# Polynucleotides. XIX.[1] Synthesis of Oligonucleotides by the Use of the *N*-Trityl-*p*-aminophenyl Group, a New Protecting Group for the Terminal Phosphate Residues

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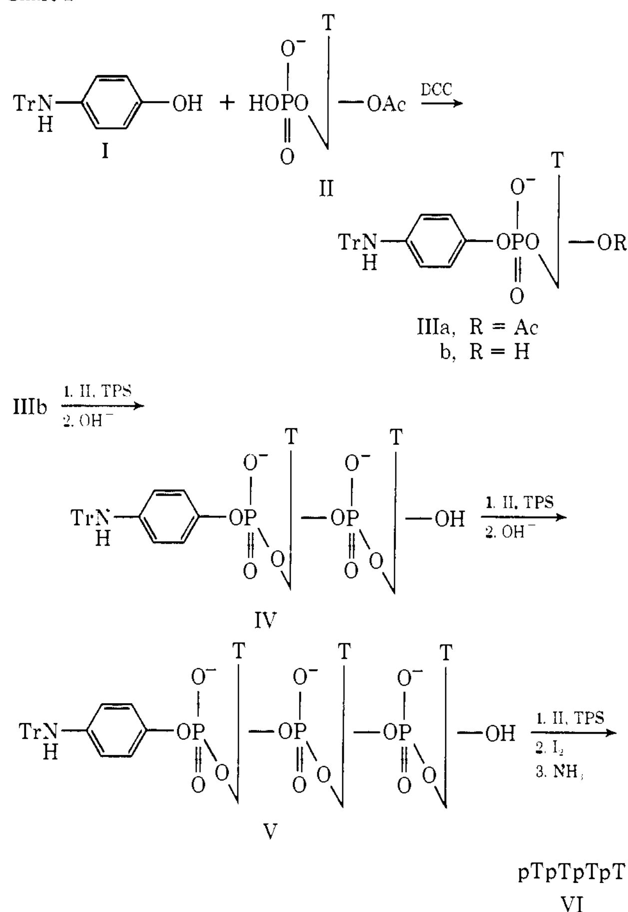
**Abstract:** *N*-Trityl-*p*-aminophenol was used for the protection of phosphomonoester groups. Condensation products derived using this material were isolated by extraction with organic solvents. pTpTpTpT was synthesized from *N*-trityl-*p*-aminophenylthymidine 5′-phosphate as the starting material by the successive condensation with 3′-*O*-acetylthymidine 5′-phosphate using dicyclohexylcarbodiimide or triisopropylbenzenesulfonyl chloride as the activating agent. The tetranucleotide was finally purified by ion-exchange chromatography. Yields in steps 1–3 were 61, 63, and 45%, respectively.

Several approaches for the protection of phosphomonoesters in the synthesis of oligonucleotides have been reported.[2–5] Aromatic phosphoramidates have been shown to be suitable for N- and O-protected ribo-[6] and deoxyribooligonucleotides.[7,8] Use of a lipophilic phosphoramidate facilitated the synthesis of deoxyribodinucleotides.[8] Previously we have synthesized phosphoro-*p*-hydroxyanilidate of nucleosides and have shown that this type of amidate is decomposed by oxidative hydrolysis, as well as by isoamyl nitrite treatment, to yield the corresponding phosphate.[9] It was also demonstrated by Todd and his coworkers that quinol phosphates were easily hydrolyzed by oxidation.[10] *p*-Aminophenyl phosphates were thought to behave similarly, and the amino group could be protected during the condensation reaction. *S*-Ethyl phosphorothioates have been used to protect phosphomonoesters. The derivatives were hydrolyzed by oxidation with iodine.[5] The present paper reports a new protecting group, *N*-trityl-*p*-aminophenyl, which is stable in anhydrous pyridine and also resistant to alkaline treatment. The di- and trinucleotides bearing the *N*-trityl-*p*-aminophenyl group were isolated by solvent extraction, and the protecting group was removed by mild oxidative hydrolysis. The resulting tetranucleotide pTpTpTpT[11] was finally purified by ion-exchange chromatography.

*N*-Trityl-*p*-aminophenol (I) was synthesized by treating *p*-aminophenol with trityl chloride in pyridine. As shown in Chart I pyridinium 3′-*O*-acetylthymidine 5′-phosphate[12] (II) and I were treated with dicyclohexylcarbodiimide (DCC) in pyridine to yield *N*-trityl-*p*-aminophenyl 3′-*O*-acetylthymidine 5′-phosphate (IIIa) in 62% yield. The product IIIa was homogeneous by paper chromatography and paper electrophoresis (Table I). The phenyl ester was inert to isoamyl nitrite under the condition in which phosphoramidates are decomposed[6] and was more stable in anhydrous pyridine than aromatic phosphoramidate.[13] This may be advantageous for condensations in

**Chart I**

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(10) V. M. Clark, D. W. Hatchinson, G. W. Kirby, and A. R. Todd, *J. Chem. Soc.*, 715 (1961).
(11) The system of abbreviations is as suggested by the IUPAC-IUB combined commission: *J. Biol. Chem.*, **241**, 531 (1966). TrNHPh-OpT-OAc refers to *N*-trityl-*p*-aminophenyl 3′-*O*-acetylthymidine 5′-phosphate.
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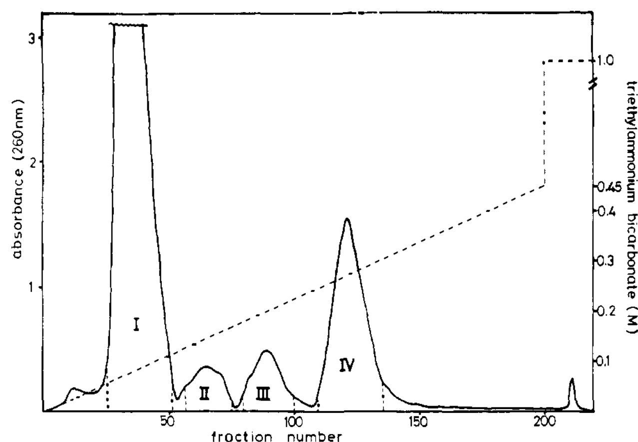


Figure 1. Chromatography of the product obtained in the synthesis of pTpTpTpT. The reaction mixture (16%) was applied to a column (1.7 × 39 cm) of DEAE-cellulose (bicarbonate form). Elution was carried out using a linear gradient of triethylammonium bicarbonate. The mixing chamber contained water (1.5 l.) and the reservoir contained 0.45 *M* triethylammonium bicarbonate (1.5 l.). Fractions of 5 ml were collected every 15 min. Peak I (861 $A_{260}$) II (82 $A_{260}$), III (115 $A_{260}$), and IV (362 $A_{260}$) contained pT, pTpT, pTpTpT, and pTpTpTpT, respectively.

**Table I**

| Compound | Paper chromatography $R_f$ Solvent A | B | C | Paper electrophoresis, relative mobility to pT (pH 7.5) |
|---|---|---|---|---|
| TrNHPhOH | 0.88 | 0.93 | | |
| TrNHPhOpT-OAc | | 0.89 | | 0.30 |
| TrNHPhOpT | 0.75 | 0.86 | | 0.41 |
| TrNHPhOpTpT-OAc | | 0.79 | | 0.66 |
| TrNHPhOpTpT | 0.67 | 0.74 | | |
| TrNHPhOpTpTpT-OAc | | 0.65 | | 0.83 |
| TrNHPnOpTpTpT | 0.58 | 0.61 | | 0.83 |
| TrNHPhOpTpTpTpT-OAc | | 0.50 | | |
| TrNHPhOpTpTpTpT | 0.36 | 0.48 | | |
| $NH_2$PhOpT | 0.45 | 0.58 | | 0.53 |
| $NH_2$PhOpTpT | | 0.49 | | 0.77 |
| pT | 0.13 | 0.27 | 0.47 | 1.00 |
| pTpT | 0.05 | 0.18 | 0.41 | |
| pTpTpT | | 0.12 | 0.33 | |
| pTpTpTpT | | 0.06 | 0.29 | |
| TpT | | 0.50 | 0.62 | 0.41 |
| TpTpT | | 0.35 | 0.50 | 0.68 |
| TpTpTpT | | 0.24 | 0.39 | 0.87 |

pyridine. Conversion of IIIa to II was achieved by oxidation with iodine or bromine in aqueous ammonium acetate (pH 7.5) at room temperature for 3 min. The yield in this process was estimated by measuring total absorption units of the protected dinucleotide TrNH-PhpTpT before and after iodine treatment. The results are summarized in Table II. Pyrophosphate formation was not detected under these conditions. Acid treatment of IIIa gave *p*-aminophenyl 3′-*O*-acetylthymidine 5′-phosphate which could be also converted to II by oxidative hydrolysis.

For the synthesis of oligonucleotides, IIIa was treated with sodium hydroxide at 0° to remove the 3′-*O*-acetyl group[14] and the product was allowed to react with 3′-*O*-acetylthymidine 5′-phosphate (II) using triisopropylbenzenesulfonyl chloride (TPS)[15] as the condensing reagent. The trityl containing mono- and dinucleotides

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**Table II.** Removal of *N*-Trityl-*p*-aminophenol from TrNHPhOpTpT (IV)

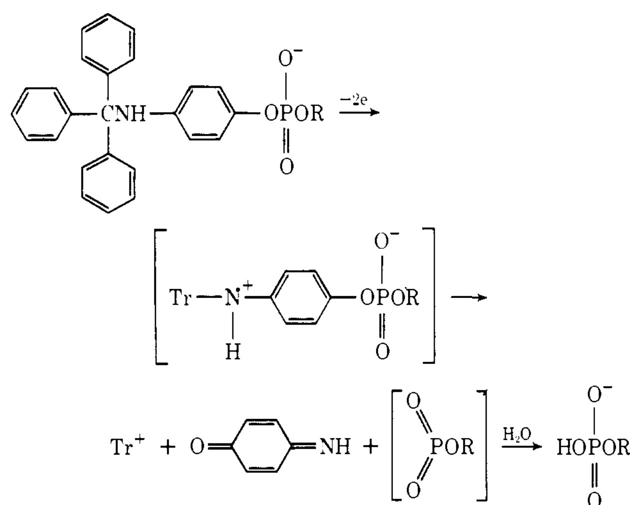
| TrNHPhOpTpT (IV) $A_{260}$ (μmol) | Solvent (ml) | Iodine (μmol) | pTpT[a] $A_{260}$ (μmol) | Yield, % |
|---|---|---|---|---|
| 102 (4.5) | DMF (0.06) | 22 | 72 (4.3) | 97 |
| 127 (5.7) | DMF (0.06) | 55 | 93 (5.6) | 98 |
| 107 (4.8) | Acetone (0.25) | 93 | 80 (4.8) | 100 |

[a] The amount of the unprotected product, pTpT, was estimated by eluting a corresponding spot with water (10 ml) from paper chromatogram (solvent A) after treatment of the protected dinucleotide (IV) with iodine at 23° for 10 hr in 1 *M* ammonium acetate (pH 7.0) (0.11 ml) together with solvent as listed.

were extracted with methylene chloride–1-butanol (7:3), and the mixture was treated as above to remove the 3′-*O*-acetyl group. The mononucleotide IIIb was removed by extraction with chloroform from the aqueous solution of the mixture. The yield of the dinucleotide (IV) was 63%. The trinucleotide (V) was synthesized similarly using IV, II, TPS, and tri-*n*-butylamine in pyridine in a yield of 34%. The partially protected trinucleotide (V) was allowed to react with II using TPS in pyridine to yield the tetranucleotide. The *N*-trityl-*p*-aminophenyl group was finally removed by iodine treatment in a mixture of DMF and 1 *M* ammonium acetate (pH 7.5) for 2 hr at room temperature. The acetyl group was removed with methanolic ammonia and the unprotected tetranucleotide (VI) was purified by ion-exchange chromatography on DEAE-cellulose. The elution pattern of an aliquot of the reaction mixture is shown in Figure 1. Peak III and IV contained the tri- and tetranucleotide, respectively, which was homogeneous in paper chromatography and paper electrophoresis. The tetra- and trinucleotides were characterized by hydrolysis with bacterial alkaline phosphatase and spleen phosphodiesterase to give the correct ratio of Tp to T. When a main portion of the reaction mixture was subjected to ion-exchange chromatography, the tetranucleotide was eluted in two peaks. Rechromatography of the combined compound from the two peaks gave a single peak and the material in the peak was characterized as above. The yield of the tetranucleotide was 45%. Thus the oligonucleotides having a phosphomonoester end group were synthesized using *N*-trityl-*p*-aminophenyl protection which seemed to be suitable for condensation in pyridine. The tritylaminophenyl ester was stable in anhydrous pyridine at room temperature and in 1 *N* sodium hydroxide at 0°. Separation of the products could be achieved up to the protected trinucleotide. When the chain length of the product increases in the stepwise condensation of mononucleotides, the difference in solubility between the product and the starting material becomes smaller. Since heterologous oligonucleotides have variable solubility according to their structure and protecting groups, conditions for extraction of oligonucleotides have to be determined in each case. The removal of the *N*-trityl-*p*-aminophenyl or *p*-aminophenyl group was achieved by mild oxidative hydrolysis. The reaction mechanism of this hydrolysis might be as shown in Chart II. Similarly benzyl-*p*-aminophenyl phosphates were hydrolyzed either by acidic conditions or by oxidation.[16] Since benzyl-*p*-

(16) Unpublished experiments, E. Ohtsuka, S. Morioka, and M. Ikehara.

Chart II



hydroxyphenyl phosphates or *p*-methoxyphenyl phosphates as well as *p*-methoxyphosphoramidates are not changed by oxidative hydrolysis, the lone pair of the para amino group might be removed in the first step of this hydrolysis. The yield in this reaction was quantitative even with a large excess of iodine (Table II). Oxidation in aqueous DMF with excess bromine caused disappearance of uv absorption due to thymidine. The pyrophosphate of the tetranucleotide was not detected by paper chromatography after treatment of the reaction mixture with iodine in aqueous DMF. Oxidation of *N*-trityl-*p*-aminophenyl or *p*-aminophenyl phosphates in anhydrous solution would cause other nucleophilic attacks on phosphorus as found in the case of the oxidative activations of *p*-hydroxyphenylphosphoramidates.[9,10] One possible advantage of the *N*-trityl-*p*-aminophenyl group as a protecting group for a terminal phosphate may be to join by oxidation an oligonucleotide having a 5′-*p*-aminophenyl phosphate aligned next to an oligonucleotide having a 3′-hydroxyl group in favorable position. Experiments along these lines are being attempted.

## Experimental Section

**General Methods.** Paper chromatography was performed by the descending technique. Solvents used were: solvent A, 2-propanol–concentrated ammonia–$H_2O$ (7:1:2, v/v); solvent B, ethanol–1 *M* ammonia acetate, pH 7.5 (7:3, v/v); solvent C, 1-propanol–concentrated ammonia–water (55:10:35, v/v). Paper electrophoresis was performed at 850 V/40 cm in 0.05 *M* triethylammonium bicarbonate, pH 7.5. Bacterial alkaline phosphatase and spleen phosphodiesterase were purchased from Worshington Biochemical Corp. For hydrolysis of oligonucleotides (*ca.* 5 $A_{260}$) bacterial alkaline phosphatase (10 $\mu$g) was incubated in 0.2 *M* ammonium bicarbonate (50 $\mu$l) at 37° for 4 hr and spleen phosphodiesterase (0.4 unit) was incubated in 0.2 *M* ammonium acetate, pH 5.7 (100 $\mu$l) at 37° for 4 hr. Venom phosphodiesterase (20 $\mu$g) was used in 0.2 *M* ammonium carbonate (100 $\mu$l) at 37° for 4 hr. The $\epsilon$ values at 260 nm of oligonucleotides were assumed as follows using values for thymidine oligonucleotides:[17] pT, 8.4 × $10^3$; TrNHPhpT, 14.3 × $10^3$; TrNHPhpTpT, 22.5 × $10^3$; TrNHPh-pTpTpT, 30.5 × $10^3$; pTpTpTpT, 31.6 × $10^3$. Other general methods were as described previously.[18]

***N*-Trityl-*p*-aminophenol.** *p*-Aminophenol (0.549 g, 5 mmol) was azeotropically dried with pyridine and dissolved in anhydrous pyridine (20 ml). Trityl chloride (5 mmol) was added to the pyridine solution in an ice bath and the mixture was stirred at 18° for 2 hr. Water (10 ml) was added under cooling and the product was extracted with chloroform (20 ml). The chloroform was washed with water and evaporated. The residue was taken up in pyridine and the solution was evaporated *in vacuo;* the residue was dissolved in cyclohexane (150 ml). The solution decanted from a small amount of undissolved tar was kept at 7° overnight and the precipitate was collected by filtration. The yield was 1.54 g, 66%, mp 151–153°. The $R_f$ value in tlc on silica gel in chloroform:ethanol (19:1) was 0.50. The product was homogeneous as detected using 30% sulfuric acid, $\epsilon_{260}$ 5.9 × $10^3$. For analysis the product was acetylated with acetic anhydride in pyridine, mp 158–160°. *Anal.* Calcd for $C_{26}H_{23}N_1O_1$: C, 82.41; H, 5.89; N, 3.56. Found: C, 82.43; H, 5.91; N, 3.47.

***N*-Trityl-*p*-aminophenyl 3′-*O*-Acetylthymidine 5′-Phosphate (IIIa).** Pyridinium 3′-*O*-acetylthymidine 5′-phosphate (1.4 mmol) and *N*-trityl-*p*-aminophenol (3 mmol) were treated with DCC (5 mmol) in pyridine (3.5 ml) for 48 hr at room temperature. Water (3.5 ml) was added and the mixture was extracted with *n*-pentane. After 16 hr the aqueous pyridine was filtered and *N*-trityl-*p*-aminophenyl 3′-*O*-acetylthymidine 5′-phosphate was extracted with chloroform. The product was precipitated with ether–pentane (3:2) from its solution in anhydrous pyridine. The yield was 12.4 × $10^3$ $A_{260}$, 0.87 mmol, 62%. The *N*-trityl-*p*-aminophenyl group was stable at least for 140 hr in anhydrous pyridine (0.1 *M* solution) at room temperature.

***N*-Trityl-*p*-aminophenylphosphorylthymidylyl-(3′–5′)-thymidine (IV).** The pyridinium salt of IIIa (8490 $A_{260}$, 0.59 mmol) was dissolved in pyridine (15 ml), and 2 *N* sodium hydroxide (38 ml) was added in an ice bath. After 5 min Dowex 50-X2 (120 ml) (pyridinium form) was added and the filtrate was evaporated with pyridine. The pyridinium salt of IIIb was precipitated with 3:2 ether–pentane from its solution in anhydrous pyridine and condensed with pyridinium pT-OAc (1.39 mmol) in pyridine (10 ml) and tri-*n*-butylamine (3.46 mmol) using TPS (4.09 mmol) for 3 hr at room temperature. Water (10 ml) and tri-*n*-butylamine (4.09 mmol) were added under cooling. After 16 hr the solution was concentrated, the residue was dissolved in 0.2 *M* triethylammonium bicarbonate (30 ml), and the solution extracted with 7:3 methylene chloride–1-butanol (30 ml) in four portions. The organic layer was evaporated to small volume and treated with pyridine (5 ml). The solution was treated with 2 *N* NaOH (15 ml) in an ice bath for 10 min. Sodium ions were removed with an excess Dowex 50-X2 (pyridinium form) resin, and the solution was passed through a small column of the same resin. The effluent and washings were evaporated with pyridine. The residue was partitioned between 0.2 *M* triethylammonium bicarbonate (30 ml) and chloroform (5 ml). The organic layer was washed with the same buffer (10 ml), and the combined aqueous layers were evaporated with pyridine. The dinucleotide was precipitated with 3:2 ether–*n*-pentane from its solution in anhydrous pyridine. The yield was 8260 $A_{260}$ units, 0.36 mmol (61%).

***N*-Trityl-*p*-aminophenylphosphorylthymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine (V).** The triethylammonium salt of IV (0.35 mmol) and pyridinium pT-OAc(1.82 mmol) were treated with TPS (5.76 mmol) in pyridine (5.0 ml) and tri-*n*-butylamine (3.6 mmol) for 4 hr at room temperature. Aqueous pyridine (50%, 5 ml) and tri-*n*-butylamine (5.76 mmol) were added with cooling. The mixture was kept overnight and evaporated with pyridine. The residue was treated with 0.2 *M* triethylammonium bicarbonate (30 ml) and the solution was extracted with 1:1 ethyl acetate–chloroform (10 ml) in three portions. The organic layer contained the dinucleotide and a small amount of the trinucleotide. The aqueous phase was then extracted with 1:1 1-butanol–methylene chloride (20 ml) in three portions. Butanol and methylene chloride were evaporated and the trinucleotide was treated with 2 *N* sodium hydroxide (20 ml) as described for the dinucleotide. The precipitated product (V) was 3421 $A_{260}$, 0.12 mmol. The yield was 34%. About 3 $A_{260}$ units of V was hydrolyzed with venom phosphodiesterase to give thymidine 5′-phosphate and *N*-trityl-*p*-aminophenol as detected by paper electrophoresis.

**The Tetranucleotide pTpTpTpT (VI).** The triethylammonium salt of the trinucleotide (V) (0.11 mmol) was allowed to react with pyridinium pT-OAc (0.51 mmol) using TPS (1.58 mmol) in pyridine (1.5 ml) and tri-*n*-butylamine (2.1 mmol) for 3 hr at room temperature with stirring. Aqueous pyridine (50%, 6 ml) and tri-*n*-butylamine (0.40 ml) were added with cooling. After 4 hr the mixture was concentrated and treated with iodine (1 mmol) in DMF (1 ml) and 1 *M* ammonium acetate (pH 7.5, 2 ml) for 2 hr. The nucleotides were precipitated with ether from their solution in anhydrous pyridine. The precipitate was dissolved in 50% pyridine and passed through a column of Dowex 50X2 (pyridinium form).

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The mixture was reprecipitated by the same technique and the precipitate was treated with 15 $N$ methanolic ammonia. The unprotected nucleotides (16%) were subjected to DEAE–cellulose column chromatography as shown in Figure 1. Aliquots of materials in peak II, III, and IV were treated with bacterial alkaline phosphatase. Dephosphorylated oligonucleotides were digested with spleen phosphodiesterase. The ratio of thymidine to Tp were: peak II, 1.00:0.93; peak III, 1.00:1.94; peak IV, 1.00:2.98. A main portion of the reaction mixture (84%) was subjected to chromatography on a column (1.7 × 65 cm) of DEAE–cellulose (bicarbonate form) using a linear gradient of triethylammonium bicarbonate (0–0.45 $M$). The total volume was 3 l. The tetranucleotide was eluted in two peaks, 871 $A_{260}$ and 409 $A_{260}$. Materials from these two peaks were identical, and the combined material was rechromatographed to give a symmetrical single peak using an identical condition.

# Analysis of a Specific Photoreaction in Oligo- and Polydeoxyadenylic Acids

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**Abstract:** In contrast to oligo- and polyriboadenylic acids, which are relatively stable against uv irradiation, oligo- and polydeoxyadenylic acids were found to be rather sensitive to uv irradiation at neutral pH. A specific photoreaction was detected in deoxyadenylic acid chains with chain lengths $n \geq 2$. The photoproduct formation was recorded both by changes in the uv spectrum and in the CD spectrum. Solutions flushed with nitrogen showed the same reaction as solutions that had not been deaerated. The major photoproduct resulting from irradiation of $d(pA)_2$ was isolated by DEAE chromatography. The uv absorption of the $d(pA)_2$ photoproduct, denoted $d(pA)_2^*$, shows a maximum extinction coefficient of $10.0 \times 10^3$ per mole of phosphorus ($\lambda_{max}$ 264 nm), which is only slightly lower than that of $d(pA)_2$, indicating the existence of an aromatic system in the photoproduct. $d(pA)_2^*$ shows a characteristic CD spectrum, which remains almost unchanged in 100% ethanol solution, indicating a rather stable asymmetric structure, which is possibly maintained by a covalent bridging bond. $d(pA)_2^*$ is characterized by a p$K$ of 7.0. One phosphate group of $d(pA)_2^*$ can be removed by the action of bacterial alkaline phosphatase. Phosphodiesterase from snake venom apparently does not have any effect on $d(pA)_2^*$. The quantum efficiency of the $d(pA)_2$ degradation by irradiation at 248 nm is around $\phi = 10^{-3}$. The quantum efficiency increases with increasing deoxyadenylic acid chain length $n$: $\phi(n = 3) = 1.7 \times 10^{-3}$, $\phi(n = 4) = 2.1 \times 10^{-3}$, $\phi(n = \infty) = 2.5 \times 10^{-3}$. The quantum yield of the $d(pA)_2$ degradation shows a relatively small dependence upon the wavelength of the irradiated light in the range of 240–280 nm. Irradiation experiments performed under different solvent conditions demonstrate that the photoreaction is solvent dependent. The base-pairing ability of irradiated deoxyadenylic acid chains to the complementary poly(U) is inhibited.

The radiation sensitivity of living organisms has been attributed mainly to radiation induced damages in the carriers of the genetic information, *i.e.*, the nucleic acids.[1–5] In the attempt to understand the radiation damages, a lot of activity has been devoted to the radiation biology and chemistry of the nucleic acids. Most of the investigations have been concerned with photoreactions of the pyrimidine bases (uracil, thymine, and cytidine) since these bases have proved to be rather sensitive to radiation. In contrast to the pyrimidine bases the purine bases have been considered to be stable against radiation, since rather high radiation doses are required to bring about any chemical change in adenine or guanine.

In a recent investigation[6] it has been demonstrated that the usually high photoresistance of adenine bases may be reduced considerably, when the adenine residues are incorporated into a specific polymer structure. Whereas the adenine bases are rather photoresistant in poly(A), a specific photoproduct is formed in poly(dA) with quantum yields of $2.5 \times 10^{-3}$ mol/einstein. This result demonstrated that photoreactions in purine bases cannot be neglected in the interpretation of photolesions in living cells. The present investigation is concerned with a characterization of the photoreaction in deoxyadenylic acid chains by using oligonucleotides of defined chain length.

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## Experimental Section

**Materials and Methods.** Poly(dA) was purchased from LP Biochemicals, Inc., Milwaukee, Wis. 53205, and was used without further purification ($s_{20,w} = 7.5$, $\epsilon_{260}$ $10.1 \times 10^3$ $M^{-1}$ cm$^{-1}$). Oligodeoxyriboadenylic acids were prepared and purified as described by Ralph and Khorana.[7] Poly(A) was obtained from Miles Laboratories (Lot No. 74) and ApA was from Zellstoffabrik Waldhof, Mannheim. Concentrations were determined according to the following extinction coefficients (all values in $10^3$ $M^{-1}$ cm$^{-1}$ at 20°): $d(pA)_2$, $\epsilon_{260}$ 12.8; $d(pA)_3$, $\epsilon_{260}$ 11.9; $d(pA)_4$, $\epsilon_{260}$ 11.3; poly(A), $\epsilon_{257}$ 10.0; ApA, $\epsilon_{259}$ 13.7. The extinction coefficient of the photoproduct was calculated from a phosphorus determination according to Eibl and Lands.[8] Alkaline phosphatase (EC 3.1.3.1) and phosphodiesterase (EC 3.1.4.1) were purchased from Boehringer, Mannheim.

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