Anionic Polymers*

I. INHIBITION OF PROSTATIC ACID PHOSPHATASE

JOHN P. HUMMEL, DARLENE O. ANDERSON, AND CHANDULAL PATEL

From the Departments of Biochemistry and Urology, College of Medicine, State University of Iowa, Iowa City, Iowa

(Received for publication, March 20, 1958)

In the course of synthesizing a number of aryl phosphate esters as substrates for prostatic acid phosphatase, polymeric phosphorylated products obtained by reaction of 4,4'-di-hydroxybiphenyl with phosphorus oxychloride were found to be potent enzyme inhibitors. This report describes the preparation of these anionic polymers and some characteristics of their inhibition of prostatic acid phosphatase which seem to resemble the inhibition of hyaluronidase and alkaline phosphatase by a number of polyarylposphate compounds (1, 2).

MATERIALS AND METHODS

Preparation and Properties of Polyarylpophosphates

Polyxenylphosphate—To 20 gm. (0.11 mole) of 4,4'-dihydroxybiphenyl (recrystallized three times from hot glacial acetic acid) in 70 ml. of dry pyridine were added, at 0° with continuous stirring, 50 ml. (0.55 mole) of freshly distilled phosphorus oxychloride in 500 ml. of dry toluene. The first one-third of the phosphorus oxychloride solution was added dropwise over a period of an hour, and a sticky yellow mass was deposited. The remaining solution was added over an additional period of an hour. After standing undisturbed at room temperature for 12 hours, the sticky material had solidified to a white resinous mass consisting of various polyxenylphosphoryl chlorides. By means of hydrolysis and fractionation as described below, polyxenylphosphates of somewhat different properties could be obtained.

The resinous mass was collected by suction, washed well with dry toluene and saved for later usage. The filtrate and washings were concentrated in vacuo and the syrupy residue was dissolved in 500 ml. of cold 10 per cent sodium bicarbonate solution. The filtered solution was cautiously acidified with concentrated hydrochloric acid to precipitate Fraction A. It was collected by suction, washed with 0.1 N hydrochloric acid, and dried in vacuo over sodium bicarbonate pellets.

The toluene-washed resin obtained earlier was crushed up and stirred with 1 liter of ice water to which were made small additions of solid NaHCO3 until gas evolution ceased. The insoluble residue was collected by suction filtration, and the filtrate was acidified with hydrochloric acid to precipitate Fraction B which was washed and dried in the same manner as was Fraction A.

Both polymer fractions were dissolved in about 200 ml. of water, adjusted to pH 7 with 0.1 N NaOH, placed in separate cellulose sausage casings, and dialyzed against 10 liters of water daily for 3 days. The dialyzed polymer solutions were filtered through diatomaceous earth.

To convert the resultant polymer preparations to the acid form or to the sodium salt form, they were passed through Amberlite IR-120 resin in the H+ or Na+ forms, respectively. The eluates and washings were concentrated in vacuo to small volumes. The acid polymer preparations precipitated as fine white powders which were filtered and dried in vacuo over P2O5; in the sodium form, the polymer solutions were freeze-dried to yield slightly hygroscopic white solids. The yields of Fractions A and B in the acid form were 1.8 and 10.5 gm., respectively.

Total phosphorus content of the polymers was determined colorimetrically (3) after Parr bomb combustion of the samples and hydrolysis of resultant pyrophosphates to orthophosphate by boiling with dilute acid. Titration curves of 15 mg. portions of the acid polymer preparations were recorded automatically during addition of 0.1 N NaOH from a syringe microburette driven at constant speed by the drum shaft of a Varian model G-10 recording millivoltmeter which traced the pH as measured by a Beckman model H-2 pH meter. A 10,000 ohm potentiometer between the recorder and pH meter permitted the pen response to be proportioned to any desired pH range. Equivalent weights were calculated from base required to titrate both primary (pKb = 1.9) and secondary (pKb = 6.8) groups. The latter were assumed to represent only terminal phosphoric acid groups.

To estimate the terminal phenol content, the difference between the absorbancy at 288 mp of the polymer preparation at pH 12 and at pH 7 was measured. The molar extinction coefficient of the phenolate was assumed to be 2.07 × 104 at 288 mp as was found with 4-hydroxybiphenyl.

4,4'-Xenylidiphosphoric Acid—To 25 gm. of POCl3 in 250 ml. of dry toluene at 3° were slowly added 10 gm. of 4,4'-di-hydroxybiphenyl in 35 ml. of redistilled 2,6-lutidine with vigorous stirring. After the preparation had been standing at room temperature overnight, lutidine hydrochloride was removed by filtration, and the filtrate was concentrated in vacuo to a syrup which soon crystallized. Ice water (100 ml) and solid sodium bicarbonate were added in portions until CO2 evolution ceased. The solution was filtered and to the cold filtrate was slowly added an excess of concentrated hydrochloric acid. A fine, amorphous precipitate was collected after standing in the cold overnight, was washed with cold 0.1 N HCl, and dried over NaOH pellets in vacuo, yield, 8 gm.

* Supported in part by grants from the Iowa Division of the American Cancer Society, and Grant No. C-3256 of the National Institutes of Health, United States Public Health Service.
Polymer solutions were calculated from the phosphorus content. Activity was also determined by direct spectrophotometric measurement of the hydrolysis of 0-carboxyphenylphosphoric acid by the method of Fiske and SubbaRow (3). Phosphatase mixtures of 0.173 ionic strength and of the desired pH, as described by Michaelis (2). The orthophosphate liberated during 30 minutes of incubation of enzyme, substrate, and other additions in the amounts indicated for each experiment was measured by the method of Fiske and SubbaRow (3). Phosphatase activity was determined also by direct spectrophotometric measurement of the hydrolysis of O-carboxyphenylphosphoric acid according to Hofstee (6).

For the polyarylphosphates, the normalities of each of the polymer solutions were calculated from the phosphorus content. For the polysulfonates and polyalkylsulfates, the weight of the repeating unit of the infinite polymer was used.

The esterase activity of chymotrypsin was measured by the method of Schwert and Takenaka (7). Pepsin was measured by the method of Anson (8), catalyzed by the method of Beers and Sizer (9), glyceraldehyde phosphate dehydrogenase by the method of Cori et al. (10), deoxyribonuclease by the Kunitz procedure (11), and ribonuclease by the method of Anfinsen et al. (12). Commercial crystalline preparations of these enzymes were used.

RESULTS

Typical PXP preparations behave as highly charged, polydisperse, small molecular weight colloids of branched and random coil structure. Variations from batch to batch were found, particularly with respect to the equivalent weights and the percentages of terminal phenol or phosphate groups. Properties of Lot 116, which was used throughout this study, are listed in Table I. Fraction A contained a higher proportion of alkali-labile triester toward alkali, Lot 116A was less stable than Lot 116B. The inhibitory effect of PXP toward a number of purified enzymes is shown in Table III. They were tested at 38° and on the acid side of the pH optimum in each case so as to favor protein polymer interaction. Prostatic phosphatase seemed to be unusually sensitive to polymer inhibition. Catalase and pepsin were virtually unaffected.

The stability of PXP to heating with acid and alkali is shown in Table II. Inhibition was measured by 30 minutes of incubation of 1 ml. of enzyme solution (0.002 per cent protein) with 5 ml. of substrate solution. Final concentration was 0.012 M β-glycerophosphate, 10⁻⁴ M PXP (repeating unit), all at pH 5.0.

0.3 showed a single broad peak corresponding to a sedimentation constant of about 1.1 S. From the partial specific volume of 0.62 and a diffusion constant of 1.2 × 10⁻⁹ cm² sec⁻¹ an approximate average molecular weight of about 6000 was estimated. Similar values were found for B fractions of other PXP preparations.

The stability of PXP to heating with acid and alkali is shown in Table II. Inhibition was measured by 30 minutes of incubation of 1 ml. of enzyme solution (0.002 per cent protein) with 5 ml. of substrate solution. Final concentration was 0.012 M β-glycerophosphate, 10⁻⁴ M PXP (repeating unit), all at pH 5.0.

<table>
<thead>
<tr>
<th>Pretreatment of PXP</th>
<th>% of inhibition of acid phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>97</td>
</tr>
<tr>
<td>1 N HCl</td>
<td>97</td>
</tr>
<tr>
<td>10 min., 100°</td>
<td>97</td>
</tr>
<tr>
<td>30 min., 100°</td>
<td>95</td>
</tr>
<tr>
<td>1 N NaOH</td>
<td>84</td>
</tr>
<tr>
<td>10 min., 100°</td>
<td>77</td>
</tr>
<tr>
<td>30 min., 100°</td>
<td>97</td>
</tr>
<tr>
<td>60 min., 100°</td>
<td>73</td>
</tr>
</tbody>
</table>

TABLE I

Analysis of typical PXP preparations

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Lot 116A</th>
<th>Lot 116B</th>
<th>Theory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorus, %</td>
<td>13.1</td>
<td>12.8</td>
<td>12.5</td>
</tr>
<tr>
<td>Terminal phenol groups, %</td>
<td>15.5</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>Terminal phosphate, % of total phosphorus</td>
<td>39.8</td>
<td>22.6</td>
<td></td>
</tr>
<tr>
<td>Equivalent weight</td>
<td>200</td>
<td>309</td>
<td>248</td>
</tr>
</tbody>
</table>

* These values were calculated for an infinite unbranched polymer.

TABLE II

Stability of PXP to acid and alkali

Inhibition was measured by 30 minutes of incubation of 1 ml. of enzyme solution (0.002 per cent protein) with 5 ml. of substrate solution. Final concentration was 0.012 M β-glycerophosphate, 10⁻⁴ M PXP (repeating unit), all at pH 5.0.

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The inhibitory effect of PXP toward a number of purified enzymes is shown in Table III. They were tested at 38° and on the acid side of the pH optimum in each case so as to favor protein polymer interaction. Prostatic phosphatase seemed to be unusually sensitive to polymer inhibition. Catalase and pepsin were virtually unaffected.

A comparison of the inhibitory potency of various polymers toward prostatic phosphatase is shown in Table IV. Polyhydroquinone phosphate, PXP, and sulfonated polysulfone were inhibitory even at concentrations of the repeating unit of 10⁻⁶ M, but other polymers with density of similar charge produced much weaker effects. Polymetaphosphate, with a mean

1 The abbreviation used is: PXP, polyxenylphosphate.
TABLE III

Comparison of inhibition of various enzymes by PXP (Lot 116B)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>pH</th>
<th>10^{-4} M</th>
<th>10^{-3} M</th>
<th>10^{-2} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatase, prostatic</td>
<td>5.3</td>
<td>5.0</td>
<td>99</td>
<td>90</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>7.4</td>
<td>6.0</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>6.7</td>
<td>6.0</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Glyceraldehyde phosphate dehydrogenase</td>
<td>8.5</td>
<td>8.0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>8.9</td>
<td>7.0</td>
<td>58</td>
<td>35</td>
</tr>
<tr>
<td>Catalase</td>
<td>7</td>
<td>6.8</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Pepsin</td>
<td>1.5</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE IV

Inhibition of acid phosphatase by various polymers

<table>
<thead>
<tr>
<th>Experimental conditions were as in Table II.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer</td>
</tr>
<tr>
<td>Hexametaphosphate*</td>
</tr>
<tr>
<td>Polymetaphosphate*</td>
</tr>
<tr>
<td>Polyhydroquinone</td>
</tr>
<tr>
<td>PXP, 116B</td>
</tr>
<tr>
<td>PXP, 116A</td>
</tr>
<tr>
<td>Polydibenzyl phosphate†</td>
</tr>
<tr>
<td>Polydiphenylmethyldimethyl methane phosphate†</td>
</tr>
<tr>
<td>Polyaacrylate†</td>
</tr>
<tr>
<td>Polyethylene sulfate§</td>
</tr>
<tr>
<td>Sulfonated polystyrene†</td>
</tr>
<tr>
<td>O-Sulfochitosan§</td>
</tr>
<tr>
<td>N-Sulfochitosan§</td>
</tr>
<tr>
<td>N-Sulfo-O-sulfochitosan§</td>
</tr>
<tr>
<td>Chondroitin sulfate§</td>
</tr>
<tr>
<td>Heparin§</td>
</tr>
<tr>
<td>Polyethylene-2-(N-methyl pyridinium iodide)</td>
</tr>
<tr>
<td>Poly(p-xylyl-N,N-dimethyl ammonium bromide)§</td>
</tr>
</tbody>
</table>

† Ing. B. Högborg, A/B Leo, Helsingborg, Sweden.
‡ Dr. F. W. Schueler, Department of Pharmacology, Tulane University, New Orleans, Louisiana.
§ Dr. Lester Coleman, The Upjohn Company, Kalamazoo, Michigan.
¶ Nutritional Biochemicals Corporation, Cleveland, Ohio.

molecular weight of about 300,000, was a poor inhibitor. Certain sulfonate or sulfate polymers exhibited a curvus maximus of inhibition at 10^{-2} N. The cationic polymers, polyethylene-2-(N-methyl pyridinium iodide) and poly(p-xylyl-N,N-dimethyl ammonium bromide) had no inhibitory action.

The degree of inhibition of prostatic phosphatase by PXP Lot 116B and by fluoride was markedly dependent upon pH (Fig. 1), being more powerful on the acid side of the pH optimum. A similar effect was previously noted by Town et al. (15) for the inhibition of urease by suramin, and by Diczfalusy et al. (1) for the inhibition of alkaline phosphatase by polyphloretin phosphate, which they interpreted as reflecting an interaction between the positively charged enzyme and negatively charged anionic polymer. If this is so, the degree of inhibition should be reduced by increasing the ionic strength of the medium. As seen in Table V, addition of NaCl significantly reduced the inhibition by PXP.

Upon prolonged dialysis of PXP, only part of the polymer remained within the dialyzing bag. The diffusible fraction was apparently of intermediate size because, like the polymers, it was inhibitory. The monomer, 4,4'-xylenediphosphoric acid, was not inhibitory even at 10^{-2} N, but was hydrolyzed by prostatic phosphatase at approximately the same rate as β-glycerophosphate.

Nonspecific interaction between PXP and protein seems to account for the reversal of PXP inhibition of phosphatase by various proteins; basic proteins were somewhat more effective than egg albumin in this respect (Table VI). The synthetic

FIG. 1. The effect of pH upon phosphatase activity in the presence of 10^{-6} N PXP Fraction B (●—●) and 5 × 10^{-4} N NaF (○—○) or without inhibitor (X—X). Conditions were as in Table II; vertical axis: milligrams of inorganic phosphate formed per mg. of enzyme N per hour.

TABLE V

Effect of salt concentration on PXP inhibition of acid phosphatase*

<table>
<thead>
<tr>
<th>NaCl concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>molar</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>96</td>
</tr>
<tr>
<td>0.04</td>
<td>93</td>
</tr>
<tr>
<td>0.08</td>
<td>90</td>
</tr>
<tr>
<td>0.4</td>
<td>54</td>
</tr>
</tbody>
</table>

* Inhibition was measured as in Table II but with 7 × 10^{-6} N PXP, Lot 116B, and omitting the Michaelis buffer (5).
cathionic polymer, poly(p-xylyl-N,N-dimethyl ammonium bromide) acted similarly to proteins in reversing PXP inhibition. The degree of reversal of PXP inhibition was essentially the same regardless of whether the PXP was preincubated with phosphatase or with globin (Table VI), demonstrating that the complex formed between PXP and phosphatase or globin was dissociable. Putrescine, cadaverine, and spermine, reported by Jeffree (16) to protect prostatic phosphatase against surface inactivation during extreme dilution, were totally inactive in reversing PXP inhibition.

In Fig. 2, the inhibition of prostatic phosphatase by unbranched PXP is shown to be of the noncompetitive type when tested by the conventional Lineweaver-Burk plot (17). The degree of inhibition was unaffected by the presence of 0.001 M Versene (Dow Chemical Company), 0.01 M citrate, 0.01 M Mg++, or of 0.01 per cent Triton X-100 (Rohm and Haas Co.), a nonionic surface-active agent employed by Tsuboi and Hudson (18) to prevent surface inactivation of prostatic phosphatase. The inhibition of phosphatase by $10^{-5}$ M PXP was 95 to 98 per cent complete regardless of whether the substrate was $\beta$-glycerophosphate (Malinekrodt), phenylphosphate (Distillation Products), 2-methyl-1,4-naphthohydroquinone diphosphate (Hoffman-LaRoche), or O-carboxyphenylphosphate (California Foundation).

**Discussion**

A comparison of the inhibitory potency of various polymers toward phosphatase indicates that factors in addition to electrostatic interaction may be implicated. Potassium polymetaphosphate (Graham's salt) of high molecular weight was a poor inhibitor despite its moderately high charge density and linear structure (19). Although the PXP preparations prepared in this laboratory were more inhibitory than the other somewhat similar polyarylamphosphates prepared by the Swedish workers, differences in the methods of synthesis may account for this, although analytical data of their preparations are scant. The lower charge density at pH 5 of polyarylamphosphate preparations of highly branched structure could explain some of the differences observed in the degree of inhibition of phosphatase by Frac- tions A and B. Although the lack of inhibition by polyelecrate (pK 6.1) is not surprising in view of its incomplete ionization at pH 5.0, the differences between the inhibition by sulfate, sulfonate, and phosphatase polymers cannot be explained on that basis. With certain of the sulfotons and polyelecrylene sulfonate, a peculiar peak of inhibitory activity was found at $10^{-4}$ M but they were generally less inhibitory than PXP or sulfonated polystyrene.

A considerable body of evidence has accumulated in favor of the concept that basic groups are essential for the activity of prostatic phosphatase. Surface inactivation of prostatic phosphatase by dilution is prevented by various polyamines (16). The high sensitivity of prostatic phosphatase to inhibition by negatively charged polyelectrolytes is best explained by blockage of a number of positively charged groups essential to the enzyme action, although not necessarily at the active center. Inasmuch as the isoelectric point of this enzyme is about pH 4.4 (20), its protein surface bears a net negative charge over most of the pH range at which the catalytically essential cationic sites are presumed to interact with polyamines. The inhibition by anionic polymers resembles that by fluoride in that in both cases inhibition is weak or absent on the alkaline side of the pH optimum in confirmation of the findings of Anagnostopoulos (21). It is also reduced by media of greater ionic strengths, or by addition of protein (22), although in a complex manner. As a consequence of these and other observations on the protective effect of fluoride on thermal inactivation of prostatic phosphatase, Reiner et al. (22) suggested that fluoride may exert its action as the dimer HF2- which could be "associated with two adjacent but independent positive centers so located that it ‘straddles’ one or more critical hydrogen bonds.” A recent steric description by London et al. (20) of the region of the active center of prostatic phosphatase pictures a cationic and a

<table>
<thead>
<tr>
<th>Addition</th>
<th>Inhibition by PXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>90</td>
</tr>
<tr>
<td>Cadaverine*</td>
<td>89</td>
</tr>
<tr>
<td>Putrescine*</td>
<td>89</td>
</tr>
<tr>
<td>Spermine§</td>
<td>90</td>
</tr>
<tr>
<td>Ovalbumin*</td>
<td>55</td>
</tr>
<tr>
<td>Salmon*</td>
<td>33</td>
</tr>
<tr>
<td>Globin*</td>
<td>33</td>
</tr>
<tr>
<td>Protamine*</td>
<td>28</td>
</tr>
<tr>
<td>Poly(p-xylyl-N,N-dimethyl ammonium bromide)§</td>
<td>52</td>
</tr>
<tr>
<td>Globin + PXP, enzyme added 15 min. later</td>
<td>33</td>
</tr>
<tr>
<td>Enzyme + PXP, globin added 15 min. later</td>
<td>28</td>
</tr>
<tr>
<td>Enzyme + globin, PXP added 15 min. later</td>
<td>28</td>
</tr>
<tr>
<td>Enzyme + globin, no PXP added</td>
<td>0</td>
</tr>
</tbody>
</table>

* Nutritional Biochemicals Corporation.
† Delta Chemical Works, New York.
‡ Generously supplied by Dr. F. W. Schueler, Department of Pharmacology, Tulane University.
hydrogen bonding site on each side of a critical seam on the enzyme surface. The extremely strong inhibition exerted by anionic polymers possibly arises from the reinforcing effect of a large number of anionic sites and a lesser extent by relatively rigid groups at distances which particularly favor attraction to these same positive centers of prostatic phosphatase. Whether the organic spacer between anionic groups affects the degree of inhibition remains to be determined.

**Summary**

The inhibitory potency of a number of polydisperse anionic polymers toward a number of enzymes was investigated. Poly-

**Acknowledgments**—We wish to acknowledge the assistance of Mrs. Lorraine H. Malek and Miss Janet Hall in this study.

**References**

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