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Diazo Coupling of Catecholamines with Poly(organophosphazenes)

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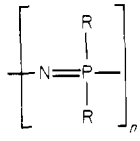
ABSTRACT: Polymer-bound catecholamines have been prepared by the diazotization of high-polymeric $[NP(OC_6H_5)_x(OC_6H_4NH_2\text{-}p)_y]_n$, followed by coupling to dopamine, *dl*-epinephrine, and *dl*-norepinephrine. These reactions were preceded by model compound studies with the cyclic trimer $N_3P_3(OC_6H_5)_5OC_6H_4NH_2$. In both cases, the aminophenoxy units were generated by reduction of 4-nitrophenoxy groups with PtO_2 and hydrogen. The phosphazene skeleton was unaffected by the reduction, diazotization, and diazo coupling processes. The physical characteristics of the trimers and high polymers are described. The polymeric azocatecholamine derivatives were purified by gel permeation chromatography, which reduced contamination by the unbound catecholamine to undetectable levels. The polymeric derivatives were found to elicit inhibition of prolactin release from rat pituitary cells in culture for 48 h.

Considerable interest exists in the synthesis of biologically active polymers.[1-4] One aspect involves the attachment of biologically active small molecules to polymers in order to confer, for example, chemotherapeutic activity on the macromolecules[5-7] themselves or to generate a controlled-release system for the active small molecule. Thus, the synthesis of macromolecules that can function as carrier species for small-molecule chemotherapeutic agents or that can selectively bind ions in aqueous media is a subject of growing importance. So, too, is the preparation of polymers that may serve as templates and supports for the covalent binding of catecholamines.

The pioneering studies by MacLeod[8,9] showed that dopamine reduces prolactin secretion from the rat anterior pituitary gland in vitro in a dose-dependent and pharmacologically reversible manner. It is now well established that this inhibitory effect by dopamine is specifically mediated via receptors on the membrane of the cell that produces prolactin.[10]

The linkage of dopamine and other catecholamines to a synthetic polymer is expected to open up a number of possibilities for new research into the mechanism of action of neurotransmitters. First, the use of such polymeric species in affinity chromatography is an important prospect. Second, the availability of a polymer-immobilized dopamine allows the study of cell response mechanisms under conditions where membrane-penetrating neurotransmitter dopamine molecules can be excluded from entering the cell. Third, polymer-bound dopamine is expected to be more stable to chemical degradation than the free active molecule. Fourth, depending on the site of linkage of the catecholamine to the polymer, it may be possible to confirm which sites on the catecholamine are responsible for the key interaction with the cell membrane.

We describe here an initial approach to the synthesis and evaluation of polymer-bound catecholamines. The carrier polymer chosen for this work was a high molecular weight poly(organophosphazene) (1). This carrier was



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selected because of the well-established suitability of this system for substitutive-type modification and the long-range prospect that a biodegradable polymer of this type might be used in pharmacological applications.

The synthesis of a wide range of poly(organophosphazenes) by the interaction of the reactive high-polymeric intermediate $(NPCl_2)_n$ with nucleophiles has been discussed in earlier work.[11-14] For example, steroidal residues[15] and a wide variety of other organic groups can be linked to a phosphazene chain.

In this paper we describe a new method that allows the attachment of biologically active catecholamine residues to a phosphazene skeleton. This pathway involves a two-step sequence in which an aromatic amino organic "spacer" side group is first attached to the inorganic skeleton. Then certain of these side groups are diazotized and the diazonium functionality is then used to bind the catecholamine.

The logic behind this approach is as follows: First, it was necessary to exclude from consideration any synthetic pathway that involved the reaction of a difunctional reagent with $(NPCl_2)_n$, because of the inevitability of

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Table I
Characterization Data for Cyclophosphazene Species

compd		microanalysis % C	% H	% N	% P	^{31}P NMR,[a] ppm	mp, °C	mol wt[b]
4	calcd	58.53	3.92	7.58	12.60	9.57	79	738
	found	58.63	3.94	7.47	12.75			738
5	calcd	61.01	4.37	7.90	13.30	10.31	102	708
	found	60.84	4.38	7.89	13.36			708
6	calcd	60.55	4.47	9.63	10.66	9.83	86-90 (dec)	872
	found	60.76	4.49	9.80	10.66			872
7	calcd	59.86	4.54	9.31	10.31	9.84	87 (dec)	902
	found	60.49	4.57	10.12				902
8	calcd	59.45	4.39	9.45	10.47	9.64		888
	found	60.58	4.55	10.17				888

[a] The ^{1}H NMR spectrum of 5 showed NH_2 protons at 3.5 ppm and aromatic protons centered at 6.8 ppm in a ratio of 29:2. [b] Mass spectral parent ion.

cross-linking reactions. Second, advantages can be foreseen for the utilization of an aminoaryloxy side-group linkage site because the amino residue provides access to a range of different linkage mechanisms for various bioactive agents. Third, this approach allows catecholamines to be distributed randomly along the polymer chain, irrespective of the amount of catecholamine employed. Fourth, linkage of the catecholamine can be accomplished under mild conditions to ensure minimal deactivation of the active species. Fifth, it provides a method for the removal of unbound catecholamine from the system before biological testing takes place. And sixth, it provides a general route for the preparation of polymer-bound catecholamines that are either organic media soluble or water soluble, depending on the nature of the cosubstituent groups employed.

In the present work, we have concentrated on the preparation of a water-insoluble system. We have also tested the ability of the polymer-bound dopamine to inhibit the release of prolactin from rat pituitary cells in culture. As in our earlier work, we have developed the new preparative procedures initially by the use of small-molecule cyclic phosphazene "model" compounds and have then extended these procedures to the high-polymeric analogues. Some anomalies were found during the comparison of the small-molecule and macromolecular systems.

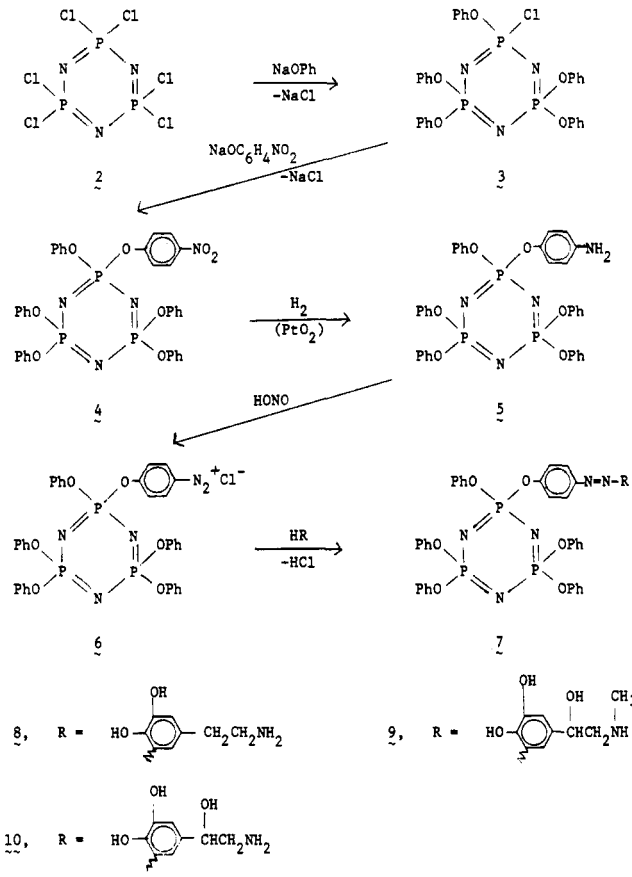
Results and Discussion

Small-Molecule Model Studies. The overall reaction sequence for the model compound studies is outlined in Scheme I. Thus, hexachlorocyclotriphosphazene (2) reacted with a fivefold excess of sodium phenoxide to yield pentaphenoxychlorocyclotriphosphazene (3), from which the remaining chlorine atom was displaced by sodium 4-nitrophenoxide to yield pentaphenoxy(nitrophenoxy)cyclotriphosphazene (4). This could be reduced catalytically to pentaphenoxy(aminophenoxy)cyclotriphosphazene (5). No evidence of catalyst poisoning by the phosphazene was found, and this was important for the later polymer synthesis work. Compound 5 was converted to the diazonium salt, 6, under standard reaction conditions and without any evidence of phosphazene skeletal cleavage. Subsequent coupling of the diazonium salt to three biologically active representative catecholamines yielded 7–10.

These model compounds were characterized by a variety of techniques. First, the characteristic colors of 7–10 were indicative of azo compound formation. Elemental analysis, ^{31}P NMR, ^{1}H NMR, melting point, and mass spectrometric data are listed in Table I. All these data are compatible with structures 7–10, and this suggested that the analogous polymeric reactions might be feasible.

Synthesis of the High Polymers. In view of the expected steric hindrance associated with groups such as

Scheme I



those in 8–10, the diazo construction sequence was carried out on a mixed-substituent high polymer that contained approximately 85% phenoxy and 15% 4-nitrophenoxy side groups. The reaction sequence is shown in Scheme II.

Hexachlorocyclotriphosphazene (2) was polymerized thermally to poly(dichlorophosphazene) (11). The phenoxy substituent groups were introduced first to form 12 because of the known ease with which nitrophenoxy groups can be displaced by a second nucleophile.[17] Subsequent treatment of 12 with an excess of sodium 4-nitrophenoxide yielded 13. Catalytic reduction of 13 to 14 was accomplished with the same techniques as those used for the cyclic oligomeric model, and subsequent treatment with nitrous acid yielded the diazonium halide 15. Coupling to the aromatic hydroxy catecholamines was then accomplished as before to yield 16–19 (see Table II for characterization).

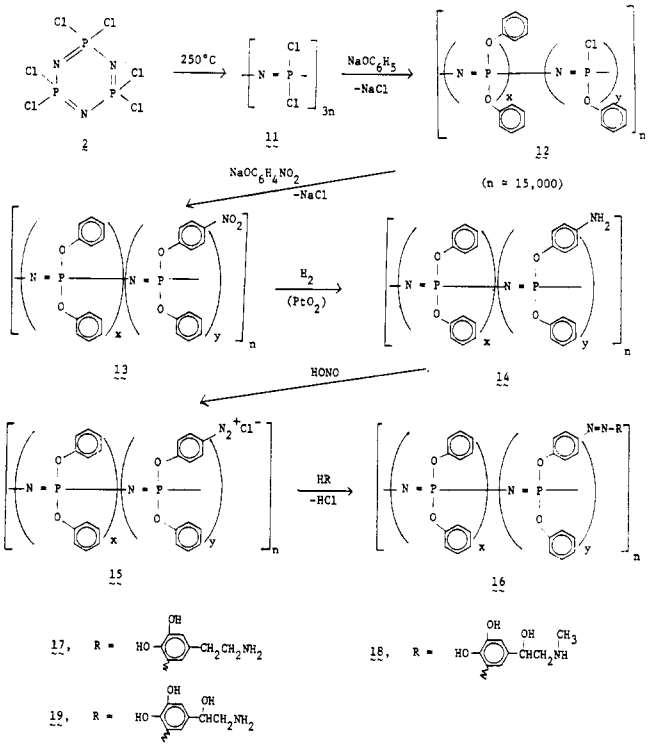
An anticipated problem with this synthesis route was the possibility that the diazonium halide (6 or 15) might undergo intra- or intermolecular diazo coupling with the phenoxy cosubstituent groups attached to the phosphazene

Table II
Characterization Data for High Polymers

compd		microanalysis % C	% H	% N[a-c]	GPC MW[d]	^{31}P NMR,[e] ppm
13	calcd	58.53	3.92	7.58	5×10^5	−19.8, −18.5
	found	58.48	4.04	7.49		
14	calcd	61.01	4.37	7.90	5×10^5	−19.5, −20.9
	found	61.17	4.14	7.90		
17	calcd	60.55	4.47	9.63	5×10^5	−19.5
	found	60.68	4.70	9.40		
18	calcd	59.86	4.54	9.31	5×10^5	−19.8
	found	60.75	4.37	10.17		
19	calcd	59.45	4.39	9.45	5×10^5	−19.4
	found	62.46	4.72	9.82		

[a] Analytical data were obtained by Galbraith Laboratories. [b] Residual chlorine (1%) was attributed to HCl bound as a salt rather than to unreacted PCl units. Evidence for this view was obtained from a correlation of the microanalyses and ^{31}P NMR data. [c] The reactant ratios had been designed to yield an 85:15 substituent ratio. [d] The values shown represent gel permeation chromatography results from identical initial synthesis. The values shown were obtained by comparison with polystyrene standards. [e] All spectra were proton decoupled and were interpreted as singlets. Chemical shift positions were relative to aqueous 85% H_3PO_4. A D_2O capillary lock was used.

Scheme II



ring or chain. However, during the course of this investigation it became evident that this side reaction did not occur to a detectable extent.

Characterization of the High Polymers. All the polymers were soluble in organic media and were, therefore, un-cross-linked. As shown in Table II, the microanalyses corresponded to structures **13–19**. (The actual ratios of the different substituent groups deduced by microanalysis are listed in Table II.) The generation of colors following the diazo coupling step provided a firm indication for the formation of **16**.

A number of questions remained about the structure of **16**. How efficient were the reduction step, the diazonium halide formation, and the diazo coupling process? What was the actual ratio of phenoxy groups to substituted phenoxy groups in **13–19**? Were the substituted aryloxy groups arrayed geminally or nongeminally along the chain? Did the substitution reactions result in detectable chain cleavage? Elemental microanalyses were compatible with a ratio of roughly 85% phenoxy and 15% substituted

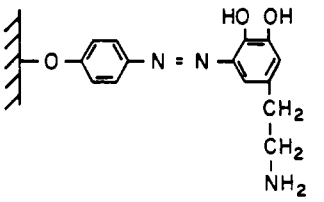


Figure 1. Proposed structure of dopamine linked to polyphosphazene. The linkage could be through either the 5′ or 6′ position.

aryloxy groups for polymers **13–16** (Table II). (Nitro groups could not be detected by infrared spectroscopy for species **14**. However, the appearance of the −N=N− "stretching" was observed in species **16**.) Hence, the reduction, diazotization, and coupling processes appeared to be virtually 100% effective. ^{31}P NMR spectra for **13–16** showed multiple peaks. A correlation of these spectra with the chemical shift changes that followed the replacement of electron-withdrawing groups (nitrophenoxy) by electron-releasing groups (aminophenoxy) favored the view that the substituted aryloxy groups are arrayed nongeminally, i.e., that the repeating groups present are $NP(OC_6H_5)_2$ and $NP(OC_6H_5)(OC_6H_4X)$, but not $NP(OC_6H_4X)_2$. This is compatible with the known nongeminal substitution pattern of $(NPCl_2)_n$ (**11**) with sodium phenoxide, a pattern presumably induced by steric factors.[14,18] The proposed structure of a catecholamine linked to polyphosphazene is shown in Figure 1. It is possible that the linkage may be through either the 5′ or 6′ position of the catecholamine aromatic ring depending on steric factors.

The GPC-average molecular weights of species **14** and **15** were in the region of 5×10^5. This value, while somewhat lower than the maximum values reported for poly[(aryloxy)phosphazenes],[11,19] is not indicative of appreciable chain cleavage during the reaction sequence shown in Scheme II. Moreover, no significant change in the shape or the width of the GPC polymer peaks occurred when **14** was diazotized and coupled. The proposed structure of the polymeric azocatecholamine derivatives is shown in Scheme II. The structure of the polymeric repeating unit cannot be specified exactly because the substitution pattern in presumed to be random. Since the diazotized aminophenoxy residues appeared to be fully substituted (supported by microanalysis), we presumed that any trace of remaining phenoxydiazonium halide groups would be hydrolyzed to phenoxy residues during the purification steps. The GPC column elution profiles for the high molecular weight polymeric azocatecholamine

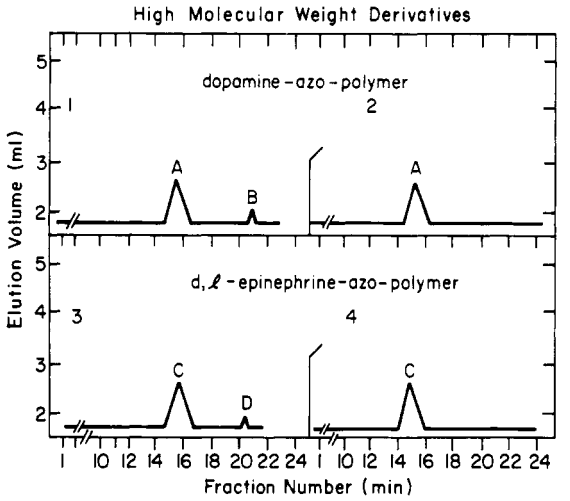


Figure 2. Purification of polymeric azodopamine and azoepinephrine derivatives as examples. Sections 1 and 3 represent the elution profiles for the separation of the products from the coupling reactions of the 5×10^5 M_n derivatives of dopamine and epinephrine, respectively. Peak assignments by thin-layer chromatography are as follows: the polymeric dopamine derivatives (A) and free dopamine (B); the polymeric epinephrine derivatives (C); and free (unbound) epinephrine (D). Sections 2 and 4 represent the second subsequent column purifications of the respective polymer peaks without (below detectable limits) the unbound catecholamines B and D. Gel permeation chromatography was performed on a μ-Styragel column with THF as eluent. Fractions were monitored by UV absorption at 254 nm.

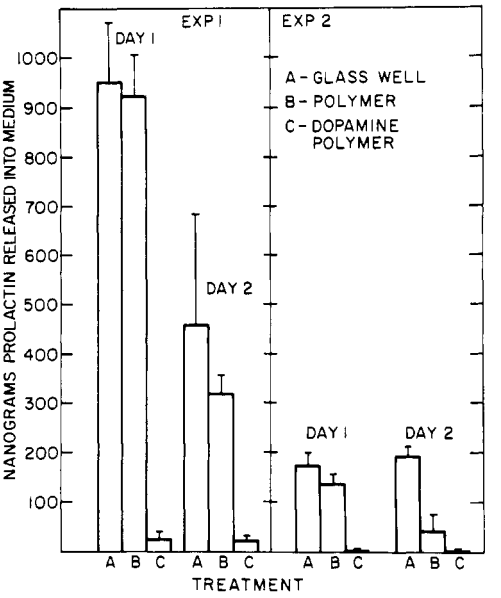


Figure 3. Release of prolactin from 200 000 pituitary cells cultured (1) in glass wells (A), (2) on poly(diphenoxyphosphazene)-coated glass wells (B), and (3) on poly(dopamine-azo-phenoxyphosphazene] coated on glass wells (C). This assay is based on the ability of prolactin to stimulate division of NB-2 rat lymphoma cells. Assay sensitivity was 50 pg/tube. Each sample was assayed in duplicate at two dilutions; there were three sample/treatment groups.

derivatives are illustrated in Figure 2. The products of the diazotization reaction mixture were separated by this method following exhaustive Soxhlet extraction and subsequent identification by thin-layer chromatography. The first GPC column purification of this derivative (Figure 2) yielded two peaks that were assigned to the polymeric derivative and free dopamine. Although photolytic cleavage of the azo linkage between the catecholamine and the polymer is possible, no 5- or 6-aminodopamine could be detected. The separation of polymeric catecholamine and free catecholamine by the GPC method allows the concentration of the free species to be reduced to undetectable levels. This is vital for the biological testing. The polymeric catecholamine derivatives proved to be stable to storage at 0 °C in the presence of ascorbic acid.

The cyclic trimeric phosphazene derivatives of dopamine, *dl*-epinephine, and *dl*-norepinephrine proved to be stable to storage at 0 °C in the solid state under vacuum, although oxidation of the catecholamine aryl ring to the quinone structure was found to occur rapidly in solution above 0 °C. The polymeric azocatecholamine derivatives, on the other hand, appeared to be less prone to oxidation of the catecholamine aryl ring.

Biological Activity. In each of two experiments, the release of prolactin from pituitary cells in culture for the first 24-h period was relatively unaffected by the presence of poly(diphenoxyphosphazene), a control polymer used to eliminate the possibility that the phenoxy residues of any of the polymers tested may be responsible for the elicited activity in the culture dish (Figure 3). The amount of hormone released from cells cultured in the presence of poly(diphenoxyphosphazene) was 80–97% of that released from cells cultured on glass. This result demonstrates that poly(diphenoxyphosphazene) by itself does not inactivate the prolactin-producing cells. Although hormone levels tended to be lower in this group during the second culture interval (48 h), the presence of active prolactin-secreting cells in the poly(diphenoxyphosphazene)-coated wells was documented.

In marked contrast, secretion of prolactin from cells in the presence of poly(dopamine-azo-phenoxyphosphazene) (17) was virtually eliminated during both culture periods (Figure 3). Although the total amounts of hormone released were different in the two experiments, the relative differences between treatment groups were maintained. These results show that the dopamine retained its biological activity in the polymer-bound form. In addition to providing experimental evidence for the biological activity of the poly(dopamine-azo-phenoxyphosphazene) (17), this result suggests the possibility that dopamine-bound polymer might prove useful for affinity separation of the prolactin-producing cells.

Following the biological testing, the catecholamine-bound polymer was retrieved from the coated wells (culture dishes) and was reanalyzed for its elemental composition. The retrieved polymer was subjected to gel permeation chromatography to remove any contamination by cellular components and to purify the poly(dopamine-azo-phenoxyphosphazene) (17). Microanalysis revealed an elemental composition virtually identical with that of the polymer used before the biological testing. This was done to eliminate the possibility that the biological activity was elicited by cellular cleavage of the dopamine from the polymer support. In addition, it is interesting to note that the polymer was apparently stable to horse serum at 37 °C for a 48-h period.

Of course, the possibility exists that the observed effect of inhibition to prolactin release may be due to undetectable levels of free dopamine in the assay media, either due to photolysis of the azo linkage or from unbound dopamine being extracted from the polymeric matrix. Therefore, a series of controls were carried out in which poly(dopamine-azo-phenoxyphosphazene) (17) was exposed to the medium (αMEM containing 20% horse serum) without the cells present. After 24 h, the serum was removed from the wells containing 17, and this medium was placed in glass vials and used to culture cells. The

assays of this study revealed the presence of prolactin at 74.7% compared to those cultured directly on glass. This suggests that the cells must be in contact with the poly(dopamine-azo-phenoxyphosphazene) (17) in order to elicit inhibition to prolactin release. The remote possibility does exist that the biological activity may be due to other factors. Nevertheless, we feel that these controls suggest that the biological activity is due to polymer-bound dopamine.

Experimental Section

A. Syntheses. Materials. Hexachlorocyclotriphosphazene (2), kindly provided by Firestone Tire and Rubber Co., was purified by sublimation at 50 °C (0.1 torr) for 24 h, recrystallized twice from hot hexane, and resublimed twice. Samples for polymerization (≃200 g) were sealed in evacuated Pyrex tubes. Phenol and 4-nitrophenol (Aldrich) were sublimed once before use. 3-Hydroxytyramine hydrobromide (Aldrich) was used as received. *dl*-Epinephrine and *dl*-norepinephrine (Sigma) were used as received. Tetrahydrofuran (THF) and dioxane (Baker) were boiled at reflux over benzophenone–sodium ketal before use. Platinum dioxide and sodium hydride (60% active, Aldrich) were used as received.

Analytical Equipment. Gel permeation chromatography (GPC) analysis was carried out with a Waters Associates ALC-201 instrument. A 132 cm × 1 cm 10^5-Å Styragel column was used with a THF solvent flow rate of 2.4 mL/min. Samples were injected at a concentration of 0.5 wt vol %. A refractive index detector was used. Approximate calibration of the columns was accomplished by means of narrow molecular weight polystyrene standards obtained from Waters Associates, together with the use of molecular weight data for other polyphosphazenes.

Synthesis of $N_3P_3Cl(OPh)_5$ (3). Phenol (13.5 g, 0.144 mol) was dissolved in THF (75 mL), and this solution was added to sodium hydride (14 g, 0.35 mol, used as an oil dispersion) suspended in THF (75 mL). When the reaction was complete, additional THF (200 mL) was added, and the reaction mixture was heated to 50 °C. The hot solution was filtered, and the warm filtrate was added dropwise to a cold (0 °C) solution of $(NPCl_2)_3$ (10 g, 0.028 mol) in THF (100 mL). The reaction mixture was stirred for 12 h at 25 °C. The solvent was removed by means of a rotary evaporator. Toluene (50 mL) was added to the residue, the solution was filtered, and the product was purified by means of high-pressure liquid column chromatography. Recrystallization from heptane yielded $N_3P_3Cl(OC_6H_5)_5$ (mp 68 °C). Mass spectrometric measurements: *m/e* 635 calcd, 635 found. A ^{31}P NMR spectrum of the product consisted of an AB_2 pattern (υ_A = 22.1 ppm, υ_B = 6.90 ppm, J = 83.1 Hz). Yield: 6.35 g, 35%.

Synthesis of $N_3P_3(OC_6H_5)_5(OC_6H_4NO_2$-*p*) (4). 4-Nitrophenol (11.0 g, 0.078 mol) was dissolved in dioxane (50 mL), and this solution was added to sodium hydride (3.15 g, 0.078 mol, used as an oil dispersion) suspended in dioxane (50 mL). When the reaction was complete, the reaction mixture was heated to 50 °C, and $(n\text{-Bu})_4NBr$ (1 g) was added to the reaction mixture as a rate accelerator. The hot solution was added dropwise to a solution of chloropentaphenoxycyclotriphosphazene (3) (25 g, 0.039 mol). During this time the solution changed from bright orange to deep brown. The mixture was then cooled and filtered via Schlenck techniques, and the filtrate was concentrated to a volume of 40 mL and added slowly to hexane (300 mL) to yield a precipitate of 4 (18 g, 62%). The white solid was recrystallized twice from ethanol. A ^{31}P NMR spectrum of the product consisted of a singlet at 9.57 ppm (mp 79 °C). Mass spectrometric measurements: *m/e* 738 calcd, 738 found.

Synthesis of $N_3P_3(OC_6H_5)_5(OC_6H_4NH_2$-*p*) (5). A 1000-mL stirrer-equipped autoclave was charged with 40.0 g (0.054 mol) of (nitrophenoxy)pentaphenoxycyclotriphosphazene (4), THF (75 mL), and 0.1 g of PtO_2 catalyst. The mixture was agitated vigorously under 50 psi of hydrogen pressure at 50 °C until no further pressure drop was recorded (≃4 h). The reaction mixture was then filtered to remove catalyst. The THF solvent was removed under reduced pressure to yield an oil, which solidified on standing. Crude 5 was recrystallized twice from ethanol to yield 34 g (88%) of pure 5, mp 104 °C. A ^{31}P NMR spectrum in THF showed a singlet at 10.31 ppm, which indicated complete conversion of NO_2 to NH_2 groups.

Synthesis of Compound 9. All the diazotizations were carried out in essentially an identical manner, and only one representative description of the method will be given. (Aminophenoxy)pentaphenoxycyclotriphosphazene (5) (2 g, 0.002 mol) was dissolved in a THF–water solution (35 mL) containing 1.5 mL of 10 N HCl. The clear solution was cooled to 0 °C in an ice bath. To this solution was added dropwise $NaNO_2$ (0.40 g, 0.005 mol) dissolved in water (10 mL) at 0 °C, and the mixture was allowed to stand at 0 °C under vacuum for 20 min in the dark. A cold solution of *dl*-epinephrine (2.05 g, 0.011 mol) in 0.25 M sodium phosphate buffer, pH 7.0, was added, the solution was titrated to pH 7.0, and the coupling reaction was allowed to proceed overnight in the dark at 0 °C. The products were separated from uncoupled *dl*-epinephrine by column chromatography in the absence of direct light. The products were dried under vacuum and were stored in the dark at 0 °C. Characterization data are included in Table I.

Synthesis of Poly(dichlorophosphazene) (11). Polymerization of $(NPCl_2)_3$ (2) was carried out in degassed, sealed glass tubes, each containing about 200 g of purified 2. The sealed tubes were heated in a Freas Model 104 thermoregulated oven at 250 °C for 120 h. The tubes were agitated during polymerization so that a rocking motion was completed about once each minute. After completion of the polymerization, residual cyclic trimer was removed by vacuum sublimation at 55 °C (0.1 torr). To reduce differences in the poly(dichlorophosphazene) samples used in the various substitution reactions, a number of polymerization tubes were filled, evacuated, and sealed at the same time with the use of trimer from one uniform batch. These tubes were stored in the dark and were polymerized as needed with the use of the same time and temperature.

Synthesis of $[NP(OC_6H_5)_{1.70}(OC_6H_4NO_2)_{0.30}]_n$ (13). The sodium salt of phenol was prepared with the use of sodium hydride (used as an oil dispersion) in dioxane solution, initially under a nitrogen atmosphere, for 1 h. A solution of sodium phenoxide (50.04 g, 0.431 mol) in dioxane (600 mL) was added via an addition funnel with a positive nitrogen pressure into a reaction vessel that contained a stirred solution of poly(dichlorophosphazene) (12) (29.5 g, 0.25 mol) in dioxane (1100 mL) under a dry nitrogen atmosphere. The addition took place over a 2-h period. The mixture was stirred for 96 h at reflux temperature, and a solution of sodium 4-nitrophenoxide (49.42 g, 0.30 mol) in dioxane (300 mL) and $(n\text{-Bu})_4NBr$ (15 g) was then added over a period of 2 h. This mixture was stirred for an additional 168 h at reflux temperature. The polymer was recovered by removal of the solvent at reduced pressure and by precipitation twice into water (5000 mL). The polymer was purified by reprecipitation twice from THF into water, once into ethanol, and twice into heptane. Yield: 60.0 g (95%). Infrared analysis confirmed the presence of a broad –P=N– "stretching" band centered near 1200 cm^{-1}.

Synthesis of $[NP(OC_6H_5)_{1.70}(OC_6H_4NH_2)_{0.30}]_n$ (14). A 1000-mL autoclave was charged with 25 g of 13, THF (500 mL), and 0.1 g of PtO_2 catalyst. The mixture was agitated vigorously and was hydrogenated under 50 psi of hydrogen pressure at 50 °C until no further pressure drop was recorded (≃25 h). The reaction mixture was filtered, and the filtrate was added dropwise to ethanol (2000 mL). Impure 14 precipitated from solution. After filtration, the crude 14 was reprecipitated twice from THF into water (2000 mL) and once each from THF into ethanol (1000 mL) and heptane (1000 mL). The polymeric material was extracted thoroughly (96 h in Soxhlet apparatus) with ethanol in order to remove any remaining traces of impurities. Yield: 21.7 g (91%) of a white polymeric material. ^{31}P NMR and ^1H NMR spectroscopy suggested a 15% 4-aminophenoxy/85% phenoxy side-group composition, with no evidence of residual 4-nitrophenoxy groups. Infrared analysis confirmed the presence of a –P=N– "stretching" band centered around 1200 cm^{-1}. The amino functionalities were detected from bands centered aroung 3500–3200 cm^{-1}.

Synthesis of Compound 18. All the polymeric diazotization reactions were carried out in an identical manner, and the following procedure is typical: Poly(15% 4-aminophenoxy/85% phenoxy)phosphazene (14) (2 g) was dissolved in THF (45 mL) containing 1.65 mL of 5 N HCl. The clear solution was cooled to 0 °C in an ice bath. To this solution was added dropwise $NaNO_2$ (0.40 g, 0.058 mol) in water (10 mL) at 0 °C. The addition

took place at a rapid rate, but one that kept the reaction temperature below 2 °C at all times and prevented precipitation of the polymer. The reaction mixture was allowed to stand at 0 °C under vacuum for 30 min, with occasional agitation. After 30 min, a cold solution of *dl*-epinephrine (1.0 g, 0.005 mol) in 0.25 M sodium phosphate buffer, pH 7, was added, the solution was titrated to pH 7, and the coupling reaction was allowed to proceed in the dark at 0 °C for 14 h. The solution was filtered cold, and the filtrate was precipitated dropwise into cold ethanol (500 mL). The polymeric solid was collected and extracted exhaustively with cold ethanol for 96 h by means of a Soxhlet apparatus. The polymer was then dried under vacuum. Yield: 1.2 g (60%). All transformations and subsequent handling of the polymer were accomplished in the absence of direct light in order to avoid photolysis of the azo linkages. The polymeric products were purified from low molecular weight material by repeated gel permeation chromatography (Figure 2), using cold THF as eluant. This was a mandatory step before assaying for biological activity. Additional characterization data for this and other products are listed in Table II.

B. Biological Materials and Methods. Preparation of Pituitary Cells. In each of three experiments, six anterior pituitary glands (Fisher, 344 female rats, 50 days old) were dissociated enzymatically into single-cell suspensions.[20] Cell yields ranged from 1.5×10^6 to 1.8×10^6 cells/pituitary, with cell viability greater than 95% as measured by trypan blue exclusion.

Preparation of Polymer-Coated Wells. A series of specially prepared glass tissue culture plates were sterilized with ethylene oxide before use. All transformations following sterilization were performed in a bacteria-free environment in the absence of direct light. Ten wells were coated (by solution-casting methods from THF) with 0.1 g of the control polymer, poly(diphenoxyphosphazene). Residual solvent was removed under vacuum for 48 h before use. An additional ten wells were coated with a film of poly(dopamine-azo-phenoxyphosphazene) (17) (by solution-casting techniques from THF), in which approximately 0.1 g of polymer was present in each well. Residual THF was removed slowly under high vacuum for 48 h to yield evenly coated wells. A series of ten uncoated glass wells were employed as controls.

Pituitary Cell Culture. Cells were maintained at 37 °C in a 95% air/5% CO_2 humidified atmosphere. The culture medium consisted of αMEM containing 20% horse serum and antibiotics. Each well contained 2×10^5 cells in a total volume of 500 μL. Under these conditions, prolactin release is maximal.[21] After 24 h, the medium was decanted (10000*g*, 5 min) to remove debris, and the supernatant fraction was frozen at −20 °C for subsequent prolactin bioassay. Fresh medium was added to the cells, and culture was continued for an additional 24 h.

Prolactin Assay. The bioassay method recently described by Tanaka et al.[22] was used to assess the amount of hormone in the culture medium. This assay is based on the ability of prolactin to stimulate division of NB-2 rat lymphoma cells. The assay sensitivity was 50 pg/tube. Rat prolactin B-1 (NIH, 20 IU/mg) was used as a standard. Each sample was assayed in duplicate at two dilutions; three sample/treatment groups were used (Figure 3). Release of prolactin from 200 000 pituitary cells cultured (1) in glass wells (A), (2) in poly(diphenoxyphosphazene)-coated glass wells (B), and (3) in poly(dopamine-azo-phenoxyphosphazene) (17) coated on glass wells (C) was studied. The data represent average and standard errors of the mean of four replicate culture/treatment groups.

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Registry No. **2**, 940-71-6; **3**, 5032-39-3; **4**, 85251-38-3; **5**, 86549-55-5; **6**, 86562-95-0; **8**, 86549-52-2; **9**, 86549-53-3; **10**, 86549-54-4; $NaOC_6H_5$, 139-02-6; *p*-$NaOC_6H_4NO_2$, 824-78-2; prolactin, 9002-62-4; dopamine, 51-61-6; *dl*-epinephrine, 329-65-7; *dl*-norepinephrine, 138-65-8.

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