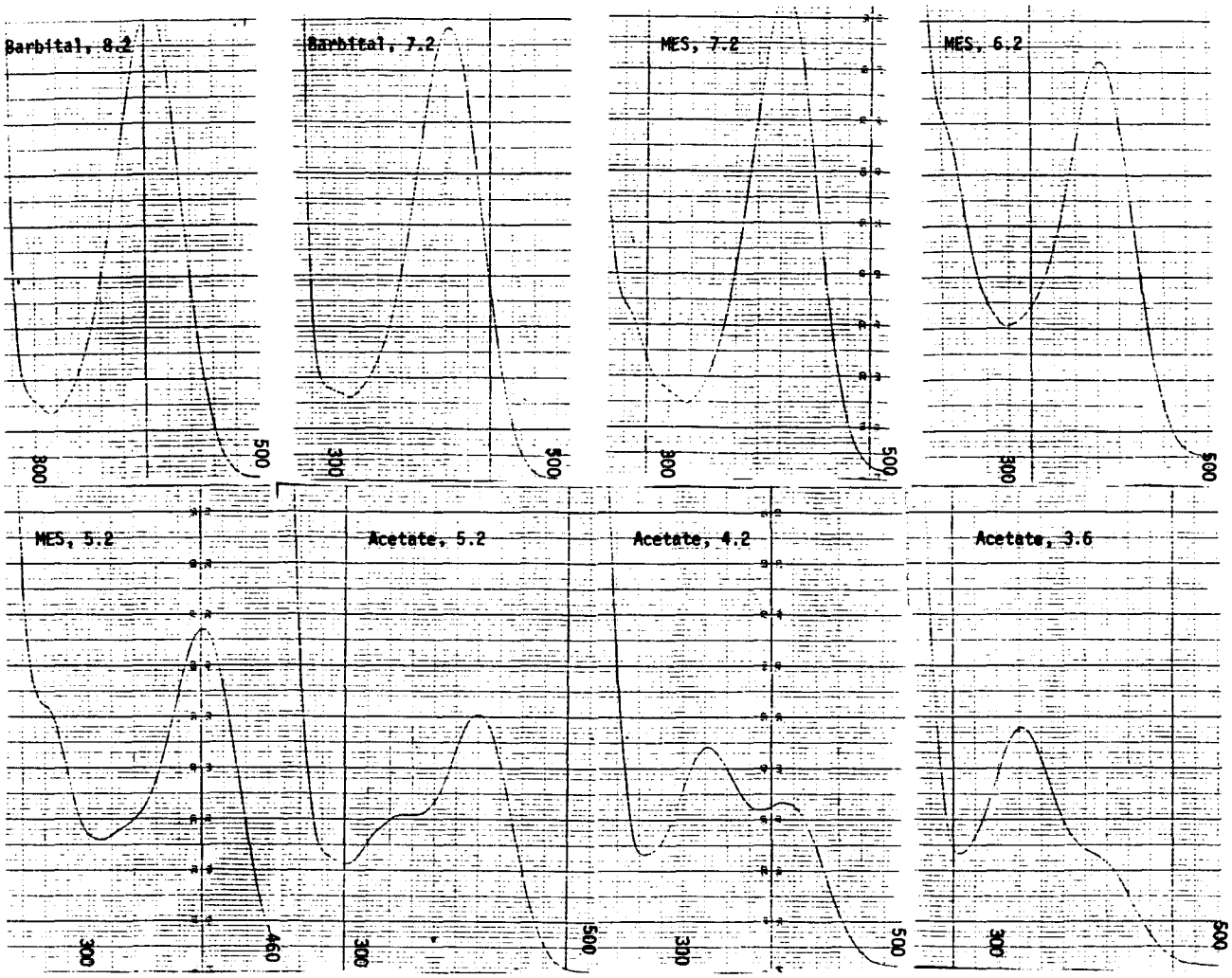


Fig. 54. 20% Lauryl Koshland I on Primary Amines Polymer conc. = 0.8 mg/ml

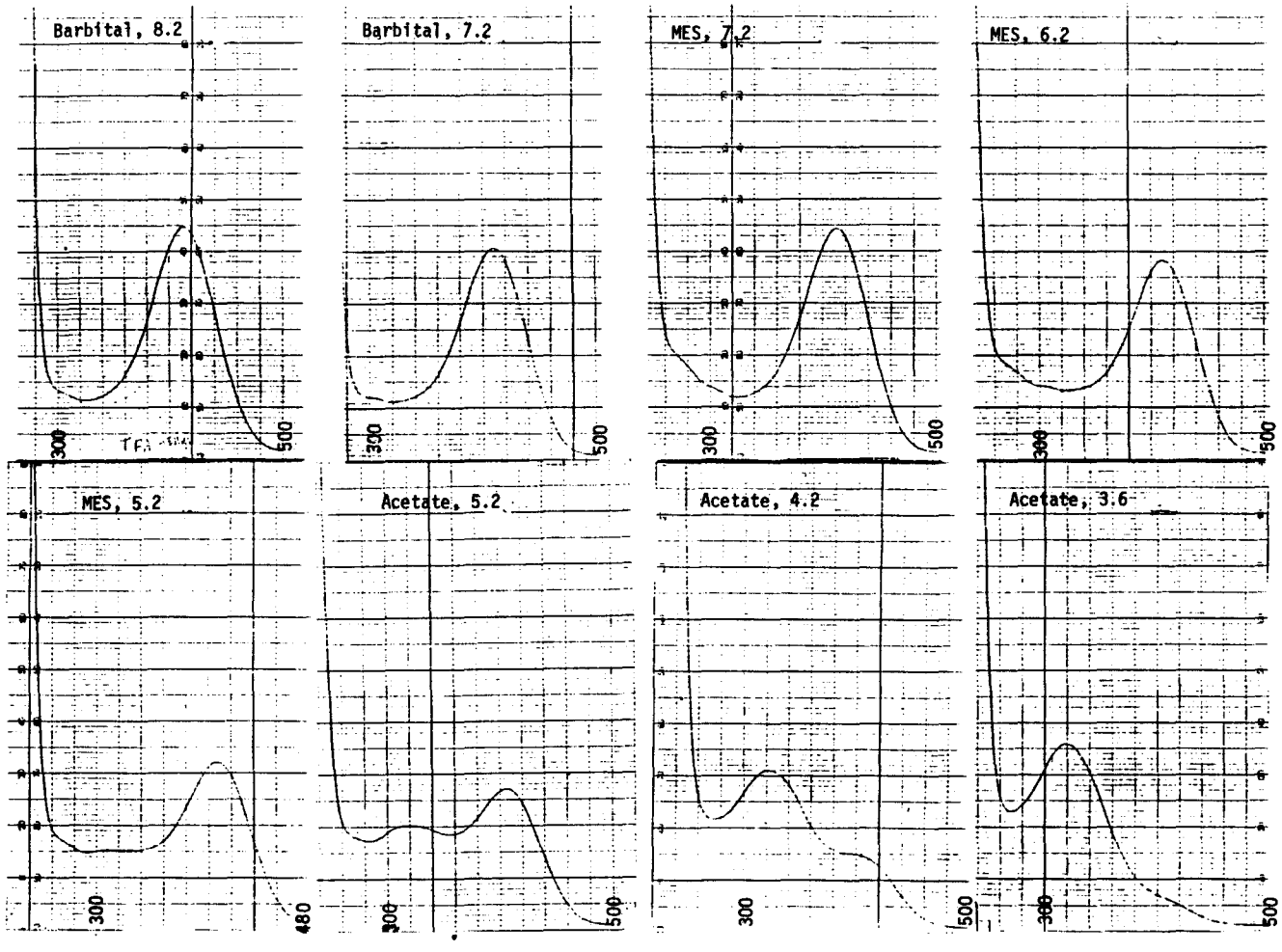


Fig. 55. Deblocked $TFA_{.7}$ $TEOC_{.05}$ 3° Dodecylbenzyl, Koshland I;
Exhaustively Methylated Polymer conc. = 0.2 mg/ml

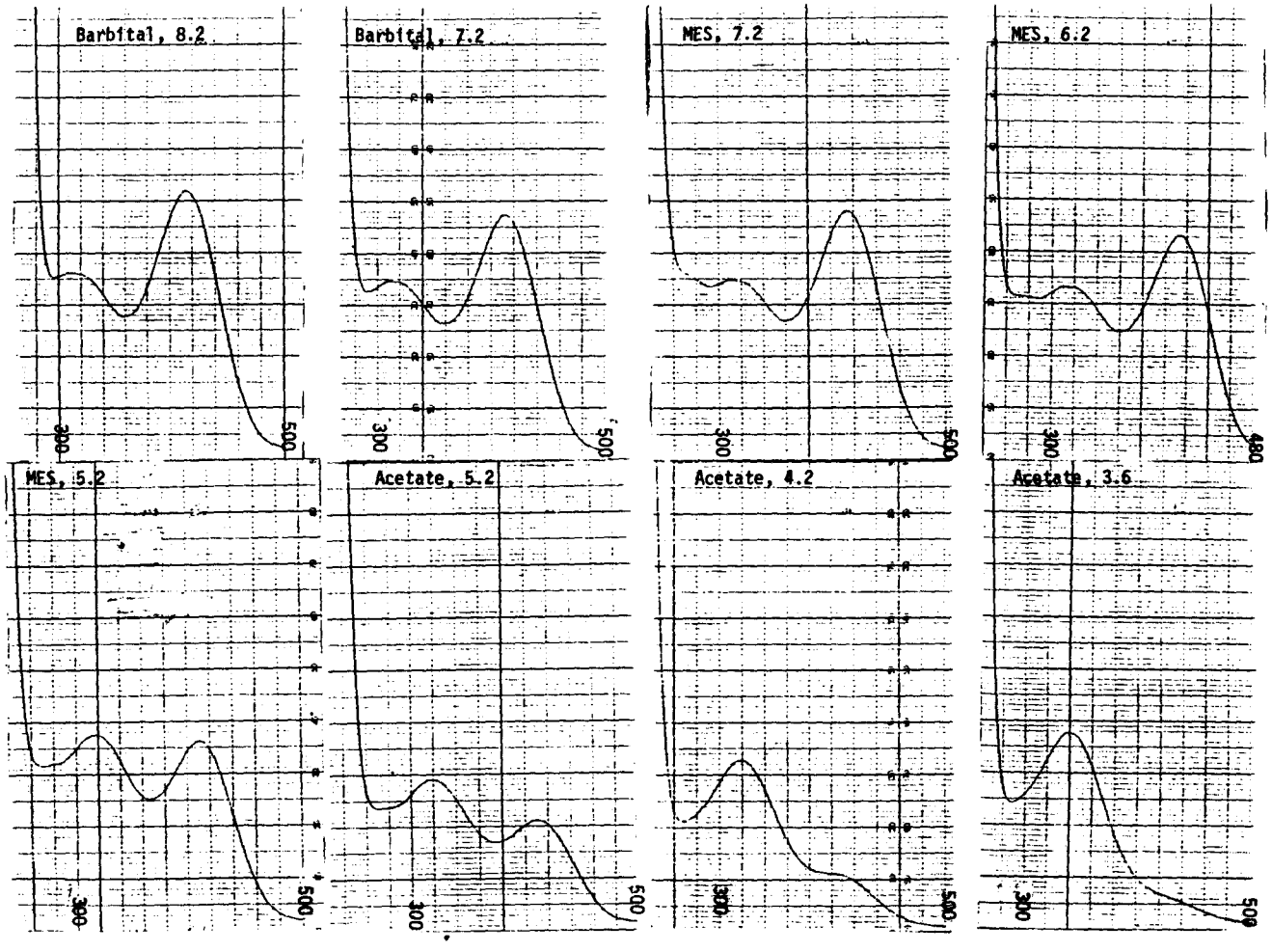


Fig. 56. Deblocked TFA_{65} Koshland I, $2°\text{-}3°C_{12}H_{25}$;

Exhaustively Methylated Polymer conc.= $\frac{0.2\ mg}{ml}$

Table 2 PEI - 3° Koshland I

Buffer, 0.02M	pH	λ max	% ionized
Barbital	8.2	405	87%
Barbital	7.2	405	83%
MES	7.2	405	86%
MES	6.2	405	81%
MES	5.2	402-5	76%
Acetate	5.2	402	75%
Acetate	4.2	395	54%
Acetate	3.6	395	34%

Table 3 PEI - 1° Koshland 1

Buffer, 0.02M	pH	λ max	% ionized
Barbital	8.2	405	73%
Barbital	7.2	405	72%
MES	7.2	405	72%
MES	6.2	405	68%
MES	5.2	398	60%
Acetate	5.2	395	58%
Acetate	4.2	390	39%
Acetate	3.6	shoulder	26%

Table 4 PEI - 3° dodecylbenzyl - 3° Koshland I
Not methylated

Buffer, 0.02M	pH	λ max	% ionized
Barbital	7.2	401	53%
MES	6.2	395	40%
MES	5.2	395	44%
Acetate	5.2	395	40%
Acetate	4.2	395	30%
Acetate	3.6	shoulder	14%

Table 5 PEI - 1° dodecylbenzyl (10%) 1° Koshland I
Not methylated

Buffer, 0.02M	pH	λ max	% ionized
Barbital	8.2	405	94%
Barbital	7.2	405	88%
MES	7.2	405	93%
MES	6.2	405	70%
MES	5.2	401	63%
Acetate	5.2	400	51%
Acetate	4.2	390	29%
Acetate	3.6	shoulder	18%

Table 6 PEI - 3° dodecylbenzyl - 3° Koshland
Exhaustively methylated

Buffer 0.02M	pH	λ max	% ionized
Barbital	8.2	405	78%
Barbital	7.2	405	69%
MES	7.2	405	73%
MES	6.2	405	64%
MES	5.2	405	52%
Acetate	5.2	405	36%
Acetate	4.2	shoulder	I4%
Acetate	3.6	shoulder	4.4%

Table 7 PEI (Partially Trifluoroacetylated) - Koshland I
dodecyl (10%) deblocked

Exhaustively methylated

Buffer, 0.02M	pH	λ max	% ionized
Barbital	8.2	405	44%
Barbital	7.2	405	42%
MES	7.2	405	42%
MES	6.2	405	45%
MES	5.2	405	33%
Acetate	5.2	405	23%
Acetate	4.2	shoulder	11%
Acetate	3.6		4.8%

The Koshland II reagent was placed on two polymers, onto primary amines of primary dodecylbenzylated PEI and onto nontrifluoroacetylated amines of 65% TFA PEI, which was subsequently laurylated and then deblocked and quarternized. These were the two polymers to be contrasted to assess in a preliminary manner whether there were major differences between the novel derivatives described herein and previously studied ones. The tertiary substituted polymer had been exhaustively methylated since some type of primary amine inactivation must be performed in order for such a derivative to be used in esterolysis. There was at best a one to two nanometer difference in the peak of maximal absorption between the two polymers. Both absorbed around 314 nm. On the basis of Koshland's work this corresponds to the polarity of a 60% dioxane, 40% water solution.[308] While this would be considered rather polar, maxima of 317 are reported in dimethyl sulfoxide. The absorbance maxima observed here are independent of pH and type of buffer used, be it barbital, MES, or acetate (Figures 57 and 58). However, absorption probes as tools for the monitoring of microenvironmental polarity have not been used extensively in proteins,[316] let alone polymers.

An alternative spectroscopic approach known to be much more sensitive to changes in dielectric is fluorescence. The NBD derivatized polymers gave emmision spectra which were very broad, sometimes having maxima that spanned 10 nm, and which were variable in their maximum wavelength of emission between seemingly closely related derivatives. Four PEI derivatives in which fluorophore and aliphatic group are on primary amines gave spectra shown in figures 59 to 62. All were excited at 464 nm, and yet

show widely varying emission maxima: 555, 570, and one so broad that no maximum could be assigned it. The tertiary substituted polymer not quaternized with methyl iodide gives a maximum of 538 (Figure 63) and after quaternization this moves to about 501 nm (Figure 64). However, the inconsistancy in results obtained from primary derivatized PEI led to abandonment of this technique in these studies.

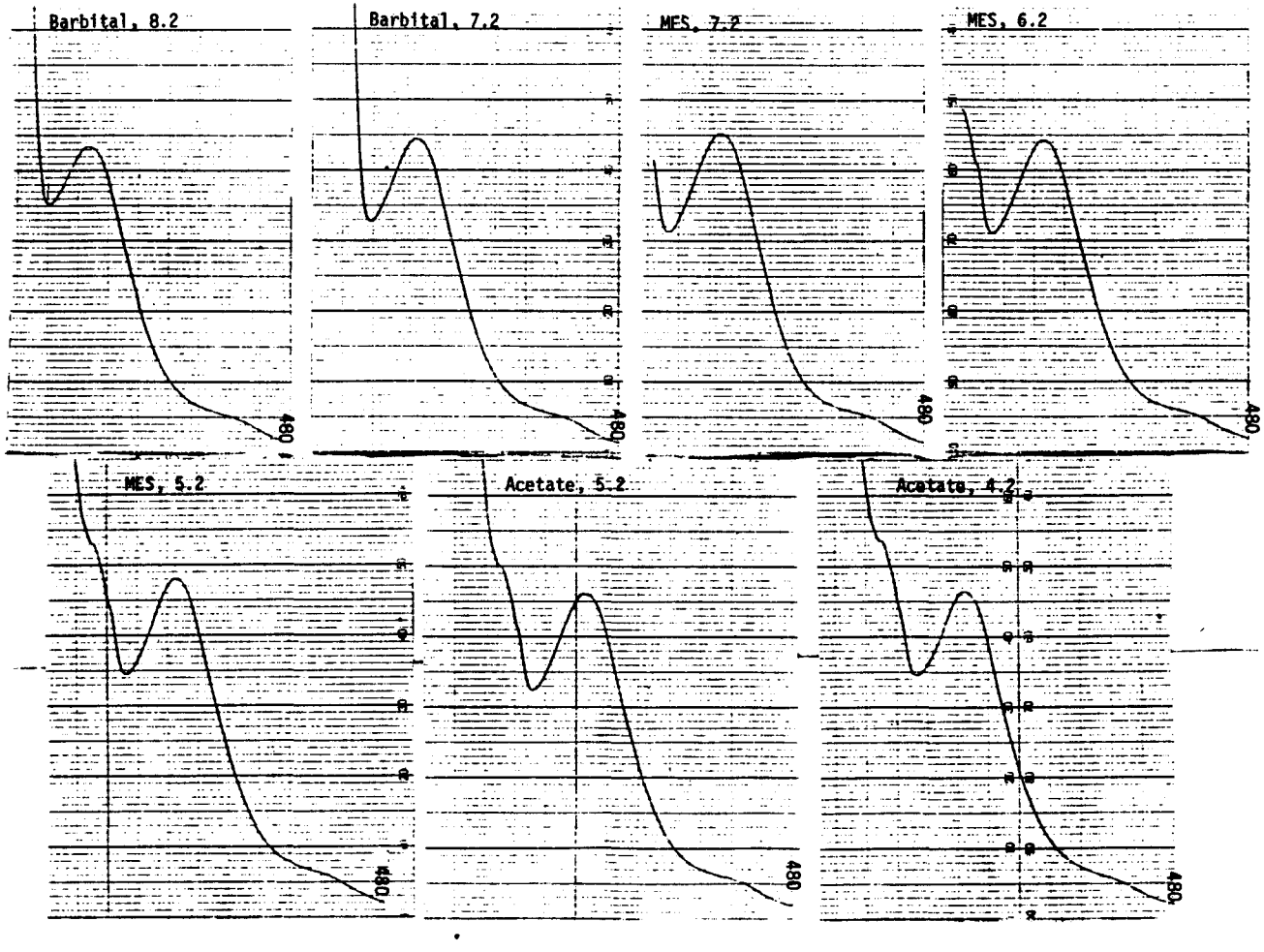


Fig. 57. Koshland II, Dodecylbenzyl (10%) on Primary Amines Polymer conc. = 0.15 mg/ml

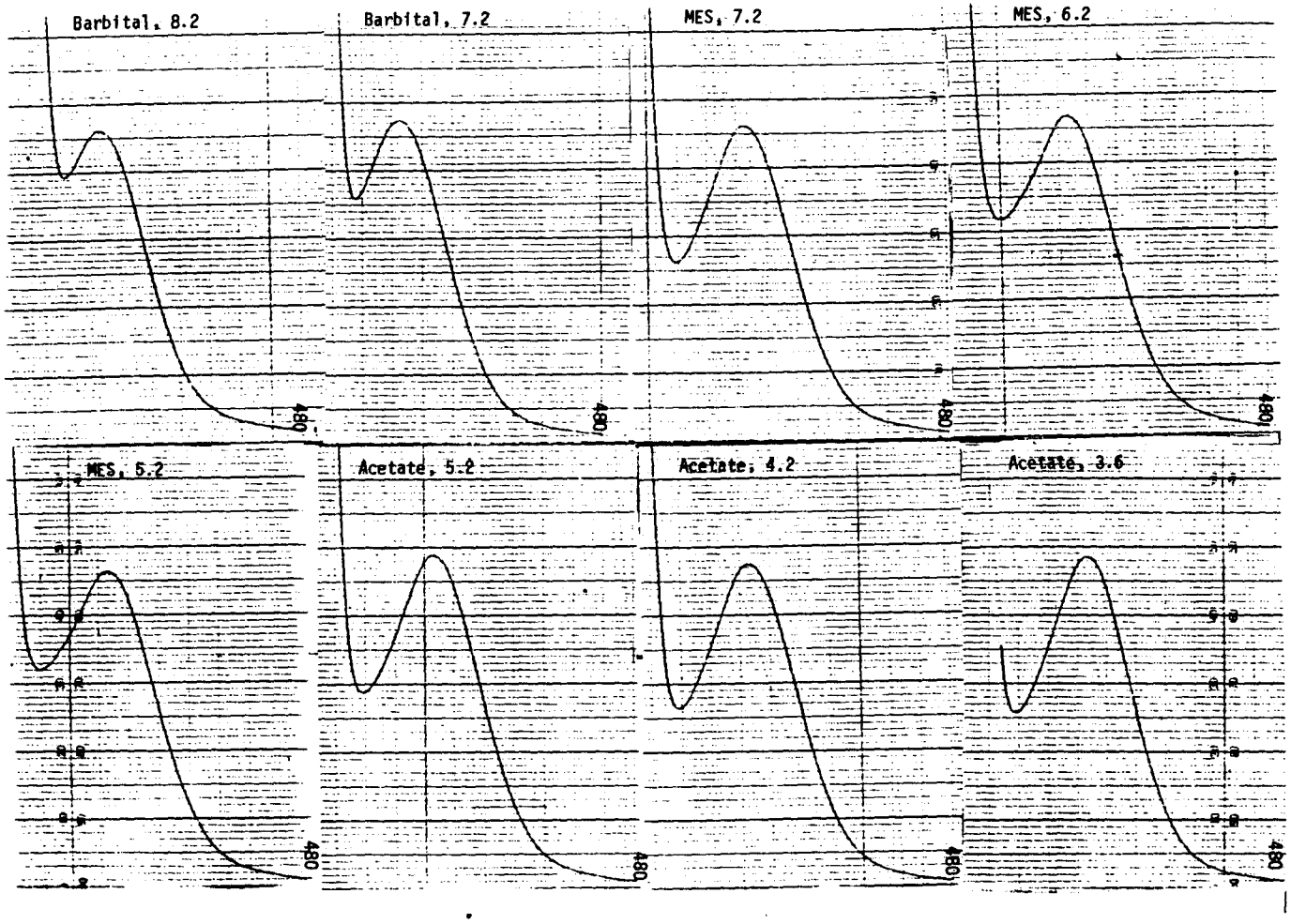


Fig. 58. $TFA_{.65}$ Koshland II 2°-3° Lauryl, Deblocked; Exhaustively Methylated Polymer conc. = 0.2 mg/ml

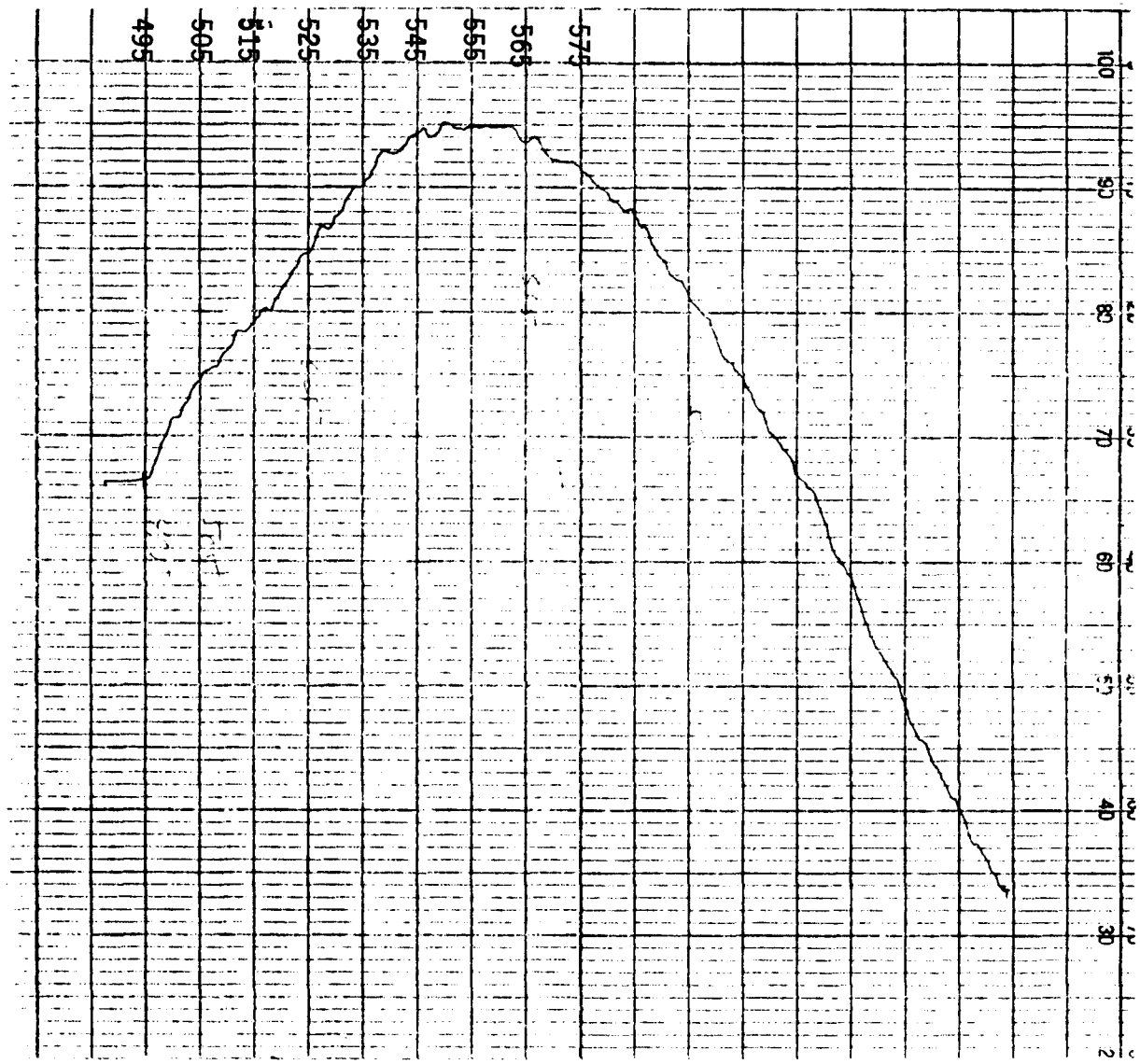


Fig. 59. Primary NBD, Primary $C_{12}H_{25}$ (10%)

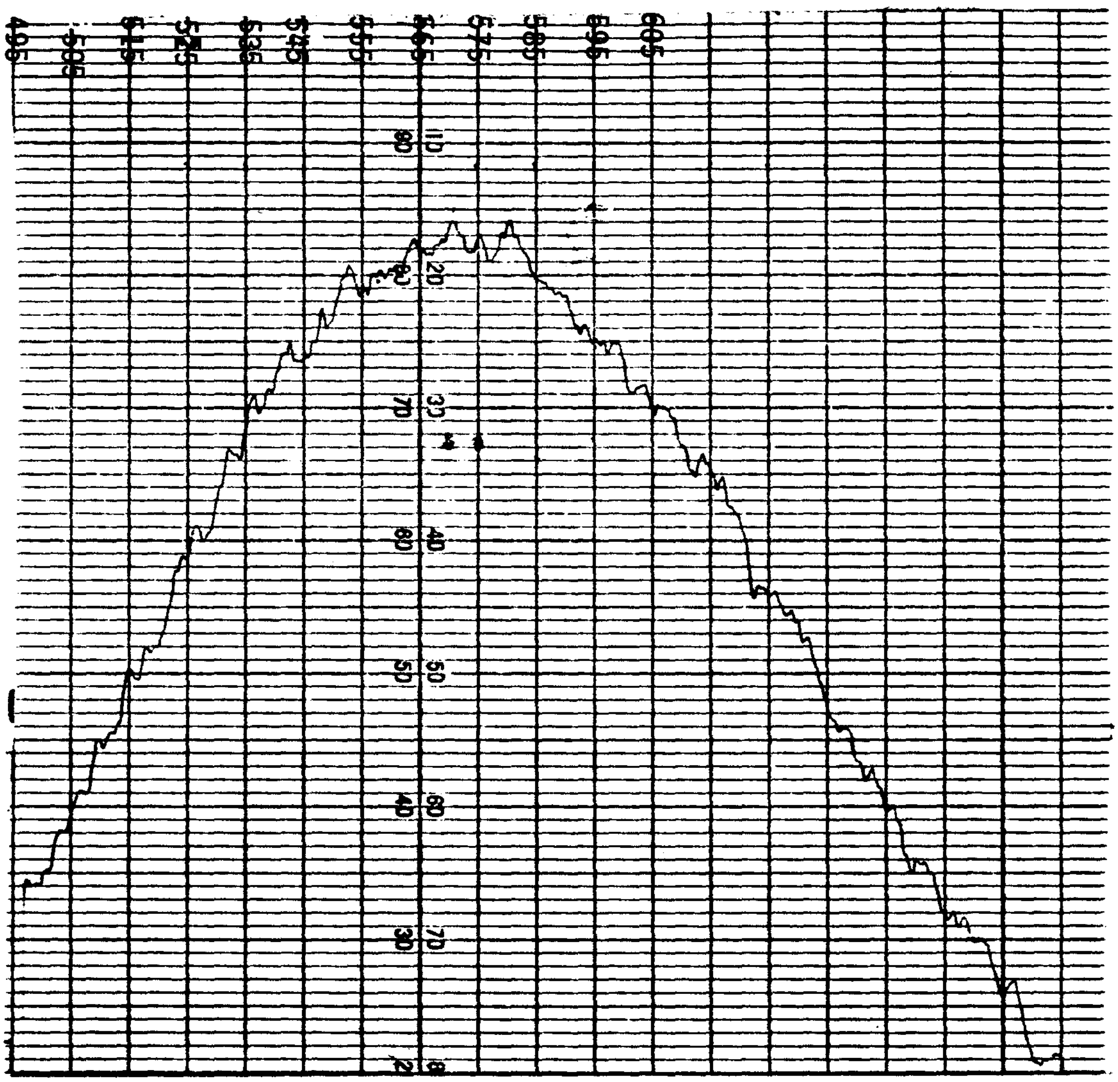


Fig. 60. Primary Decyl, Primary NBD

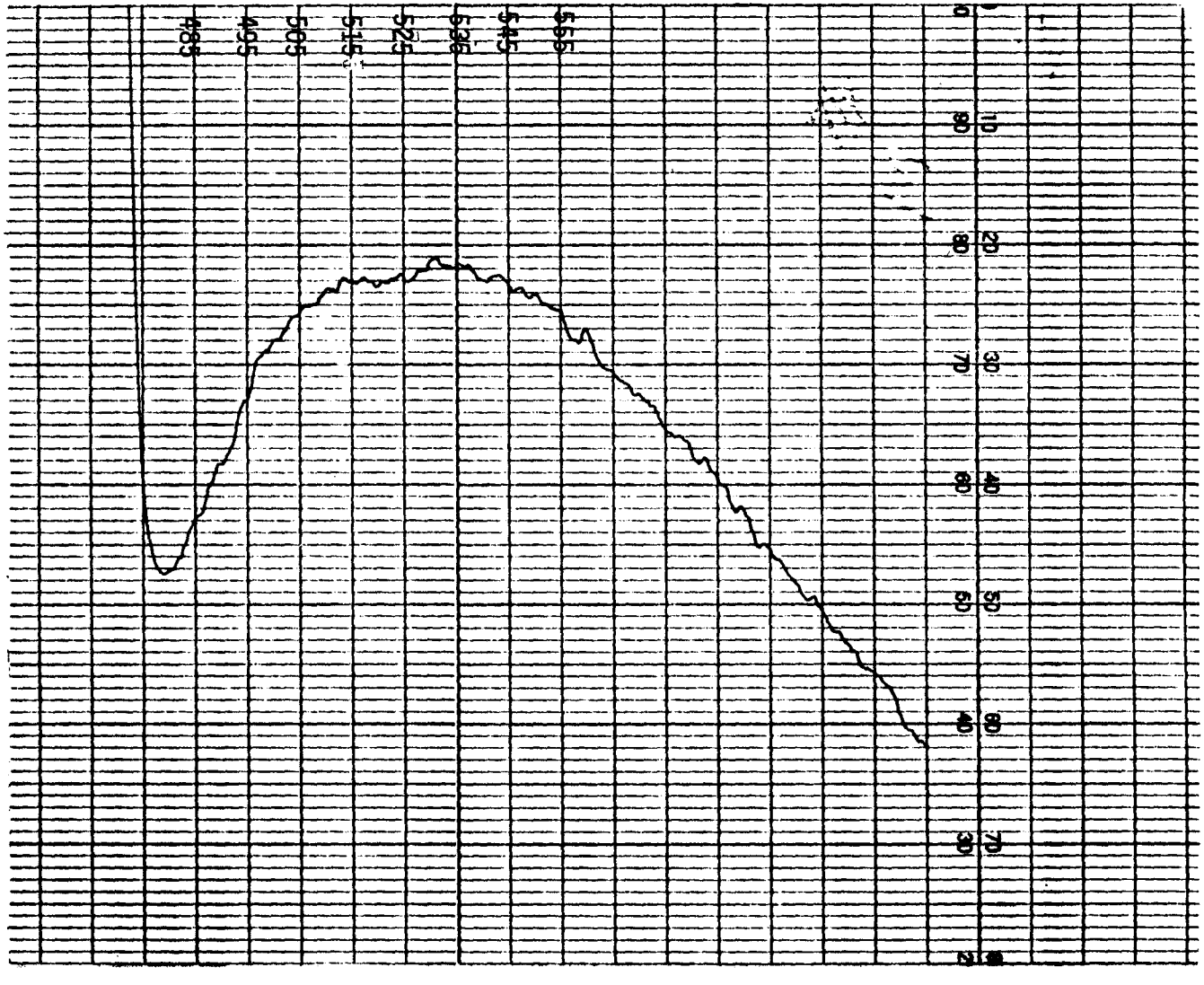


Fig. 61. Primary NBD, Primary Dodecyl Benzyl (10%)

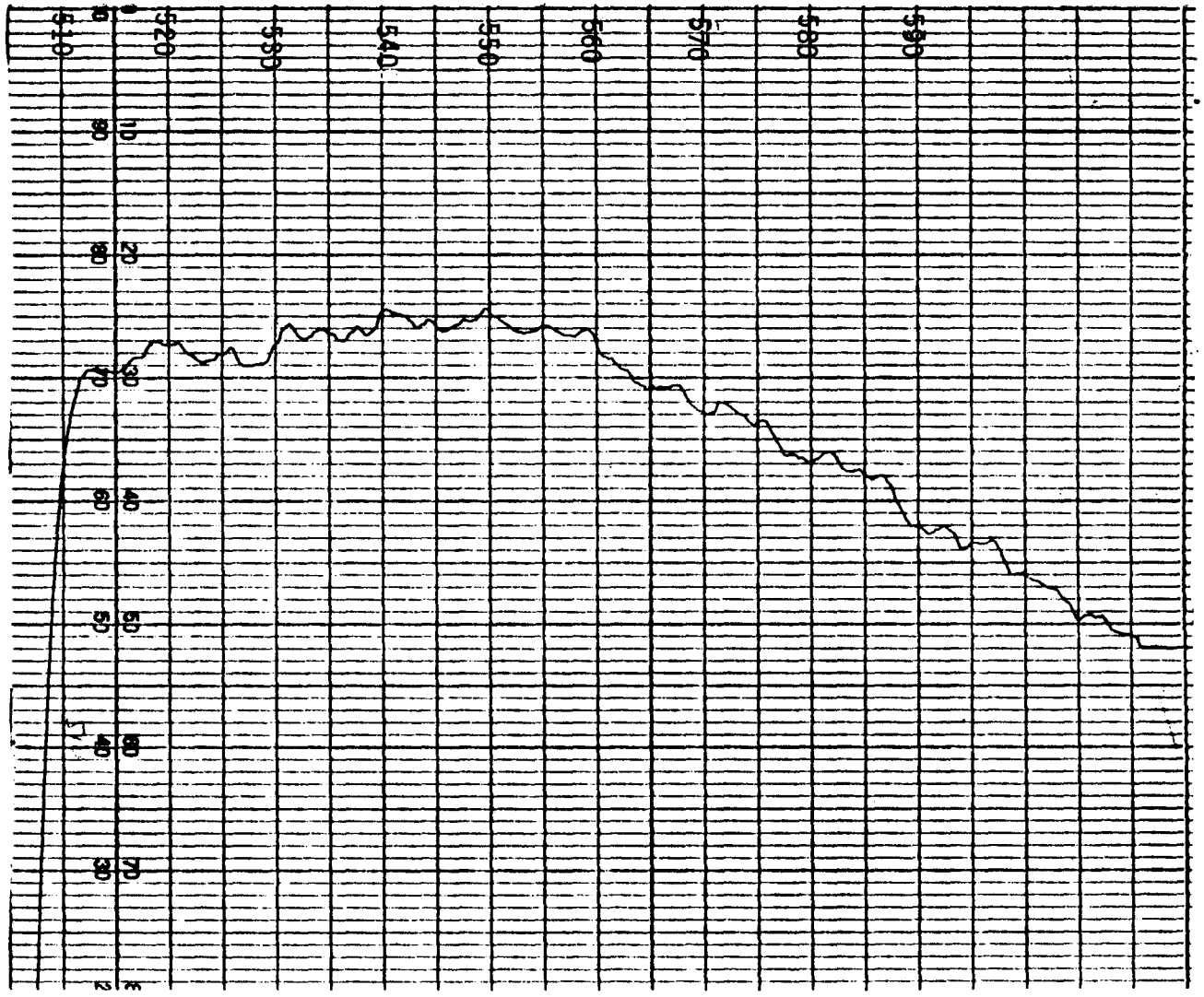


Fig. 62. Primary Dodecyl Benzyl (10%), Primary NBD

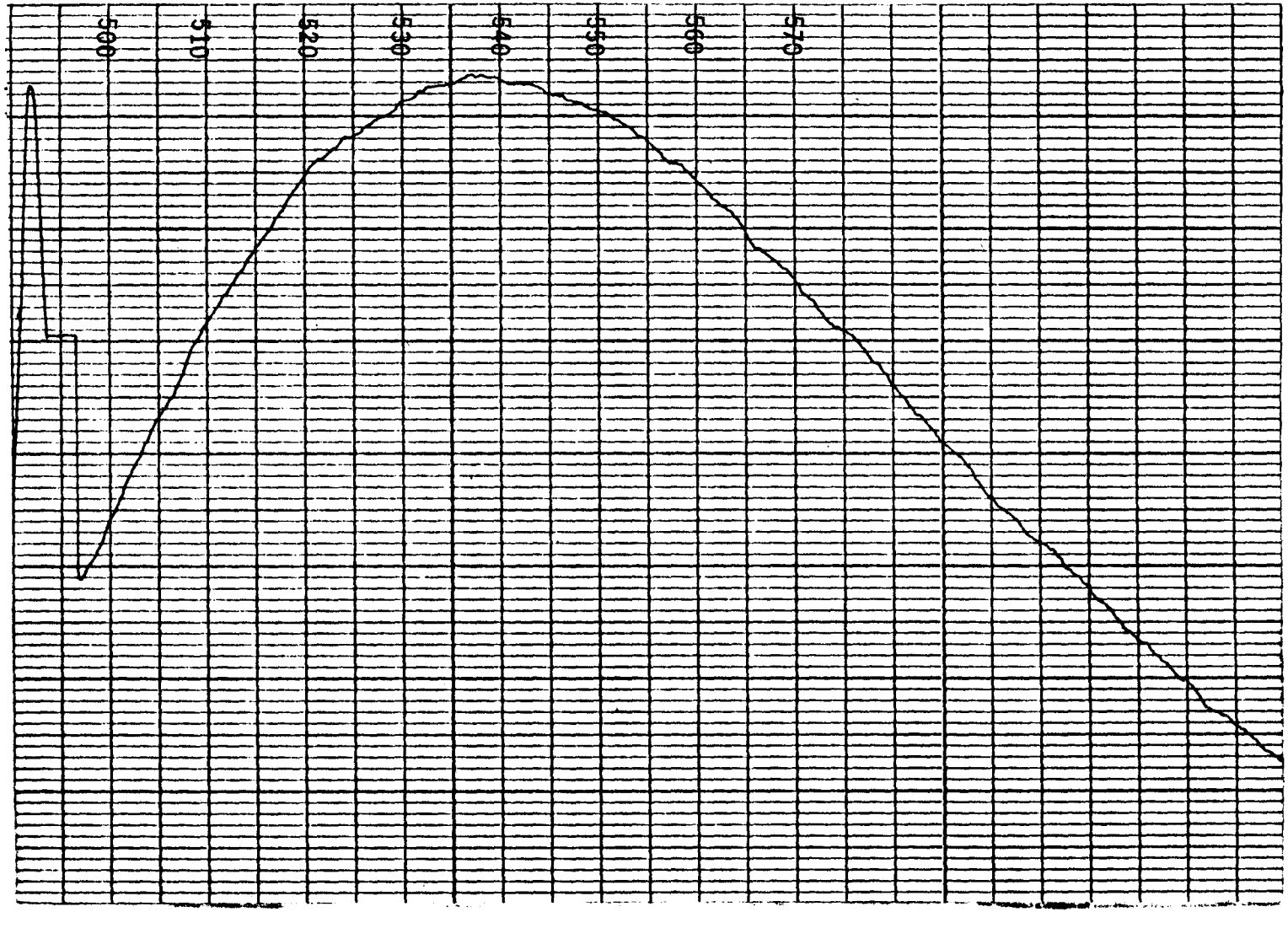


Fig. 63. $TFA_{.7}$ Dodecyl Benzyl (10%) 3° NBD

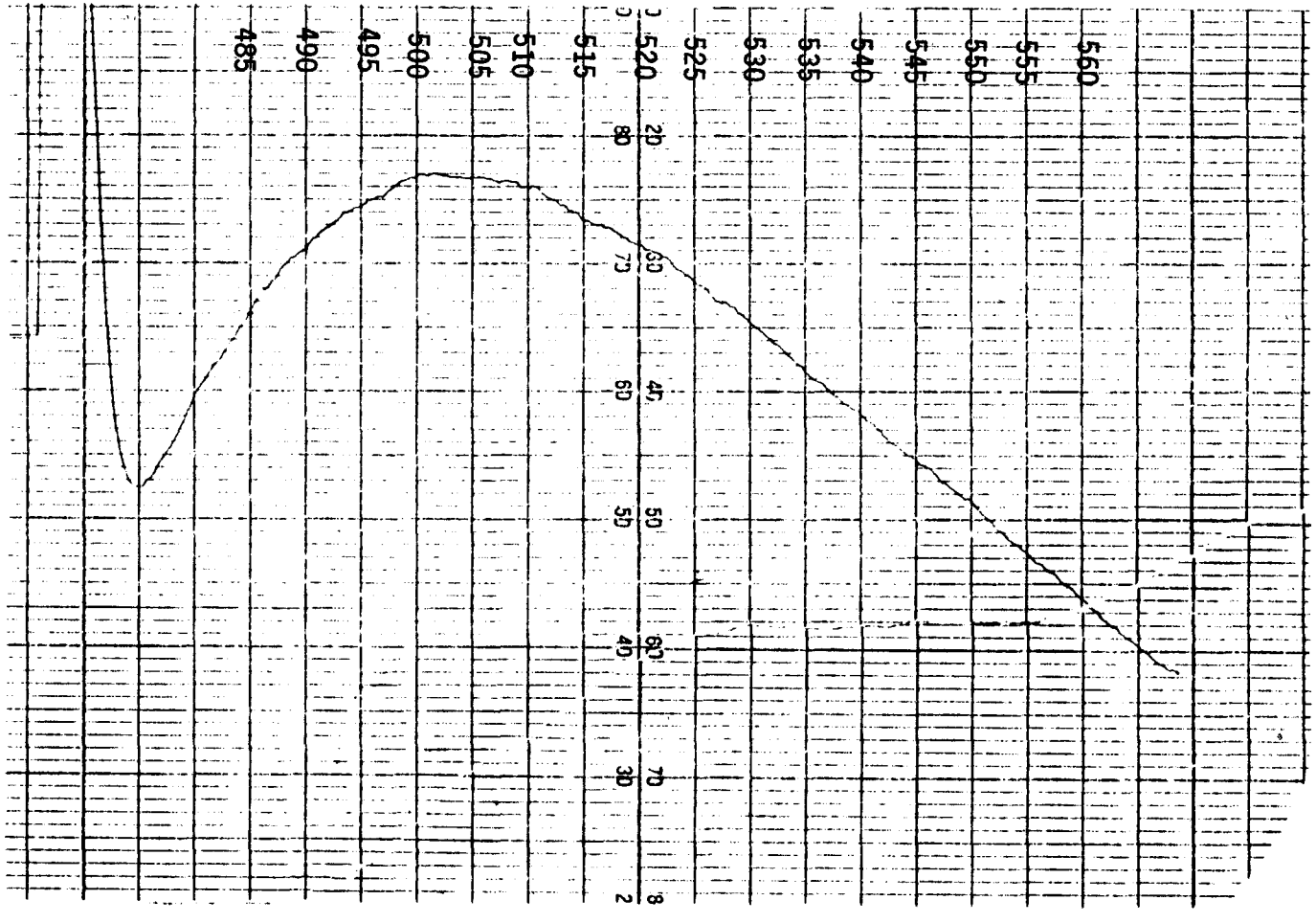


Fig. 64. $TFA_{.7}$ Dodecyl Benzyl (10%) 3° NBD;
Exhaustively methylated

DISCUSSION

Synthesis of PEI Derivatives

In order to further the use of this polymer as a synthetic catalyst, the efficacy of stepwise and site directed chemical modifications had to be established. The work described in this dissertation clearly demonstrates that multistep site directed synthesis is feasible with PEI, a polyelectrolyte of MW = 60,000. Exotic catalysts or unusually reactive reagents were not necessary and all reactions could be monitored and quantitatively assessed by rather simple spectroscopic and analytical techniques. Reactions and products cited in this work are readily reproducible. Clearly, the vast recent advances in NMR spectroscopy and synthetic methods can now be applied to generate sophisticated catalytic models in the near future. The prevailing sentiment regarding polymer modification is that the degree of substitution is rather limited and extremely difficult to follow. My work demonstrates a different picture at least where PEI is concerned.

The removal of trapped reactants and side products in the polymeric matrix could present a difficult experimental complication, but is absolutely necessary in obtaining good extents of modifications during stepwise synthesis. The combination of gel permeation chromotography (LH-20), ion exchange chromotography, and dialysis was employed successfully to purify the compounds mentioned in this dissertation. Most reactions of

the polymeric amines are exothermic reactions and ideally can be performed at low temperatures (-4°C). It is advantageous to use the most active of acylating, alkylating, and deblocking agents at low temperatures to circumvent amine oxidation or polymer degradation. The latter problem is particularly troublesome when it comes to quaternized polymers, where a large local concentration of positive charge is involved.

The synthetic work of my dissertation had a two-fold purpose: a) The introduction of a nucleophile on a pre-determined locus, one which is removed from the polymer-water interface. Such a compound would more closely approach an enzymic model as opposed to a micellar model and b) To covalently introduce reporter groups in various domains of the polymer, probing micropolarity and the pK perturbation of both the probe and the amine to which the probe has been attached. Based on experiments done in my dissertation (Figure 30),and elsewhere[296], the terminal primary amines are the first to react in both alkylation and acylation reactions. The remaining secondary amines are presumably more buried in the polymer.

To date the overall structure and conformation of the polymer, as well as the complexity of matrix branching, remain unknown. Progress regarding the determination of a structural unit of PEI has been reported recently by Lukovkin.[298] Nevertheless, his work, useful as it is, does not provide insight regarding the higher order structural organization of the polymer. Undoubtedly, the structural features of the polymer must account for its exceptional binding and catalytic capacities.[210] Where the amines have been inactivated through quaternization there is still exceptional activation of non-covalently bound nucelophiles.[292 293]

Sulfhydryl nucleophiles play a significant role in enzymic catalysis.[117] However, because of -SH instability[317], very few polymeric models have been studied to date.[318] In my work, I strived to synthesize a polymeric model where the sulfhydryl group would be relatively stable and, if possible, placed in domains isolated from one another and from the water interface. Sulfhydryl isolation precluded intermolecular and intramolecular disulfide formation which could have crosslinked the polymer and/or led to its precipitation.

To introduce the sulfhydryl group on the polymer, we followed the general scheme outlined on page 105. (See synthetic strategy.) Preliminary experiments indicated that the trifluoroacetyl group is the most suitable for blockage of all primary as well as a good part of the secondary amines. In addition, the TFA group allowed further blockage of remaining secondary amines and, above all, the alkylation with various hydrophobic groups of the tertiary amines. Deblockage of TFA groups can occur either under slightly basic or acid conditions according to synthetic needs. As a secondary blocking group, either the t-boc or the interesting TEOC groups were used. Both differential blocking groups are stable to base but can be removed in acid. Interestingly, the TEOC group proved to be stable to acid hydrolysis on our polymer derivatives, contrary to reports in the literature.[303] Polymer derivatives dissolved in acetic acid to which a threefold excess (with regard to blocking group) of trifluoroacetic acid has been added proved to be stable. Under the same experimental conditions, the t-boc is quantitatively removed. In other words, t-boc and TEOC blocking groups can be used side by side in synthetic schemes with subsequent

selective removal of the t-boc group under mild acidic conditions. The TEOC group can be easily removed in the presence of naked fluoride in HMPA/DMF.

After blockage of both primary and secondary amines with appropriate groups, the tertiary amines were modified through alkylation with various hydrophobic moieties. Mostly dodecyl and dodecyl benzyl groups were introduced on the tertiary amines. Based on information supplied by the manufacturers, there are about 25 to 30% tertiary amines. My work indicates that up to 25% substitution with long hydrophobic groups can be achieved. The extraordinary degree of substitution of the polymeric matrix strongly indicates that the highly branched polymer to be reactive despite steric considerations and charge density.

At this stage, the principal blocking group, the trifluoroacetyl group, was removed under mildly basic conditions (See p. 100). Exceptional care was taken not to degrade the polymer through Hoffman degradation reactions since we are now dealing with a partly quaternized polymer. The deblocked primary and secondary amines are now quaternized mostly with methyl iodide. At this stage of the synthesis, we have a compound where a) the primary and secondary amines have been rendered catalytically inactive, b) the charge of the polymer is constant over a wide pK range, which in turn assures conformational stability and lack of kinetic complex behavior and c) our model is presumably closer to a globular protein with hydrophobes located in the inside surrounded by a more hydrophilic exterior.

The differential blocking group is then removed and the exposed secondary amines become loci for the introduction of the particular nucleophile

of choice. In my case, we chose the introduction of the sulfhydryl group, a nucleophile playing a prominent role in enzymic hydrolysis reactions.[117] The sulfhydryl group was introduced via an episulfide, propylene sulfide. From work cited[319], the product is secondary thiol. Unlike the SPDP reagent, a sulfhydryl is generated directly. Therefore, one need not add a reducing agent and clean the polymer from it prior to assaying with DTNB. The episulfide reaction is one step, versus three steps (including thiol ester hydrolysis) for the -OH to -SH conversion used by Polgar.[311] Due to the minute amounts present (0.2% of the total amine), a qualitative or quantitative determination of the sulfhydryl content by NMR spectroscopy is not practical. The percentage of sulfhydryl was determined by the indirect method of Butterworth et al.[313] The complications of the direct method have been extensively discussed in page 147 . Our final product, unlike other sulfhydryl polymers[318], exhibits stability to thiol oxidation or disulfide formation over a prolonged period of time. The percentage of free sulfhydryl remained constant (0.2%) when assayed with DTNB over a period of two to three weeks. Such a polymer can now be used for further kinetic studies.

Analytical Determinations

In view of the fact that the polymer has three kinds of amines, primary, secondary, and tertiary in a ratio of 1:2:1, it has always been a difficult task to follow which amine type reacted with a particular reagent. In my work, NMR spectroscopy has been used extensively to resolve ambiguities regarding site substitution. Of particular importance has been carbon-13 NMR spectrometry. The carbonyl region of acylated polymers in ^{13}C NMR has

been informative. The carbonyl carbon of the trifluoroacetyl group appears as two sets of quartets around 160 ppm, corresponding to those on primary versus secondary amines. Partial removal of the trifluoroacetyl group indicates that it is the primary amine population that most resists deblockage (Figure 25). The more upfield quartet continues to appear after partial hydrolysis.

The acetyl and TEOC carbonyls are not very useful and each appears as a singlet at 173 and 180 ppm, respectively. (Fig. 29 & 12) On the other hand, the t-boc carbonyl is resolved into two peaks appearing at 164 and 167 ppm (Figure 11).

Based on the ^{13}C NMR spectrum of TFA$_{.7}$ t-boc$_{.05}$ PEI (Figure 13), I was able to conclude that it is secondary amines that remain unblocked following trifluoroacetylation, not primary ones. This was imperative to demonstrate if I was to be certain that our derivatives would be distinct from earlier ones, which were primary-substituted. Corroborating this was the shift of the tertiary carbon of the t-boc group. When reacted with a primary amine, this doublet is centered at 85.5 ppm, and when on a secondary amine it is at 88 ppm (Figure 11). A single doublet is present in differentially blocked TFA PEI, this being at 88 ppm and therefore corresponding to the t-boc being on secondary amines.

^{1}H NMR was used in a routine manner to follow all kinds of modifications on the polymer. Before the results section I have included a table with chemical shifts of groups placed on the polymer. Therefore I shall not elaborate here. Suffice it to say that PMR was very important in quantitating the degree of hydrophobe substitution and in following acylation

reactions. TEOC-O-Np as an acylating reagent is especially suitable since its shift is far upfield at 0 ppm. There are two points in the synthetic scheme at which TEOC quantitation was very useful:

1. in determination of the number of secondary amines that have resisted trifluoroacetylation and therefore are available for differential blockage (Figure 5).

2. in proving that there is an imperceptible number of amines below the tertiary state after quaternization of the polymer and prior to removal of the differential blocking group so that nucleophile addition would indeed be onto those amines targeted for this substitution (Figure 48).

Also, TEOC acylated polymers have a chemical shift that is well separated from the backbone peak. This serves as an internal reference against which the polymer backbone may be integrated prior and subsequent to exhaustive methylation. Using this technique, it was established that the degree of quaternization of the polymer went to 93% completion. While we were aware that NMR spectroscopy is an insensitive technique, it is environment-independent, which is of great advantage when working with as complex a system as a polymer.

Spectroscopic Findings

An effective means of monitoring the microscopic dielectric of the tertiary substituted polymer before and after quaternization was not achieved in this work. The fluorescence spectra are complex, and it is the suspicion of the author that the fluorophores are in slightly differing environments enhancing the probability of fluorophore-fluorophore coupling and leading to band broadening. Absorption probes suffer from a general lack of sensitivity but are free of the complications arising in fluorescence spectroscopy.

The pK perturbation studies with Koshland reagent I have been quite informative. When placed on either primary or tertiary amines of unmodified PEI, the Koshland reagent shows substantial pK perturbation of the p-nitrophenol (Tables 2 & 3). Considering first those polymers having no hydrophobes, it is evident that even at pH 5.2, which is 1.8 pH units below the unperturbed reporter group's pK, over 50% have not yet protonated, be they on the primary amines or the tertiary amines. This anion stabilization is indeed in the direction predicted from consideration of the polyelectrolyte effect. The pKs are perturbed appreciably more on the tertiary substituted polymer than on the primary substituted one.

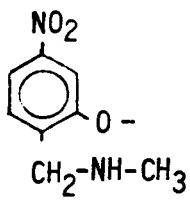
The polymers were then modified by placement of hydrophobes onto the same amine population as that containing the reporter group. At pH values of 7.2 to 5.2 a greater percentage of reporter groups remain ionized when on primary derivatized PEI as opposed to tertiary derivatized PEI (Tables 4 & 5). The hydrophobic character of the tertiary substituted polymer impedes ionization of the probe. Yet the hydrophobicity introduced to the inner core of the polymer is not sufficient to promote ionization via ion pair formation. Possibly the ammonium groups are not posed for this due to a lack of steric constraints. Increased hydrophobicity is consistent with the decreased (percent) ionization of the probe in tertiary substituted PEI. The introduction of dodecylbenzyl groups to the primary amines leads to a more compact structure of the polyelectrolyte as established by viscosity measurements (Klotz, Scarpa, unpublished results). Compactness results in approximation of the positively charged amines, which in turn induce ionization of the nitrophenol. Such overall changes of conformation of the

polymer have not been observed when the same percent of hydrophobe substitution was performed on tertiary amines (Klotz and Scarpa, unpubl. results).

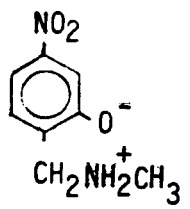
The tertiary Koshland polymer containing dodecyl groups was then quaternized (Table 6). This polymer had been blocked prior to alkylation with both the TFA and TEOC groups for a more site directed substitution of the hydrophobes and reporter groups onto tertiary amines. Dodecylbenzylation preceeded reporter group placement. Although I could not be certain that the Koshland reagent would be on tertiary amines with the analytical techniques at hand, by first putting on TFA, TEOC, and hydrophobes, I thought that the chances were good that reporter group placement would indeed be there. In Table 7 are presented results from a polymer which had been trifluoroacetylated to the extent of 60-65% total amine. The Koshland reagent was then reacted with it in hopes of placing a good portion of them onto the remaining secondaries. Lauryl groups were then introduced onto the polymer; it was then deblocked and exhaustively methylated. Both of these exhaustively methylated polymers would be expected to have rather open structures. This would allow counterious to enter the polymeric network and associate with the ammonium cations. The nitrophenolate's pK thus returns to its unperturbed value. A shielding of the reporter groups does not occur. It is notable that only one-fifth to one-fourth of the reporter groups remain ionized at pH 4, whereas in other polymer derivatives, a greater fraction of reporter groups which had been ionized at neutral pH retain this ionization at pH 4.2. Thus to assume that quaternization stabilizes the polymer against pH dependent conformational changes may well be incorrect.

All hydrophobe-containing polymers show a definite sensitivity to buffer as the buffer is changed from MES to acetate. In all four laurylated polymers acetate impeded phenol ionization. However, no buffer effect is seen in the more neutral range when the change is from barbital to MES.

The scanning spectra with the Koshland reagent show phenolate maxima at greater that 400 nm only for the two exhaustively methylated polymers. This indicates that over a wide pH range there is no change in the ionization of the nitrogen to which the reporter group is attached. In the case of quaternized polymers, the maximum corresponds to that of the first structure of the model compound studied by Koshland, not the second one.[314]



$\lambda_{max} = 405$



$\lambda_{max} = 395$

Since the Koshland reagents are inert to quaternization with methyl iodide, their placement onto the polymer preceeded methylation. The placement of hydrophobe onto polymer amines was also by alkylation, not acylation. Therefore, unlike blocked amines (i.e. amides), those nitrogens having the Koshland reagent may still be quaternized, if indeed reporter group placement is not itself a quaternization.

Westheimer has hypothesized that the sensitivity of the phenolate's maximum absorption to the ionization of the nitrogen to which it is attached is due to hydrogen bonding between the oxyanion and ammonium's proton. That the quaternized polymers absorb at 405 nm verifys his hypothesis. Despite

quaternization, which fixes the positive charge on essentially all nitrogens, the maximum is at the maximum of the amine derivative, not that of the ammonium salt. Yet no hydrogens are on these nitrogens. Thus it is the presence of absence of a proton on the ammonium nitrogen which determines the phenolate's exact absorption maximum.

The amines to which the Koshland I reagent has been reacted titrate between 6.2 and slightly below 5.2 in that polymer containing only the reporter group on primary amines. The tertiary Koshland I polymer shows complete titration of the amines when the solution has been acidified to 4.2; yet no protons would be on these nitrogens if the reporter group had truely been put on them. Therefore, the Koshland I reagent has either reacted with a population of secondaries that have resisted two attempts at blockage, or there is hydrogen bonding between the phenolate and a neighboring ammonium group which is not the site of attachment. While I favor the second explanation, no definitive answer can be made at this point. Primary Koshland I, primary dodecyl benzyl PEI shows a broad titration of the amines to which the reporter group has been attached. Complete titration is not achieved until pH 4.2. This contrasts sharply with tertiary dodecyl benzyl, tertiary Koshland I PEI, where most amines interacting with the phenol oxyanion are protonated when the pH is 6.2. Since it is possible that the ammonium proton hydrogen bonding with the phenolate is not the nitrogen of reporter group attachment, definitive statements about PEI amine pKs cannot be made. Nonetheless, the results reported herein cast some doubt on the assumption that the lowest pKs are restricted to the primary amines, as was concluded from colloidal titration studies.[261]

The conclusions reached from the pK perturbation studies are that:

1. In an extended conformation, pK perturbations are not appreciably different between primary and tertiary amines and it is in this form that the polymer induces the greatest perturbation.
2. Upon introduction of the hydrophobe, a smaller fraction of reporter groups has highly perturbed pKs but a greater fraction of the total remain ionized when substitution has been onto the surface amines.
3. Surprisingly, exhaustive methylation of the polymers is not conducive to maintenance of highly perturbed pK values.

Regarding the third point, several explanations are possible:

a) exhaustive quaternization opens up the polymer due to coulombic repulsions, returning the pKs to their original values.

b) hydrogen bonding is responsible for the extreme perturbation of the pK values.

ABSTRACT

Polyethylenimine is a highly branched polyamine which has been shown to be very effective matrix for catalytic and binding studies. Previously reported work dealt mostly with placement of catalytic and binding groups onto the outer surface of the matrix, resulting in a stable micelle-like macromolecule. In contrast, in the work described herein catalytic, binding, and reporter groups were covalently placed on the inner core of the polymer in an attempt to mimic microenvironments found in globular proteins.

The incorporation of a sulfhydryl group in a domain of the polymer which would not lead to inter- or intramolecular crosslinking was achieved by the following route. Surface (primary) and all secondary amines are blocked with two types of blocking groups, trifluoroacetyl and trimethylsilyl ethoxycarbonate (TEOC). Only a small number of secondary amines (10%) were protected with the TEOC group. Through alkylation, hydrophobic groups are then introduced onto the tertiary amines, these being the inner core amines. After removal of the trifluoroacetyl group, the primary and secondary amines thus regenerated are rendered cationic and inert by quaternization with methyl iodide. The TEOC blocking group is now removed with "naked" fluoride, revealing the small number of secondary amines which serve as loci for the attachment of a sulfhydryl group. This was attempted by two different routes:

1. the introduction of a hydroxyl group via an epoxide for later conversion to a sulfhydryl
2. the direct introduction of the sulfhydryl via propylene sulfide

The latter of the two techniques was successful. 0.2% total amine of sulfhydryl was placed on a polymer in a position which did not result in crosslinking either intra- or intermolecularly. All steps described above were followed primarily by spectroscopic techniques. Proton and ^{13}C NMR techniques proved invaluable. The sulfhydryl content was determined by the indirect method of Butterworth using Ellman's reagent. Koshland reagent I, a titratable reporter group, was placed on primary, secondary, and tertiary amines using synthetic methods similar to those reported above. Koshland I had been used successfully to determine pK_a perturbation in the active site of acetoacetate decarboxylase. Depending on the ionization state of the amine, spectra were recorded at 395 or 405 nm. Extinction coefficients used in this work were obtained from Westheimer's studies with small amine models. The following conclusions were reached from this study:

1. In an extended conformation, pK perturbations are not appreciably different between primary and tertiary amines of PEI and it is in this form that the polymer induces the greatest perturbation.

2. Upon introduction of the hydrophobe, a smaller fraction of reporter groups has highly perturbed pKs but a greater fraction of the total remain ionized when substitution has been onto the nonsurface amines.

3. Surprisingly, exhaustive methylation of the polymers is not conducive to maintenance of highly perturbed pK values.

ABBREVIATIONS

ANTI	3-acetoxy-N-trimethylanilinium iodide
As Cys	n-cetyl dimethyl (2-ethylamino) ammonium chloride, amidated with cysteine
BSA	bovine serum albumin
C12	n-dodecyl
C18	n-octadecyl
CTABr	n-cetyl trimethyl ammonium bromide
CTACl	n-cetyl trimethylammonium chloride
CMC	critical micelle concentration
DIEA	diisopropyl ethylamine
DNPS	dinitrophenyl sulfate
DTNB	5,5' dithiobis-(2-nitrobenzoic acid)
EtOH	ethanol
HMPA	hexamethylphosphoramide
NABA	4-acetoxy-3-nitrobenzoic acid
NABS	4-acetoxy-3-nitrobenzene sulfonate
NaLS	sodium lauryl sulfate
NBD-Cl	nitrobenzoxadiazole chloride
NCS	nitrocatechol sulfate
PAA	polyacrylic acid
PEI	polyethylenimine
PEI^+	quaternized polyethylenimine
PMA,PMAA	polymethacrylate
PMES	polymethacryloxyethylsulfonate
PNP	paranitrophenol
PNPA	paranitrophenyl acetate
$PNPC_n$	paranitrophenyl alkanoate with n carbons
PNPOx	paranitrophenyl oxalate
PNPPr	paranitrophenyl propionate
PNPS	paranitrophenyl sulfate
PVA	polyvinyl amine
PVBMA	polyvinyl benzylmethacrylate
PVIm	polyvinyl imidazole
PVP	polyvinylpyridine
PVP^+	quaternized polyvinyl pyridine
PVS	polyvinyl sulfonate
SDS	sodium dodecyl sulfate
SNPA	N-succinimidyl paranitrophenyl acetate
SPDP	N-succinimidyl 3- (2-pyridyldithio) propionate
t-boc	tert-butyloxy carbonyl
TEOC-O-Np	2- (trimethylsilyl) ethyl carbonate, nitrophenyl ester
TFA	trifluoroacetyl, trifluoroacete
THF	tetrahydrofuran
TMAC	trioctylmethylammonium chloride

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the

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