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Derivatization of unprotected polynucleotides

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ABSTRACT

A simple and efficient method for attaching amines to the terminal 5'-phosphate of unprotected oligonucleotides or nucleic acids in aqueous solution is described. The method is applicable to low molecular-weight amines, polypeptides, or proteins.

The terminal 5'-phosphate of an oligonucleotide or nucleic acid reacts with a water-soluble carbodiimide in imidazole buffer at pH 6 to give good yields of the 5'-phosphorimidazolide. Exposure of the phosphorimidazolide to amine-containing molecules in aqueous solution results in the production of a wide range of stable phosphoramidates in high yield.

The exposure of polynucleotides to carbodiimide does not result in significant breakage of phosphodiester bonds or damage to nucleoside bases. The biological activity of a drug resistant plasmid is not affected.

The direct condensation of polynucleotides with amines in 1-methylimidazole buffer is also possible. However, it is not a satisfactory preparative method if the ligand is sensitive to carbodiimide.

INTRODUCTION

A general procedure for the synthesis of well-defined covalent adducts of a polynucleotide with any one of a wide range of organic molecules would find many applications in biochemistry and DNA technology. Ideally, the reaction should proceed in aqueous solution without requiring protection of the bases and should be applicable to polynucleotides independently of their composition, sequence, or molecular weight.

With this in mind, we have made an introductory study of the condensation of amines with polynucleotides using a water-soluble carbodiimide as the coupling agent. The use of water-soluble carbodiimides as condensing agents in oligo- or polynucleotide chemistry has already received considerable attention. Gilham and his coworkers, for example, have used carbodiimides to attach nucleic acids to paper via their 5'-terminal phosphate

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groups (1) and, more recently, to synthesize nucleic acid-sorbose adducts (2). However, the danger of side reactions between the carbodiimide and nucleotide bases or phosphodiester groups has prevented a fuller exploitation of this method.

We find that treatment of nucleotides, oligonucleotides or nucleic acids with a water-soluble carbodiimide in imidazole buffer leads to the rapid and efficient synthesis of the corresponding 5'-phosphorimidazolide. The latter activated derivative can then be isolated and treated with an excess of an amine to obtain a 5'-phosphoramidate. The method is suitable for the attachment of oligonucleotides, DNA or RNA to small molecules such as ethylenediamine, polymers such as polylysine, or proteins. Direct coupling of amines to polynucleotides in 1-methylimidazole (1-MeIm) or 2-morpholinoethanesulfonic acid (MES) buffer is also possible, but is not applicable when the ligand reacts with carbodiimides.

MATERIALS AND METHODS

Reagents and Enzymes

Nucleotides and polynucleotides were obtained from Sigma or from P-L Biochemicals, 1-ethyl-3,3-dimethylaminopropylcarbodiimide (CDI) from Calbiochem, and γ-^{32}P-ATP and ^{14}C-ethylenediamine from Amersham. ^{14}C-nucleotides were purchased from Amersham or Schwarz/Mann and purified by paper chromatography in Solvent A (see below) before use. Non-labelled and ^{14}C-labelled nucleotide 5'-phosphorimidazolides (ImpNu;I) were prepared by a slightly modified version of a procedure described by Mukaiyama and Hashimoto (3) and Lohrmann and Orgel (4). Decathymidylic acid, $(pT)_{10}$, was obtained from Sigma and tetradecadeoxyadenylic acid, $(pdA)_{14}$, from P-L Biochemicals.

Mixtures of oligo(U)'s, oligo(C)'s or oligo(A)'s were prepared from homopolymers as follows. About 1.3 mgs of poly(A) or poly(C) were treated with .03 units P_1 nuclease in 0.2M sodium citrate at pH 6 and 37°C for 15 minutes (5). Poly(U) degradation was carried out similarly, but at pH 4.5. HPLC chromatography on a RPC-5 column indicated that the polynucleotides had been degraded largely to 5'-phosphate terminated oligomers 2-15 in length. Oligo(G)'s were prepared by template-directed synthesis as

previously described (6).

BacF alkaline phosphatase was obtained from Worthington Biochemicals, P_1-nuclease from P-L Biochemicals, polynucleotide kinase from Miles Laboratories. All other reagents and solvents were obtained from commercial sources.

We used two E. coli plasmid DNA's. MUA3 is approximately 5,000 bp long and contains a single Eco R1 cleavage site (7). PMK20 is approximately 3,600 bp long, has a Col El replicon and confers kanamycin and tetracycline resistance on the E. coli in which it replicates (8).

Chromatography and Electrophoresis

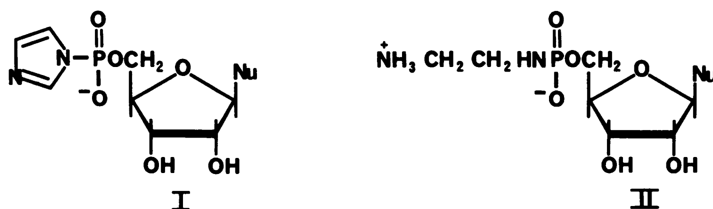
Paper chromatography was performed by the descending method on Whatman No. 3 paper in Solvent A (MeOH:NH_3:H_2O 7:1:2) or Solvent B (n-PrOH:NH_3:H_2O 55:10:35). Paper electrophoresis was performed at pH 7.1 in 0.02M phosphate buffer. Radioactive spots were located and estimated using a Nuclear Chicago strip scanner with integrator. HPLC of oligonucleotides and their derivatives was performed on RPC-5 at pH 8 or 12, using a perchlorate gradient as previously described (9). Gel electrophoresis of DNA was performed on 0.7% agarose, pH 7.4 at 7 V/cm and the DNA was quantitated by staining with ethidium bromide.

Reactions of Nucleotides

Formation of phosphorimidazolides A 0.001M solution of a ^{14}C-labelled nucleoside-5'-phosphate (pNu) (0.4 μCi/μmole) was reacted with 0.1M CDI in 0.1M imidazole buffer at pH 6. After 15, 30, and 60 minutes, aliquots were co-chromatographed with authentic samples of the corresponding nucleoside-5'-phosphorimidazolides(I) in Solvent A. The papers were then run through a Nuclear Chicago strip scanner with integrator. The identification of the phosphorimidazolides was confirmed by co-chromatography against authentic markers in Solvent B and by co-electrophoresis at pH 7.1 in 0.02M phosphate buffer (See Table 1 for chromatographic and electrophoretic mobilities). Furthermore, samples of the phosphorimidazolides hydrolyzed cleanly to the corresponding nucleotides in three hours at pH 2 and 37°C, and gave a positive reaction in the Ehrlich diazo-test (10).

Displacement reactions between phosphorimidazolides and nucleophiles

(a) ethylenediamine - A 0.0025M solution of 2-^{14}C-ImpU (2 μCi/μmole)



Nu = U, C, A or G

was reacted with 0.1 or 0.25M ethylenediamine (EDA) in 0.25M 2-6-lutidine buffer at pH 7.5 for 2, 4, 7 or 24 hours. After these time periods, aliquots were chromatographed in Solvent A and the chromatograms run through a strip scanner with integrator. The product(II) was then eluted and divided into 2 aliquots. One aliquot was chromatographed in Solvent B; only one radioactive spot with R_f=0.50 was detectable. The other aliquot was run in HVE at pH 7.1;the only radioactive spot was close to the origin of the chromatogram, indicating an uncharged molecule. The product gave a positive

Table 1. Chromatographic and Electrophoretic Mobilities

	Solv. A.	Solv. B	HVE pH 7.1* (mobility relative to 5'-nucleotide)
pA	0.12	0.35	1.0
ImpA	0.51	0.63	0.44
pC	0.09	0.36	1.0
ImpC	0.49	0.65	0.48
EDA-pC	0.19	0.45	-0.08
pG	0.09	0.28	1.0
ImpG	0.36	0.53	0.42
pU	0.12	0.40	1.0
ImpU	0.46	0.64	0.52
EDA-pU	0.20	0.47	-0.12

*Negative values indicate that the compound runs toward the cathode.

reaction with ninhydrin, hydrolyzed to pU after 4 hours at pH 2 and 37°C, and was resistant to alkaline phosphatase.

The corresponding derivative of pA was prepared in the same manner. It was isolated by elution from paper with water, followed by lyophilization. The product was then purified by precipitation from dry pyridine with dry ether. The NMR spectrum in D_2O taken on a Varian EM-390 90 MHZ spectrometer showed the characteristic proton resonances of adenine H-2 and H-8 at 8. 1 and 8. 4 ppm and the resonances at 6. 0 and 4. 4-3. 9 ppm characteristic of ribose H-1 and H-3, H-4 and H-5. In addition a multiplet integrating to 4 protons at 2. 9-2. 7 ppm was observed. This position is characteristic of the ethylene protons of ethylenediamine (11, 12), and of the α-CH's of phosphoramidates (13).

The composition of the product was established by condensing ImpA with ^{14}C-ethylenediamine. The observed specific activity correspond to .92 moles of ethylene diamine for each mole of pA. Hydrolysis studies at 50°C indicated that the compound was stable at pH's above 7. At pH 6 and pH 5, 8% and 22%, respectively, was hydrolyzed to pA after 24 hours.

(b) poly-L-lysine (PLL) - A 0. 0025M solution of 2-^{14}C-ImpU (2μCi/μmole) was reacted with 0. 1, 0. 5, 1. 5M PLL (concentration of lysine residues) of molecular weight approximately 3,000 in 0. 25M lutidine buffer at pH 7. 5. After 4, 7 or 24 hours, aliquots were analyzed by paper electrophoresis in 0. 02M phosphate buffer at pH 7. 1. The ^{14}C-pU-PLL complexes migrated toward the cathode. The percentage of ^{14}C-pU attached to PLL was estimated using a strip scanner with integrator.

(c) bovine serum albumin (BSA) - A 0. 0025M solution of 2-^{14}C-ImpU (2 μCi/μmole) was reacted with 0. 005 or 0. 0003M BSA (roughly equivalent to 0. 05M or 0. 003M lysine) in 0. 2M lutidine buffer at pH 7. 5. After 4, 7 and 24 hours, aliquots were removed and dialyzed against 0. 05M lutidine buffer at pH 9 with several changes of dialysate until there was no detectable radioactivity in the dialysate. Then dialysis was continued for 30 minutes against H_2O. The resulting solution was lyophilized.

The total pU attached to the protein at the end of the dialysis was determined by measuring the weight and specific activity of the lyophilized material. The amount of pU attached by labile bonds or non-covalently was

determined by chromatography in Solvent A, in which protein-bound pU remains at the origin and is separated from free pU.

Direct condensation of ^{14}C-pC with ethylenediamine - A 0.001M solution of ^{14}C-pC (0.4 μCi/μmole) was reacted with 0.2M CDI and 0.25M EDA in either 0.1M 1-MeIm or MES buffer at pH 6 for 2, 4 and 24 hours. Aliquots were then analyzed by paper chromatography, as described above.

Reactions of Oligonucleotides

Formation of imidazolides Solutions of $(pT)_{10}$ or $(pdA)_{14}$ (0.05-0.5 μM) were treated with 0.1M CDI in 0.1M imidazole buffer at pH 6 for 15 or 60 minutes. Samples were then analyzed by HPLC on RPC-5 at pH 12 on a perchlorate gradient.

Mixtures of oligo(U)'s, oligo(C)'s, oligo(A)'s or oligo(G)'s in the size range 2-15 at a concentration of 4×10^{-4}M in nucleotide were treated with 0.1M CDI in 0.1M imidazole buffer for 15 and 60 minutes. Samples were analyzed by HPLC on RPC-5, using a perchlorate gradient at pH 8 for oligo(U)'s, oligo(C)'s and oligo(A)'s or pH 12 for oligo(G)'s.

Displacement of imidazole by ethylenediamine The imidazolide of $(pT)_{10}$ was collected from an RPC-5 column run at pH 12, and was then brought to pH 7.8 with perchloric acid, and treated with 0.25M EDA for 3 or 24 hours at room temperature, or 1 or 3 hours at 50°C. The resulting solutions were then chromatographed on RPC-5 at pH 12.

Direct condensation of oligodeoxynucleotides with CDI and ethylenediamine 0.05-0.1 μM solutions of $(pT)_{10}$ were reacted with 0.1M CDI and 0.25M EDA in 0.1M 1-MeIm or MES buffer at pH 6 for 4 and 24 hours. Aliquots of the reaction solution were analyzed by HPLC on RPC-5 as described above.

Addition of CDI to the bases of $(pT)_{10}$ at pH 8.1 In order to study the addition of CDI to the bases of $(pT)_{10}$ under conditions that favor the addition reaction (14), a 0.15 μM solution of $(pT)_{10}$ that had previously been purified by HPLC on an RPC-5 column was reacted with 0.1M CDI in 0.05M lutidine buffer at pH 8.1 and 30°C. After 20 minutes, 2 hours or 3 1/2 hours aliquots were analyzed by HPLC on an RPC-5 column as described.

Reactions of DNA and Effect of CDI on Hybridization and Biologic Activity of DNA

Presumptive formation of 5'-phosphorimidazolide of a high M. W. DNA We prepared a double-stranded DNA fragment (Eco RI cleaved MUA3) about 5,000 base-pairs in length and with protruding 5'-phosphate termini (7). The terminal phosphates were removed with alkaline phosphatase and replaced by ^{32}P-labelled phosphate (3000 Ci/mmole) using polynucleotide kinase. An aliquot of this DNA (2 x 10^6 cpm/ml, 0.26 pmole PO_4^{-3}/ml) together with carrier herring sperm DNA (300 μg/ml) was treated with 0.1M CDI in 0.1M imidazole buffer at pH 6 for one or two hours at room temperature.

The DNA was isolated by ethanol precipitation, dissolved in 0.2M imidazole buffer at pH 8 and treated with bacterial alkaline phosphatase at 37°C for 30 minutes. An aliquot of the enzyme reaction mixture (1-2 x 10^4 cpm) was added to 100 μg carrier DNA (0.1 ml). The number of cpm in this aliquot was determined accurately by counting a 5 μl sample on a GF/C filter in liquifluor in a Beckman scintillation counter. The remainder of the sample was precipitated with 2 ml 10% TCA, filtered on a GF/C filter and counted. The number of cpm found on the filter, as a percentage of the total counts, represents the proportion of DNA whose 5'-$^{32}PO_4$ termini had been protected from phosphatase by conversion to a phosphorimidazolide. An identical experiment using DNA that had not been exposed to CDI served as a control.

Effect of 0.1 or 0.2M CDI on DNA phosphodiester bonds PMK 20 plasmid DNA (3,600 bp; 95% Form I supercoiled, 5% Form II open circular) (8) (25 μg/ml) was treated with 0.1 or 0.2M CDI in 0.1M imidazole buffer at pH 6 for 1 hour at room temperature. Samples (100 ngs) were then analyzed by 0.7% agarose gel electrophoresis at pH 7. The introduction of one single-stranded break converts Form I of the plasmid to Form II; this can be detected by the different mobilities of Form I and Form II on agarose gels during electrophoresis (15). The percentage conversion of Form I to Form II was quantitated after ethidium bromide staining.

Effect of 0.1M CDI on DNA plasmid biological activity The plasmid PMK 20 (8) confers kanamycin resistance on E. coli in which it multiplies. PMK 20 (95% Form I, 5% Form II) at a concentration of 250 μg/ml, was reacted with 0.1M CDI in 0.1M 1-MeIm buffer at pH 6 and 12-14°C for 1 hour, 4

hours, or 19 hours. A control experiment was carried out omitting the CDI. After the indicated times, the plasmid DNA from each experiment was precipitated with EtOH, washed with 70% EtOH and resuspended in 0.01M Tris 0.001M EDTA at pH 7.5. Either 1 or 10 ngs PMK 20 DNA from each reaction tube was then allowed to multiply in kanamycin sensitive E. coli and plated onto kanamycin containing agar plates. After overnight growth at 37°C, the number of kanamycin resistant bacterial colonies on each plate was counted.

Hybridization of MUA3 DNA after treatment with carbodiimide Unlabelled MUA3 DNA was denatured by heating in 0.3M NaOH at 65°C for 1 hour, neutralized by adding 1 volume of 2M NH_4OAc, and filtered onto BA83 nitrocellulose paper through thin slots cut in a plastic manifold. The nitrocellulose bound DNA was baked for 2 hours at 80°C in a vacuum oven and then hybridized with 2 x 10^5 cpm/ml of ^{32}P-labelled MUA3 DNA (which had or had not been treated with CDI). The hybridization conditions were those described previously (16). The hybridized filters were then washed once for 0.5 hour in 0.1 x SSPE*, 0.1% SDS at 42°C and once for 0.5 hour in 0.1 x SSPE*, 0.1% SDS at 65°C. The filters were blotted dry and autoradiographed at -70°C for 4 hours using a Lightning plus intensifying screen.

RESULTS

Reactions of Mononucleotides

Formation of nucleoside 5'-phosphorimidazolides The half-lives for the conversion of nucleotides to their 5'-phosphorimidazolides(I) on treatment with 0.1M CDI in 0.1M imidazole buffer at pH 6 and 25°C were about 30 minutes. The nature of the base had little effect on the rate of the reaction. The yield of product after 1 hour is about 75% for pU, pC, pA and pG. Very similar results were obtained with pT.

Only in the case of pU (and pT) could we detect sideproducts. They amounted to about 3% of the input after a reaction time of 1 hour. The R_f's of these side products in Solvent A were 0.76 and 0.84 for the adducts of pU and pT, respectively. We believe that these compounds may be the phosphorimidazolides of the carbodiimide adducts to the bases of pU and pT

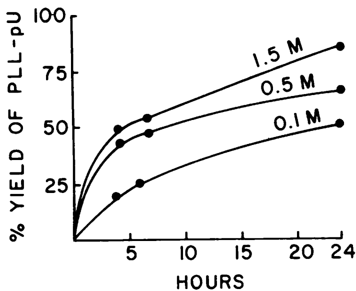


Fig. 1 Reaction of ImpU with Polylysine. The percentage conversion of ImpU to the polylysine adduct of pU when 0.0025M ImpU is treated with 0.1M, 0.5M or 1.5M polylysine (molarity given as equivalent concentration of monomeric lysine) in 0.2M lutidine buffer at pH 7.5 and 25°C.

that have previously been reported (14). We did not investigate them further.

Displacement reactions of ImpU

(a) The reaction of ^{14}C-ImpU with ethylenediamine. The half-life for transamidation at room temperature is 3-4 hours with 0.1M EDA and about 2 hours with 0.2M EDA. The yield of the amine(II) after 24 hours is almost quantitative. Very similar results were obtained with ImpA.

(b) The reaction of ^{14}C-ImpU with polylysine. The yields of adducts obtained after various times in the reaction of ^{14}C-ImpU with polylysine (MW 3000) are illustrated in Fig. 1. The yield of adduct obtained after 24 hours is about 50% with 0.1M (lysine) PLL, and increases with the concentration of PLL. A yield as high as 85% was obtained with a concentrated (1.5M lysine) solution of PLL.

(c) The reaction of ^{14}C-ImpU with serum albumin. The yields of stable BSA-pU adducts obtained after 4 or 7 hours in the reaction of .0025M ^{14}C-ImpU with .005M BSA (roughly .05M in lysine) are 24% and 31%, respectively. After 24 hours at room temperature, the percentage of input pU stably attached to protein was about 50%, corresponding to roughly 1 pU molecule for every 2 BSA molecules. The number of pU molecules bound by each BSA molecule could be increased by increasing the ratio of ImpU to BSA in the reaction mixture. In a control experiment, using ^{14}C-pU in place of ^{14}C-ImpU, no radioactivity was bound to the albumin.

Direct condensation of ^{14}C-pC with ethylenediamine The yields of adduct in the direct condensation of ^{14}C-pC with 0.2M EDA and 0.2M CDI in 1-MeIm or MES buffer at pH 6 are about 85% after 1 day. The reaction is half com-

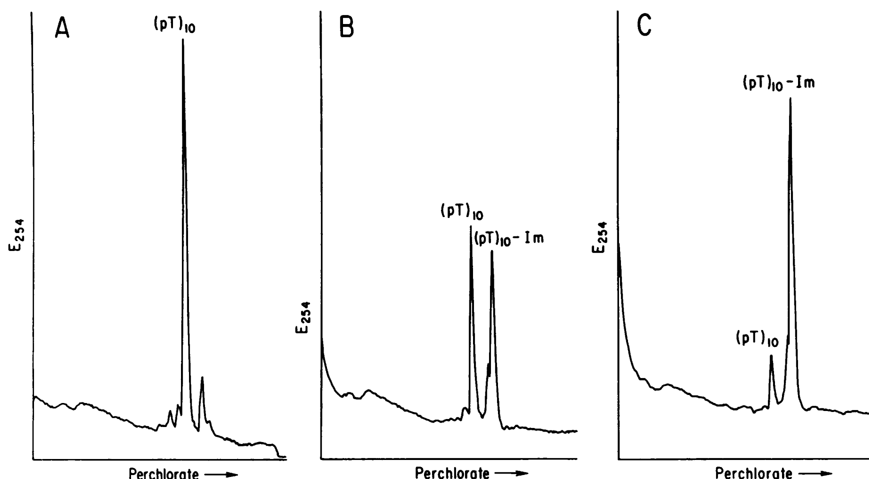


Fig. 2 Conversion of $(pT)_{10}$ to a Phosphorimidazolide $(pT)_{10}$-Im. Elution profiles from an RPC-5 column using a perchlorate gradient at pH 12 of:- (a) starting material, $(pT)_{10}$; (b) products formed after 15 minutes treatment of $(pT)_{10}$ with 0. 1M CDI in 0. 1M imidazole buffer at pH 6 and 25°C; (c) after treatment for 1 hour. The peak with longer retention time than $(pT)_{10}$ in (a) is due to an impurity in the commercial sample. The impurity does not react with CDI and gives rise to a small side peak in (b) and (c).

pleted in about 2 hours. Thus, this reaction is significantly slower than the direct condensation with imidazole.

Reactions of Oligonucleotides

Formation of oligonucleotide 5'-phosphorimidazolides The HPLC elution profiles of $(pT)_{10}$ and of the products from the reaction of $(pT)_{10}$ with 0. 1M CDI in 0. 1M imidazole buffer after 15 and 60 minutes are shown in Fig. 2. The yield of the 5'-phosphorimidazolide after 1 hour is about 85%. In an exactly similar experiment $(pdA)_{14}$ was converted to the 5'-phosphorimidazolide in 85-90% yield after 1 hour (HPLC profile not shown).

A survey of a much wider range of oligomers was achieved by subjecting mixtures of homoligomers in the size range 2-15 to the same treatment. Oligo(U)'s, oligo(C)'s, oligo(A)'s, oligo(G)'s and oligo(T)'s all responded in the same way to treatment with 0. 1M CDI in 0. 1M imidazole buffer, giving at least 70% of 5'-phosphorimidazolides after 1 hour. The conversion of oligo(G)'s to phosphorimidazolides is illustrated in Fig. 3.

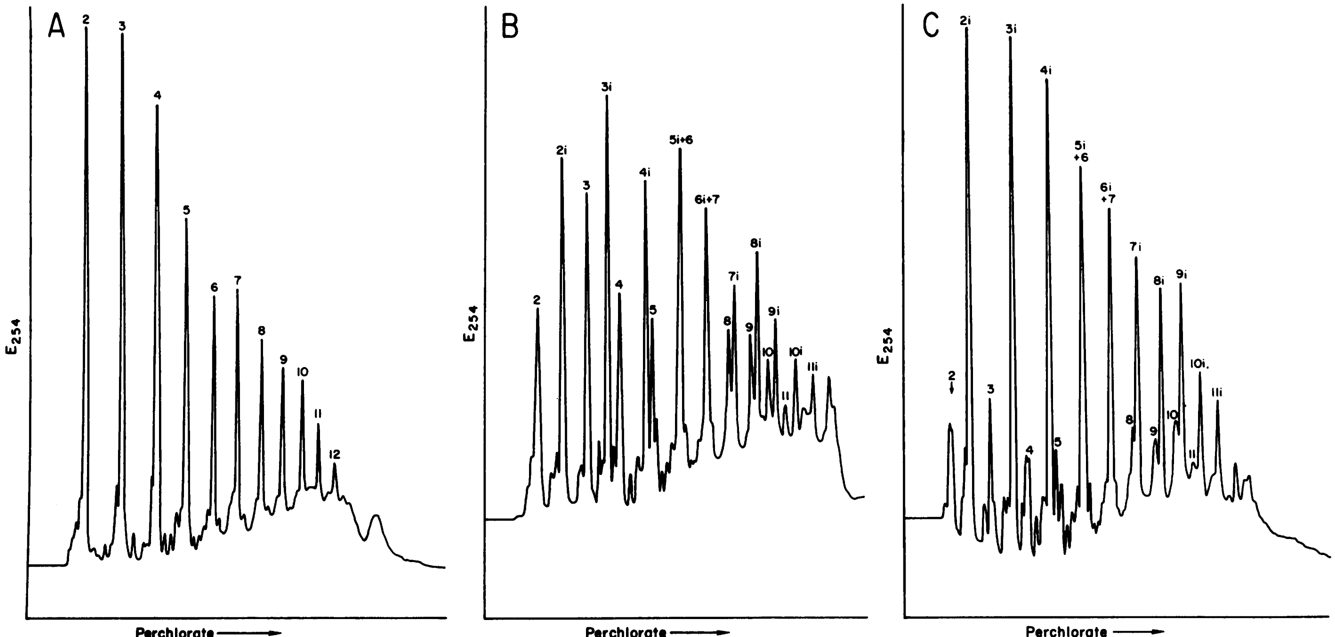


Fig. 3 Conversion of a Mixture of Oligo(G)'s (9) to Phosphorimidazolides. Elution profiles from an RPC-5 column using a perchlorate gradient at pH 12:–(a) starting mixture; (b) after treatment with 0.1M CDI in 0.1M imidazole buffer at pH 6 for 15 minutes; (c) after 1 hour. Peaks corresponding to phosphorimidazolides are indicated by an "i".

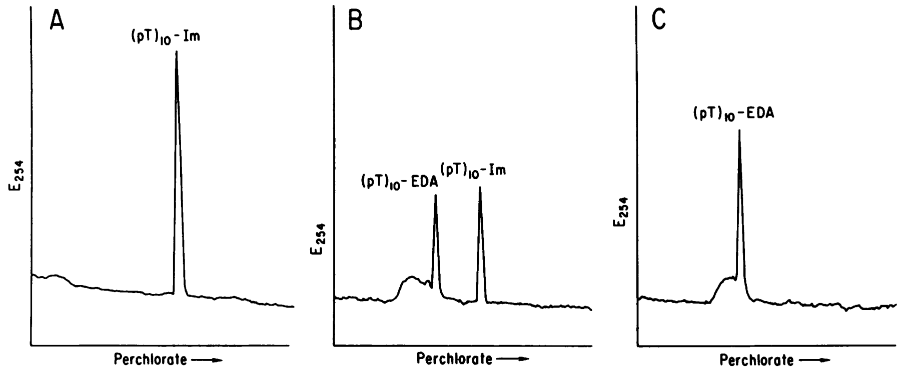


Fig. 4 Formation of the Ethylenediamine Adduct EDA-$(pT)_{10}$ of $(pT)_{10}$. Elution profiles from an RPC-5 column using perchlorate gradient at pH 12 of:- (a) starting material, $(pT)_{10}$-Im; (b) after treatment with 0.25M EDA in 0.1M lutidine buffer at pH 7.7 for 3 hours at 25°C; (c) after 24 hours.

The new peaks have retention times agreeing with those determined previously for the corresponding imidazolides of oligo(G)'s (9).

Displacement of imidazole from the imidazolide of $(pT)_{10}$ by ethylenediamine

When the phosphorimidazolide of $(pT)_{10}$ is eluted from an RPC-5 column and treated directly with a 0.25M solution of ethylenediamine at pH 7.7 a new product is formed which is resolved from $(pT)_{10}$ and its imidazolide on rechromatography. The isolated yield is almost quantitative. The half-life for the conversion is about 4 hours at room temperature (Fig. 4) and the reaction is almost complete after 24 hours. At 50°C, the conversion is virtually complete after 1 hour. By analogy with the corresponding products from mononucleotides, we believe that the product must be the phosphoramidate derived from EDA and the terminal phosphate of $(pT)_{10}$.

Direct reaction of $(pT)_{10}$ with CDI and ethylenediamine After 4 hours in 1-MeIm or MES buffer at pH 6 the yield of the EDA adduct from $(pT)_{10}$ and 0.25M EDA was about 50%. After 24 hours the yield had increased to 90%, but a side product was present in 8-10% yield.

Reactions of DNA

Formation of the 5'-phosphorimidazolide of double-stranded DNA In control experiments, we found that the 5'-phosphates of mononucleotides and the 5'-terminal phosphate of MUA3 DNA are removed by treatment with alkaline

phosphatase, but that mononucleoside-5'-phosphorimidazolides are resistant to this enzyme. Pretreatment of MUA3 DNA with 0.1M CDI in 0.1M imidazole buffer progressively protects the DNA from dephosphorylation by alkaline phosphatase. After 1 hour, under our standard conditions, about 70% protection is achieved. These data strongly suggest that DNA is converted to a phosphorimidazolide in about 70% yield. The 70% conversion is very similar to the yields of imidazolides obtained with the nucleotides and with a variety of oligonucleotides.

Effect of CDI on physical and biological properties of DNA No increase in the proportion of nicked PMK 20 DNA (Form II) (8) from supercoiled DNA (Form I) could be detected by electrophoresis on agarose gels (15) after treatment for 1 hour with 0.1 or 0.2M CDI in 0.1M imidazole buffer at pH 6. This shows that treatment with CDI does not lead to breaks in the DNA backbone.

The ability of plasmid PMK 20 DNA to multiply in E. coli (8) and to confer kanamycin resistance was not affected by treatment with 0.1M CDI in 1-MeIm buffer at pH 6 for 1 hour. Thus our treatment neither inhibits the ability of the plasmid to replicate, nor does it inactivate the gene that confers kanamycin resistance.

Treatment of MUA3 DNA with 0.1M or 0.2M CDI in imidazole buffer at pH 6 for 1 or 2 hours did not affect hybridization at 42°C under standard conditions (16). Hence our treatment does not modify a sufficient number of bases to destabilize DNA double-helices and interfere with hybridization.

DISCUSSION

We have converted each of the mononucleotides, a variety of short homopolynucleotides and the termini of a double-stranded DNA about 5 kilobases in length (with exposed ends) to 5'-phosphorimidazolides. The reaction with 0.1M CDI in 0.1M imidazole buffer proceeds in aqueous solution at pH 6 and 25°C and does not require protection of the nucleotide bases. Neither the rate of reaction nor the final yield of about 75% is strongly dependent on the length of the oligonucleotide. These findings are consistent with the expectation that the reactivity of the 5'-terminal phosphate of a mononucleotide or oligonucleotide should not depend strongly on the moiety to

which the phosphate is attached, provided that access of the reagent is not blocked.

The displacement of imidazole from a phosphorimidazolide intermediate by an amine should also be relatively insensitive to the nature of the nucleotide sequence. We find that the reactions of 0.25M ethylenediamine with the imidazolides of a mononucleotide, pU, or a polynucleotide, $(pT)_{10}$, proceed at roughly equal rates. Both give almost quantitative yields after 24 hours. Our results on the displacement of imidazole by ethylenediamine are similar to those reported for the reaction of glycine (17) and 2'-amino-2'-deoxyuridine (18) with ImpU. Similar reactions with 3'-amino-3'-deoxynucleosides have been used to synthesize oligonucleotide analogues (19).

These observations suggest that a wide range of nucleophilic displacements that are well-known in the chemistry of mononucleotides can be carried out equally easily at the 5'-terminal phosphate of any non-recessed oligo- or polynucleotide. The use of HPLC chromatography on RPC-5 provides a particularly advantageous method of purifying and isolating products derived from oligonucleotides in the size range 2-15. As little as 0.01 ODU of very pure product can be obtained in this way. We have not, as yet, prepared DNA adducts, but are developing the methods needed to isolate them.

The attachment of nucleotides or polynucleotides to complicated polypeptides or to proteins is, of course, a much less defined reaction than coupling to simple amines, since attachment may occur at any nucleophilic group. Attachment to lysine would be expected to result in a stable adduct, but attachment to histidine or cysteine produces an adduct that would decompose relatively rapidly in aqueous solution at room temperature and neutral pH. The 15-35% of labile nucleotide obtained after coupling ImpU with serum albumin must have formed by the hydrolysis of adducts with residues other than lysine. We have not studied adducts between oligonucleotides or nucleic acids and proteins. While the coupling reaction between the phosphorimidazolide of a polynucleotide and a protein should proceed normally, we anticipate that the analysis of such adducts will require the development of novel separation techniques and novel methods of analysis.

The obstacles to the use of CDI as an activating agent are less severe than has been anticipated. We find that treatment of a 5'-^{32}P-DNA plasmid

with 0.1M CDI in imidazole buffer for 1 hour at pH 6 converts more than 70% of this DNA to a derivative that is resistant to alkaline phosphatase, and which we believe to be the 5'-phosphorimidazolide. When supercoiled (Form I) PMK 20 DNA is treated the same way, we are unable to detect any conversion to the relaxed form. This implies that, on average, much less than one break is introduced in a 3,600 b.p. double stranded DNA sequence. Thus attack on the phosphodiester backbone followed by chain cleavage is not a significant reaction.

Surprisingly, the biological activity of a drug resistance plasmid is little affected by our standard procedure. This implies that the formation of base-adducts cannot be very extensive, and that any deleterious adducts that are formed can be repaired in rec A^- E. coli. The undiminished efficiency of hybridization of DNA after treatment with CDI also points to a rather low level of base-modification.

Experiments in which $(pT)_{10}$ was exposed to 0.1M carbodiimide at pH 8.1 and 30°C, conditions that favor CDI addition to the bases (14), lead to a similar conclusion. After 2 hours, about half of the $(pT)_{10}$ had been converted to material which moved as a rather broad band on RPC-5, with a retention time substantially longer than that of the starting material (Data not shown). After 4 hours, the starting material had been further depleted and a new very broad band with even longer retention time had appeared. We believe that the first band represents a mixture of the 10 isomers obtained by adding one carbodiimide molecule to $(pT)_{10}$, while the second band represents a mixture of the 45 di-adducts. If this is so, we may conclude that the half-life for addition of CDI to an individual T residue in a single-strand polynucleotide is about 20 hours at pH 8.1.

The addition of carbodiimide to thymine is much faster than the addition to other bases. The reaction is also rapid at pH 8.1 and much slower at pH 6 (14). Thus the half-life for the derivatization of an individual pT residue in a single-stranded oligonucleotide at pH 6 and 25°C must be many days. Residues other than T are attacked more slowly, if at all, and, furthermore, addition of CDI to the bases is likely to be slower in double-stranded than in single-stranded DNA. Thus both the biological and the chemical evidence suggests that modification of the bases is not extensive when DNA (or RNA)

is treated with 0.1M CDI for 1 hour at pH 6.

The indirect synthesis of phosphoramidates via an intermediate phosphorimidazolide is a generally applicable method. It is also possible to carry out the coupling reaction between a polynucleotide and an amine directly in MES or 1-MeIm buffer if the ligand does not react with carbodiimide. Proteins cannot be used as ligands in the direct coupling reaction since they are extensively cross-linked by treatment with carbodiimide. Simple amines can be coupled directly, but the reaction is slower, and requires a longer exposure of the polynucleotide to CDI.

Our results suggest that a wide variety of useful oligonucleotide and nucleic acid adducts can be prepared from amine-containing molecules using a water-soluble carbodiimide as condensing agent. When the ligand is sensitive to CDI, it is necessary to isolate a phosphorimidazolide intermediate and react it with the amine. Otherwise a direct condensation in a 1-MeIm or MES buffer may be possible. The use of diamines $NH_2(CH_2)_nNH_2$ as linkers will also permit the attachment of carboxylic acids to oligonucleotides. These methods could be used, for example, to prepare biotin adducts as non-radioactive probes (20), EDTA adducts as specific reagents for cleaving DNA in the presence of Fe^{2+} ion (21), protein adducts that might be internalized into cells (22), or affinity columns for molecules that interact specifically with polynucleotides.

Since the original submission of this paper, we have learned that Shabarova and coworkers have applied essentially the same chemistry to prepare the 3'-phosphorimidazolides of oligodeoxynucleotides and have used them as substrates for non-enzymatic ligation (23, 24).

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*SSPE: .18M NaCl, 0.010M NaH_2PO_4, 0.001M EDTA, pH 7.4

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