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Enhanced Esterolytic Activities of Several Derivatives of Polyethyleneimine[1]

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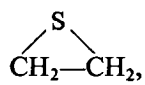
Mercaptoethylation of polyethyleneimine introduces nucleophilic —SH groups into the polymer. Rates of esterolysis of nitrophenyl esters in the presence of such polymer derivatives show increases of 2–3 orders of magnitude.

INTRODUCTION

Exceptionally strong binding of small organic molecules is manifested by derivatives of polyethyleneimine containing apolar side chains (*1*, *2*). Furthermore, it is evident that this increased binding can contribute strongly to kinetic steps also, for it has been found that water-soluble derivatives of polyethyleneimine containing residual primary amine groups enhance markedly the rates of aminolysis of nitrophenyl esters (*3*). The primary aim of the present investigation has been to introduce an external nucleophile, such as an —SH group, into polyethyleneimine to see if further rate enhancements can be obtained.

EXPERIMENTAL

Ethylene sulfide,



was chosen for the mercaptoethylation reaction with polyethyleneimine since this sulfide reacts readily with aliphatic and aromatic amines (*4*). To avoid undesirable polymerization of the sulfide, a frequent side reaction in basic alcoholic or aqueous media, we used the aprotic solvent *N*-methylpyrrolidinone.

Materials

Polyethyleneimine (PEI-600),[3] 33% in water (*5*) was obtained from Dow Chemical Company. Dry PEI was prepared by dissolving the commercial polymer in absolute ethanol (10 g/100 ml) and evaporating to dryness in a rotary evaporator. This was repeated three times. The final product was dissolved in absolute ethanol and kept as a 5% stock solution. The solvent 1-methyl-2-pyrrolidinone was purchased from Eastman-Kodak and ethylene sulfide from Aldrich. *p*-Nitrophenyl laurate and *p*-nitrophenyl caproate were products of Sigma, and dithiothreitol of Calbiochem.

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[3] The number following the acronym PEI, multipled by 100, indicates the molecular weight of the polymer (5).

Preparation of Lauroyl Derivatives of PEI-600

The lauroyl derivatives of PEI-600, containing 5%, 10%, and 15%, respectively, of this acyl group conjugated to primary amine residues of the polymer, were prepared as follows: To 10 ml of the 5% stock solution of PEI-600 in absolute ethanol an appropriate stoichiometric amount of *p*-nitrophenyl laurate in 5 ml absolute ethanol was added. The solution was stirred at room temperature overnight. The lauroyl polyethyleneimine (L-PEI) was separated from the *p*-nitrophenol by exclusion chromatography on a Sephadex LH-20 column (6 × 50 cm) using absolute ethanol as eluting agent. The emergence of the substituted, still-basic, polymer, which appeared right after the holdup volume, was followed with Alkacid test ribbon. The ethanolic L-PEI effluent was concentrated to dryness in a rotary evaporator, dissolved in 1-methyl-2-pyrrolidinone, and kept as a 0.14 residue-*M* solution (with respect to the primary amines of the original PEI) for further operations.

Mercaptoethylation

A 4-ml aliquot of either 0.14 residue-*M* PEI or L-PEI was placed in a 10-ml flask with a ground glass stopper. The solution was flushed with nitrogen, 3 droplets (~30 mg) of ethylene sulfide were added, the solution was flushed again with nitrogen, and the flask was stoppered and closed tightly with parafilm ribbon. The reaction was allowed to proceed with constant stirring at room temperature for 2–3 hr. (In this period of time, polymerization of ethylene sulfide did not occur.) The preparation was usually assayed either directly or after addition of an equivalent amount of dithiothreitol. For comparative purposes some of the samples were dialyzed against water containing dithiothreitol to remove extraneous materials (other than PEI–ethylmercaptan or L-PEI–ethyl mercaptan).

Determination of Thiol Groups

This was performed with Ellman's (*6*) reagent [5,5-dithiobis (2-nitro-benzoic acid), DTNB] as described by Zahler and Cleland (*7*).

Determination of Primary Amines

Attempts to estimate primary amines with 2,4,6-trinitrobenzenesulfonic acid (TNBS) failed because of interfering turbidity that appeared on addition of this reagent to the L-PEI solution. Therefore the use of ninhydrin reagent (*8*) was adopted. The assay was performed in 20-ml test tubes on 0.1 ml solution (containing 0.2–0.5 mg PEI) to which 1 ml of the ninhydrin reagent (*8*) was added. The tubes were stoppered, kept in the dark at room temperature overnight and then diluted with water to 5–10 ml. Their absorbances at 570 nm were then determined. For each set of determinations appropriate controls of the participating reagents and of reference standards of PEI-600 were assayed as well. To avoid interference from free SH groups (*9*) the thiolated polymer was diluted 10-fold with 0.01 *M* Tris buffer, pH 8.6, and left overnight at room temperature for oxidation of thiol groups. Under these conditions oxidation was essentially complete. The ninhydrin reaction was then performed as described.

Kinetic Studies

Rates of cleavage of the substrate *p*-nitrophenyl caproate at 25°C were followed spectrophotometrically by the appearance of *p*-nitrophenolate ion, as reflected by

increased absorbances at 400 nm measured with a Cary Model 14 spectrophotometer. The reactions were performed at pH 7 in one of the following 0.02 *M* buffers: tris (hydroxymethyl) aminomethane (Tris), *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES), or glycylglycine. At pH 9, 0.02 *M* Tris buffer was used. A stock solution of substrate, 2×10^{-3} *M*, was made in acetonitrile as solvent. The reaction mixture for catalytic assay had a final volume of 3 ml. It was prepared by adding 0.1 ml of polymer, previously diluted with 0.02 *M* Tris buffer, pH 6.6, to the desired concentration, to 2.75 ml of buffer. The reaction was started by further addition of 0.15 ml of stock solution of substrate. The initial substrate concentration in the 3-ml reaction mixture was 1×10^{-4} *M*. In each rate experiment the reference cell contained appropriate controls to measure the cleavage of nitrophenyl ester in the presence of all other constituents except the polymer. Dithiothreitol was added to the thiolated polymers to protect the —SH groups in the latter from oxidation. Control experiments with and without dithiothreitol showed no effect on the rate-enhancements produced by the polymer.

RESULTS

The hydrolysis of *p*-nitrophenyl caproate by PEI-600, to which different amounts of the apolar lauroyl groups have been attached, is shown in Fig. 1. For comparative purposes the rate of release was followed at pH 7 (Fig. 1A) as well as at pH 9 (Fig. 1B),

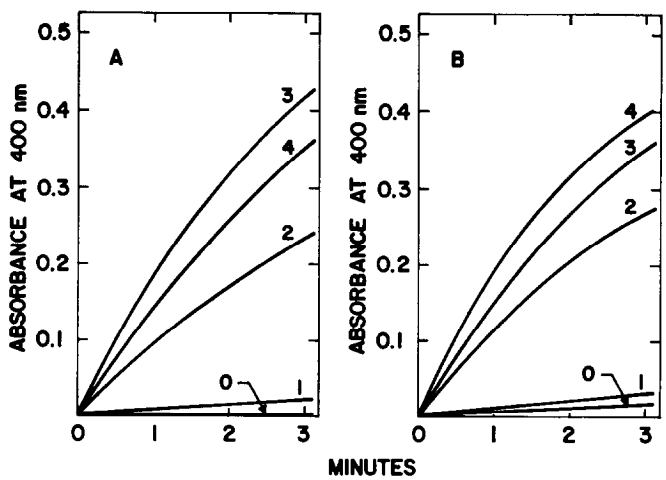

FIG. 1. Comparative rates of release of *p*-nitrophenol from *p*-nitrophenyl caproate, at pH 7 and pH 9, in the presence of PEI-600 and lauroyl (L)–PEI-600 derivatives. Polymer concentrations are expressed in molarity of total primary amines (free and lauroylated). (A) Reactions performed at pH 7 in 0.02 *M* Tris buffer and polymer concentrations of 1×10^{-3} *M*. (B) Reactions performed at pH 9 in 0.02 *M* Tris buffer and polymer concentrations of 1×10^{-4}. (0) Substrate + buffer control; (1) PEI-600; (2) L(5%)–PEI-600; (3) L(10%)–PEI-600; (4) L(15%)–PEI-600.

the latter being more favorable for deacylation. At both pH values, esterolysis increases markedly with increasing content of apolar side chain. The slight drop in rate for L(15%)–PEI-600 at pH 7 may arise from incomplete solubility of this preparation in the aqueous reaction mixture.

The enviromental effect on the kinetics for three different buffers has also been compared (Fig. 2). It is obvious that the glycylglycine buffer furnishes the most favorable conditions for hydrolysis at pH 7. Neither the glycylglycine buffer nor HEPES buffer

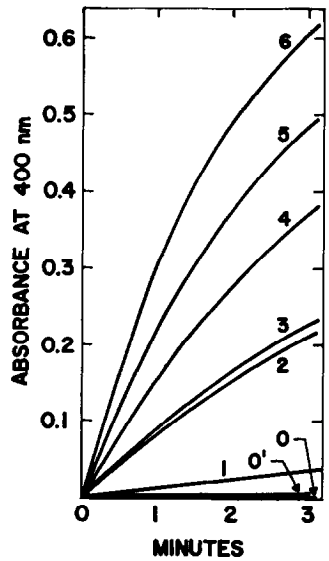

FIG. 2. Effect of different buffers on rate of release of *p*-nitrophenol from *p*-nitrophenyl caproate at pH 7 in the presence of PEI-600 and lauroyl–PEI-600 derivatives, at a concentration of 1×10^{-3} *M* total (free and laurolyated) primary amines. (0) substrate + buffer control (0.02 *M* Tris or 0.02 *M* HEPES); (0′) substrate + buffer control (0.02 *M* glycylglycine); (1) PEI-600 in glycylglycine; (2) L(5%)–PEI-600 in Tris; (3) L(5%)–PEI-600 in HEPES; (4) L(5%)–PEI-600 in glycylglycine or L(10%)–PEI-600 in Tris; (5) L(10%)–PEI-600 in HEPES; (6) L(10%)–PEI-600 in glycylglycine.

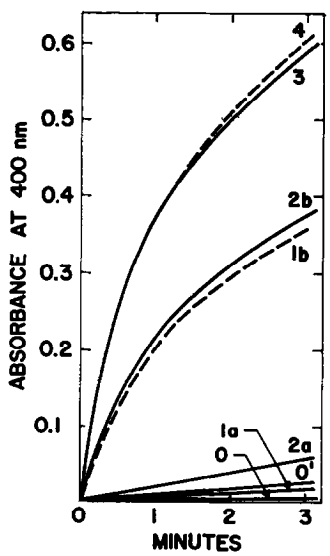

FIG. 3. Effect of PEI-600-SH(2.5%) and L-(10%)–PEI-600-SH(2.5%) on rate of release of *p*-nitrophenol from *p*-nitrophenyl caproate. (0) substrate + buffer (0.02 *M* glycylglycine) control at pH 7; (0′) substrate + buffer (0.02 *M* Tris) control at pH 9; (1a) PEI-600 at a concentration of 3×10^{-4} *M* primary amines in 0.02 *M* glycylglycine buffer, pH 7; (1b) PEI-600-SH(2.5%) at a concentration of 3×10^{-4} *M* total primary amines (free and thiolated) in 0.02 *M* glycylglycine buffer, pH 7; (2a) L(10%)–PEI-600 at a concentration of 1×10^{-4} *M* total (free, lauroylated, and thiolated) primary amines in 0.02 *M* glycylglycine buffer, pH 7; (2b) L(10%)–PEI-600-SH(2.5%) at a concentration of 1×10^{-4} *M* total primary amines (free, lauroylated, and thiolated) in 0.02 *M* glycylglycine buffer, pH 7; (3) same as (2b) but in 0.02 *M* Tris buffer at pH 9; (4) same as (1b) but in 0.02 *M* Tris buffer, pH 9.

TABLE 1

RATE CONSTANTS FOR THE HYDROLYSIS OF *p*-NITROPHENYL CAPROATE BY DERIVATIVES OF POLYETHYLENEIMINE

Polymer preparation	pH of assay	Molar concentration of nucleophiles in reaction mixture		Molar concentration of lauroyl groups in reaction mixture	v_0[a] moles/liter / min	$k\left(\frac{\text{liter}}{\text{mole min}}\right)$		
		—NH_2	—SH			Calcd in terms of —NH_2 residues present	Calcd in terms of —SH residues present	Calcd in terms of imidazole residues present
PEI-600	7	1×10^{-3}	—	—	1.11×10^{-6}	11.1	—	
PEI-600	9	1×10^{-3}	—	—	2.77×10^{-6}	27.7	—	
L(5%)PEI-600	7	8×10^{-4}	—	2×10^{-4}	1.35×10^{-5}	169	—	
L(5%)PEI-600	9	8×10^{-5}	—	2×10^{-5}	0.61×10^{-5}	762	—	
L(10%)PEI-600	7	6×10^{-4}	—	4×10^{-4}	2.7×10^{-5}	450	—	
L(10%)PEI-600	9	6×10^{-5}	—	4×10^{-5}	0.8×10^{-5}	1330	—	
PEI-600-SH(2.5%)	7	5.4×10^{-4}	0.6×10^{-4}	—	3.4×10^{-5}	—	5 700	
PEI-600-SH(2.5%)	9	2.7×10^{-4}	0.3×10^{-4}	—	2.15×10^{-5}	—	7 200	
L(10%)PEI-600-SH(2.5%)	7	1.25×10^{-4}	0.25×10^{-4}	1×10^{-4}	3.3×10^{-5}	—	13 200[c]	
L(10%)PEI-600-SH(2.5%)	9	0.625×10^{-4}	0.125×10^{-4}	0.5×10^{-4}	2.0×10^{-5}	—	16 000[c]	
Imidazole[b]								20
Chymotrypsin[b]								10 000
Pentapeptides[b] (with His)								92 to 147
Polyhistidine[b]								10

[a] Values of v_0, in moles per liter of nitrophenol released per minute, were calculated from absorbances, using a molecular extinction coefficient of 9 000 at pH 7 and of 18 000 at pH 9. The initial substrate concentration, S_0, was 1×10^{-4} *M*.

[b] Taken from Refs. (10) and (11). The substrate in these cases was nitrophenylacetate.

[c] Small-molecule organic thiols (including β-mercaptoethylamine) at corresponding pH show *k* values of 10–300. See Refs. (13–15).

could be used for kinetic studies at pH 9 since in both of them the spontaneous hydrolysis of *p*-nitrophenyl caproate is very fast.

The data in Figs. 1 and 2 indicate that of the three *N*-lauroyl–PEI preparations, L(10%)–PEI-600 is the most convenient with respect to activity and solubility in aqueous solutions. It has been used, therefore, as "carrier" polymer for the mercaptoethylation. Typical esterolysis curves of *p*-nitrophenyl caproate, in the presence of L(10%)–PEI-600-SH (2.5%), i.e., containing 2.5% of the nitrogen residues holding $-CH_2CH_2SH$ groups, are shown in Fig. 3, for reactions in glycylglycine buffer at pH 7, and in Tris buffer at pH 9.

For quantitative comparison, the rates were also expressed as follows: From the initial rate, v_0, for disappearance of nitrophenyl ester substrate, S,

$$v_0 = -(dS/dt)_0 \tag{1}$$

one can compute a pseudo first-order rate constant, k_1,

$$k_1 = -(1/S_0)(dS/dt)_0 \tag{2}$$

A "catalytic constant" (*10*) k may then be calculated from the equation

$$k_1 = k(P) \tag{3}$$

where (P) is the concentration of polymeric additive expressed in residue moles of various nucleophilic groups. Table 1 summarizes the results of such calculations for the polyethyleneimine derivatives examined here and compares their effectiveness with other substances described in published literature (*10*, *11*).

DISCUSSION

From the kinetic measurements, most readily compared in terms of the values of k in Table 1, we see immediately that introduction of apolar binding sites (lauroyl groups) leads to a marked enhancement in the rate of cleavage of the nitrophenyl ester. This rate enhancement is substantially greater at pH 9 than at 7, especially if we keep in mind that the glycylglycine buffer, used to determine k values at pH 7, is more effective than Tris (Fig. 2).

It should be pointed out, however, that no "turnover" is observed at either pH. Once all of the amino groups have been acylated the effect of the polymer disappears. In the absence of deacylation, it is clear that the observed accentuations in rates of ester cleavage are entirely increases in rates of acylation. Thus, the faster rate at pH 9 must reflect primarily the increase in concentration of $-NH_2$ groups, as contrasted to $-NH_3^+$.

Similarly the higher rates in glycylglycine buffer, as compared to Tris, are not due to the higher concentration of $-NH_2$ groups provided by the former buffer, for deacylation of the polymer is not observed in any of the buffers used. It seems likely, therefore, that the effect of buffer is primarily a reflection of its relative binding by the polymer. Differences in binding of buffer molecules by proteins are well known (*12*). The more strongly buffer ions are bound by polyethyleneimine, the more they will decrease the uptake of ester substrate. A decrease in binding constant for the substrate would diminish the rate of aminolysis by pendant amines of the polymer.

Very remarkable enhancements in cleavage rates were obtained with both polyethyleneimine derivatives containing —SH groups (Table 1). The constants k, for these derivatives have been calculated on the assumption that the amine nucleophiles do not contribute to the rate enhancements. If the amine groups in PEI-600-SH (2.5%)

contribute to the same extent as they do in unmodified PEI-600 (top of Table 1), then k at pH 7 should be reduced from 5700 to 5600. Correspondingly for L(10%)–PEI-600-SH-(2.5%) the reduction in k would be to 11 000. Nevertheless, even the reduced rate constants correspond to remarkable enhancements in esterolysis. Once again it should be mentioned, however, that "turnover" and regeneration of mercaptan groups were not observed. Once the quantity of substrate added exceeded the total moles of —SH and primary $—NH_2$ provided by the polymer, further addition of substrate led to cleavage at rates essentially the same as controls without polymer.

It is clear, therefore, that the limiting factor is the rate of deacylation of the acyl-S-bond. In the presence of the primary amine groups of the polyethyleneimine, deacylation of the acyl-S group must be occurring rapidly since the original content of —SH groups is only 20% that of the free $—NH_2$ and the extent of fast substrate esterolysis corresponds to the sum of the content of both of these groups. Once the amine groups have been acylated, however, the rapid esterolysis disappears. It seems, therefore, that if a general base that would not remain acylated were introduced near the mercaptan groups, cleavage of the acyl-S linkage might continue at a fast rate.

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