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Macromolecule–Small Molecule Interactions. Strong Binding and Cooperativity in a Model Synthetic Polymer*

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ABSTRACT: Exceptionally strong binding of organic anions is exhibited by polyethylenimine derivatives with apolar side chains. Conformationally compact, water-soluble polymers have been prepared with pendant butyryl, hexanoyl, or lauroyl aliphatic groups, or with carbobenzoxytyrosine or carbobenzoxytryptophan aromatic residues. All of these complex much more extensively with methyl orange than do proteins such as serum albumin or β-lactoglobulin.

The dependence of binding on concentration of small anion as well as the spectra of the complexes show that strong cooperative interactions appear with increased uptake of small molecule. These polymers offer attractive macromolecules for insertion of catalytic sites in addition to binding sites.

The binding of small molecules and ions by serum albumin has been of interest for many years (Klotz *et al.*, 1946; Klotz, 1949) because these complexes provide an insight into general biomacromolecular interactions with substrates and modifiers. Stoichiometric and energetic quantities characteristic of these interactions were obtained readily, particularly by equilibrium–dialysis techniques. However, an understanding of the detailed molecular nature of binding has been more elusive, although it has been clear that a combination of apolar and ionic interactions contribute to the strength of the complexes (Klotz, 1946).

If one truly understands a biochemical interaction it should be possible to reproduce it *de novo* with materials of nonbiological origin. Thus one might expect water-soluble synthetic polymers containing suitable apolar and ionic side chains to exhibit strong affinities for small molecules. Many such polymers have been examined (for references see Klotz and Sloniewsky, 1968). In our previous experience, however, no linear-chain type of water-soluble polymer was found to bind small molecules with an avidity comparable to that of serum albumin.

Such polymers, however, have highly swollen, extended conformations in aqueous solution, as is evident from their high intrinsic viscosities. In contrast serum albumin with an intrinsic viscosity near 4 ml/g must be relatively compact. It seemed possible, therefore, that if one could create a polymer with a high *local* concentration of apolar and ionic

* From the Biochemistry Division, Department of Chemistry, Northwestern University, Evanston, Illinois 60201. *Received July 25, 1969.* This investigation was supported in part by a grant from the National Science Foundation. It was also assisted by support made available by a U. S. Public Health Service training grant (5T1-GM-626) from the National Institute of General Medical Sciences.

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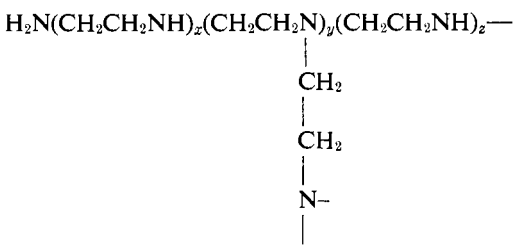
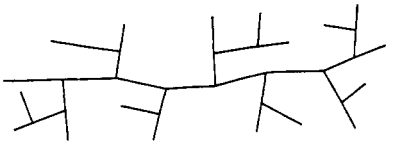
groups but still water soluble, one might produce a macromolecule with strong binding properties. A class of such polymers has been produced in derivatives of polyethylenimine.

Polyethylenimine is a highly branched water-soluble polymer (Davis, 1968), a segment of which may be described as



Approximately 25% of the nitrogens are primary amines, 50% secondary, and 25% tertiary (Davis, 1968). The branching of the polymer may be represented schematically as



The primary amine groups form a very suitable locus for the attachment of apolar groups to the polymer. We have prepared, therefore, a number of derivatives with different side chains attached to a portion of the primary amine groups. These modified polymers show remarkable binding properties.

Experimental Section

Experiments described in this paper were all carried out with derivatives of PEI-600, a commercially available (Dow Chemical Co.) polyethylenimine with a molecular weight near 50,000.

Two types of acyl derivatives were prepared, one containing long-chain hydrocarbon groups and the other containing aromatic amino acid substituents.

The long-chain aliphatic acyl derivatives were prepared in the following manner. Water from the 33% aqueous solution of PEI-600 was removed in a rotary evaporator, the residue dissolved in dry ethanol, and the solvent again removed. The polymer was then dissolved in absolute ethanol to give a 35% solution. A measured quantity of the methyl ester of the appropriate acid (lauric, hexanoic, butanoic, or isobutyric) was then added to the solution of polymer. This mixture was refluxed, with stirring, for 48–72 hr. At the conclusion of the reaction, the mixture was diluted with ethanol to two to three times its original volume. Gaseous HCl was bubbled in to precipitate the polymer as its hydrochloride salt, which was washed with large quantities of ethanol containing dissolved HCl. The product was then dried for several days under vacuum at 45°. Drying was continued until the product showed no significant amount of ethanol as judged from its nuclear magnetic resonance spectrum. Nuclear magnetic resonance was also used to determine the extent of acylation of the polymer. For this purpose 15% solutions of the hydrochloride salt of the polymer were dissolved in D_2O.

The amino acid derivatives were made with the appropriate nitrophenyl ester reagent. Measured quantities of a dry alcoholic solution of the nitrophenyl ester of either carbobenzoxytryptophan or carbobenzoxytyrosine were added dropwise with stirring to a dry alcoholic solution of PEI-600. The progress of the reaction could be followed by the appearance of the yellow color of the released nitrophenolate ion. The modified polymer was purified by gel filtration on Sephadex LH-20 with ethanol as solvent. The polymer appeared in the void volume of the eluent whereas nitrophenol and any unreacted ester emerged much later. Ethanol in the polymer fraction was removed by evaporation and an aqueous solution of the polymer was lyophilized to dryness. The content of carbobenzoxytryptophan or of carbobenzoxytyrosine was determined from the absorbance of the polymer derivative at about 280 nm. The extinction coefficients employed were those determined for carbobenzoxytyrosine propylamide and carbobenzoxytryptophan propylamide, 1600 and 5700, respectively. These reference amides were prepared from the appropriate nitrophenyl ester and propylamine.

The extent of binding of methyl orange was measured by equilibrium dialysis following procedures previously described (Klotz *et al.*, 1946; Hughes and Klotz, 1956; Rosenberg and Klotz, 1960). In this procedure, a pair of test tubes are filled with a measured quantity (10 ml) of buffer solution containing methyl orange at a known concentration. A dialysis bag containing (10 ml) polymer at a known concentration in buffer solution is immersed in one tube; a bag containing (10 ml) buffer only is immersed in the other. The tubes are shaken in a mechanical device overnight. The solutions external to the bag are then analyzed for equilibrium concentrations of methyl orange. From these measurements, the moles of bound small molecule/10^5 g of polymer can be readily computed, after corrections have been made for binding of the dye by the cellulose bag. All experiments were carried out in a 0.1 M Tris-cacodylate buffer at pH 7 and at 25°. Since binding by the polymers, especially the lauroyl derivative, was so exceptionally high, polymer concentrations within the dialysis bag were reduced from the 0.2% commonly used with serum albumin to as low as 0.001%. Most of the measurements with the lauroyl derivative were made at polymer concentrations of 0.005–0.1%, with the hexanoyl derivative at approximately 0.025%, and with the butyryl derivatives at 0.04%. At the very lowest concentrations used, near 0.001%, binding measurements gave lower results which we attribute to significant depletion of the polymer from the extremely dilute solutions by absorption on the cellophane membrane. With lauroylpolyethylenimine, when the moles of bound methyl orange/10^5 g of polymer exceeded approximately 470, an insoluble complex of macromolecule and small molecule was detected within the dialysis bag. Pronounced precipitation was observed as the extent of binding increased further.

Absorption spectra of the bound methyl orange were obtained by recording the difference spectrum between the inside of a dialysis bag and the external solution containing free methyl orange in equilibrium with the bound dye (Klotz and Shikama, 1968). When necessary, cells of very small light path were used and the values of absorbances were then normalized to a 1.0-cm path length.

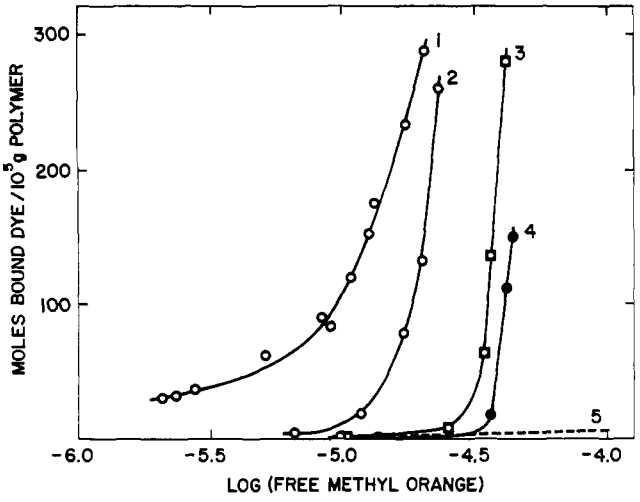


FIGURE 1: Extent of binding of methyl orange at pH 7.0 and 25° as a function of free (nonbound) dye concentration. (1) Polyethylenimine with 8.4% of residues acylated by lauroyl groups. (2) Polyethylenimine with 11.5% of residues acylated by hexanoyl groups. (3) Polyethylenimine with 10% of residues acylated by butyryl (○) or isobutyryl (□) groups. (4) Polyethylenimine, PEI-600. (5) Bovine serum albumin.

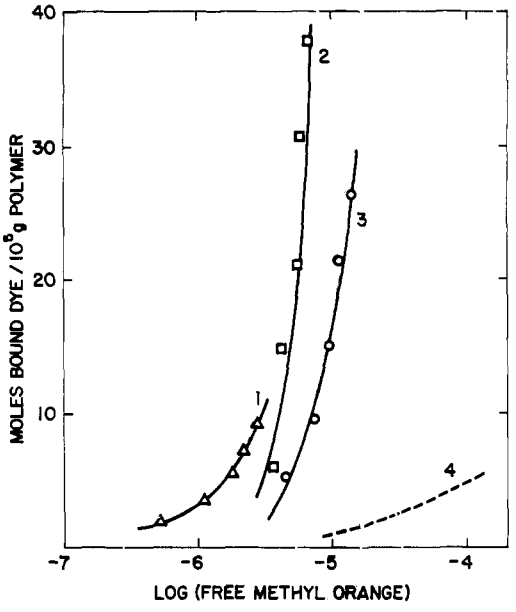


FIGURE 2: Extent of binding of methyl orange at pH 7.0 and 25° as a function of free (nonbound) dye concentration. (1) Polyethyleneimine with 10% of residues acylated by carbobenzoxytyrosine. (2) Polyethylenimine with 8% of residues acylated by carbobenzoxytryptophan. (3) Polyethylenimine with 2% of residues acylated by carbobenzoxytryptophan. (4) Bovine serum albumin.

Results

To facilitate comparison of different macromolecules the extent of binding has been expressed in terms of a common unit of weight of 10^5 g of polymer or protein. Figure 1 illustrates the tremendously greater extent of binding by the acylpolyethylenimines as compared with serum albumin. At a free methyl orange concentration of 10^{-5} M, the lauroyl derivative binds over 100 moles of small dye molecule, the hexanoyl about 10, and the butyryl about 1, whereas earlier studies with bovine albumin (Klotz *et al.*, 1946) lead to values just below 1. Admittedly, lauroylpolyethylenimine has a very long apolar side chain. On the other hand, less than 10% of its residues are acylated, whereas serum albumin contains nearly 40% nonpolar amino acid residues. Furthermore, the hexanoyl and butyryl derivatives of polyethylenimine, in which side-chain lengths are comparable to those in proteins, show substantially greater binding of organic anions than does albumin.

Also remarkable in comparison with albumin is the very steep rise in binding with increasing concentration of methyl orange. In Figure 1 this is most strikingly apparent in the smaller chain derivatives of the polymer (curves 4, 3, and 2, compared with 5) but, as more sophisticated graphical analyses (Klotz, 1953), based on various linear transformations of the fundamental binding equations, indicate, it is equally true of the lauroyl derivative.

Figure 2 illustrates the extent of binding of methyl orange by polyethylenimine derivatives with aromatic amino acid acylating groups. Again it is immediately evident that these synthetic polymers exhibit much greater avidity for organic anions than does serum albumin. Particularly striking, perhaps, is the substantial increase in binding upon introduction of carbobenzoxytryptophan groups on only 2% of the ethylenimine residues.

In connection with widespread interest in the effect of urea on proteins and their interactions it seemed relevant to examine binding by substituted polyethylenimines in the presence of this denaturant. These results, together with an extension of the concentration range for binding in the absence of urea, are shown in Figure 3. Obviously the presence of urea markedly reduces the binding affinity of lauroylpolyethylenimine.

The spectra of bound methyl orange are shown in Figure 4. Free, nonbound methyl orange has an absorption maximum at 464 nm. When the moles of bound methyl orange/10^5 g of polymer is near 100 or below, the spectrum of the bound dye shows a peak near 420 nm. At higher ratios of bound anion, particularly above 150 moles/10^5 g, a new sharp peak appears near 375 nm.

Discussion

It is obvious from the results shown in Figures 1 and 2 that acylated derivatives of polyethylenimine, in which the acyl group is apolar, possess very strong affinities for organic anions. The extent of binding exceeds substantially that observed with serum albumin, which is the best of the proteins in general binding ability (Klotz and Urquhart, 1949b). At the lowest experimental level this is strikingly shown by the fact that a 0.001% concentration of lauroylpolyethylenimine (8.4%) is as effective as a 0.2% concentration of bovine albumin in depleting a solution of free methyl orange anions.

In principle it is possible to evaluate the first binding constant, k_1, for anion–macromolecule complexes by suitable extrapolation of binding data to infinitely dilute small molecule concentration (Klotz and Urquhart, 1949a), even when there are strong interactions between successively bound small molecules. For methyl orange–serum albumin complexes, k_1 is of the order of 5×10^4 (Klotz *et al.*, 1946). For the corre-

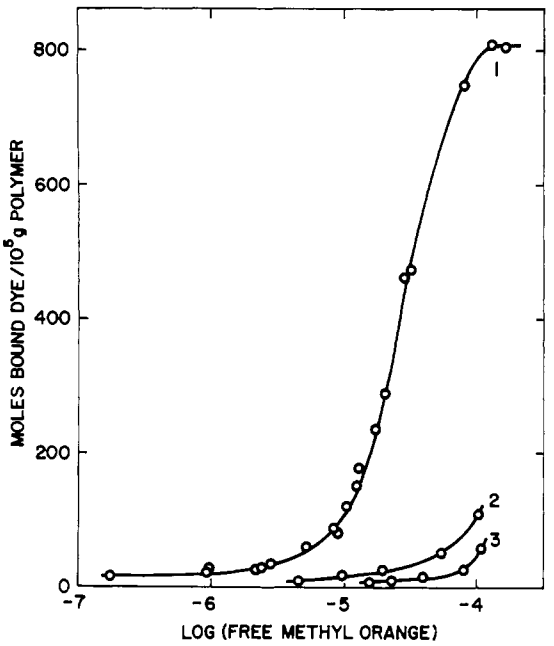


FIGURE 3: Effect of urea on extent of binding of methyl orange, at pH 7 and 25°, by polyethylenimine with 8.4% of residues acylated by lauroyl groups. (1) Tris-cacodylate buffer, 0.1 M. (2) Buffer and 6.0 M urea. (3) Buffer and 9.0 M urea.

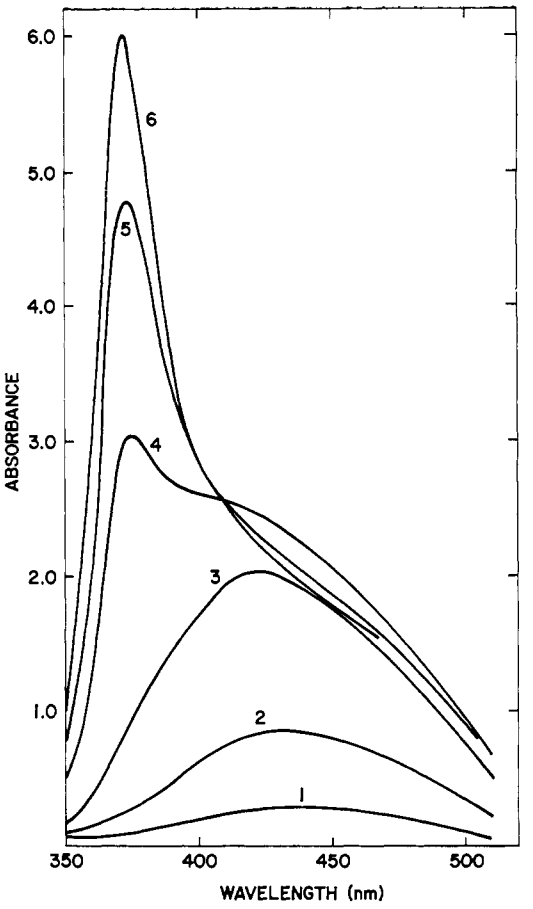


FIGURE 4: Absorption spectra of *bound* methyl orange on lauroylpolyethylenimine (8.4%) in Tris-cacodylate buffer (pH 7.0) and at 25°. Polymer concentration 0.0100%. Moles bound of methyl orange/10^5 g of polymer varied as follows: curve 1, 9.1; curve 2, 28.3; curve 3, 82.4; curve 4, 174; curve 5, 233; curve 6, 287.

sponding complex with lauroylpolyethylenimine, $k_1 \gg 10^6$. In fact the stability constant for the polymer–dye complex is so great that it has not been possible to evaluate it. As Figure 1 shows, even at free methyl orange concentrations of $\sim 10^{-6}$ M, the moles of bound dye are $\sim 10/10^5$ g of polymer. In practice to evaluate k_1 one must find the range of free methyl orange concentration at which the moles of bound dye are $\leq 1/10^5$ g of polymer. The results in Figure 1 suggest that this concentration of free methyl orange will lie well below 10^{-7} M, a range too low for customary procedures using equilibrium dialysis.

The marked increment in binding produced by the tryptophan derivatives (Figure 2) is of interest in connection with the possible involvement of this aromatic residue in binding by proteins. X-Ray diffraction has established the presence of tryptophan in the cleft in which substrate is held in lysozyme (Blake *et al.*, 1967; Phillips, 1967). There is also optical evidence that tryptophan residues are at the binding site of avidin (Green, 1963), antibody γ-globulins (Little and Eisen, 1967), and serum albumin (Reynolds *et al.*, 1967; Herskovits and Sorensen, 1968).

Also of relevance to the molecular basis of protein behavior are the observations (Figure 3) that urea strongly suppresses the binding ability of lauroylpolyethylenimine, just as it does that of serum albumin (Klotz *et al.*, 1948). Classically the effects of urea on proteins have been attributed to the disruption of peptide hydrogen bonds by this solute. In view of the present results with polyethylenimines, however, it seems unlikely that the mechanism of urea action in proteins involves disruption of N–H···O=C bonds. Similar conclusions have been reached previously from studies of the effect of urea on binding of anions by polyvinylpyrrolidone (Klotz and Shikama, 1968) and on the acid–base behavior of organic molecules attached to proteins and polymers (Klotz and Stryker, 1960). Also it has been shown (Hammes and Schimmel, 1967) that urea perturbs the ultrasonic attenuation in aqueous solutions of polyethylene glycol, and that it decreases the solubilization affinity of (nonionized) polymethacrylic acid for hydrocarbons (Barone *et al.*, 1967). Since urea has similar effects on aqueous solutions of synthetic and biopolymers of such very different molecular structure and conformation, it seems unlikely that it exerts its perturbing effects by direct combination with these different macromolecules. The very fact that large concentrations of urea are necessary for all of these effects indicates that its action is due to a change in the character of the solvent environment of the macromolecule, *i.e.*, *alloplastic* factors (Klotz, 1966) are involved.

The spectra of bound methyl orange on polyethylenimines (Figure 4) are distinctly different from those previously seen with this colored probe (Klotz, 1946; Klotz *et al.*, 1952; Klotz and Shikama, 1968). The free dye in bulk aqueous solution has a peak near 464 nm, in various organic solvents (ranging from benzene to ethanol to substituted acetamides) near 420–430 nm. Methyl orange bound to bovine albumin or to β-lactoglobulin shows an absorption maximum in the range 420 to 435 nm, presumably a reflection of the apolar character of the binding region. On the other hand, the dye

bound to the polymers polyvinylpyrrolidone or polylysine has a peak near 470 nm; evidently the open swollen conformation of these macromolecules exposes the bound dye to a largely aqueous milieu.

Bound to lauroylpolyethylenimine, methyl orange has a spectrum which depends on the number of anions attached to the polymer (Figure 4). When the moles of bound dye/10^5 g of polymer is less than 100, the spectrum has a peak near 420 nm, a position in consonance with the apolar environment of pendant lauroyl groups. When the moles bound/10^5 g reaches about 150, the 420-nm absorption maximum begins to be replaced by an entirely new and unique peak near 375 nm, which becomes more intense and sharper as the moles of bound dye increases even further.

The fact that the blue shift in the spectrum of bound dye occurs near 150 moles of bound dye/10^5 g of polymer is noteworthy, for 150 is also (approximately) the number of lauroyl groups attached to (10^5 g of) the polyethylenimine. It seems, therefore, that up to this point, each methyl orange anion is exposed largely to a lauroyl environment. Above 150, however, bound dye anions go to sites that already have one (or more) bound methyl orange molecules. A new type of interaction thus arises between the aromatic rings of neighboring azobenzene molecules, similar to interactions which lead to stacking in other systems, and this is reflected in the appearance of a peak at 375 nm, not previously encountered.

It is, furthermore, noteworthy that the transition concentration for the spectroscopic blue shift is also that at which cooperativity in binding manifests itself. With lauroylpolyethylenimine, appropriate graphical analysis using linear transformations of the fundamental binding equations (Klotz, 1953) shows that cooperative binding of methyl orange sets in at a free concentration (see Figure 1) near 10^{-5} M, that is when the moles of bound dye/10^5 g of polymer approaches 150. Cooperativity is more evident the smaller the aliphatic acyl side chain of the polymer (Figure 1). In fact the rise in binding is sharpest with unmodified polyethylenimine (curve 4 of Figure 1). In this case each methyl orange anion as it is bound simultaneously creates a new stronger apolar site for further binding of additional anions, as their concentration is increased. This interpretation is also supported by the spectra of this series of complexes. When about 2 moles of dye is bound per 10^5 g of unmodified polyethylenimine, the absorption maximum is at 470 nm; in the neighborhood of 15 moles (and perhaps even lower) a sharp peak at 380 nm is observed.

Thus cooperativity may reflect not only "conformational adaptability" (Karush, 1950) of a macromolecule but also modifications in local environment which are a consequence of the mere presence of bound small molecule.

Summarizing, we see that a compact, branched water-soluble polymer with apolar pendant groups shows very strong binding affinity for small molecules. Having introduced binding sites into polyethylenimines, we are now inserting potential catalytic groups also. It may then be possible to achieve rate enchancements by such synthetic polymer catalysts which may approach those observed with enzymes.

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