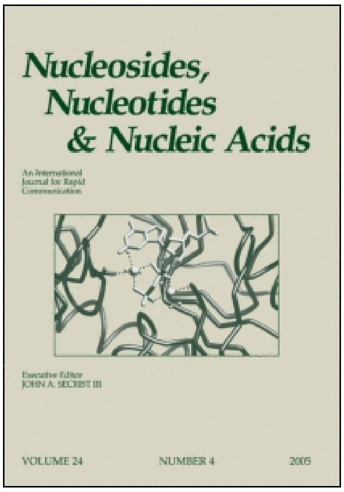

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Intraduplex Photo-cross-linking of p-Azidoaniline Residue and Amino Acid Side Chains Linked to the Complementary Oligonucleotides via a New Phosphorylating Intermediate Formed in the Mukaiyama System

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INTRADUPLEX PHOTOCROSS-LINKING OF P-AZIDOANILINE RESIDUE AND AMINO ACID SIDE CHAINS LINKED TO THE COMPLEMENTARY OLIGONUCLEOTIDES VIA A NEW PHOSPHORYLATING INTERMEDIATE FORMED IN THE MUKAIYAMA SYSTEM.

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ABSTRACT: Oligonucleotide derivatives carrying a side chain of either lysine or histidine at the 3'-end and their complementary oligonucleotides having photoreactive groups a p-azidophenyl-NH$(CH_2)_n$NH- (n = 4, 6) residue at the 5'-end were prepared by using new phosphorylating species formed by treatment of oligonucleotides with Ph_3P and $(PyS)_2$ or $(PyrS)_2$. in DMF, DMSO or their mixture. Efficient cross-linking of duplexes occurred under UV-irradiation (λ > 300 nm).

INTRODUCTION

Oligonucleotide derivatives have been widely used in various fields of molecular and cell biology. Reactive derivatives have been studied as antisense devices to inactivate irreversibly definite nucleic acids targets (1). Oligonucleotides carrying fluorescent haptens or biotin residues capable of binding to specific proteins (antigens or streptavidin conjugated with appropriate enzymes) have been used as sensitive probes in DNA diagnostics. Oligoribonucleotides having reactive groups allowed to localize mRNA binding sites of ribosomes (2) and use as the primers in transcription to determine the exit corridor of nascent RNA at the

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Dedicated to the memory of famous scientist in the field of the nucleic acids chemistry and charming person Professor Tsujiaki Hata

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Escherichia coli RNA polymerase (3). The use of reactive oligodeoxyribonucleotides opened the possibility to estimate quantitatively the thermodynamics of interaction of DNA with prokaryotic and eukaryotic DNA polymerases (4). However, there are some other interesting possibilities of application of oligonucleotide derivatives. Attachment of two functional groups to the 3'- and 5'-ends at one terminal site of duplexes between two complementary oligonucleotides permits to draw them together and thus to study both chemical and noncovalent interactions in these duplexes (5).

A few years ago a simple method was proposed to derivatize oligonucleotides by using of the terminal phosphate activation of oligonucleotides with Mukaiyama reagent (6), i.e. a mixture of triphenylphosphine and 2,2'-dipyridyl disulfide in the presence of N-methylimidazole or N,N-dimethylaminopyridine, which gave relatively stable reactive phosphorylating intermediates (7). The latter may be used for conjugation of oligonucleotides with various groups including those which decrease the activity of primary condensing reagents (e.g. basic amines (8)), or are incompatible with the Mukaiyama reagent (e.g. photoreactive azido groups with triphenylphosphine (9)) when the original procedure is employed.

In the present paper we describe our first experiments dealing with intraduplex photochemical reactions of oligonucleotides having azido groups with their complementary oligonucleotide fragments having typical amino acid residues. Since we use widely the above approach to conjugate oligonucleotides with different non-nucleotide components we describe also a simplified version of this reaction recently elaborated by our group.

EXPERIMENTAL

Materials. N,N-dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetone, acetonitrile were purified and dried according to standard procedures. 4-(N,N-dimethylamino)pyridine (DMAP) was purchased from Bergkamen (Berlin), triphenylphosphine, 2,2'-dipyridyl disulfide, histamine were purchased from Fluka AG (Switzerland). Cetyltrimethylammonium bromide and TLC aluminium sheets (silica gel 60 F_{254}) were purchased from Merck (Germany). Other reagents were analytical grade. 4,6-Dimethyl-2,2'-dipyrimidyl disulfide $(PyrS)_2$ and diphenyl disulfide were prepared according to ref. (10). γ-ATP-p-azidoanilide was obtained by the method described in (11). The oligonucleotides pd(GTG) (I), pd(ATGA) (II), d(GGTATC)p (III), pd(GATACCAA) (IV) and pd(TGATAC) (V) were prepared by the phosphoramidite approach using an automated synthesizer "Victoria", Russia (12). The oligonucleotide (III) and its phosphoramide derivatives (IIIa, IIIb) were labeled at the 5'-end using T4-polynucleotide kinase (NIKTI BAV, Russia) and [γ-^{32}P]ATP (>3000 Ci/mmol) obtained by D. Semenov (Biosan, Russia). The ^{32}P labeled oligonucleotides (III), (IIIa), (IIIb) used as the targets in photochemical experiments were subjected to electrophoretic purification in 20% polyacrylamide gel under denaturing conditions (8 M urea, 89 mM Tris-H_3BO_3, 40 °C).

General Methods. ^{1}H and ^{31}P NMR spectra were recorded on a Bruker AC-200 spectrometer (200 and 81.015 MHz respectively). The ^{31}P chemical shifts are reported using an external standard of 85% H_3PO_4. Infra-red spectra were recorded on a Specord M80 (Karl Zeiss, Yena) in KBr and H_2O. UV-absorption spectra were recorded on a Specord M40 (Karl Zeiss, Yena). Molar extinction coefficients of oligonucleotides at 260 nm were estimated according to ref. (13). The coefficients of oligonucleotides (IVc) and (IVd) were estimated as a sum of the oligonucleotide and the aryl azide (λ = 260 nm, ε = 11000 $M^{-1}cm^{-1}$, pH 9) molar extintion coefficients (9). Optical melting curves of oligonucleotide complexes were obtained by Ju. Markushin on the home-made device with UV detector of a Milichrom liquid chromatograph and thermoregulated microcell (2μL). Isolation and purification of the oligonucleotide derivatives were performed by reversed-phase HPLC using a LiChrosorb RP-18, 10 μm (Merck, FRG), 4.6x250 mm column, a Waters 600E chromatograph and a Waters 484 tunable absorbance detector (USA). A linear gradient (2 mL/min) from 0 to 30% of acetonitrile in 0.05 M $LiClO_4$ (pH 7.5) was used. Analytical anion-exchange microcolumn chromatography was performed on the Milichrom chromatograph (Russia) using a Polisil-SA, 15 μm (Vector, Russia), 2.5x30 mm column. A linear gradient (flow rate 50 μL/min) from 0 to 1 M of NaCl in 7 M urea and 0.01 M Na_2HPO_4 (pH 8.9) was employed.

Synthesis of Oligonucleotide Phosphoramide Derivatives in Organic Solution. A reaction mixture containing 0.01 - 0.1 μmol of the cetyltrimethylammonium salt of the oligonucleotides (I)-(V), 15 μmol of Ph_3P, 15 μmol of $(PyS)_2$ [either $(PhS)_2$ or $(PyrS)_2$] in 0.1 mL DMF [or DMF/DMSO (1:1)] was incubated for 15 min at room temperature. The oligonucleotide derivatives were precipitated with 2% $LiClO_4$ in acetone. Thirty μmol of an appropriate amine [1,3-diaminipropane, 1,6-diaminohexane, morpholine, histamine, N-(p-azidophenyl)-1,6-diaminohexane] in 50 μl of water was added to the oligonucleotide derivative precipitated and the solution was incubated for 0.5 h at 25 ^{o}C. The resulting phosphoramide formed was isolated by reversed-phase HPLC. The product was precipitated by addition of 2% $LiClO_4$ in acetone. The yield was 90-97%.

Synthesis of Oligonucleotides Phosphoramide Derivatives in Aqueous Solution. A reaction mixture containing 0.01-0.1 μmol of the Li salt of an oligonucleotide in 5μL H_2O, 15 μmol of Ph_3P, 15 μmol of either $(PyS)_2$ or $(PyrS)_2$ in 0.1 mL DMF/DMSO (10:9) was incubated for 15 min at room temperature. The oligonucleotide derivatives were precipitated by addition of 2% $LiClO_4$ in acetone. Thirty μmol of an appropriate amine (1,3-diaminopropane, 1,6-diaminohexane, morpholine, histamine) in 50 μL of water was added to the oligonucleotide derivative precipitate and solution was incubated for 0.5 h at 25 ^{o}C. The phosphoramide oligonucleotide formed was isolated as described above. The yield was 90-97%.

Kinetics of Hydrolysis of Synthetic Intermediates of Oligonucleotides Formed by the Oxidative-Reductive Condensing Agent. The phosphorylating intermediate of pd(TGATAC) obtained by precipitation with a 2% $LiClO_4$ acetone solution was dissolved in buffer pH 9.93, containing 0.05 M $NaHCO_3$ and 0.05 M Na_2CO_3, and was incubated at 37 ^{o}C. At 15 min intervals, 5 μL aliquotes were taken, treated with 30 μL of a 0.5 M aqueous solution of 1,3-diaminopropane and analyzed by anion-exchange chromatography. The concentrations of the hydrolysis products were determined from the corresponding UV peak areas. To determine the rate constants, the kinetic data were treated using pseudofirst order time course equation and nonlinear regression program.

Photochemical Behavior of Arylazide-linked Oligodeoxyribonucleotide Derivatives. A $5 \cdot 10^{-5}$ M solution (20-40 μL) of photoreagent (IVc) or (IVd) and its complementary oligonucleotide (III) or its histamine or 1,6-diaminohexane derivative (IIIa or IIIb) in 0.15 M NaCl and 0.02 M Na_2HPO_4 (pH 9) were pipetted into 96-well polystyrene plate at 4 °C. Irradiation was carried out by exposing the plate for 5 min to filtered light (300-365 nm) emitted by a high-pressure mercury lamp with an intensity of 10^{15} quanta•cm^{-2}•s^{-1}. The irradiated mixtures were subjected to anion-exchange chromatography or electrophoresis in 10% denaturing PAGE visualized by radioautography. Quantitative treatment of radioautographs was carried out using Ultrascan laser densitometer (LKB).

The Reaction of γ-ATP-p-Azidoanilide with Imidazole. The $7 \cdot 10^{-3}$ M solution of γ-ATP p-azidoanilide in the 1M aqueous solution of imidazole was irradiated for 20 min by filtered light (300-365 nm) emitted by a high-pressure mercury lamp with an intensity of 2 10^{15} quanta•cm^{-2}•s^{-1}. The reaction mixture was incubated 12 h at -4 °C, evaporated at 35-40 °C using rotary evaporator , and the rest was dissolved in 0.5 ml of water. The aqueous solution was added dropwise to 10 ml of acetone and the mixture was incubated for 3 h at -15 °C. The precipitate formed was separated by centrifugation (-10 °C, 10 min, 40,000 rpm), washed twice with 10 ml of cold acetone and dissolved in 2 ml of twice-distilled water. The solution was put on the 1x5 cm Chelex (Na^+) column and eluted with twice-distilled water (30 ml/h). Fractions absorbing at 260 nm were collected and dried using rotary evaporator. The product was homogeneous as revealed by TLC in the system dioxane-ammonia-water 6:1:4 (v/v) with R_f 0.29 and by anion-exchange chromatography with retention time corresponding to three negative charges.

RESULTS AND DISCUSSION

Mechanism of the oxidative-reductive condensation by a mixture of triphenylphosphine

Ph_3P and dipyridyl disulphide $(PyS)_2$ still remains unestablished. M. Hashimoto *et al.* (14) have reported that 3'-*O*-acetylthymidine-5-phosphate by treatment with this mixture in anhydrous pyridine was rapidly converted to the S,S'-dipyridyl phosphorodithioate derivative which can be stored in solution and behave as an active phosphorylating species. Later, it was demonstrated that a highly reactive phosphorylpyridinium derivative is produced in this reaction mixture (15). Both could explain the role of the relatively stable intermediate in the oxidative-reductive condensation.

We have tried to isolate the intermediate formed by treatment of oligonucleotides with Ph_3P and $(PyS)_2$ in DMF. A mixture of 0.005-0.1 µmole of cetyltrimethylammonium salt of an oligonucleotide (I-V) was incubated with 15 µmole each of Ph_3P and $(PyS)_2$ for 15 min in 0.1 mL DMF at room temperature. The resulting oligonucleotide derivative was precipitated by addition of an 2% acetone solution of $LiClO_4$. The precipitate was carefully washed with acetone and diethyl ether, and treated with an aqueous solution of 1,3-diaminopropane. As a result, the corresponding oligonucleotide phosphoramide derivative was nearly quantitatively formed as shown by anion-exchange chromatography and the regeneration of the parent oligonucleotide by its acid treatment. The use of dipyrimidyl disufide $(PyrS)_2$ and diphenyl disulfide $(PhS)_2$ in place of $(PyS)_2$ resulted in similar phosphorylating intermediates under the same conditions. With the latter reagent the reaction proceeded significantly slowly. Similar derivatives were obtained in DMSO solution and in a 1:1 mixture of DMF and DMSO. In this mixed solvent it was demonstrated that the Li salt of oligonucleotide to be activated can be added in aqueous solution. To obtain reasonable yield in the latter case the water content in the final solution should not exceed 10%. This avoided the preparation of the cetyltrimethylammonium salt of oligonucleotide.

The aqueous solution of a similar phosphorylating intermediate obtained from d(pGpTpG) was studied using ^{31}P NMR spectroscopy. The ^{31}P NMR spectrum of the solution is presented in FIG. 1. It is seen that the spectrum consists of two main signals at

-1.7 ppm (singlet) and -0.898 ppm (broad singlet). The latter corresponds most probably to the internucleotidic phosphates. The former may be tentatively assigned to an activated phosphate. After incubation of this solution for 20 h these signals were transformed to three signals at 3.95 ppm, -0.97 ppm and -14.18 ppm.

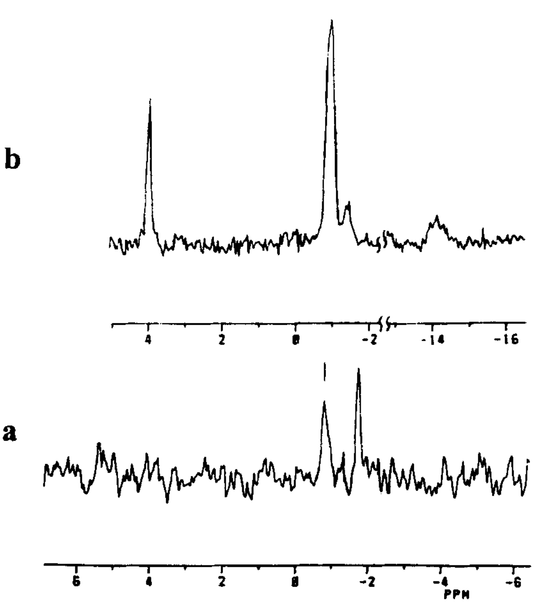
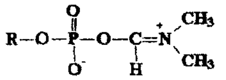


FIG. 1. ^{31}P NMR spectra in H_2O of 10^{-3}M solution of the phosphorylating derivative of d(pGpTpG) after 60 min(a) and after 20 h of incubation after dissolution.

They satisfactorily correlate with those expected for the 5'-terminal phosphate, internucleotidic phosphates and internal cyclic pyrophosphate, formed between the 5'-terminal and one of internucleotidic phosphates (16). Chromatographic behavior of the components of aqueous solution subjected to incubation is in accordance with the above interpretation of ^{31}P NMR spectrum: in the course of anion-exchange chromatography, one of the products is eluted similar to parent oligonucleotide. The alkaline phosphatase treatment of the mixture resulted in the conversion of this compound to a product with the negative charge decreased by two units. Another product is eluted as a compound having one-unit less negative charge than the parent oligonucleotide in accordance with the expected behavior of the structure in which the 5'-phosphate and one of internucleotidic phosphates are connected by an anhydride bond.

Active derivatives of oligonucleotides are rather stable in aqueous solution. For the derivative of pd(TGATAC) (V) obtained by treatment with Ph_3P and $(PyS)_2$ in DMF solution half time of hydrolysis at 37 ^{o}C and pH 10 was found to be about 117 min as compared with 330 min for the corresponding 4-N,N-dimethylaminopyridinium derivative.

An attempt to elucidate the structure of these initially formed derivatives was done for those obtained in DMF solution using ^{1}H NMR spectroscopy. Reactive derivative of pdT was used for this goal to simplify the spectrum. This derivative seems to be less stable than the corresponding oligonucleotide derivatives as revealed by reaction of its precipitated derivative with morpholine. Only 70% of P-morpholide of pdT is formed. In this case the remaining part consists of the symmetric pyrophosphate derivative $O(pdT)_2$ and the parent mononucleotide. Therefore, the ^{1}H NMR spectrum was recorded after 2 h incubation of the aqueous solution to deal with the hydrolysis product of the initially formed reactive derivative (FIG. 2). No signals were observed in the range of 7.2-8.5 ppm which is typical of aromatic protons of PPh_3 and PyS fragments. This means that the derivative did not contain any fragments of the pair $PPh_3/(PyS)_2$, and consequently, does not represent neither the S-pyridyl phosphorothioate bond obtained in (14), nor the phosphorylphosphonium salt (Ph_3P^+—O—PO_2^-—OR) postulated in (6). Two new signals were observed as compared with pdT solution. One of them at 7.99 ppm is close to a signal at 7.90 ppm of CH fragment of dimethylformamide and according to the integral intensity corresponds to one H atom. Two other new signals at 2.90 ppm and 3.05 ppm both with intensities corresponding to three H atoms each are close to the signals of two methyl groups of pure DMF (2.79 ppm and 2.94 ppm). These signals do not appear if pdT is subjected to the same procedure of precipitation from DMF solution and subsequent extensive washing. This indicates that DMF fragment is the part of the reactive phosphorylating derivative formed in DMF solution. At present only tentative structure (A) may be ascribed to this type of derivatives similar to proposed in (17) for the product of the reaction of diethylchlorophosphate with DMF.



$$\mathrm{R{-}O{-}\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}{-}O{-}\underset{\underset{\displaystyle H}{|}}{C}{=}\overset{+}{N}(CH_3)_2}$$

(A)

The modified phosphorylation procedure was used to prepare the components of the duplexes for the study of the intraduplex photochemical cross-linking. Two amines as the models of amino acid side chains were used to link to oligonucleotides. Histamine as a

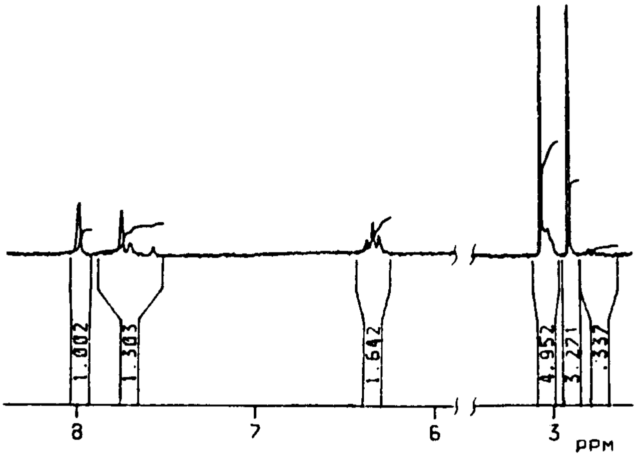
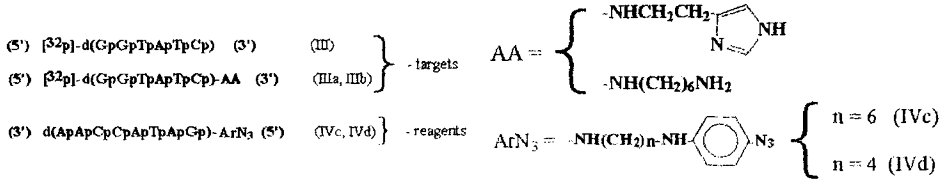


FIG. 2. ^{1}H NMR spectra of 10^{-3} M solution of the phosphorylating derivative of pT in D_2O after 360 min of incubation after dissolution.

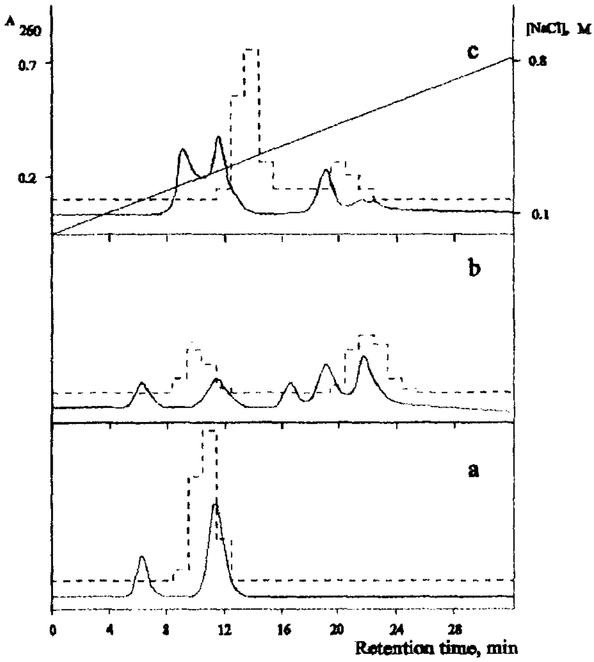
model of the histidine residue and 1,6-diaminohexane as a model of the lysine side chain. These residues were attached to the 3'-terminal phosphate of oligonucleotide d(GGTATC)p (III) which served as a target in the intraduplex modification. These derivatives were labeled by introduction of a 5'-terminal [^{32}P]-phosphate group using [γ-^{32}P]ATP and polynucleotide kinase. N-p-(Azidophenyl)-1,4-diaminobutane and N-p-(azidophenyl)-1,6-diaminohexane were used as photoreactive moieties attached to the 5'-terminal phosphate of octanucleotide pd(GATACCAA) (IV) containing a hexanucleotide sequence complementary to the target sequence. The p-Azidophenylamino group was chosen as a photoreactive one since this photo-activated species was previously shown to be unable to modify oligonucleotide moities of the target (5) and at the same time readily reacted with proteins in chromatin (18). This type of photoreactive group was previously shown to react via intermediate formation of quinonediimine derivatives. Consequently, any cross-linking in the duplex could be ascribed to reaction with the imidazolylethyl or aminohexyl fragments. After phosphorylation of these amines with the reactive derivative of oligonucleotide IV the corresponding phosphoramides were purified by HPLC to homogeneity (as revealed by anion-exchange chromatography). The presence of azido group in the products was proved by IR spectroscopy (the presence of band at 2100 cm^{-1})

To elucidate whether phosphorylation of histamine by phosphorylating reagent used proceeds selectively at aliphatic amino group and does not touch imidazole ring. The ^{31}P NMR spectrum of the product resulted from phosphorylation of histamine with the activated derivative of tetranucleotide pd(ATGA) was recorded. Such a shorter oligonucleotide was used in this case to make identification of the signals easier. In the NMR spectrum the single signal besides signals at -0.29 ppm typical of internucleotidic phosphates was observed at 8.38 ppm in the area typical of aliphatic phosphoramide groups. The signal of the imidazolylphosphoramide group should be expected at -11 ppm (16).

Intraduplex photochemical cross-linking was studied at 4 °C. The reaction mixture contained 10^{-5} M of each oligonucleotide derivative in 0.16 M NaCl, 0.02 M Na_2HPO_4, pH 8.9. The duplexes subjected to investigation are presented in the scheme:



To estimate the content of the duplex in these conditions melting temperature was measured for a mixture of oligonucleotides III and IV, as well as for mixtures of IIIa with IV and IIIa with IVc. The Tm values were found to be 26 °C, 28 °C and 30 °C, respectively. Since the ΔH value estimated for the complementary sequences in accordance with (19) is 37.7 kcal/mol the association constant value for the duplex at 4 °C calculated using Vant-Hoff equation is significantly below 100. Consequently, in all reaction mixtures duplexes strongly predominate over single stranded components. All the duplexes formed were subjected to irradiation by the filtered light of a mercury lamp at 300-365 nm. The irradiated samples were subjected to anion-exchange chromatography in 7 M urea to provide dissociation of non-covalent duplex. The samples were put on the column after 6-12 h to escape modification of the amino groups of the sorbent with quinone diimine intermediate. These chromatograms are presented in FIG. 3. To identify



the products resulting from interaction of the reagent with the target, a small amount of 5'-^{32}P labeled target was added to the bulk of the target derivative lacking a 5'-terminal phosphate. Therefore, the positions of UV and radioactivity profiles do not coincide the latter being eluted by higher ionic strength of the eluent.

FIG. 3. Profile of the analytical anion-exchange microcolumn chromatography of the parent duplex (IIIb) + (IVc) (a), of the irradiated duplex (IIIb) + (IVc) (b) and irradiated duplex (IIIa) + (IVc) (c). Solid line - A_{260}, broken line - radioactivity counts/min.

No new products derived from the target appeared after irradiation of (III) in the presence of (IVc) or (IVd) (data not shown). On the contrary, irradiation of (IIIb) (FIG. 3b) and (IIIa) (FIG. 3c) in the presence of the photoreagents resulted in the appearance of new products with the retention time exceeding that of the starting target. In all cases the A_{260} profiles contain peaks lacking radioactivity. These peaks most probably correspond to the products of the reagent transformation. Further, we analyzed only the peaks containing the target moiety. In all cases, the peaks corresponding to the parent target are clearly seen. At the same time the radioactivity profiles in FIG. 3b and FIG. 3c showed the fractions containing the products with the retention times significantly exceeding those of unmodified targets. In both cases the peaks corresponding to the cross-linking product appeared. However, simultaneously additional fractions corresponding to the products of larger size were obtained. The total yields of the covalent adducts were estimated as the

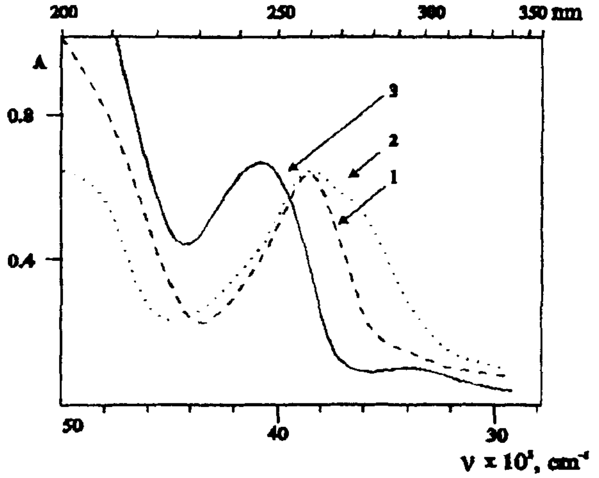


FIG. 4. UV-spectra of aqueous solutions of ATP γ-p-azidoanilide (1), the product of its photolysis under argon after 90 s irradiation (2), and of the product of the interaction of the latter with 1 M imidazole.(3).

ratio of radioactivity of the all fractions to the total radioactivity and were found to be 25% for IIIa + IVc, 67% for IIIb + IVc and 43% for IIIb + IVd.

It seems reasonable to suggest that cross-linking within the duplex proceeds via intermediate formation of the quinone diimine derivative. In this case, an initial product may be obtained by 1,4-addition of the nucleophilic center of the target to the photolysis product accompanied by aromatization of the benzene ring of the reagent (20). Such a product is known to be easily oxidized to a quinoid structure capable of the second addition reaction.

Consequently, the larger cross-linking product may be formed by addition of two target molecules to the photolized reagent. The ratio of radioactivity and UV intensity at 260 nm in these peaks qualitatively coincide with this suggestion.

To prove such a possibility at least in the case of the histamine fragment we have studied a model photochemical reaction of γ-ATP-p-azidoanilide with imidazole. In accordance with the previously established mechanism of photolysis of p-azidoanilides similar conversions occurred both under irradiation of γ-ATP-p-azidoanilide in 1 M

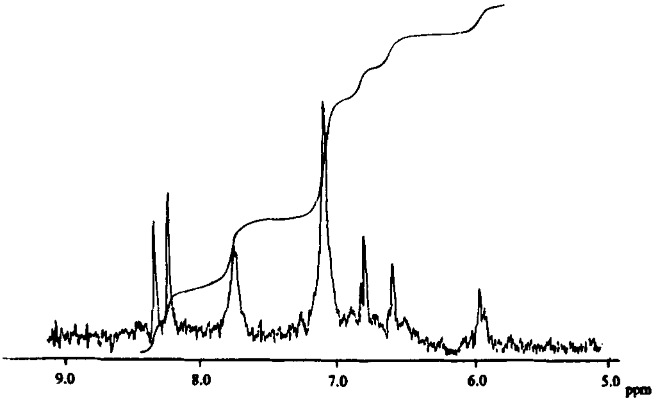


FIG. 5. ^{1}H NMR spectra of the $2\cdot10^{-3}$ M D_2O solution of the product of irradiation of ATP γ-p-azidoanilide in 1 M imidazole solution

imidazole solution and after addition of irradiated solution of the ATP derivative to 1 M imidazole in the dark as shown by the changes of UV spectra.

The ^{1}H NMR spectrum of the isolated product is presented in FIG. 5. It is clearly seen that the ratio of intensities of the adenine ring signals of H-2 and H-8 protons (at 8.22 and 8.32 ppm) are approximately two times lower than signals of H-2 imidazole protons at 7.76 ppm and four times lower than those of H-4 and H-5 imidazole protons at 7.1 ppm. Two unsplitted signals corresponding to protons of the phenyl ring (at 6.6 and 6.8 ppm) as well as their intensities suggested that one phenyl residue is present per two imidazole rings and these protons are separated by others substituents. These results strongly indicated the formation of γ-ATP 2,5-bis-imidazolyl-4-aminoanilide.

These data support the assumption that two target molecules may cross-link with one complementary target. If this suggestion is correct, this should mean that dissociated target molecules formed due to high dissociation rate constant of rather short duplex under investigation may react with the first cross-linked target.

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