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Synthetic Derivatives of Polyethyleneimine with Enzyme-Like Catalytic Activity (Synzymes)

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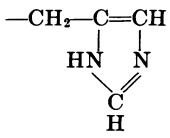
ABSTRACT **A synthetic polymer has been prepared which contains dodecyl groups (to bind small substrate molecules) and methyleneimidazole side chains (as nucleophilic catalytic sites) linked to a polyethyleneimine framework. This macromolecule, with a high local concentration of binding and catalytic groups, catalyzes the hydrolysis of *uncharged* nitrophenyl esters in water at pH 7 with rates markedly greater than previously observed with any other synthetic substances under similar conditions.**

Numerous attempts have been made to prepare synthetic polymers with catalytic activity mimicking that of enzymes, particularly the hydrolytic ones [1–6]. Especially interesting would be water-soluble macromolecules that are able to act upon *uncharged* small molecules, since apolar interactions play an important role in enzyme–substrate complexes.

Our approach to this objective has been to first modify polymers and mold their conformation to increase markedly their affinity for small molecules. This has been achieved with derivatives of polyethyleneimine [7, 8] and of modified polypeptides [9]. Furthermore, water-soluble derivatives of polyethyleneimine containing free amino groups have been found to enhance markedly the rates of aminolysis of nitrophenyl esters [10]. We have now discovered that substitution of the primary amines of polyethyleneimine with suitable nucleophilic groups creates a polymer with remarkable catalytic properties in the actual hydrolysis of nitrophenyl esters to acid and phenol.

MATERIALS AND METHODS

A derivative of polyethyleneimine, PEI-600 [11], was prepared containing 15% of its nitrogen residues alkylated with methyleneimidazole



side chains and 10% with dodecyl groups. The former provided nucleophilic catalytic sites; the latter, binding sites [8]. The imidazole function was introduced by alkylation of water-free PEI-600 with chloromethyl imidazole in the presence of KI and potassium *t*-butoxide as catalysts. Imidazole content in the modified polymer was determined both by chemical analysis [12] and by nmr spectroscopy. The primary amines remaining after introduction of methyleneimidazole groups were alkylated with stoichiometric amounts of dodecyl iodide. This reaction was quantitative, as judged by thin-layer chromatography to detect residual alkyl halide and by ninhydrin to detect residual primary amines. The absence of any ninhydrin color indicates preferential reaction of alkyl iodide with primary amines of the polymers.

The cleavage of *p*-nitrophenyl caproate was followed by the appearance of *p*-nitrophenolate ion, as detected by increased absorbance at 400 nm (measured with a Cary model 14 spectrophotometer). The reactions were run in 0.02 M Tris·HCl (pH 7.3) containing 1.25% acetonitrile, at 25°C.

RESULTS

That the cleavage of nitrophenyl caproate by the polymer is truly hydrolytic was demonstrated in two ways. In the first, repeated additions were made of 1×10^{-4} M substrate, up to 5 times the concentration of polymer imidazole groups (6×10^{-5} residue molar); nitrophenol was released completely each time. At lower polymer concentrations it was also possible to add a single injection of substrate, and in every case, the molar amount of nitrophenol released was substantially in excess of the concentration of imidazole groups on the dodecylpolyethyleneimine (Fig. 1).

To compare the catalytic effectiveness of our polymer with that reported for other substances that accentuate nitrophenyl ester cleavage, we have carried out a series of experiments (at pH 7.3) in which the residue molar concentration

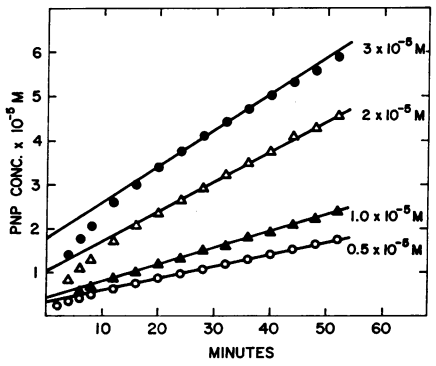


FIG. 1. Rates of esterolysis of *p*-nitrophenyl caproate at pH 7.3 and 25°C in presence of a derivative of polyethyleneimine (PEI-600) containing 10% of its residues alkylated with dodecyl groups and 15% alkylated with methyleneimidazole substituents. The numbers shown adjacent to each curve are the residue molar concentrations of imidazole groups in solution. Initial concentration of substrate was 1×10^{-4} M.

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[P-Im] of polymer imidazole groups was substantially in excess of [PNPAc], the concentration of substrate, *p*-nitrophenol acylate. Pseudo first-order rate constants k_1' were determined as previously described [10] at each of a number of polymer concentrations. Under these conditions k_1' was found to be linear with $[\text{P-Im}]_0$, the initial residue concentration of methyleneimidazole groups:

$$k_1' = k[\text{P-Im}]_0 \qquad (1)$$

Values of a "catalytic constant" (1), k, computed from Eq. 1, are listed in Table 1. It is obvious that the imidazole-substituted dodecylpolyethyleneimines are more than 100 times as effective as the simple imidazole molecule itself. The "catalytic constant" for the imidazole-dodecyl-polymer (V) in fact approaches that of the enzyme chymotrypsin. Accentuations were found with two other PEI-600 derivatives (III, IV), but without indications of regeneration of catalyst.

DISCUSSION

The shapes of the curves in Fig. 1 are consistent with a two-step pathway, analogous to that of a hydrolytic enzyme such as α-chymotrypsin [13], in which an initial acylation "burst" is followed by a slow deacylation reaction. If we insert also an equation for a pre-equilibrium binding step, this pathway may be presented as follows:

$$\text{P-Im} + \text{PNPAc} \overset{K_A}{\rightleftharpoons} \text{P-Im}\cdot\text{PNPAc} \qquad (2)$$

TABLE 1. *Relative effectiveness of various substances in cleavage of nitrophenyl esters*

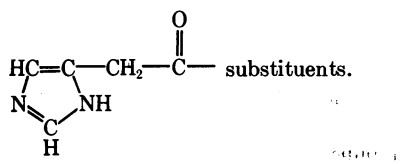
Substance	"Catalytic constant," k (liter/mol per min)
I. Imidazole	10*,†
II. α-Chymotrypsin	10,000*,‡
III. PEI(600)-HA(25%)§	420
IV. PEI(600)-HA(8%)-L(8%)-Im(6.6%)¶	3,100
V. PEI(600)-D(10%)-Im(15%)**	2,700

* The substrate used was *p*-nitrophenylacetate, at a pH near neutrality.

† Taken from Bruice, T. C., and G. L. Schmir, *J. Amer. Chem. Soc.*, **79**, 1663 (1957).

‡ Taken from ref. 1.

§ In this derivative of polyethyleneimine 600, 25% of the amine residues were alkylated with $-CH_2CON(OH)CH_3$ groups. This hydroxamate by itself has been studied by Gruhn, W. B., and M. L. Bender, *J. Amer. Chem. Soc.*, **91**, 5883 (1969).

¶ This derivative of PEI(600) had also 8% of its residues alkylated with hydroxamate, 8% acylated by lauroyl groups, and 6.6% acylated with  substituents.

** This preparation of PEI(600) had 10% of its residues alkylated with dodecyl (i.e., lauryl) substituents and 15% alkylated with methyleneimidazole groups.

$$\text{P-Im}\cdot\text{PNPAc} \xrightarrow{k_1} \text{Ac-Im-P} + \text{PNP} \qquad (3)$$

$$\text{Ac-Im-P} \xrightarrow{k_2} \text{P-Im} + \text{Acid} \qquad (4)$$

where Ac represents the acyl group and the PNP the nitrophenolate.

The linear portions of the curves in Fig. 1 are assumed to reflect the steady-state situation for Ac-Im-P that can occur when $k_1 K_A \gg k_2$. Under these circumstances, if [PNP-Ac] $\gg [\text{P-Im}]_0$, the completion of step 4, with release of P-Im, is immediately followed by regeneration of Ac-Im-P. Thus one may write

$$\frac{d[\text{Acid}]}{dt} = \frac{d[\text{PNP}]}{dt} = k_2[\text{Ac-Im-P}] = k_2[\text{II}] \qquad (5)$$

where [II] is the initial concentration of reactive imidazole sites. It can be shown that [II] is the intercept on the ordinate axis of the extrapolation of the linear portion [14] of any one of the curves in Fig. 1. Thus a graph of d[PNP]/dt, from the linear portions of Fig. 1, against [II] yields the deacylation rate constant k_2. For our imidazole-dodecyl-polyethyleneimine, we find $k_2 = 0.06\ \text{min}^{-1}$, a value appreciably larger than the rate constant of 0.01 min for the hydrolysis of acetyl imidazole [15].

In summary, we have prepared several derivatives of polyethyleneimine which markedly enhance the rate of cleavage of nitrophenyl esters. At least one of these, a synthetic polymer containing imidazole nucleophiles and dodecyl apolar binding sites, is also a true catalyst (a *synzyme*), with turnover or regeneration of catalyst, and is effective at pH 7. Exploratory mechanistic studies indicate that the hydrolysis proceeds along a pathway involving acylation and deacylation of the macromolecule, just as it does for some hydrolytic enzymes.

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